THE LHRH-RECEPTOR PROTEIN: CHARACTERISATION AND STRATEGIES FOR ITS PURIFICATION

by

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This Thesis is dedicated to my parents, who made possible for me what they only dreamt of achieving. Thank you.

I declare that the studies presented in this Thesis are the result of my own independent investigation with the exception of the chemical synthesis of LHRH analogues which was carried out by Dr. Christine Bladon.

The work has not been and is not being currently submitted for candidature for any other degree.

Some of the results presented in this Thesis have been published as follows(denoted *)

Mitchell, R. and Ogier, S.-A. (1985) A novel stimulatory action of somatostatin on prolactin secretion. Brit. J. Pharmacol. 86: 698P.

Mitchell, R. and Ogier, S.-A. (1986) Investigation of the mechanism of somatostatin action on growth hormone secretion in rats. J. Physiol. 373: 75P.

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ABSTRACT OF THESIS

Experiments were carried out using ligand-binding techniques to investigate extensively a variety of aspects of the LHRH receptor, both in situ in the cell membrane and in a solubilised state.

The possibility of the close involvement of a K^+ channel with the LHRH receptor protein gained no support from results presented in this Thesis. However, the possible role for a G-protein in the LHRH receptor in primary transducing system is supported by these studies. A direct interaction between ligand binding to the LHRH receptor and a G-protein has not been demonstrated previously.

Solubilisation of the LHRH-receptor has been achieved with a very high efficiency for the first time, using the combination of a zwitterionic detergent (CHAPS) and high ionic strength. Experiments with CHAPS and a broad range of other detergents, with varying NaCl concentrations and protein: detergent ratios were not able to reveal another method capable of giving comparable results.

The conditions required for maximal solubilisation of the LHRH-receptor were found to inhibit the binding of LHRH to the receptor protein. A polyethylene glycol (PEG) precipitation step was sufficient to remove the CHAPS/NaCl solution and allow optimal binding of LHRH and its analogues. Ligand binding experiments to such PEG- precipitated preparations revealed that the solubilised LHRH-receptor had very similar binding properties to that of the membrane bound receptor. These results strongly suggest that the entity solubilised by the CHAPS/NaCl method was an undenatured LHRH-receptor.

To try to investigate further the solubilised LHRH-receptor using ligand binding techniques conditions were sought that would maintain both the solubility of the protein and its affinity for

LHRH analogues. Many possible solutions to this problem were investigated, but no such condition has been found.

In order to overcome this obstacle, a new approach was devised. LHRH-biotin analogues were designed that would allow the interaction of both the LHRH and the biotin moieties. Chemical crosslinking and photoaffinity techniques were investigated to optimise the specific crosslinking of these bi-functional ligands to the anterior pituitary membrane preparation. Optimal results were obtained using the homo-bifunctional chemical crosslinker ethylene glycolbis (succinimidylsuccinate)(EGS). Whilst further refinement of this method of covalently labelling the membrane bound LHRH-receptor protein with a biotin molecule is necessary, preliminary results using a streptavidin-affinity column suggest that it is a viable approach to the purification of specifically covalently-labelled membrane proteins.

Further characterisation of the solubilised LHRH-receptor was achieved using the technique of gel filtration. As this is a non-denaturing technique under the conditions utilised in this study, the results may be interpreted as revealing the apparent molecular size of the non-denatured LHRH-receptor. A molecular weight of approximately 100,000-160,000 was revealed. Whilst this is approximately twice that shown by other workers using the denaturing technique of sodium dodecyl sulphate polyacrylamide gel electrophoresis, (SDS PAGE) it is of a similar magnitude to that revealed in situ by the radiation inactivation technique.

These results suggest that the LHRH-binding protein solubilised with high efficiency from anterior pituitary membranes is the LHRH-receptor and that this is maintained in solution as the same molecular complex as that of the LHRH receptor in situ in the cell membrane.

CONTENTS

Chapter 1

Introduction

1.1 The identification of a hypothalamic factor influencing pituitary gonadotrophin secretion.

The culmination of many years of investigation into the brain's influence on reproduction (see Sawyer, 1969; Harris, 1972 for reviews) was the discovery of an unidentified factor extracted from the median eminence of the hypothalamus which, on infusion into the anterior pituitary gland, induced an ovulatory response (Campbell, Feurer, Garcia and Harris, 1961; Campbell, Feurer and Harris, 1964; McCann, 1962). For many years previously, it had been clear that somatosensory stimuli and many other environmental factors such as day length, light, temperature and olfactory stimuli could modify reproductive function (Sawyer, 1969). The association of hypothalamic lesions and tumours with many gonadal and other sexual disorders, such as precocious puberty or amenorrhea, led early workers to suggest that a neural mechanism within the hypothalamus may have a role in regulating the reproductive status of man and other mammals (for reviews see Riddoch, 1938; Sawyer, 1969). The influence of the hypothalamus over the pituitary (pars nervosa, pars intermedia and pars distalis or anterior pituitary) was initially thought to be exerted via a neural link (Le Gros Clark, 1938; Beattie, 1938). However, experiments revealed that the anterior pituitary was insensitive to direct electrical stimulation (Markee, Sawyer and Hollinshead, 1946; Harris, 1948). Identification (Popa and Fielding, 1930) and further investigation of (Wislocki and King, 1936) a system of fine capillaries in the pituitary stalk connecting two capillary networks (one in the hypothalamus and one in the anterior pituitary gland) revealed a flow of blood from the hypothalamus to the pituitary gland. In the hypothalamus the

capillary plexus was in the median eminence, the area found to be most sensitive for an electrically stimulated ovulatory response (Saul and Sawyer, 1957). Severence of the pituitary stalk had been found to block irreversibly the ovulatory response to electrical stimulation (Westman and Jacobsohn, 1936; Brooks, 1938) and result in gonadal atrophy (Westman and Jacobsohn, 1938; Westman and Jacobsohn, 1940). Experiments involving the transplantation of the pituitary from its position under the hypothalamus resulted in an abolition of gonadotrophin secretion (Harris and Jacobsohn, 1952; Harris, 1964), except when the pituitaries were reimplanted in situ when normal functioning was subsequently observed (Greep, 1936; Harris and Jacobsohn, 1952), a phenomenon that was coincident with the regeneration of the portal vessels (Harris and Jacobsohn, 1952). In a series of experiments, Nikitovitch-Winer and Everett (1957, 1958, 1959) demonstrated that the retransplantation of a pituitary from a site under the kidney capsule back to its original position resulted in the restoration of all pituitary functions, including the release of gonadotrophins. Taken together these results led to the hypothesis of a neurosecretory mechanism for the hypothalamic control of the anterior pituitary (Harris, 1969). That is the release of substances from nerve terminals in the median eminence into the portal plexus which, on reaching the anterior pituitary via the portal system, affected the activity of the pituitary gland.

The effect of various neurotransmitters on the gland was investigated by Markee, Sawyer and Hollinshead (1946). No classical neurotransmitter could be shown to exert a direct effect on the anterior pituitary and it was not until its extraction in 1961

(Campbell, Feurer and Harris) that the hypothalamic factor mediating neural control over anterior pituitary gonadotrophes was identified (Sawyer, 1969; Harris, 1972).

In subsequent years, the median eminence ovulatory factor was partially purified, being identified as a polypeptide, distinct from other known hypothalamic factors (such as corticotrophin releasing factors and thyrotrophin releasing factors) which could be extracted from the median eminence but not the cerebral cortex (Mittler and Meites, 1964; Endroczi and Hilliard, 1965). Porcine hypothalami were the source of the first isolated homogenous preparation of this factor (named luteinizing hormone releasing hormone, LHRH: Schally, Arimura, Baba, Nair, Matsuo, Redding and Debeljuk, 1971) and it was subsequently sequenced (Matsuo, Baba, Nair, Arimura and Schally, 1971). A similar peptide could be purified from ovine hypothalami (Amoss, Burgus, Blackwell, Vale, Fellows and Guillemin, 1971). Very rapidly, chemical synthesis of the decapeptide (pGlu, His, Trp, Ser, Tyr, Gly, Leu, Arg, Pro, Gly-NH_2) was achieved (Matsuo, Arimura, Nair and Schally, 1971). This product was shown to have effects identical to those of the isolated peptide, that is inducing ovulation in rabbits and causing LH release from the anterior pituitary gland, confirming its authenticity as the hypothalamic factor for gonadotrophin release.

1.2 The physiological role of LHRH and its clinical applications

LHRH (or an LHRH-like substance) has been found to occur in the CNS, predominantly in hypothalamic regions but also to a lesser extent in other brain areas such as cortex (Sternberger and Hoffman, 1978; Krey and Silverman, 1983), and in non-neural tissues such as the placenta (Lee, Seppala and Chard, 1981; Tan and Rousseau 1982; Hseuh and Jones 1983), the gonads (Paull, Turkelson, Thomas and Arimura, 1981; Hseuh and Jones, 1983; Sharpe, 1984) and the lactating mammary gland (Sardar and Nair, 1981; Smith-White and Ojeda, 1983). It acts at the level of the pituitary to stimulate the release of the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk and White, 1971). Radioimmunoassay techniques have been used to determine LHRH levels in portal blood (Carmal, Araki and Ferin, 1976; Fink and Jamieson, 1976; Sarkar and Fink, 1980) and the results of such studies suggest that changes in LHRH secretion can account for most alterations in LH and FSH secretion. In this way LHRH appears to exert a controlling influence on reproduction (see Fink, 1979, for review).

An array of additional LHRH actions have been suggested, not all of which are fully elucidated as yet. One seemingly well established role of LHRH in the CNS is that of facilitating mating behaviour. LHRH facilitates the lordosis response in ovariectomised, oestrogen-treated female rats when given systemically or by intracerebral infusions into medial preoptic area, arcuate area and mesencephalic central gray (McCann and Moss, 1975; Moss, 1977; 1979). Conversely, intracerebral infusions of anti-serum to LHRH abolishes the lordosis response in these

animals (Sakuma and Pfaff, 1983). Iontophoresis of LHRH or its analogues results in an increase in electrical activity of up to 50% of neurones in the arcuate ventromedial complex (Moss 1977) and mesencephalic central gray (Samson, McCann, Chud, Dudley and Moss, 1980), and electrical stimulation of mesencephalic central gray in oestrogen treated, ovariectomised rats results in a facilitation of the lordosis response (Sakuma and Pfaff, 1979). In the medial preoptic area the membrane responses to iontophoresis of LHRH and its analogues have been shown to be modulated by ovarian hormone pretreatment (Moss and Dudley, 1978). These results all suggest a physiological role of LHRH in the brain in determining sexual responsiveness, according to hormonal status. There is also some evidence of an ultra-short feedback mechanism of LHRH on LHRH release into portal blood (De Paulo, King and Carrillo, 1987).

In the gonads LHRH (or an LHRH-like substance: Ying and Guillemin, 1980) has been found to inhibit gonadotrophin-stimulated steroidogenesis (Arimura, Serafini, Talbot and Schally, 1979; Clayton, Harwood and Catt, 1979; Hseuh and Erickson, 1979(a); 1979(b); Hseuh and Ling, 1979; Ying and Guillemin, 1979; Bambino, Schreiber and Hseuh, 1980; Hseuh, Wang and Erickson, 1980). Less well established actions of LHRH include its role in the differentiation of various pituitary cell types (Begeot, Hemming, Martinet, Dubois and Dubois, 1983; Aubert, Begeot, Winiger, Morel, Sigoneako and Dubois, 1985) and in the early, post natal sexual differentiation of the brain (Charlton, 1986). At puberty a change in the pulse frequency of LHRH release into the hypophysial portal blood system is thought to be the key factor in controlling the increased gonadotrophic hormone output required for sexual

maturation (Yen, Tsai, Naftolin, Vanden Berg and Ajabor, 1972; Carmel, Arai and Ferin, 1976; Brook, Jacobs, Stanhope, Adams and Hindmarsh, 1987). Evidence of this pivotal role of the LHRH gene in the postnatal development and functioning of the reproductive system has been provided by experiments using the hypogonadal (hpg) mouse. Homozygous hpg mice have a deletional mutation of 33.5 kilobases in the gene encoding for pre-pro LHRH, including the third and fourth exon of the gene (Mason, Hayflick, Zoeller, Young, Phillips, Nikolics and Seeburg, 1986). Although they produced a shortened version of LHRH mRNA, they are unable to generate any functional LHRH (Mason, Hayflick, Zoeller, Young, Phillips, Nikolics and Seeburg, 1986). Such hpg mice show normal foetal development but postnatally their reproductive system fails to develop further (Cattanach, Iddon, Charlton, Chiappa and Fink, 1977). Using techniques of genetic engineering a 13.5 kilobase gene encoding for LHRH and its flanking sequences have been introduced into ova from heterozygous hpg mice. Selective breeding resulted in offspring that were homozygous hpg but transgenic for the LHRH gene (Seeburg, Mason, Stewart and Nikolics, 1987). These offspring exhibited normal reproductive development and function (Seeburg, Mason, Stewart and Nikolics, 1987), apparently confirming the central role of LHRH in coordinating both sexual maturation and the processes involved in maintaining reproductive capacity.

Clinically LHRH and its analogues appear to have wide application (Ziporyn, 1985). Initially it was thought that long acting agonist analogues would be useful in treating hypogonadal states (Schally, 1973). However repeated administration of LHRH analogues was found to result in a paradoxical

desensitisation and inhibition of gonadotrophin release (see Ziporyn 1985; McLachlan, Healy and Burger, 1986; Fraser and Baird, 1987 for reviews). As this inhibition of the pituitary-gonadal axis was induced by peptides of high potency with no apparent metabolic side effects and which were rapidly metabolised (Sandow, Jerabek-Sandow, Kraus and Stoll, 1982),the clinical application of this antigonadotrophic action was rapidly investigated. The successful clinical uses of LHRH analogues include the treatment of precocious puberty (Sandow, 1983; Ziporyn, 1985; Fraser and Baird, 1987) and many steroid-dependent disorders. These include hormone-dependent tumours such as prostatic and some mammary carcinomas (Sandow, 1983; McLachlan, Healy and Burger, 1986; Fraser and Baird, 1987) and many other oestrogen-dependent gynaecological diseases such as endometriosis, fibriomyomata and menstrual disorders (Sandow, 1983; McLachlan, Healy and Burger, 1986; Fraser and Baird, 1987). Much research has also been directed towards the use of LHRH analogues for contraception, both in males and females (Sandow, 1983; Ziporyn, 1985; McLachlan, Healy and Burger, 1986; Fraser and Baird, 1987). As yet it appears unlikely that LHRH analogues offer significant overall advantages, but their use is indicated in certain cases (such as in lactating women or older women for whom steroids are contra-indicated: McLachlan, Healy and Burger, 1986; Fraser and Baird, 1987).

When LHRH, or its analogues, are administered in frequent but small pulses (such as by a pulsatile infusion pump) a pro-gonadotrophic action can be achieved without the paradoxical desensitisation. This type of regime has been successfully used to treat conditions such cryptorchidism (Sandow, 1983; Ziporyn, 1985;

Fraser and Baird, 1987) and hypogonadotrophic hypogonadism, in both men and women (Sandow, 1983). In infertility in women due to a disturbance of rather than deficiency of gonadotrophins, LHRH agonists have been used to induce a hypogonadal state, facilitating induction of follicular development and ovulation by exogenous gonadotrophins (Fraser and Baird, 1987). Such regimes have been applied to induce multiple follicular development for in vitro fertilisation techniques.

The actions of LHRH are all thought to be mediated via specific membrane-bound receptor proteins (Conn, Marian, McMillan, Stern, Rogers, Hamby, Penna and Grant, 1981). Gonadal and pituitary gland LHRH binding sites have been shown to exhibit similar pharmacological characteristics (Clayton, Katikineni, Chan, Dufau and Catt, 1980; Reeves, Seguin, Lefebvre, Kelly and Labrie, 1980), whereas those in placental tissue have a lower affinity for LHRH analogues (Currie, Fraser and Sharpe, 1981) and the potency ratios of analogues for behavioural and electrophysiological responses in the CNS differ from those in the pituitary gland (Moss and Dudley, 1978; Sakuma and Pfaff, 1983). Possibly different but related receptors for LHRH (or an LHRH-like substance) may be involved in the placental and central actions of the peptide; however, there is no evidence of a heterogeneous LHRH receptor population in the anterior pituitary gland (see Chapter 3). The clinical responses to LHRH administration are therefore all likely to be receptor mediated - by one or possibly two related receptors. Certain aspects of the paradoxical clinical effects of LHRH and its analogues can be explained in terms of known properties of these receptors and their signal tranduction mechanisms. For example, using an in vitro

superfusion system, Badger, Loughlin and Naddaff (1983) demonstrated that whilst continuous exposure of dispersed anterior pituitary cells to LHRH at a high dose rapidly produces a desensitisation of LHRH induced LH release, administration in pulses of a few minutes at hourly intervals was able to prevent this effect. Such a result may in part explain the paradoxical inhibitory action of LHRH agonists used clinically at high doses (although extrapituitary actions of LHRH have also been suggested to be involved in this effect; Fink, 1986) and the pro-gonadotrophic action of a pulsatile LHRH dose regime. The way in which this desensitisation is brought about has not yet been elucidated, although it is not thought to be directly linked to a reduction in the number of receptors available for interaction, but possibly the result of a change in the coupling of the LHRH receptor to its effector mechanisms (Conn, McArdle, Andrews and Huckle, 1987).

The structure of the LHRH-receptor protein and the way in which this affects the release of LH and FSH from gonadotrophes are largely unknown, although some progress towards understanding its transducing mechanism has been made (see 1.4 and Chapter 3). Further insights into these aspects of the LHRH receptor may well facilitate understanding of its functioning in vivo. This could in turn lead to the design of improved analogues, or enable manipulation of cellular processes regulating the receptor and its responses, so improving and/or extending the clinical applications of LHRH analogues.

1.3 Analysis of membrane receptor proteins by biochemical and molecular biological techniques

The first membrane receptor to be biochemically and structurally characterised in depth was the nicotinic acetylcholine receptor (nAChR) from the electric organ of rays (Torpedo species) or eels (Electrophorus species). The availability of the receptor in such a concentrated form, making up as much as 30-45% (by mass) of Torpedo electric organ preparations (Changeux, Giraudat and Dennis, 1987), was fundamental to its successful molecular characterisation. Biochemical techniques were initially used to isolate and physically characterise the nAChR (Karlin, 1980), revealing a complex of four different glycosylated polypeptide subunits, each relatively hydrophobic in nature (Vandlen, Wu, Eisenach and Raftery, 1979). Each complex was found to consist of five subunits: α_2, β, γ, δ, with the one complex containing both the ligand binding sites and the Na^+ channel (Anholt, 1981). The integral nature of the nAChR and Na^+ channel greatly facilitated the study of functional aspects of the receptor complex. Some understanding of the structural basis of the receptor complex has been achieved by the application of molecular biological techniques. Cloning and sequencing of the cDNA for the four different subunits (Noda, Takahashi, Tanabe, Toyosata, Kikyotani, Furutani, Hirose, Takashima, Inayama, Miyata and Numa, 1983; Changeux, Devillers-Thiery and Chemoulli, 1984) revealed a high degree of homology (both of amino-acid sequence and hydropathicity profiles) between them, leading to the suggestion that they all arose from a common ancestral protein (Changeux, Giraudat and Dennis, 1987). Previous biophysical techniques - such as measurement of the Stokes radius

(Meunier, Olsen and Changeux, 1972), neutron scattering of the solubilised receptor complex (Wise, Schoenborn and Karlin, 1979) and electron microscopy (McCarthy, Earnest, Young, Chloe and Stroud, 1986; Changeux, Giraudat and Dennis, 1987) had resulted in a detailed model of the overall shape of the nAChR complex. The sequence homology between the four subunits led to the suggestion of a similar arrangement across the membrane for all subunits and several models of the tertiary and quaternary configuration of the nAChR complex with respect to the membrane have been put forward (Guy and Hucho, 1987).

Affinity labelling studies of isolated nAChRs or subunit cDNA expressed in oocytes (Gurdon, Lane and Woodland, 1971), had shown that the two α subunits of the complex were involved in ligand binding, the two sites apparently not being equivalent (McCarthy, Earnest, Young, Chloe and Stroud, 1986; Changeux, Giraudat and Dennis, 1987). As both α subunits were thought to be derived from a single gene, differential post-translational modifications (such as glycosylation) or an influence of neighbouring subunits was suggested as being responsible for these differences (McCarthy, Earnest, Young, Choe and Stroud, 1986; Changeux, Giraudat and Dennis, 1987). Site directed mutagenesis techniques (Mishina, Kurosaki, Tobimatsu, Marimoto, Noda et al., 1984) revealed components of the α helix essential for ligand binding, and others involved in mediating the agonist induced ion flux, possibly in forming the wall of the ion channel. Molecular biological techniques would appear to have contributed significantly to understanding certain aspects of the functional operation of the nAChR (Changeux, Giraudat and Dennis, 1987). In addition to this,

molecular biological techniques have revealed the existence of several different nAChR-subunit-like polypeptides. A novel ϵ subunit, thought to be involved in the functional alteration of nAChR during muscle development (Mishina, Takai, Imoto, Noda, Takahashi, Numa, Methfessel and Sakmann, 1986) was identified by cloning and sequencing cDNA from calf muscle (Takai, Noda, Mishina, Schimizu, Furutani, Kayano, Ikeda, Kubo et al. 1985). In neural tissue two putative α subunit species have been identified (Goldman, Deneris, Luyten, Kochhar, Patrick and Heineman, 1987). The implication of different nAChR complexes predominating at different developmental stages or in different tissues gives rise to the possibility of the development of selective drugs as well as contributing to the understanding of the function and mechanisms of nAChR under these different conditions.

Whilst the nAChR complex was the first, and probably the most extensively studied receptor to be investigated using molecular biological techniques, such procedures have recently been applied to many different membrane receptors and other proteins (Strosberg, 1987). This has led to the identification of different families of membrane proteins, exhibiting homologies of amino-acid sequence, hydropathicity, glycosylation sites and hypothesised topography. For example the bovine brain $GABA_A$ receptor complex (Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg and Barnard, 1987) and rat glycine receptor strychnine binding subunit (Grenningloh, Rienitz, Schmitt, Methfessel, Zensen, Beyreuther, Gundelfinger and Betz, 1987) have both been recognised to share features with the nAChR. These include the existence of four hydrophobic α helices in similar positions, similar postulated ion channel charge distribution and a

highly conserved Cys domain in the extracellular region (the presumptive ligand binding regions). The similarities are thought to be coincident with the related functions of the three receptors - that is, as ligand gated ion channels (Grenningloh, Rienitz, Schmitt, Methfessel, Zensen, Beyreuther, Gundelfinger and Betz, 1987; Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg, and Barnard, 1987). Similarly homologies can be seen with other ion channel-type proteins (for review see Unwin, 1986) such as the gap junction polypeptide (Paul, 1986; Kumar and Gilula, 1986), the voltage sensitive sodium channel (Noda, Shimizu, Tanabe, Takai, Kayano, et al., 1984),the potassium channel (Schwarz, Tempel, Papazian, Jan and Jan, 1988) and the postulated voltage-sensitive calcium channel (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose and Numa, 1987).

Membrane receptors not associated with ion channels have also been recognised as belonging to families by virtue of their sequence homologies and functional similarities. One such family is that of the growth-stimulating peptide receptors, all of which appear to be substrates for tyrosine specific protein kinase (Ullrich, Coussens, Hayflick, Dull, Gray, Tam, Lee, Yarden et al., 1984; Ullrich, Bell Chen, Herrera, Petruzzelli, Dull, Gray, Coussens et al., 1985; Caoussens, Van Beveren, Smith, Chen, Mitchell, Isacke, Verma and Ullrich, 1986; Yarden, Escobedo, Kuang, Yang-Feng, Daniel, Tremble, Chen et al., 1986). Another group of membrane receptor proteins for which functional similarities appear to be reflected in sequence and probable structural homologies are those known to interact with G-proteins. Indeed, it is this superfamily of receptors acting

through a G-protein mediated production of diffusible second messengers to which the LHRH receptor is likely to belong (Conn, McArdle, Andrews and Huckle, 1987; Chapter 3). Examples of such receptors that have been sequenced include rhodopsin (Ovichinnikov, 1982), β_1-adrenergic (Frielle, Collins, Daniel, Caron and Lefkowitz, 1987), β_2-adrenergic (Dixon, Kobilka, Strader, Behovic, Dohlman, Frielle, Volanowski et al., 1986), and muscarinic cholinergic receptors M_1 (Kubo, Fukuda, Mikami, Maeda, Takahashi, Mishina, Haga, Haga et al., 1986; Bonner, Buckley, Young and Brann, 1987), M_2 (Kubo, Maeda, Sugimoto, Akiba, Mikamim Takahashi, Haga, Haga et al., 1986; Peralta, Winslow, Peterson, Smith, Ashkenazi, Ramachandran, Schimerlik and Capon, 1987; Gocayne, Robinson, Fitzgerald, Chung, Kerlavage, Lentes, Lai, Wang, Fraser and Venter, 1988) and two pharmacologically uncharacterised muscarinic receptors, m_3 and m_4 (Bonner, Buckley, Young and Brann, 1987). In all cases the receptors are members of families of related proteins, non-identical in either pharmacological profile or type of second messenger activated (Hall, 1987). However, high sequence identity (20-30%) exists between the three families (that is the rhodopsin, β adrenergic and muscarinic receptor protein families) which also exhibit very similar hydropathicity profiles. Two other G-protein associated receptors recently cloned and shown to exhibit sequence and hydropathicity homologies with the three above families are the $5HT_{1C}$ receptor (Julius, McDermott, Axel and Jessell, 1988) and the substance K receptor (Masu, Nakayama, Tamaki, Harada, Kuno and Nakanishi, 1987). This has resulted in the suggestion that these G-protein linked receptors all exhibit a similar transmembrane topography with seven membrane spanning hydrophobic sequences (Hall, 1987; Kerlavage, Fraser and Venter, 1987). Homologous glycosylation

sites and putative positions of the ligand binding site of the different receptors types have also been suggested (Hall, 1987; Kerlavage, Fraser and Venter, 1987). The coincidence of sequence and functional similarities (that is the interaction of the proteins with G-protein of one type or another) might suggest that the G-protein binding site is also conserved in all such receptors. One suggestion is that the cytoplasmic loop between hydrophobic segments I and II (a highly conserved region in all known sequences for G-protein-linked receptors) is a likely site for the G-protein interaction (Hall, 1987). Deletion experiments on the hamster β adrenergic receptor amino acid sequence suggest that the cytoplasmic loop from segment V to VI may be involved in the receptor interaction with G-proteins (Dixon, Sigal, Rands, Register, Candelore, Blake and Strader, 1987). The idea of a conserved site of interaction is further supported by the finding that all known G-proteins, although capable of interacting with different second messenger systems, (Stryler and Bourne, 1986) have a topologically conserved amphipathic α helix at their carboxy-terminal thought to be involved in their interaction with receptor proteins (Sullivan, Miller, Masters, Beiderman, Heideman and Bourne, 1987).

One obvious conclusion that can be drawn from these findings is that the function of a protein is closely related to its primary structure. Knowledge of the primary structure of a membrane receptor may therefore provide some insight into its function. Even when its mechanism of action is not known and its primary structure shows no homologies with other known receptor proteins - as in the case of the growth hormone receptor (Leung, Spencer, Cachianes, Hammonds, Collins, Henzel, Barnard, Waters and Wood, 1987) - the application of other molecular biological techniques (such as site

directed mutagenesis) may be fruitful for its elucidation. Knowledge of the mechanism of action of a receptor may in turn be used to isolate the cDNA encoding for it. For example the screening of the human genome with a full length β_2 adrenergic probe (under conditions of low stringency) revealed a putative protein, differentially expressed in several tissues, with the structural features of a G-protein coupled receptor (Kobilka, Frielle, Collins, Yang-Feng, Kobilka, Francke, Lefkowitz and Caron, 1987). A similar technique has been used to identify possible cDNA clones of proteins related to steroid receptors (Giguera, Yang, Segui and Evans, 1988). So, in these ways, molecular biological techniques can be used not only to directly determine primary sequences but also to help in the elucidation of the function and tertiary structure of a receptor protein (as in the case of the nAChR) and even in the identification of other receptors utilising similar mechanisms.

1.4 Cellular signalling by LHRH as a clue to its possible receptor structure.

The mechanism of action of the LHRH receptor has not yet been fully elucidated. Whilst a role for calcium in LHRH-induced gonadotrophin secretion appears to have been established (Conn, McArdle, Andrews and Huckle, 1987), an increase in intracellular calcium levels seems to be a virtually ubiquitous signal for exocytosis in many cell types (Baker and Knight, 1986) and may not necessarily be a very direct consequence of LHRH receptor activation. Nevertheless, recent results (see Chapter 3) suggest that this increase in intracellular calcium, in response to LHRH, may be derived from non-mitochondrial intracellular stores. In other cell types where agonist binding results in the release of calcium from such non-mitochondrial intracellular stores, inositol 1,4,5-trisphosphate has been implicated as the second messenger (Berridge, 1984; Irvine, 1986).

Inositol 1,4,5-trisphosphate has been shown to act in a specific and concentration-dependent manner over a μM range - which is considered capable of being reached physiologically (Streb, Irvine, Berridge and Schulz, 1983; Joseph, Thomas, Williams, Irvine and Williamson, 1984). It is thought to be derived from membrane inositol phospholipids; phosphatidyl inositol and the 4-phosphate and 1,4-bisphosphate derivatives, by an enzymic hydrolysis that is regulated by receptor:G-protein interaction (see reviews, Michell, 1975; Hawthorne, 1983; Berridge, 1984; Putney, 1987). A membrane bound phospholipase C, specific for the polyphosphatidyl inositols, inactive under normal cell conditions but activated by a G-protein at unstimulated intracellular calcium concentrations has been

described (Cockcroft, 1987). The concomitant production of diacylglycerol by the action of this enzyme may also subserve a regulatory role in cellular signalling by means of its activation of protein kinase C (Nishizuka, 1984).

In support of such a mechanism for the LHRH receptor, rapid, agonist-specific, concentration dependent stimulation of inositol phospholipid hydrolysis has been shown in response to LHRH (Schrey, 1985; Andrews and Conn, 1986; Huckle and Conn, 1987). This effect has been found to be largely independent of calcium ion concentration (Andrews and Conn, 1986; Huckle and Conn, 1987). Experiments using ^{32}P labelling of membrane phospholipids show that LHRH increases the labelling of phosphatidyl inositols whereas labelling of other phospholipids is unchanged (Naor, Molcho, Zakut and Yavin, 1985). Analysis of the inositol phosphates produced as a result of LHRH stimulation of pituitary cells has confirmed the increased production of inositol 1,4,5 trisphosphate (Morgan, Chang and Catt, 1987). The implication from all this evidence is that the LHRH receptor belongs to the same superfamily as the muscarinic cholinergic, β adrenergic and rhodopsin families, $5HT_{1C}$ and substance K receptors (see 1.3). That is, receptor proteins that interact with G-proteins on activation by an agonist. Consistent with this idea is recent evidence that introduction of GTP analogues into permeabilised gonadotrophes can facilitate LHRH-induced LH secretion (Andrews, Staley, Huckle and Conn, 1986).

1.5 Strategy of this study

In order to investigate further the transducing mechanisms of the LHRH receptor-including the possible involvement of a G-protein, experiments were carried out to assess any (previously undescribed) influence of guanine nucleotides on ligand binding to the LHRH receptor. Together with extensive pharmacological characterisation of the native binding site in pituitary membranes for LHRH analogues, these experiments are described in Chapter 3.

The success of molecular biological techniques in helping to determine functional and topological aspects of other membrane receptors (1.3) suggests that this would be a fruitful approach to understanding the structure and function of the LHRH receptor. One of the most commonly used strategies for molecular biological characterisation of membrane proteins involves their solubilisation with detergents. Purification and isolation of the solubilised protein can be followed by partial protein sequencing to produce oligonucleotide probes (Strosberg, 1987) for the screening of cDNA libraries for long stretches of receptor sequence. This approach was adopted in these studies of the LHRH receptor.

In Chapter 4, various detergents were investigated in order to optimise conditions for the solubilisation of the rat anterior pituitary LHRH receptor. The high yielding non-denaturing solubilisation enabled several biochemical and pharmacological properties of the solubilised receptor to be determined (Chapter 5). In order to overcome the inhibition of specific LHRH analogue binding in the presence of solubilisation solution, an affinity labelling strategy to incorporate a biotin moiety onto the LHRH receptor was developed (Chapter 6). This approach allows for

subsequent purification and isolation of the LHRH receptor protein utilising a biotin-avidin affinity column.

Biochemical techniques have been used both to further the understanding of the way in which the LHRH receptor functions in situ and reveal aspects of the detergent-solubilised receptor. Although the strategy of receptor solubilisation and isolation did not provide sequencable amounts of the LHRH receptor (due in part to the low tissue concentration of the LHRH receptor), the results obtained should facilitate future attempts to isolate and sequence the LHRH receptor protein.

Chapter 2

Methods

2.1. Animals

Animals used for the majority of experiments were male Wistar rats (200-250g) supplied by Edinburgh University (Department of Occupational Medicine) or purchased from Charles River, UK Ltd (Margate, Kent). They were maintained under controlled lighting (lights on 05.00-19.00h) and temperature (22°C) and had free access to tap water and diet 41B (Oxoid, Basingstoke, Hants).

Bovine pituitaries (obtained from slaughter house steers) were purchased from Imperial Labs (Europe) Ltd (Andover, Hampshire). These had been plunged into liquid nitrogen immediately after dissection and shipped on dry ice. On arrival they were stored at -20°C. The porcine pituitaries were generously supplied by Dr R Jones (Department of Pharmacology, Edinburgh University), from farmyard reared young boars.

2.2. Dissection of tissues

Rats were stunned, decapitated and their brains removed. The pituitary gland was then removed from the base of the skull and the neurointermediate lobe separated from the anterior pituitary with fine forceps. The hippocampus was dissected from the posterior portion of the brain after making a midline sagittal section. The bovine pituitaries were dissected into anterior and neurointermediate lobes using blunt dissection, after removal of the surrounding connective tissue. The porcine pituitaries whose morphology were similar to that of the bovine pituitaries (Cummings and Habel, 1965) were dealt with in a similar manner.

2.3. Peptide Iodination

Iodination was carried out by the chloramine-T method (Hunter

and Greenwood, 1962) as described for LHRH (Niemann and Sandow, 1973) and buserelin ([D-Ser(Bu^t)^6, des Gly^{10}]-LHRH ethylamide; Sandow and Konig, 1979) with modifications. The procedure is detailed in Appendix 1. Briefly 37 MBq Na ^{125}I was added to 5µg of peptide in a 0.5M phosphate buffer pH 7.5. On addition of chloramine-T the $Na^{125}I$ is oxidised and the radioiodine incorporated into the tyrosyl residue (Hunter and Greenwood, 1962). After 20 secs the remaining chloramine-T was then reduced by the addition of excess sodium metabisulphite. In the case of LHRH the reaction yield is > 90% (Niemann and Sandow, 1973), the ratio of di-iodo- to mono-iodo-LHRH increasing with the degree of iodination. Chromatography on a Sephadex G-25 fine (Pharmacia Fine Chemicals, Milton Keynes, Bucks) column separated free ^{125}I from the iodinated peptide and the mono-iodo- from the di-iodo-peptide (as shown by Niemann and Sandow, 1973). The free ^{125}I was initially eluted using 0.01M acetic acid and the iodinated peptide with 3% BSA/0.01M acetic acid in the case of ^{125}I-buserelin, 6% BSA/0.01M acetic acid for the other LHRH analogues. For use in the crosslinking reactions ^{125}I-XBAL was separated from free ^{125}I using HPLC, as detailed in Appendix 1, the ^{125}I-XBAL eluting in 60% MeOH/0.2% TFA.

2.4. LHRH-Receptor Binding

LHRH exerts its gonadotrophin (LH and FSH)-releasing and regulatory actions on gonadotrophes via interaction with a membrane bound receptor (Vale, Rivier, Brown, Leppaluoto, Ling, Monahan and Rivier, 1976; Hopkins and Gregory, 1977). Whilst initial studies using radiolabelled native LHRH suggested the existence of two

populations of receptors, one of high affinity but low capacity and one of low affinity and high capacity, further experiments using degradation-resistant analogues of LHRH labelled to a high specific activity with ^{125}I confirmed the existence of a single class of specific high affinity LHRH receptors (reviews - Clayton and Catt, 1981; Conn, Marian, McMillian, Stern, Rogers, Hamby, Penna and Grant, 1981; Chapter 3). In the majority of the ligand binding experiments in this thesis the radiolabelled analogue used was the LHRH superagonist buserelin, generously donated by Hoechst AG (Frankfurt, FRG).

2.4.1. Rat Equilibrium Binding Assay

The method is outlined in Fig.2.1.

2.4.1.1. Membrane preparation

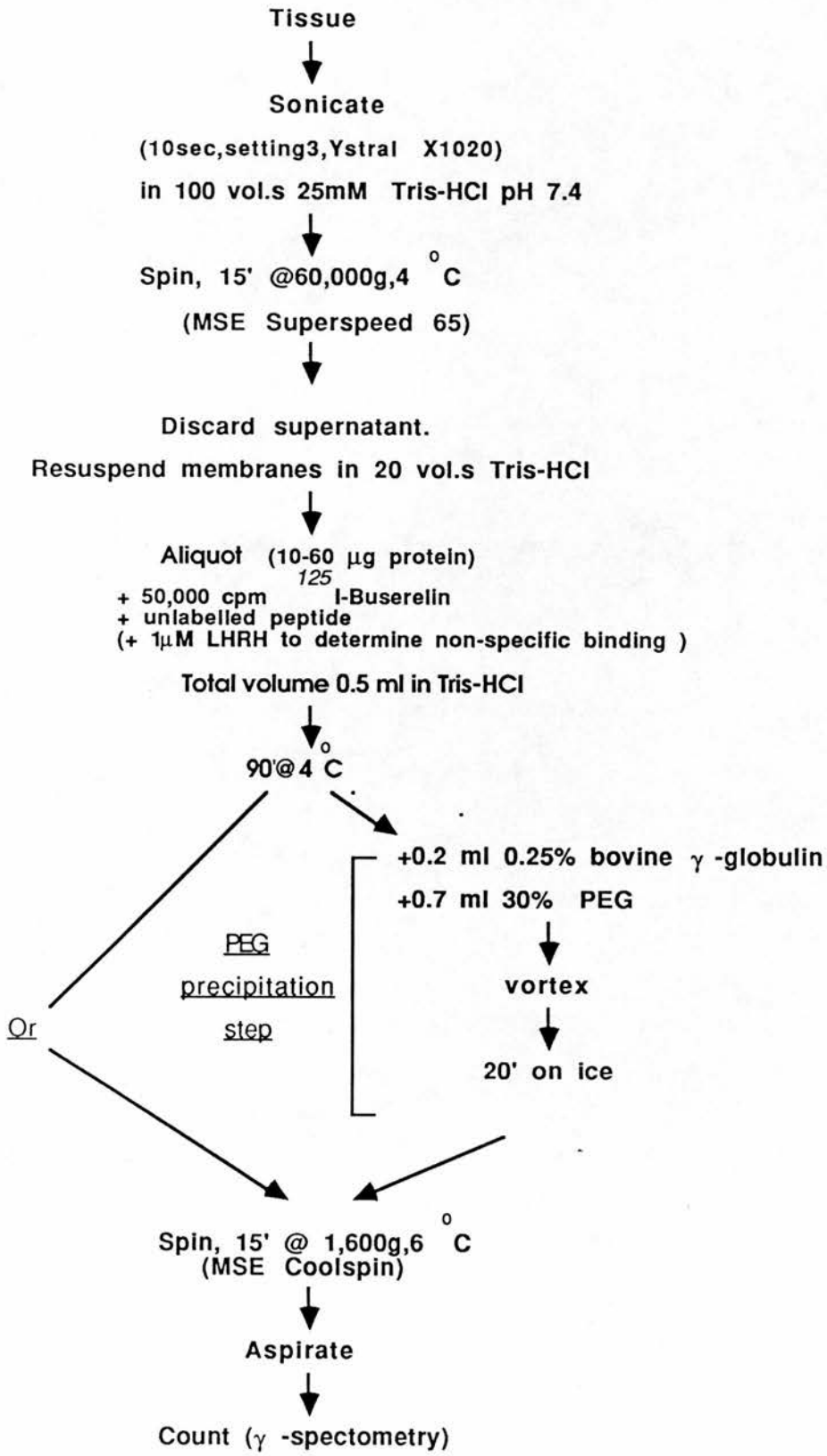
Dissected anterior pituitaries (or hippocampus) were placed in 100 volumes ice-cold Tris-HCl (25mM, pH 7.4) and homogenised using a high frequency homogeniser (Ystral X1020) for 5 seconds at setting 3 followed by centrifugation at 60,000g for 15 minutes at 4°C (MSE Superspeed 65). The supernatant was then discarded and the washed membranes resuspended into 20 volumes of Tris-HCl (25mM, pH 7.4)/0.1% BSA (Tris/BSA).

2.4.1.2. Ligand binding

Assays were usually carried out in triplicate, in a volume of 500µl containing 10-60 µg protein, using either a membrane or solubilised preparation. Iodinated analogue diluted in Tris/BSA was added to give ~50,000cpm per tube and unlabelled LHRH analogue was present in the appropriate concentrations. In all cases non-specific binding was determined in the presence of 1 µM LHRH. After incubation for 90 minutes at 4°C, equilibrium had been reached (Fig.2.2). The

Figure 2.1

Ligand Binding Assay Method



bound radioiodinated ligand was then separated from the free by centrifugation at 1,600g, 6°C for 20 minutes (MSE Coolspin) followed by careful aspiration of the supernatant, or by a polyethylene glycol 8000 (PEG) precipitation (see below). The resulting pellet was counted by γ-spectrometry (Berthold Mag 310 Gamma counter) to determine the amount of ^{125}I-LHRH analogue bound.

A linear relationship was found between the amount of tissue used in the binding assay and the specific binding seen (Fig.2.3). In all cases the total binding was 20.8 ± 1.2% (± SEM, n = 8) of the total radioligand added, and non-specific binding 28 ± 3% (± SEM, n = 8) of total binding, similar to values previously reported for iodinated LHRH analogues (Marshall and Odell, 1975). Specific activity of the ^{125}I-buserelin was determined by self-displacement (Clayton and Catt, 1980; Millar, Garritsen and Hazum, 1982; Loumaye, Wynn, Coy and Catt, 1984) to be approximately 40,700 GBq (1100 Ci/mmol).

2.4.1.3. PEG precipitation

To ensure consistent precipitation of the membrane preparation and the solubilised preparation (2.6.) a PEG precipitation step was included. PEG was found by Polson, Potgieter, Largier, Mears and Joubert (1964) to be the most suitable of a number of linear polymers tested for protein precipitation. It has been shown to have little tendency to denature, or otherwise interact with proteins (Ingham, 1984). The rapid precipitation is thought to be a result of the proteins becoming concentrated in the extrapolymer space until their solubility limit is exceeded. This effect is relatively insensitive to temperature, pH or ionic strength and dependent both on the proteins' molecular weight and solubility

Figure 2.2

^{125}I-Buserelin binding to rat anterior pituitary gland tissue. Each point is the mean ± SEM for assays carried out in triplicate. 50,000cpm ^{125}I-buserelin was added to each tube, non-specific binding was determined in the presence of 1μM LHRH, in a total volume of 500μl 25mM Tris-HCl, pH7.4/0.1% BSA. Free radioligand was separated from that bound to the tissue preparation at various time intervals by a PEG precipitation step.

a) Binding to membrane preparation. Tissue equivalent to one eighth of an anterior pituitary gland was present in each assay tube.

b) Binding to membrane preparation solubilised with 5mM CHAPS, 1.5M NaCl (shaken in 30 vols for 90' at 4°C, then centrifuged 2 hrs at 60,000g, 4°C). Aliquots of the supernatant (equivalent to one sixth of an anterior pituitary gland) were used in each assay tube. 0.25mM CHAPS, 75mM NaCl were present in the assay mixture.

Figure 2.3

Relationship between amount of tissue (membrane preparation) and specific ^{125}I-buserelin binding detected. Each point is the mean value from four determinations. Assays were carried out as described (2.4.1.2) in a total volume of 500µl 25mM Tris-HCl pH7.4/0.1% BSA with 50,000cpm ^{125}I-buserelin; non-specific binding was determined in the presence of 1µM LHRH. After incubation for 90' at 4°C samples were subjected to a PEG precipitation to separate free ^{125}I-buserelin from that bound.

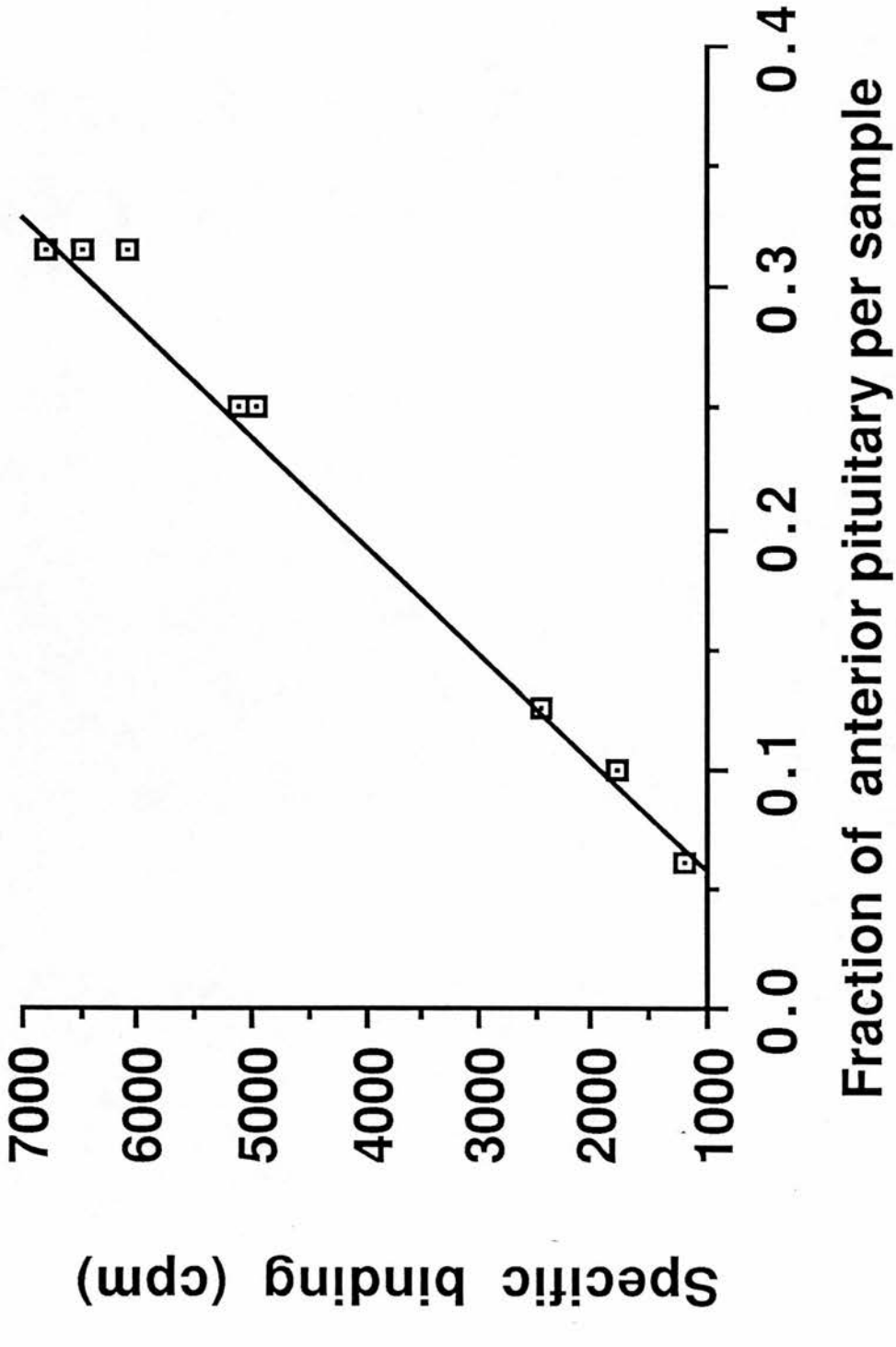

Specific binding (cpm)
7000
6000
5000
4000
3000
2000
1000
0.0
0.1
0.2
0.3
0.4
Fraction of anterior pituitary per sample

Figure 2.3

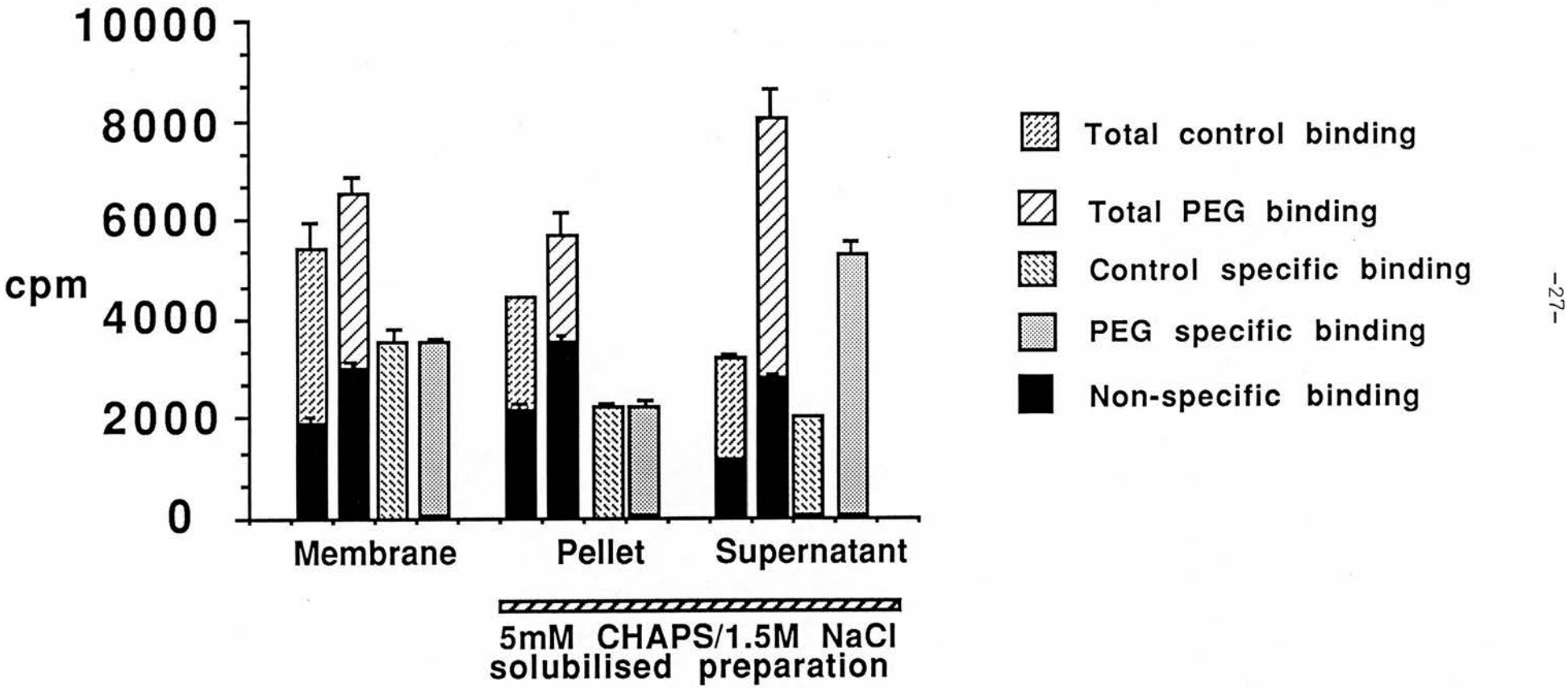
(Ingham, 1984). Small soluble peptides (such as free LHRH analogues) would therefore be expected to have little or no tendency to precipitate under conditions where membrane proteins would. PEG precipitation therefore represented a simple, reproducible and quick method of protein precipitation, allowing both for separation of bound and free radioligand and also the solubilised preparation from the detergent solution. Other methods of protein precipitation (ammonium sulphate and trichloroacetic acid, Ratcliffe, 1974) tend to denature proteins, therefore subsequent binding assay of the precipitated protein may not have been possible. Separation of bound from free ligand by charcoal absorption would not be of similar use for removal of the detergent solution prior to binding assay, and is said to be adversely affected by high ionic strength (Ratcliffe, 1974). These two factors suggest it to be less suitable than the PEG precipitation technique. Another method that has been used to separate free and bound radiolabel is gel chromatography, but its relatively low sample capacity and therefore unsuitability for large numbers of samples (Ratcliffe, 1974) again suggests PEG precipitation to have advantages over it here.

Using PEG, effective precipitation of LHRH binding sites has been shown without any change in their binding characteristics (Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert, 1983;Hazum, 1983; Perrin, Haas, Rivier and Vale, 1983). Briefly PEG was added here to a final concentration of 15% and bovine γ-globulin to 0.035%, both in Tris-HCl (25mM, pH 7.4), to give a total volume of 1.5ml. This was left on ice for 20 minutes and then centrifuged for 15 minutes at 1,600g, 6°C. After careful aspiration the resulting pellet of membrane protein with bound radioligand was counted by γ-spectrometry.

Figure 2.4

^{125}I-Buserelin binding to membrane and solubilised preparations both with and without the inclusion of a PEG precipitation step before the ligand binding assay. All values are shown as mean ± SEM of three determinations. Ligand binding assay was carried out as described (2.4.1.2) with 50,000cpm ^{125}I-buserelin in a total volume of 500µl 25mM Tris-HCl pH7.4/0.1% BSA and 50µg/ml soybean trypsin inhibitor, 400KIU/ml aprotinin, non-specific binding being determined in the presence of 1µM LHRH. After reaching binding equilibrium (90' at 4°C), samples were subjected to a PEG precipitation step to separate free ^{125}I-buserelin from the tissue preparation. Tissue equivalent to one eighth of an anterior pituitary gland was used in the membrane binding experiments, tissue equivalent to one quarter of an anterior pituitary gland for the solubilised preparation. Membranes were solubilised in 20 vols of the 5mM CHAPS, 1.5M NaCl solubilisation solution (agitated for 90' at 4°C, centrifuged for 2hrs at 60,000g, 4°C) and both pellet and supernatant (either with or without an initial PEG precipitation step) were used in the binding assays.

Figure 2.4


10000
8000
6000
cpm
4000
2000
0
Membrane
Pellet
Supernatant
5mM CHAPS/1.5M NaCl
solubilised preparation
Total control binding
Total PEG binding
Control specific binding
PEG specific binding
Non-specific binding

Initial experiments in which membrane preparations were used in ligand binding experiment both before and after a PEG precipitation step suggested that no change in binding of LHRH-analogues resulted from inclusion of this technique (Fig.2.4). Similar results were obtained using the pellet obtained after solubilisation of the membrane preparation with 5mM (3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate)/CHAPS/1.5M NaCl, see Chapter 4 (Fig.2.4).

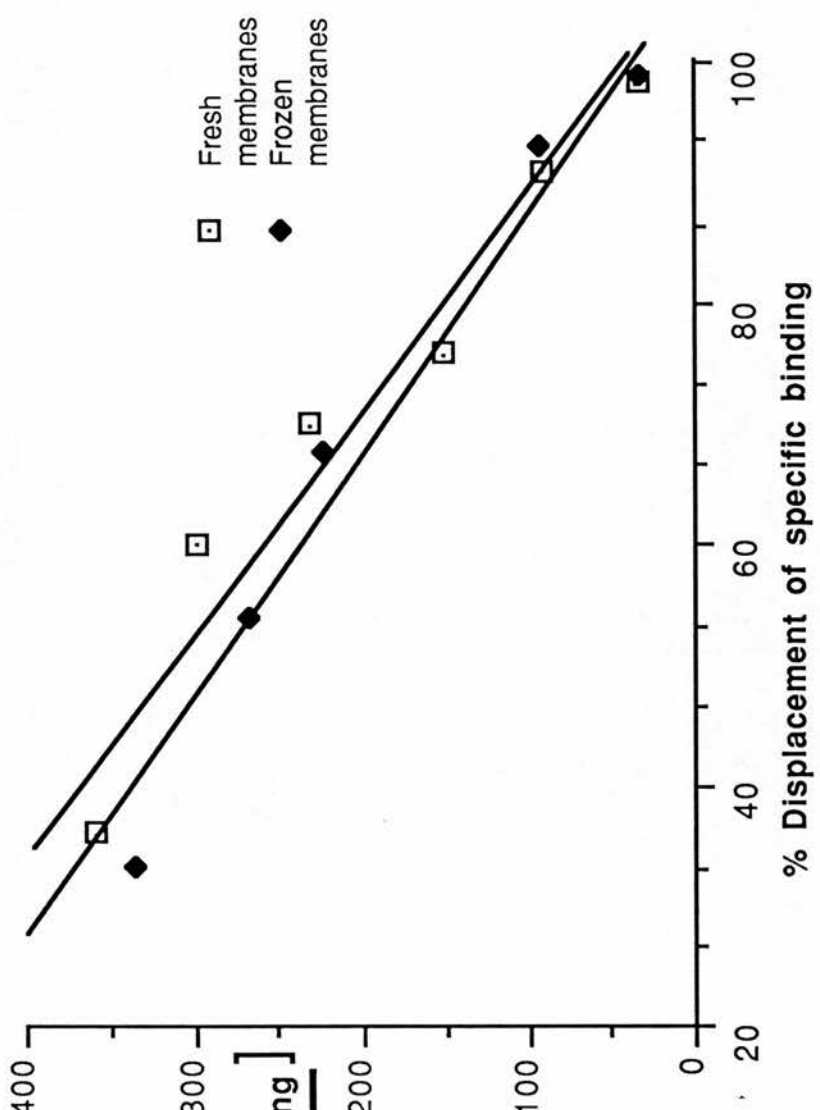
2.4.1.4. Use of fresh rat anterior pituitary tissue versus frozen, for ligand binding assays and solubilisation

Initial experiments comparing the displacement of ^{125}I-buserelin by cold buserelin from freshly prepared rat anterior pituitary membranes and membranes that had been frozen overnight at -20°C showed there was no difference in the binding characteristics of the two preparations (Fig.2.5). Hofstee analysis revealed a binding constant (K_i) of 0.31nM in both cases (single experiment, triplicate samples). In initial experiments on LHRH receptor solubilisation (Chapter 4), fresh and frozen membrane preparations did not give similar results. Whilst fresh membrane preparations treated with 20 volumes of 5mM CHAPS/1.5M NaCl solution as described (4.2.2.1) resulted in 56% of LHRH binding sites being found in the supernatant, only 34% were solubilised from frozen tissue using similar conditions (single experiment, triplicate samples). Freezing the tissue had also resulted in an apparent overall 33% loss of LHRH binding sites (as determined by combining binding of ^{125}I-buserelin seen in both the pellet and supernatant of the solubilised preparation). It was therefore decided that fresh rat anterior pituitary preparations should be used in all experiments, in case the reduction in LHRH binding sites and amount

Figure 2.5

Comparison of ^{125}I-buserelin binding to anterior pituitary gland membranes prepared from fresh or frozen tissue. Ligand binding assay was carried out as previously described (2.4.1.2) in a total volume of 500µl 25mM Tris-HCl pH7.4/0.1% BSA with 50,000cpm ^{125}I-buserelin and various concentrations of unlabelled buserelin. Non-specific binding was determined in the presence of 1µM LHRH. Tissue equivalent to one eighth of an anterior pituitary gland was used in each sample. Points are means of triplicate samples. Data was analysed according to the Eadie-Hofstee method using an error weighted programme (see 2.5). A best-fitted K_i of approximately 0.31nM was found for both preparations.

Figure 2.5


Fresh membranes
Frozen membranes
% Displacement of ^{125}I-Buserelin specific binding
[Buserelin added (nM)]
% Displacement of specific binding
400
300
200
100
0
20
40
60
80
100

solubilised reflected an adverse affect of freezing on the LHRH receptor.

2.4.2. Bovine Equilibrium Binding Assay

2.4.2.1. Membrane preparation

Bovine anterior pituitaries were weighed and homogenised in 100 volumes of Tris-HCl (25mM, pH 7.4) with 10 strokes in a glass-teflon homogeniser. The homogenised tissue preparation was carefully decanted to remove any remaining pieces of connective tissue. Following centrifugation (as for the rat tissue, 15 minutes at 60,000g, 4°C) the anterior pituitary gland membranes were resuspended in to 20 volumes Tris/BSA containing 50µg/ml soybean trypsin inhibitor and 400 KIU/ml aprotinin (Tris/BSA.peptidase inhibitors).

2.4.2.2. Binding assay

Assays were carried out in triplicate in a total volume of 500µl containing 50µl of the washed membrane preparation. Unlabelled analogue and 50,000cpm of radio-iodinated analogue, both in Tris/BSA/peptidase inhibitors, were added as appropriate. Non-specific binding was determined in the presence of 1µM LHRH. Equilibrium was reached after incubation at 4°C for 16 hrs, an incubation period considered to be optimal in earlier studies by Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert (1983) (Fig.2.6). After this, free radioligand was separated from the membrane preparation by centrifugation, 20 minutes at 1,600g, 6°C. Bound radioligand was counted by γ-spectrometry. Of the radiolabelled buserelin added, 12 ± 3% (± SEM, n = 6) was bound, 28 ± 3% (± SEM, n = 6) of this total binding being specific.

Figure 2.6

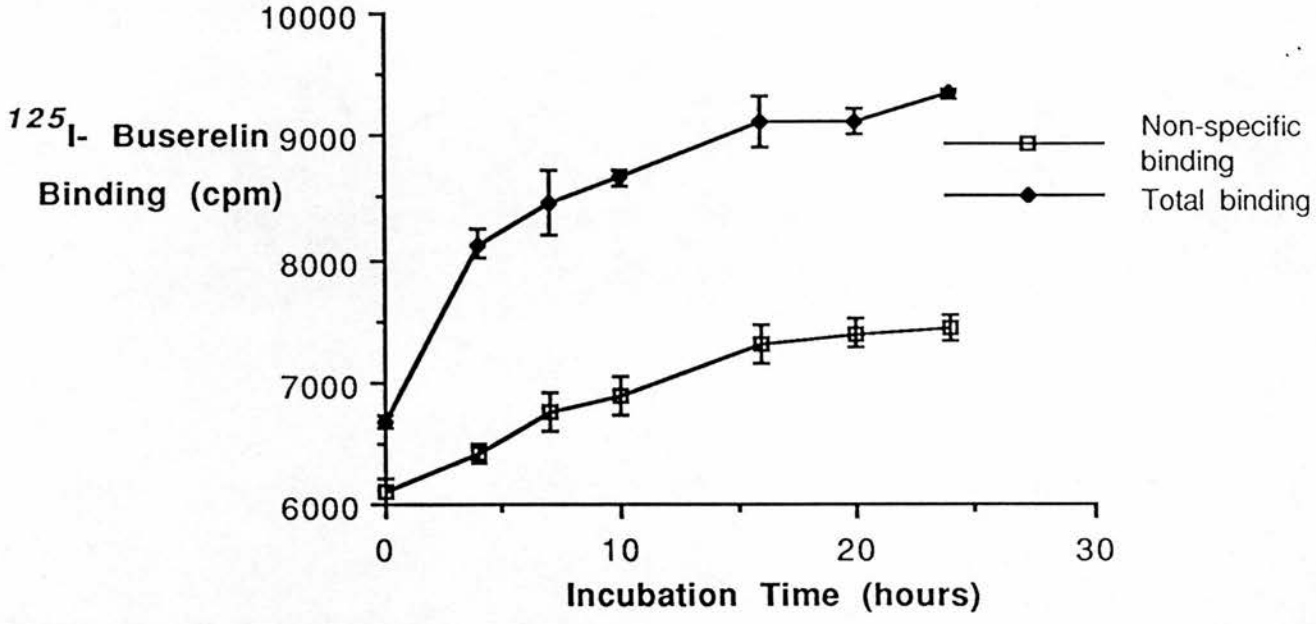


Figure 2.6

^{125}I-Buserelin binding to bovine anterior pituitary gland membranes. Assays were carried out as described (2.4.2) using ~2.5mg of tissue per sample, 50,000cpm ^{125}I-buserelin in a total volume of 500µl 25mM Tris-HCl pH7.4/0.1% BSA/50µg/ml soybean trypsin inhibitor/400KIU/ml aprotinin. Non-specific binding was determined in the presence of 1µM LHRH. Points are mean ± SEM of four determinations. At various time intervals free ^{125}I-buserelin was separated from that bound to the membrane sample by centrifugation (15' at 1,600g, 9°C).

2.5. Analysis of Binding Data

Displacement data were analysed according to the Eadie-Hofstee method (Eadie, 1942; Hofstee, 1952). The saturation data were analysed after graphical transformation according to Scatchard (1949) using an error-weighted programme (Zivin and Waud, 1982) on a Texas TI58C calculator or by linear regression using a Casio fx-5200P calculator.

2.6. Affinity Chromatography

Once membrane constituents have successfully been solubilised a method of protein purification is required. Ideally this method should be based on a property that is specific to the protein of interest - in this case the LHRH receptor. The usual method of choice for membrane protein purification is affinity chromatography (Strosberg, 1984). Ligands specific for the desired protein are covalently linked to inert supports - such as agarose or sephadex. On passing a solubilised membrane preparation down a column of the ligand-gel complex, specific interaction between the ligand and membrane protein result it in being retained whilst other membrane constituents are washed out. Addition of excess free ligand to the gel will in turn elute the membrane protein.

The first use of this technique was for the separation of antibodies from other serum constituents using a cellulose-antigen complex (Lerman, 1953). Basically unchanged, except for the development of improved inert gels (Cuatrecasas, Wilchek and Anfinsen, 1968), the method has been used for the purification of many different proteins and other molecules exhibiting specific and selective binding (Wilchek, Miron and Kohn, 1984).

Essential to the technique is the retention of specific binding under the conditions used. For solubilised proteins these conditions should be such that the protein is retained in solution. In the case of the LHRH receptor, extensive surveying of detergent conditions (Chapter 4) showed the two requirements apparently to be incompatible for high yielding solubilisation conditions. An alternative approach was therefore needed. Hofmann and Finn (1985) and Kohanski and Lane (1985) described a method using the biotin-streptavidin interaction for affinity chromatography of insulin receptors bound with biotin-containing analogues of insulin. Using LHRH analogues incorporating a biotin molecule and crosslinking the analogue to the receptor site, the method could be adapted for the LHRH receptor protein. Solubilised LHRH receptors could then be purified using the biotin-streptavidin interaction down a biotin affinity column.

2.6.1. Biotin-Avidin affinity column matrix and characterisation of binding both in the absence and presence of 5mM CHAPS/1.5M NaCl

Affinity columns were constructed using streptavidin-agarose (streptavidin attached via a 6 carbon spacer to 4% cross-linked beaded agarose; Sigma Chemical Co. Ltd., Poole, Dorset) or avidin-Sepharose CL-4B.

The method used for coupling the avidin to the Sepharose CL-4B was that of Beaty and Lane (1982) and is detailed in Appendix V. Briefly, the Sepharose CL-4B was activated by cyanogen bromide (March, Parikh and Cuatrecasas, 1974), washed with 0.1M $NaHCO_3$ pH 8.5, water and 0.1M Na/PO_4 buffer, pH 7.0 and then mixed with avidin for 24 hours. After this time, uncoupled avidin was removed by washing and unreacted sites were blocked using 0.1M 2-amino-ethanol.

Binding of [^{3}H]-biotin to the avidin-Sepharose CL-4B was studied both in the presence and absence of the detergent solution containing 5mM CHAPS/1.5M NaCl in order to determine the effect of the detergent solution on the biotin-avidin interaction. [^{3}H]-Biotin (10^{-8}M, 155 GBq/mmol purchased from Amersham International plc, Bucks) and 50µl of the avidin-Sepharose CL-4B preparation in a total volume of 500µl of 0.01M Na/PO_4 pH 7.0, 0.1% BSA (or 5mM CHAPS/1.5M NaCl in Tris-HCl 25mM pH 7.4) with various concentrations of unlabelled biotin, were incubated for 60 minutes at 4°C (Kohanski and Lane, 1985(b)). Non-specific binding was determined in the presence of 0.1mM biotin. Following a wash step (by centrifugation 1,600g, 10 minutes at 6°C, and resuspension in fresh buffer) the [^{3}H]-biotin bound to the pelleted avidin-Sepharose CL-4B was determined by liquid scintillation counting in 10mls NE-265 (Nuclear Enterprises, Edinburgh).

Similarly, the displacement of [^{3}H]-biotin by the biotinylated LHRH analogue PBL* (2.9) in the CHAPS/NaCl solution was determined.

Displacement of [^{3}H]-biotin by unlabelled biotin from the avidin-Sepharose CL-4B revealed an IC_{50} value of 7µM in the Na/PO_4 buffer and 3µM in the CHAPS/NaCl solution (Fig. 2.7). These results are of a similar order to those found by Kohanski and Lane, (1985(b)) of a K_D = 0.2µM. The biotinylated LHRH analogue, PBL, displaced [^{3}H]-biotin from the avidin-Sepharose CL-4B in the presence of CHAPS/NaCl with an IC_{50} of 11µM. These results suggest that the binding of biotin to avidin-Sepharose CL-4B is of an affinity suitable for use in affinity chromotography (Kohanski and Lane, 1985(b)), is not affected by the high NaCl/detergent solution used in LHRH binding site solubilisation (Chapter 4) and is

* [D Lys^6 [N$^{\varepsilon}$-biot Phe(N_3)]]-LHRH

Figure 2.7

Binding of biotin and PBL to the avidin-Sepharose CL-4B. Assays were carried out as described (2.6.1) using 50µl of the avidin-Sepharose CL-4B preparation, 10^{-8}M ^{3}H-biotin and various concentrations of unlabelled biotin or PBL in a total volume of 500µl. Non-specific binding was determined in the presence of 0.1mM biotin. After 60' at 4°C samples were washed (centrifuged for 10' at 1,600g 6°C, resuspended in 500µl fresh buffer, recentrifuged) and the bound ^{3}H-biotin determined by liquid scintillation.

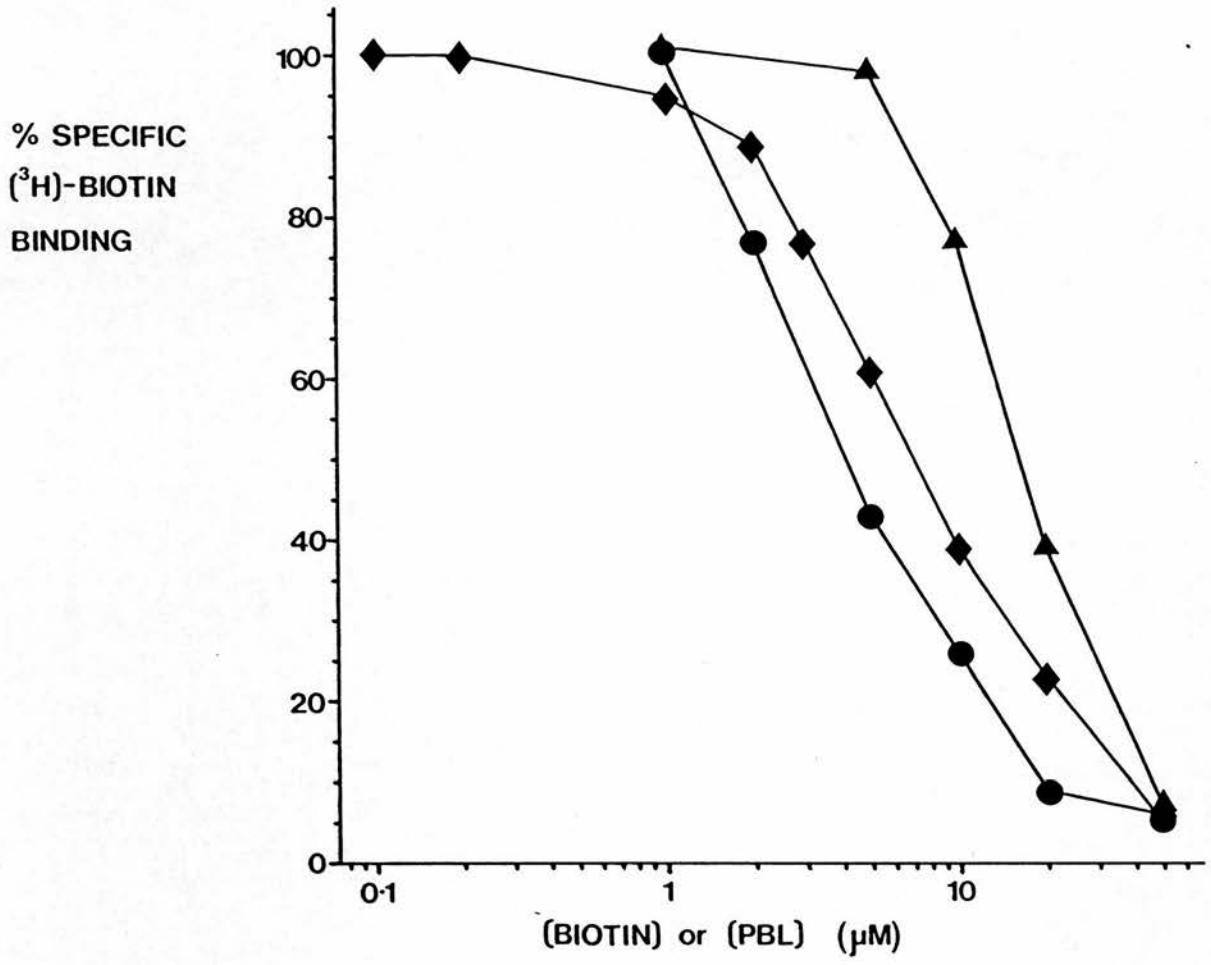
(●) Biotin binding in 0.01M Na/PO_4 pH7/0.1% BSA. IC_{50} 7µM.

(◆) Biotin binding in 5mM CHAPS, 1.5M NaCl/25mM Tris-HCl pH7.4. IC_{50} 3µM.

(▲) PBL binding in 5mM CHAPS, 1.5M NaCl/25mM Tris-HCl pH7.4. IC_{50} 11µM.

Points are means of triplicate samples.

Figure 2.7


% SPECIFIC
(3H)-BIOTIN
BINDING
100
80
60
40
20
0
0·1
1
10
(BIOTIN) or (PBL) (μM)

not adversely affected by covalent attachment to an LHRH analogue.

2.6.2. Method of affinity chromatography

The method is outlined in Fig.2.8.

Anterior pituitary membranes covalently labelled (6.2.2) with a radioiodinated, biotin-containing LHRH analogue (2.9) were solubilised as described (4.2.2) using 5mM CHAPS/1.5M NaCl. The method for biotin/streptavidin affinity chromatography used was based on that described by Kohanski and Lane, 1985(b). The labelled solubilised preparation was preincubated (1 hr at room termperature) with 1ml streptavidin-agarose in the CHAPS/NaCl solubilisation solution. After this time, the column, (in a 1ml syringe plugged with glass wool) was constructed and washed through for an hour with the 5mM CHAPS/1.5M NaCl solubilisation solution at 4°C. A constant head reservoir was used, and the columns ran under gravity at 8mls/hr. Two minute fractions were collected. After 1 hr the eluting solution was changed to the CHAPS/NaCl solution containing 2mM biotin. Two minute fractions were again collected. 100µl aliquots of the fractions were then counted using γ-spectrometry.

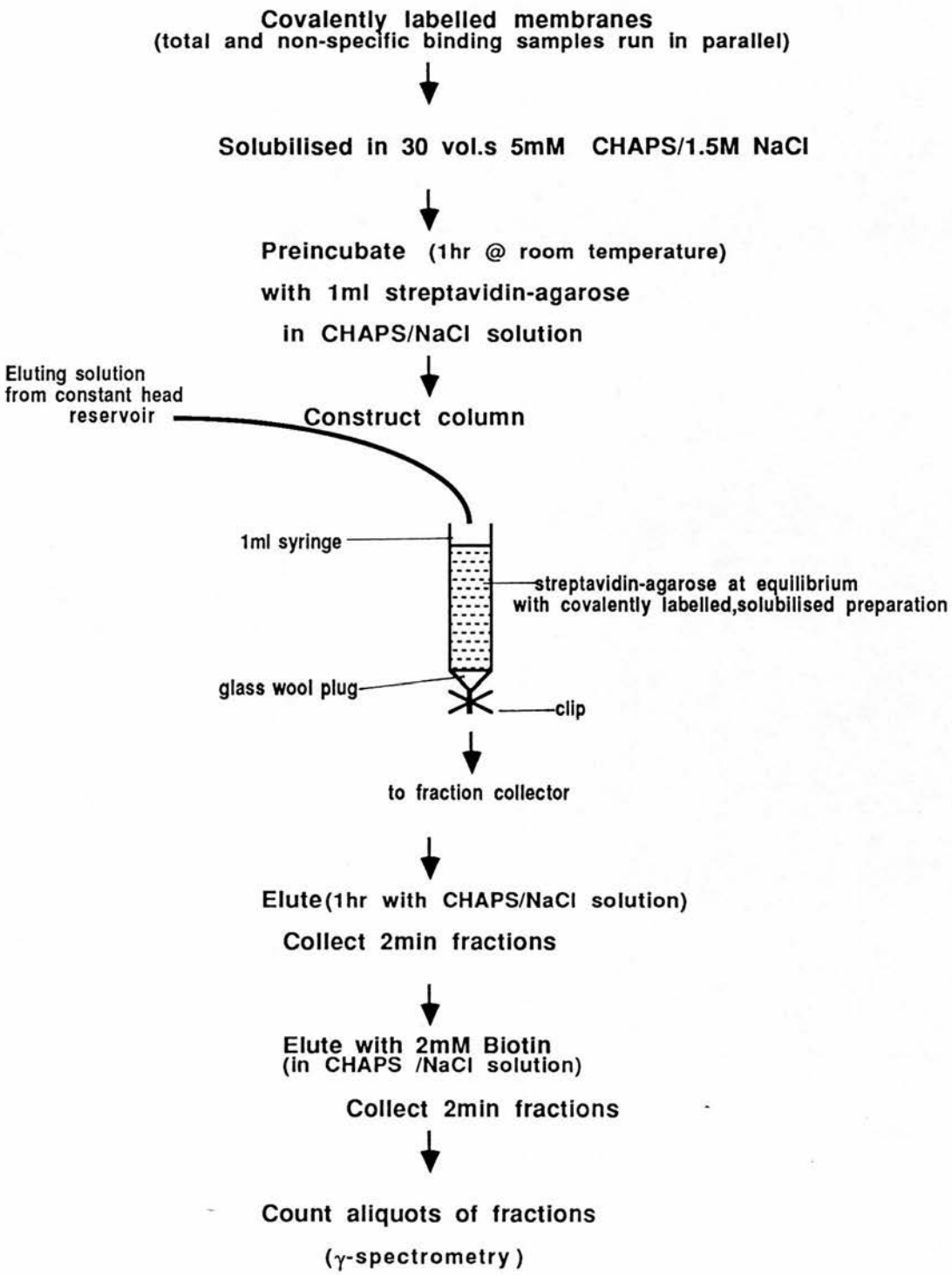
Two affinity columns were run in parallel. One was loaded with solubilised membranes labelled with the iodinated biotin-LHRH analogue, one with solubilised membranes that had been bound and cross-linked in the presence of 1µM LHRH (non-specific binding conditions). Comparison of the two patterns of eluted radiolabel indicated the specificity of the method for LHRH receptor protein purification.

2.7. Estimate of the Molecular Weight of the LHRH receptor protein by Gel filtration.

The separation of molecules by gel filtration according to their

Figure 2.8

Affinity Chromatography Method



molecular size was first described by Lathe and Ruthven (1956) using a column of starch. Methods have since been improved with the development of more suitable inert gels such as the crosslinked dextran (Sephadex: Porath and Flodin, 1959) and beaded agarose (Sepharose: Hjerten, 1964). The separation depends on the ability of the molecule to move out of the solution into the stationary phase of the gel (Ackers, 1975). Smaller molecules may spend a high proportion of their time in the stationary phase and therefore move slowly through the gel. Larger molecules unable to enter the gel stationary phase move much more rapidly. Assuming a similar relationship between molecular weight and molecular size for all molecules, there is a constant relationship between molecular weight and elution volume.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Ve = elution volume for the molecule

Vt = total bed volume of column

Vo = void volume of column

and the calculated Kav = the fraction of the stationary gel volume available for diffusion of the molecule.

A plot of Kav against log (molecular weight) for several standard substances of known molecular weight, provides a calibration curve for a particular column (Ackers, 1975). This can be used to determine unknown molecular weights using the calculated Kav values. The molecular weight value is, however, only an apparent value, for whilst denatured proteins (and to some extent soluble proteins) may have good correlation between their molecular radius (Stokes radius) and molecular weight, this does not

necessarily hold for solubilised membrane proteins (Ackers, 1975; Tanford and Reynolds, 1976).

Gel filtration has the advantage over other forms of molecular weight determination in the wide range of conditions under which it can be used. It does not require pure samples of the protein (as is needed for the more accurate methods of sedimentation equilibrium and velocity determination, Tanford and Reynolds, 1976). Denaturing of proteins is not essential (Fischer, 1980) as is the case for SDS PAGE techniques (2.10). This means that bioassays - such as ligand binding - can be used in the construction of an elution profile for the protein of interest. Gel filtration under mild conditions has been used to help characterise many proteins, including the muscarinic acetylcholine receptor (Peterson, Rosenbaum, Broderick and Schimerlik, 1986) and has in some instances revealed or confirmed the existance of receptor binding sites in high molecular weight complexes, for example the insulin receptor complexes found by Sepharose CL-6B gel filtration to have apparent molecular weights of 370K and 140K, which under denaturing SDS PAGE conditions dissociate into 130K/82K and 47K subunits respectively (Im, Fraugakis, Meezau, DiBona and Kim, 1982). Gel filtration of CHAPS solubilised GABA receptors have revealed the benzodiazepine binding site to exist on the same protein complex (Stephenson, Watkins and Olsen, 1982). High molecular weight complexes of porcine LH/hCG receptors (Wimalasena, Moore, Wiebe, Abel and Chen, 1985) and bovine mammary gland prolactin receptors (Ashkenazi, Madar and Gertler, 1987) have been demonstrated using gel filtration. In several cases a putative receptor-G-protein complex has been identified using this technique (Nissenson, Mann, Winer, Teitelbaum and Arnaud, 1986;

Dattatreyamurtyl, Figgs and Reichert, 1987; Kamada, Rondon, Frohlich and Cole, 1987; Paul and Said, 1987) as has the apparent dissociation of a multimeric mineralocorticoid receptor complex as a result of receptor activation (Eisen and Harmon, 1986). Gel filtration therefore appears to be a suitable technique for partial characterisation (giving an apparent molecular weight estimate) of non-denatured, solubilised proteins under mild conditions.

In this case it has been used to determine the apparent molecular weight of the solubilised LHRH receptor protein. Gel filtration has been shown not to be adversely affected by the presence of detergents (Tanford, Nozaki, Reynolds and Makino, 1974) or high salt concentrations (Petrovic, Petrovic, Markovic and Knezevic, 1974). The LHRH receptor protein molecular weight can therefore be determined whilst held in solution in the 5mM CHAPS/1.5M NaCl.

2.7.1. Method

A gel filtration column (70cm x 2.6cm) was prepared using Sepharose 6B (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) and kept at 4°C. After packing for 3 column volumes at 25ml/hr the column was run at 19.5ml/hr. The eluting solution used was the 5mM CHAPS/1.5M NaCl solubilisation solution. Void volume was determined using Dextran 2000 (1mg/ml) and a calibration curve constructed using high and low Molecular Weight Gel Filtration Calibration Kits (Pharmacia) (Fig.2.8). Eluted proteins were detected using an LKB Uvicord II 8300 and UV absorbtiometer 8303A detector unit and recorded on a LKB Chopper Bar Recorder 6520-4 (LKB Instruments, Stockholm, Sweden). Sucrose (50µg/ml) was added to the samples before applying them to the column to facilitate their entry into

Figure 2.9

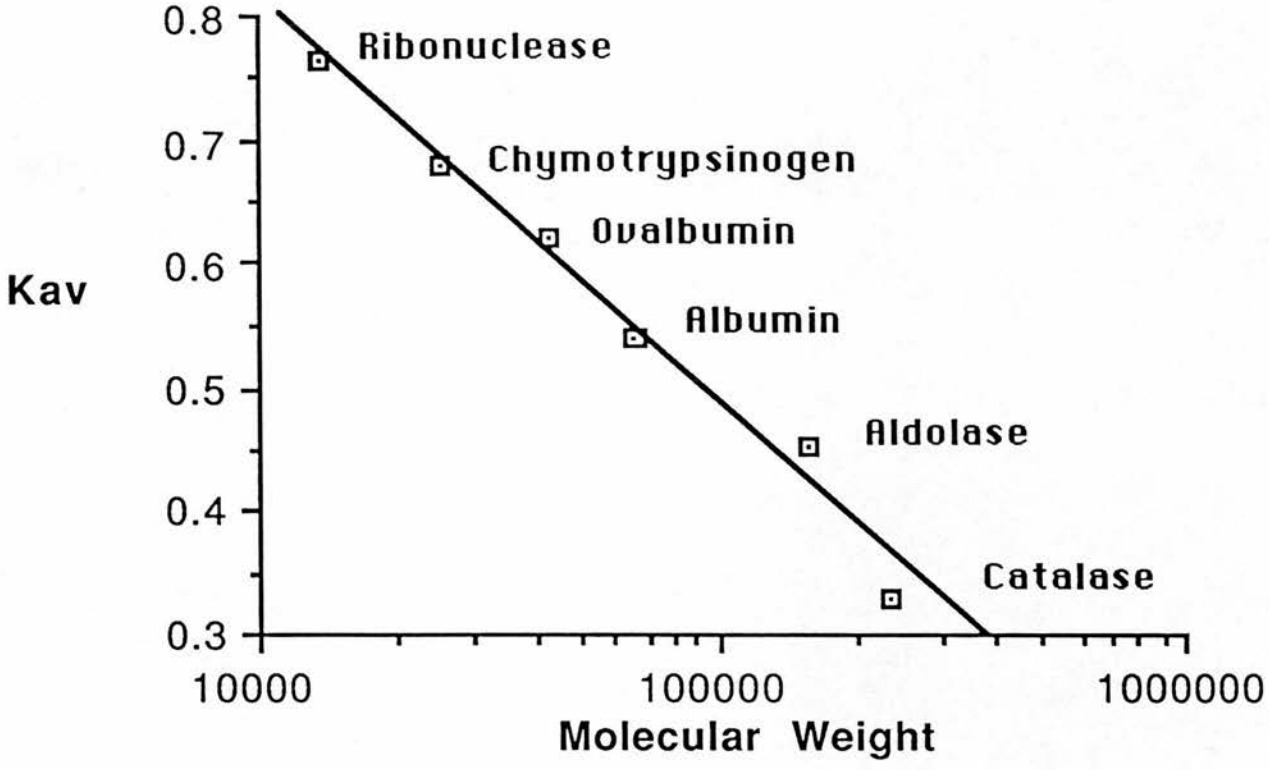


Figure 2.9

Calibration curve for Sepharose 6B (70cm x 2.6cm) gel filtration column. Kav values, calculated as described (2.7) are means of two determinations. Void volume was determined using Dextran 2000 (1mg/ml) before each sample run. The column was run as described (2.7.1) at 19.5ml/hr with the eluting solution (5mM CHAPS, 1.5M NaCl, 25mM Tris-HCl pH7.4, 50µg/ml soybean trypsin inhibitor, 400KIU/ml aprotinin) and eluted proteins detected using an LKB Uvicord II 8300 and UV absorbtiometer 8303A detector unit.

the gel bed as a compact band and therefore improve the resolution of the separation.

The supernatant of anterior pituitary membranes solubilised as previously described (Chapter 4) using 5mM CHAPS/1.5M NaCl were applied to the calibrated column and eluted (19.5ml/hr) using the CHAPS/NaCl solution. Fifteen minute fractions were collected using a LKB Ultrorac 7000 fraction collector. From these 1.3ml aliquots were taken for PEG precipitation (as previously described but scaled up proportionally to a total volume of 35mls). After standing for 20 minutes on ice the precipitated proteins were separated from the detergent solution by centrifugation for 30 minutes at 15,000 g 4°C, and then resuspended in 1.8 ml Tris-HCl (25mM, pH 7.4). Aliquots of 300 µl were used in ^{125}I-buserelin binding assays (2.4) to determine the elution volume (Ve) of the proteins responsible for specific binding of LHRH ligands. Using the calibration curve (Fig.2.9) and the calculated Kav for the protein its apparent molecular weight could be estimated.

2.8. SDS Polyacrylamide gel electrophoresis (PAGE)

Whereas gel filtration (2.7) can be used to give an estimate of the apparent molecular weight of the solubilised LHRH binding protein, any contribution to this by individual polypeptides or subunits may not be revealed. Dissociation of the receptor protein into its constituent polypeptides (if more than one) may not occur under the mild conditions used for solubilisation and gel filtration, more extreme conditions may be required to reveal these. The anionic detergent sodium dodecyl sulphate has been shown (Nielsen and Reynolds, 1978) to dissociate many water soluble proteins into their constituent polypeptide chains (in the presence

of a reducing agent). Approximately 1.4g SDS is found to bind per gram of protein (Nielsen and Reynolds, 1978) which is said to be a number of SDS molecules approximately equal to half the number of amino-acid residues (Weber and Osborn, 1975). This is thought to result in an almost constant negative charge per unit mass of protein (for proteins not initially possessing a high overall charge; Weber and Osborn, 1975). The binding of SDS to the proteins was also shown to induce a conformational change, resulting in rod-like complexes of constant width but of length proportional to their molecular weight (Reynolds and Tanford, 1970).

This denaturing of proteins by SDS has enabled electrophoretic techniques to be used to estimate their molecular weights (Weber and Osborn, 1975; Nielsen and Reynolds, 1978). Whilst the electrophoretic mobility of proteins is dependent on their molecular charge and size, no rigorous relationship exists between electrophoretic mobility and molecular weight (Nielsen and Reynolds, 1978). For protein-SDS complexes in polyacrylamide gel electrophoresis (PAGE, first introduced by Raymond and Weintraus, 1959), the high degree of SDS binding and conformational changes result in an apparent relationship between the logarithm of the molecular weight of protein and its electrophoretic mobility (Shapiro, Vinuela and Maizel, 1967; Weber and Osborn, 1969). The empirically established relationship has not however been shown to have any theoretical basis (Nielsen and Reynolds, 1978) and exceptions exist, such as proteins of high intrinsic charge, for example histones (Weber and Osborn, 1975) or glycoproteins (Pitt Rivers and Impiombato, 1968). Grefrati and Reynolds (1974) found that the erythrocyte glycoprotein (a transmembrane protein consisting of 55% carbohydrate) whilst dissociating into monomers in

the presence of SDS, binds an abnormally high amount of SDS (5 - 7g of SDS/g protein, or 2.5 - 3.4g SDS/g glycoprotein). Similarly the hydrophobic region of the intrinsic mitochondrial membrane protein cytochrome b_5 has been shown to bind ~3g SDS/g protein whereas the hydrophilic, extramembrane portion of the protein binds only ~0.7g SDS/g protein (Robinson and Tanford, 1975). In both cases these proteins might be expected to show non-standard mobilities in SDS-PAGE, and this is thought to be the case for many intrinsic membrane proteins (Nielsen and Reynolds, 1978).

The technique of SDS-PAGE has however been used both to show the existence of polypeptide subunits making up a membrane associated complex, and to determine the approximate molecular weights of the constituents. For example the mitochondrial membrane cytochrome C oxidase complex has been shown to contain six polypeptide subunits (Briggs, Kamp, Robinson and Capaldi, 1975) or seven in the case of the yeast complex (Rubin and Tzagoloff, 1973). The acetylcholine receptor (Lindstrom, Merlie and Yogeeswaran, 1979), insulin receptor (Im, Frangakis, Meezan, DiBona and Kim, 1982) prolactin receptor (Yamada and Donner, 1985; Mitani and Dufau, 1986; Haldosen and Gustafsson, 1987) and the LH/hCG receptor (Wimalasena, Moore, Wiebe, Abel and Chen, 1985) have all been shown by SDS-PAGE to be complexes of more than one polypeptide subunit. Electrophoresis of samples containing many different proteins, such as a sample of rat anterior pituitary membranes, requires some method of identifying specific proteins. In the case of several receptor-types including the somatostatin (Lewis and Williams, 1987; Zeggari, Viguerie, Susini, Garnier, Esteve and Ribet, 1987) TSH (Furmaniak, Haskim, Buckland, Petersen, Beever, Howells and Rees Smith, 1987) angiotensin II

(Guillemette, Guillon, Marie, Balestre, Escher and Ward, 1987; Laribi, Allard, Vincent and Simonnet, 1987) and adenosine receptors (Stiles and Jacobson, 1987), this has been achieved by the covalent labelling of radioiodinated analogues of specific ligands to the receptor proteins prior to electrophoresis. This method has previously be used in the identification of LHRH receptors on SDS-PAGE (Hazum, 1981(b); Iwashita and Catt, 1985). Ligand binding to the LHRH protein after electrophoresis has also been used (Eidne, Hendricks and Millar, 1985), but the technique of covalently labelling with a radioiodinated specific ligand was that used in this thesis (Chapter 6).

The method of SDS-PAGE used here was that of discontinuous gel electrophoresis first described by Laemmli (1970). Estimation of the unknown molecular weights of proteins is by calculation of relative mobilities (R_f) - the migration distance of protein divided by that of dye front - and consulting a standard curve of R_f values for proteins of known molecular weight against the log of their molecular weight (Weber and Osborn, 1969).

2.8.1. Method

Separating gels of 7% acrylamide were used. The solutions used are listed in Appendix II. Slab gels (20cm x 20cm x 0.7mm) were poured by hand. After polymerisation was complete (~1 hr at room temperature) a stacking gel (4.75% acrylamide) was applied, using well formers of 100µl capacity.

Samples dissolved in sample buffer (see Appendix II) were heated at 90-100°C for five minutes and 5µl of 1% bromo-phenol blue solution added to each. ^{14}C-labelled molecular weight standards (Amersham International plc, Bucks, England; molecular weights

Figure 2.10

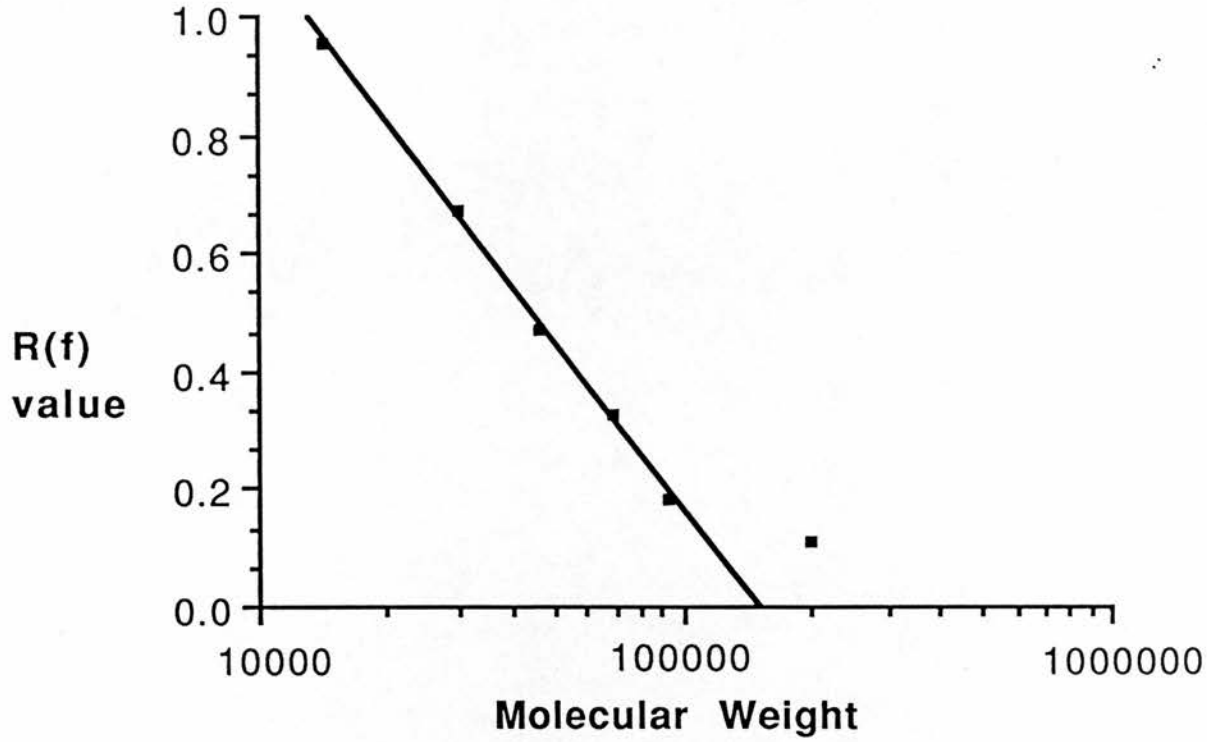


Figure 2.10

Representative calibration curve for an SDS 7% polyacrylamide gel with a stacking gel of 4.75% acrylamide. ^{14}C-Labelled standards (Amersham International plc) dissolved in sample buffer as described (2.8.1 and Appendix II) were applied and run at 40mA for 10-12hr at 10°C. The gel was then fixed and dried and exposed to X-Omat S film for 4 weeks at -70°C (see Appendix II). The resulting autoradiograph was used to calculate Rf values (the migration distance of protein divided by that of the dye front) and so construct a standard curve.

2,350-200,000) were run with each set of samples and could be used to construct a standard curve, as above (Fig.2.10). Electrophoresis was carried out for 10-12 hr at 40 mA (constant current) using the GE-214 LS vertical electrophoresis apparatus (Pharmacia Fine Chemicals, Ltd) and LKB 2197 power supply. The running buffer (see Appendix II) was continuously circulated and cooled to 10°C with the LKB 2219 Multitemp II thermostatic circulator.

2.9. Design and Synthesis of LHRH Analogues

For the affinity chromatography of the LHRH receptor using biotin affinity columns (2.6) analogues of LHRH containing a biotin molecule and some means of covalently attaching the peptide to the receptor site are required. It is also necessary for efficient affinity labelling of the LHRH receptor, that the analogues have a high specific:non-specific binding ratio, a slow rate of dissociation from the LHRH receptor site and resistance to enzymatic degradation. In order to try and satisfy these requirements, a literature survey of the conformational and binding characteristics of LHRH and its analogues was undertaken (2.9.1). Similarly, information in the literature was used to try to optimise the structure of the biotin containing groups chosen (2.9.2).

2.9.1. Choice of Peptide Backbone

Conformational studies of LHRH (Momany, 1976(a)) and analogues of LHRH (Momany, 1976(b)) have shown the lowest energy confirmation of the decapeptide to be hinged, forming a β turn at the Gly^6 residue (Fig.2.11). Generally it was shown that changes in the amino-acids of LHRH which resulted in a change in the backbone shape (in order to maintain a lowest energy conformation) lead to a loss

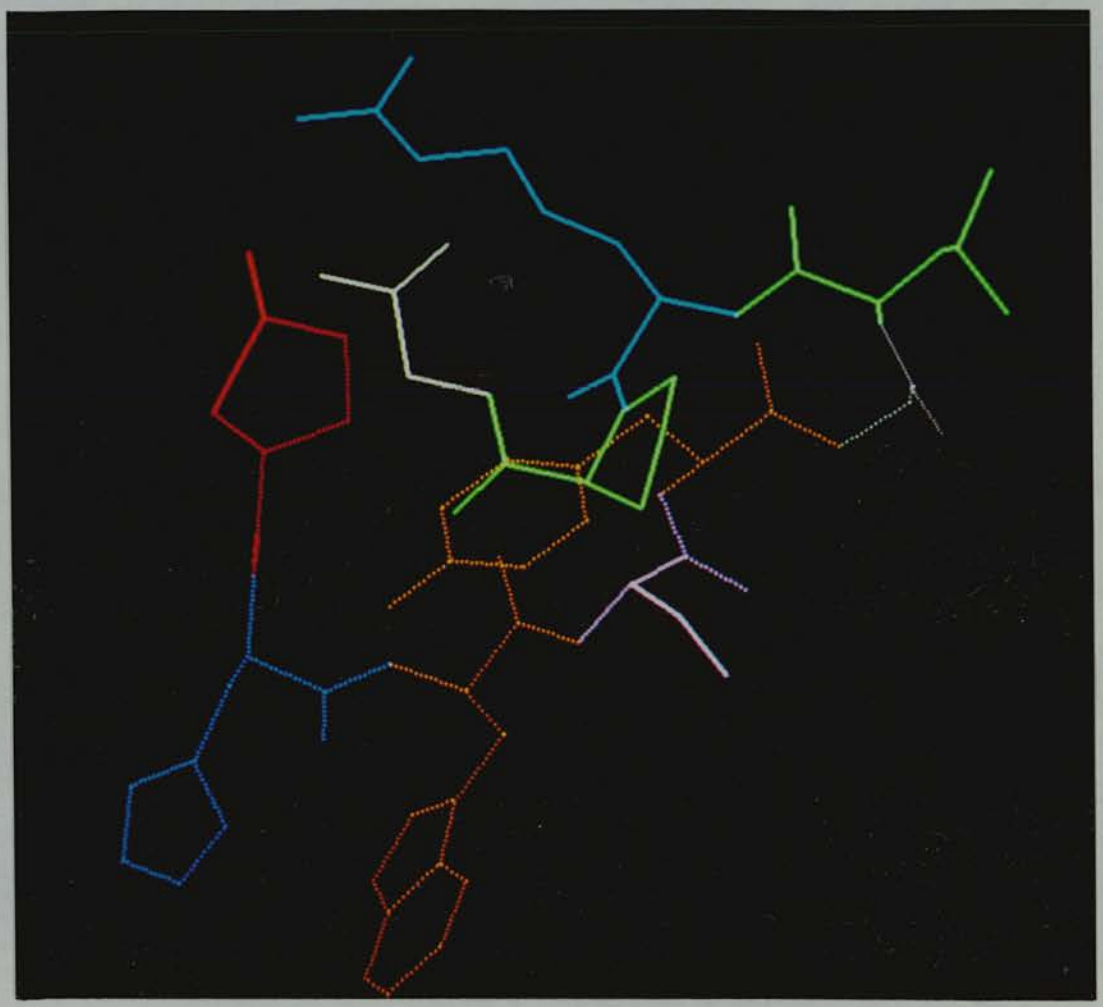


Figure 2.11

Conformation of LHRH as proposed by Momany (1976(a),(b)). Positions of hydrogen atoms are not shown for clarity. Structure modelled using "Interchem" by Dr P Bladon (Strathclyde University).

of biological activity. Some changes which did not involve a change in backbone shape also resulted in decreased biological activity and these have been suggested to indicate the moieties important for receptor-peptide interaction. Those residues required for biological activity would appear to be the Glu^1, His^2 and Pro^9 residues, with aromatic residues at Trp^3, Tyr^5 positions. The amino-acid in position 6, as long as it retained the β turn formation, was unimportant. A D-isomer in this position however stabilises the β turn of the molecule (Momany, 1976(b)) and such analogues are more active than the native LHRH (Coy, Vilchez-Martinez, Coy and Schally, 1976).

As position 6 is some distance away from the part of the LHRH molecule considered to interact with the receptor it may be a good candidate as the position on which to substitute bulky groups - such as the biotin molecule. Replacement of Gly^6 with D-Lys^6 would not only stabilise the low energy β turn conformation but provide a free amino group at position 6 for substitution.

Arimura, Vilchez-Martinez, Coy, Coy, Hirotsu and Schally (1974), Fujino, Fukunda, Shinagawa, Kobayashi, Yamazaki, Nakayama, Seely, White and Rippel (1974) and Dericks-Tan, Hammer and Taubert (1977) showed that LHRH analogues altered not only by a D-amino acid substitution at position 6 but also at their C-terminal to des Gly^{10} ethylamide were far more potent, biologically, than either native LHRH or the $D(x)^6$ LHRH analogue. Binding experiments using iodinated analogues (Clayton, Shakespear, Duncan and Marshall, 1979a) showed that these [$D(x)^6$ des Gly^{10}]-LHRH ethylamine analogues bound largely to one high affinity site, non-specific and low affinity site binding being much less than that seen for native LHRH. However, the increase in biological activity over native LHRH

observed could not be accounted for entirely by the increase in affinity.

Marks and Stern (1974) using whole brain extracts and Koch, Baram and Chobsieng (1974) using hypothalamic and cerebrocortical extracts showed that native LHRH was rapidly degraded, largely by the cleavage of the Gly^6-Leu^7 peptide bond. Clayton, Shakespear, Duncan and Marshall (1979(b)) showed also that an LHRH degradation enzyme (of an unspecified type) was associated with purified pituitary plasma membranes. Further studies by Elkabes, Fridkin and Koch (1981) suggested that the main cleavage sites for the plasma membrane bound enzymes were the Gly^6-Leu^7 and Trp^3-Ser^4 bonds of LHRH. However, LHRH analogues with a D-amino acid at position 6 were shown to be resistant to degradation by these enzymes (Marks and Stern, 1974; Koch, Boram, Hazum and Fridkin, 1977; Clayton and Shakespear, 1978; Clayton, Shakespear, Duncan and Marshall, 1979(b)), with the increase in potency of these analogues of a similar order to their resistance to enzymatic degradation. A slower, C-terminal inactivation of LHRH was seen by whole brain extracts (Marks and Stern, 1974) and this was blocked by the modification of the C-terminal to des Gly^{10}-ethylamide. The higher biological activity of LHRH analogues [$D(x)^6$, des Gly^{10}]-LHRH ethylamide therefore could possibly be explained in terms of both an increased affinity and a resistance to enzymatic degradation. Such analogues have been shown *in vivo* to bind to specific sites in the pituitary to a greater degree and for a longer time than native LHRH (Reeves, Tarnavsky, Becker, Coy and Schally, 1976). As the half-lives of the analogues in serum were found to be similar to that of LHRH (Reeves, Tarnavsky, Becker, Coy and Schally,

1976), this has been suggested (Clayton and Catt, 1981) to be due to the slower dissociation of these analogues from the LHRH receptor than native LHRH. This is in agreement with *in vitro* data on the dissociation of radiolabelled LHRH and its analogues from rat anterior pituitary tissue (Clayton and Catt, 1981).

In summary, LHRH altered to a D-amino acid at position 6, on to which the biotin containing group can be added, and with a C-terminal alteration to des Gly^{10} N-ethylamide should optimise all the conditions required for the peptide backbone for a biotin containing affinity ligand for the LHRH receptor. That is:-

(1) a high affinity LHRH analogue with a high specific:non-specific binding ratio,

(2) resistance to enzymatic degradation,

(3) and slow dissociation from the LHRH receptor site.

A D-Lys^6 residue, as mentioned above, provides a free amino group onto which the biotin containing molecule can be reacted.

2.9.2. Choice of Biotin containing group

As the biotin-streptavidin interaction was to be used in the affinity purification of the covalently labelled LHRH receptors it was important that the biotin group binding was not sterically impeded. The 4-carbon chain, between the part of the biotin molecule important in streptavidin binding and its attachment to the rest of the side chain (Fig. 2.12), was thought to be sufficient to ensure this.

Two methods of covalent labelling of the receptor site were to be used - photoaffinity and bifunctional chemical crosslinkers.

2.9.2.1. Choice of Photoaffinity Moiety

A photoaffinity ligand may in theory enable a high specificity

Figure 2.12

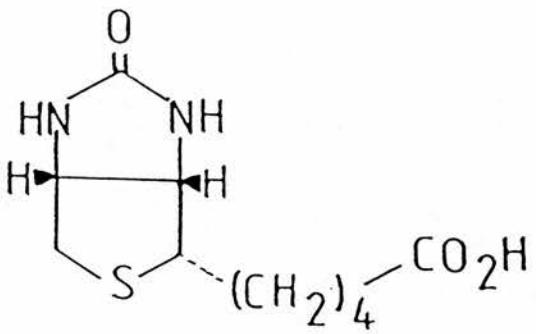
Chemical structure of a) Biotin, and the novel, biotin-containing reagents prepared by Dr C M Bladon for coupling LHRH analogues -

b) the aryl azide, photoaffinity compound

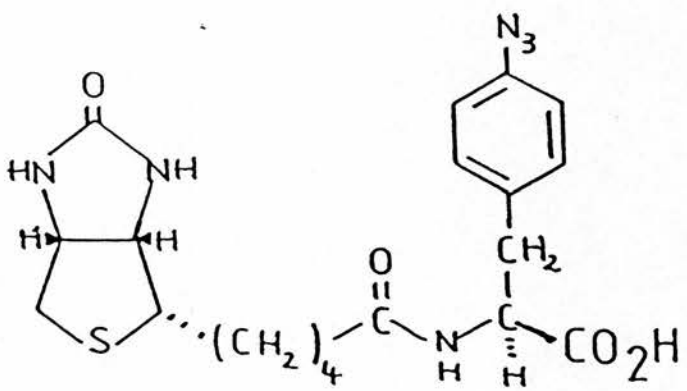
c) the free amino containing compound

Figure 2.12

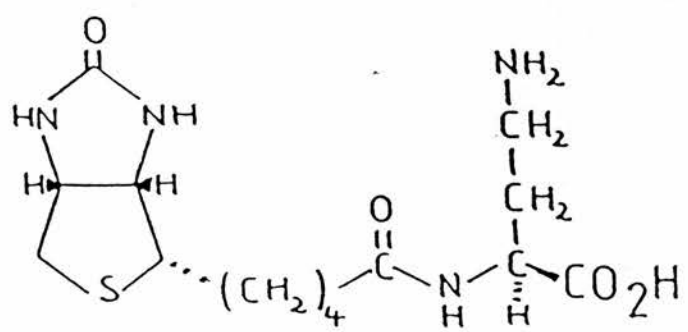
a **Biotin**



b **Photoaffinty side chain**



c **Side chain incorporating a free amino group**



of labelling in the region of the receptor site (Bayley and Knowles, 1977; Das and Fox, 1979; Ji, 1979). This is thought to be dependent on the specificity of the ligand binding at equilibrium with the membrane preparation, the close proximity of the photoaffinity group to the receptor and the rapid and non-selective reactivity of the photoactivated group (Das and Fox, 1979; Ji, 1979).

Criteria have been proposed that a suitable photo-reactive group should fulfil (Baley and Knowles, 1977), they are:-

1) a readily synthesised precursor that is chemically stable in an aqueous solution for at least the time required to reach a binding equilibrium.
2) a group susceptible to photolysis at wavelengths that will not result in photo-oxidative or other photochemical damage to the membrane constituents.
3) a group which on exposure to light gives rise to a highly reactive species of short half-life.

Early experiments using photoreactive ligands were attempts to map the active site of the enzyme chymotrypsin and utilised diazo compounds (Singh, Thornton and Westheimer, 1962). Photoactivation of the diazo group generates a carbene which is capable of reacting very rapidly and non-selectively (Bayley and Knowles, 1977). Unfortunately diazo compounds are susceptible to rearrangements (Bayley and Knowles, 1977; Das and Fox, 1979) resulting in a loss of photoreactivity or a loss in the non-selective, rapid reactivity of the photogenerated group (Bayley and Knowles, 1977; Das and Fox, 1979). They therefore would not appear to be ideal photoreactive compounds. A second group of photoreactive compounds are the aryl azides. Aryl azides are very stable in aqueous solution (in the

dark) but have a low activation energy and are activated by light of the long wavelength UV/visible range ~300-400nm (Ji, 1979; Das and Fox, 1979). On activation an aryl nitrene is formed which is a highly reactive species capable of reacting with primary, secondary and tertiary carbon-hydrogen bonds or with an oxygen-hydrogen bond. Only the amino-acid glycine has been suggested to be resistant to reaction with them (Ji, 1979). The half-life of the reactive species has been found to be of the order of 10^{-4}sec, the actual value dependent on substituents on the group (Das and Fox, 1979). Aryl azides would appear to comply with the criteria suggested for useful photoreactive groups, and have been used in preference to diazos in many studies (Bayley and Knowles, 1977).

Early photoaffinity ligands incorporating aryl azide groups included acetylcholine receptor and esterase ligands (Kiefer, Lindstrom, Lennox and Singer, 1970; Ruoho, Kiefer, Roeder and Singer, 1973) and derivatives of opioids (Winter and Goldstein, 1972) insulin (Levy, 1973) and gastrin (Galardy, Craig, Jamieson and Caron, 1974). Whilst initial studies revealed high non-specific labelling with the photoaffinity ligands (Ruoho, Kiefer, Roeder and Singer, 1973; Winter and Goldstein, 1972), their use in combination with histochemical (Rostene, Mazella, Dussaillant and Vincent, 1986) and SDS-PAGE techniques (Furmaniak, Hashim, Buckland, Petersen, Beaver, Howells and Rees Smith, 1987; Stiles and Jacobson, 1987; Amlaiky, Berger, Chang, McQuade and Caron, 1987; Robichon, Kuks and Besson, 1987) have shown specific covalent labelling of the receptor site. Efficiencies as high as 40% of all receptors covalently labelled have been reported (Guillenette, Guillon, Marie, Balestre, Escher and Jard, 1987). Aryl azide compounds were therefore chosen

as the photoreactive group in photoaffinity LHRH analogues used in this Thesis.

2.9.2.2. Choice of the Substrate for Chemical Crosslinker Affinity Labelling

Another method for covalently linking a ligand to its receptor site is that of chemical crosslinking. Bifunctional reagents are used to introduce a chemical bridge between the two molecules (Ji, 1979). The first attempts to crosslink ligands to their receptors used glutaraldehyde to covalently bind insulin and epidermal growth factor to their respective binding sites on liver membranes (Sahyoun, Hook and Hollenberg, 1978). The bond formed by the glutaraldehyde was unstable and required reducing before the resultant complex could be further examined. More suitable chemical crosslinkers for ligands and receptors were found in the bisimidates (Dutton, Adams and Singer, 1966 ; Hartman and Wold, 1966). These react (with high specificity) with primary and secondary amino groups to form stable amide bonds. Unfortunately they have been found to be unstable in water and hydrolyse rapidly to their free acid, particularly below pH 8 and low temperatures (0-4°C) where these side reactions are said to occur at a far greater rate than the amide bond formation (Ji, 1979; Pilch and Czech, 1984). Lomant and Fairbanks (1976) suggested that bis(succinimidyl) esters would prove more useful as chemical crosslinkers in biological systems. They showed them to be reactive with primary and secondary amino groups with the amide bond reaction proceeding at a far higher rate than hydrolysis at pH7, 23°C. Homobifunctional succinimidyl esters have been widely used in the crosslinking of ligands to their receptors, for example receptors for insulin (Pilch and Czech, 1979)

M.S.A. (Massague, Guillette and Czech, 1981) human chorionic gonadotropin (Rebois, Omedeo-Sale, Brady and Fishman, 1981) platelet derived growth factor (Glenn, Bowen-Popeand Ross, 1982) growth hormone releasing hormone (Zysk, Cronin, Anderson and Thorner, 1986) and angiotensin II (Laribi, Allard, Vincent and Simonnet, 1987).

An alternative to the homobifunctional crosslinkers are heterobifunctional crosslinkers. These are often constructed to contain a succinimidyl ester and a photoreactive group (such as an aryl azide: Ji, 1976; Ji, 1979). These have the advantage over homobifunctional N-hydroxy succinimidyl esters of being able to react with the ligand by amide bond formation prior to binding with the receptor and then, after ligand binding equlibrium has been reached, undergo photoactivation to crosslink the ligand to the receptor (Ji, 1979). Again many ligands and receptors have been crosslinked using these types of reagent including LHRH (Hazum, 1981; Iwashita and Catt, 1985), somatostatin (Lewis and Williaks, 1987; Zeggari, Viguerie, Susini, Garnier, Esteve and Ribet, 1987), thyroid stimulating hormone (Furmaniak, Hashim, Buckland, Petersen, Beever, Howells and Rees-Smith, 1987), adenosine (Stiles and Jacobson, 1987), dopamine D_1 receptors (Amlaiky, Berger, Chang, McQuade and Caron, 1987) and vasoactive intestinal peptide (VIP: Robichon, Kuks and Besson, 1987). In the case of VIP the derivatisation of the ligand was carried out whilst it was bound to the receptor in order to protect those parts of the ligand required for binding affinity.

Comparison of studies of insulin receptors using chemical crosslinkers, photoaffinity ligands and specific antibodies reveal no major differences in the apparent structure of the receptor found

(Czech, Massague and Pilch, 1981). This suggests that, at least in the case of the insulin receptor, neither photoaffinity nor chemical crosslinking techniques results in significant crosslinking of non-related membrane proteins, supporting the hypothesis that they are valid techniques for the identification of receptors for specific ligands.

All of the chemical crosslinkers used in this Thesis, both homobifunctional and heterobifunctional, contained an N-hydroxysuccinimide ester group. This is reactive with free amino groups (see above) and so the biotin-containing group of LHRH analogues used in the chemical crosslinking experiments were required to have a free amino group. In order to increase the chance of this free amino group being available for reaction with the chemical crosslinker and not subject to excessive steric hinderance by the ligand-receptor complex a 2-carbon spacer arm was incorporated (Fig.2.12).

2.9.3. Biotin containing LHRH-analogues

The biotin-LHRH-analogues chosen to be synthesised were [biotinyl-p-azidophenylalanine-D-Lys^6]-LHRH (PBL), [biotinyl-p-azidophenylalanine-D-Lys^6, des Gly^{10}]-LHRH ethylamide (PBAL) and [4-amino-2-biotinyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide (XBAL) (Fig.2.13(a,b,c)).

2.9.4. Method of Synthesis of Biotin-containing LHRH-analogues

All peptide synthesis was undertaken by Dr C.M. Bladon of the MRC Brain Metabolism Unit. [D-Lys^6, des Gly^{10}]-LHRH ethylamide was synthesised by the solid-phase method using Sheppard's Fmoc-t-butyl-polyamide chemistry (Eberle, Atherton, Dryland and Sheppard, 1986). Details of the techniques and methods appear in

Figure 2.13

Probable conformations of the biotinylated LHRH-analogues.

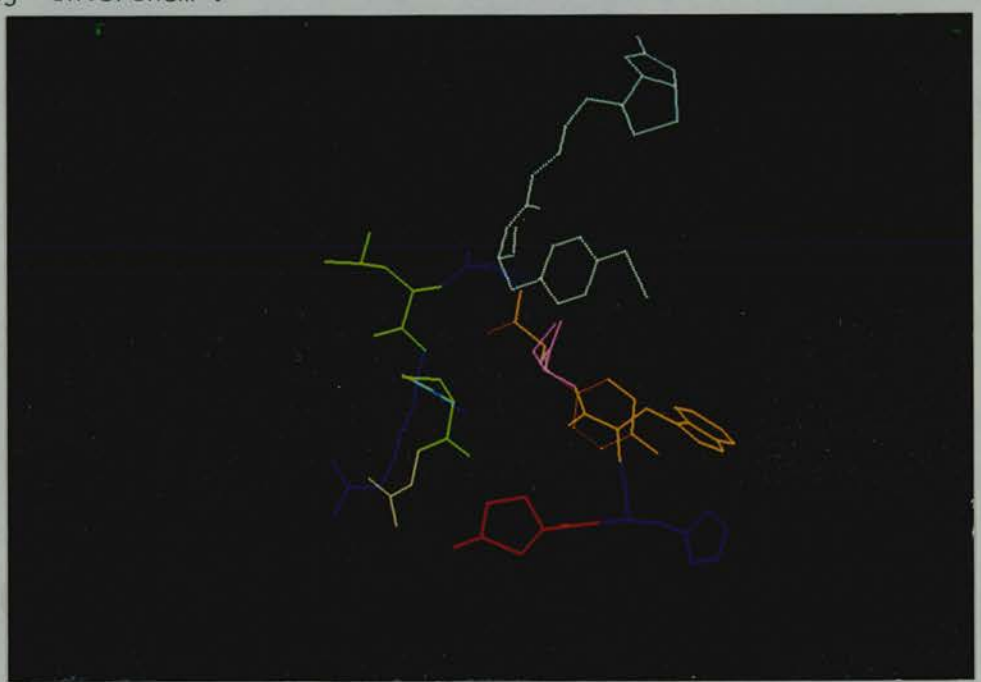
a) PBL

b) [DLys^6]-LHRH

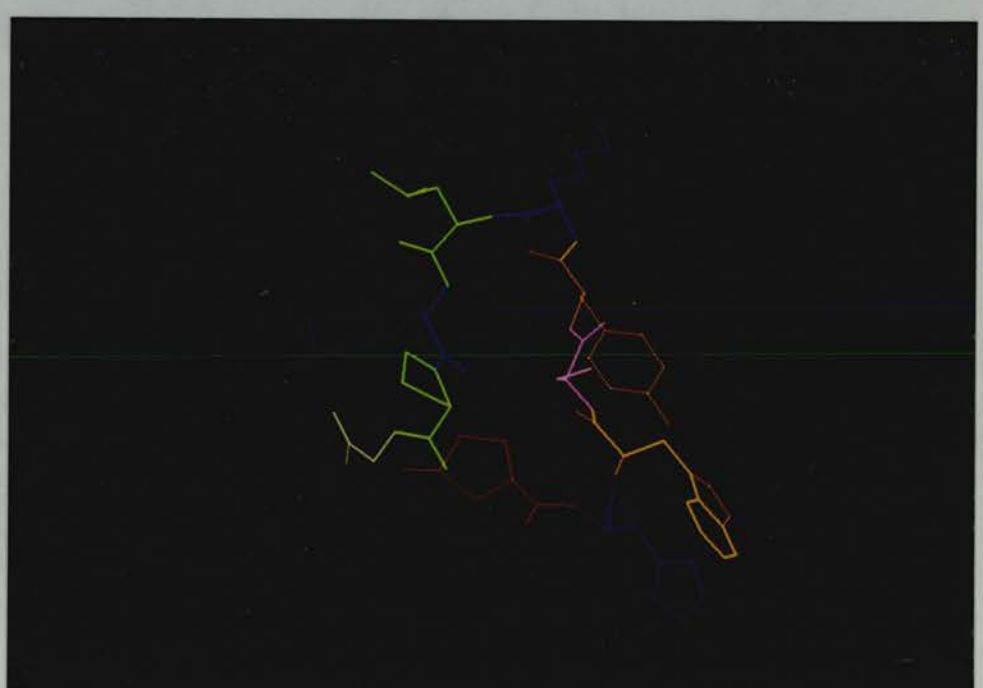
c) XBAL

Structures were modelled by Dr P Bladon (Strathclyde University) using "Interchem".

a



b



C

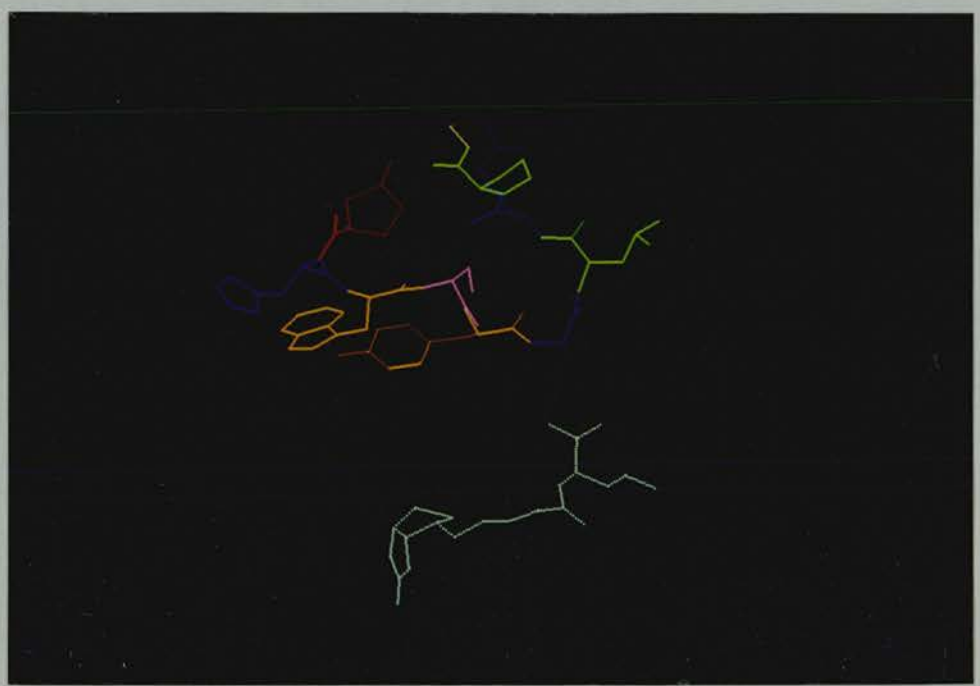


Table 2.1

Analogue	Amino Acid										
	pGlu	His	Ser	Tyr	DLys	$PheN_3$*	But	Leu	Arg	Pro	Gly
PBL	1.07	1.00	0.81	1.07	1.01	0.30	-	1	1.00	0.96	1.04
PBAL	1.05	0.96	0.83	0.95	1.02	0.20	-	1	0.89	0.91	-
XBAL	1.09	1.01	0.91	1.01	1.07	-	0.94	1	0.96	0.99	-

* largely decomposed on hydrolysis.

Table 2.1

Amino-acid analysis of LHRH analogues (performed by Dr C M Bladon). Samples were hydrolysed in distilled, constant boiling hydrochloric acid containing a trace of phenol for 18hr at 110°C. The hydrolysate was evaporated and the residue was partitioned between citrate buffer (pH2.20) and methylene chloride. The aqueous layer was filtered through a nylon-66 membrane and an aliquot subjected to amino-acid analysis (LKB LH50 Alpha amino acid analyser).

Each conjugate gave an amino acid analysis consistent with the expected sequence, though tryptophan was not determined due to its normal degrading under these conditions.

Appendix III. [D-Lys^6]-LHRH was purchased from Peninsula Laboratories Europe Ltd (Merseyside).

The novel ligands (Fig.2.12) were prepared in several steps (see Appendix III) from biotin and either p-nitrophenylalanine or 2,4-diaminobutyric acid. These ligands were converted to their N-hydroxysuccinimide esters prior to coupling to the ε-amino group of the D-Lys^6 of the peptide backbone. The coupling reactions were performed in demethylformamide in the presence of triethylamine and the conjugated products were purified by reverse-phase hplc. Details of the reactions are shown in Appendix III. Each conjugate gave an amino acid analysis (Table 1) consistent with the expected sequence, though tryptophan was not determined due to the expected degeneration during analysis.

2.10. Protein Assay

All protein assays in this Thesis were carried out according to the method of Geiger and Bessman (1972). Samples in Tris or Hepes buffers were first precipitated using TCA and then resuspended into 0.03M NaOH. Aliquots of samples or bovine serum albumin standards (range 4-20μg) were made up to 200μl with distilled water in 3ml polystyrene (LP_3) tubes and 500μl alkaline copper reagent was added. After 20 minutes 1ml of diluted Folin-Ciocalteau reagent was added. Absorbance of the solution was measured, at 725nm using a Gilson model 250 spectrophotometer, after 20 minutes when the colour formation had gone to completion. Details of the method are given in Appendix IV. Values for samples were read off from the standard curve, which showed linear absorbance against protein concentration over the range used (Fig.2.14).

Figure 2.14

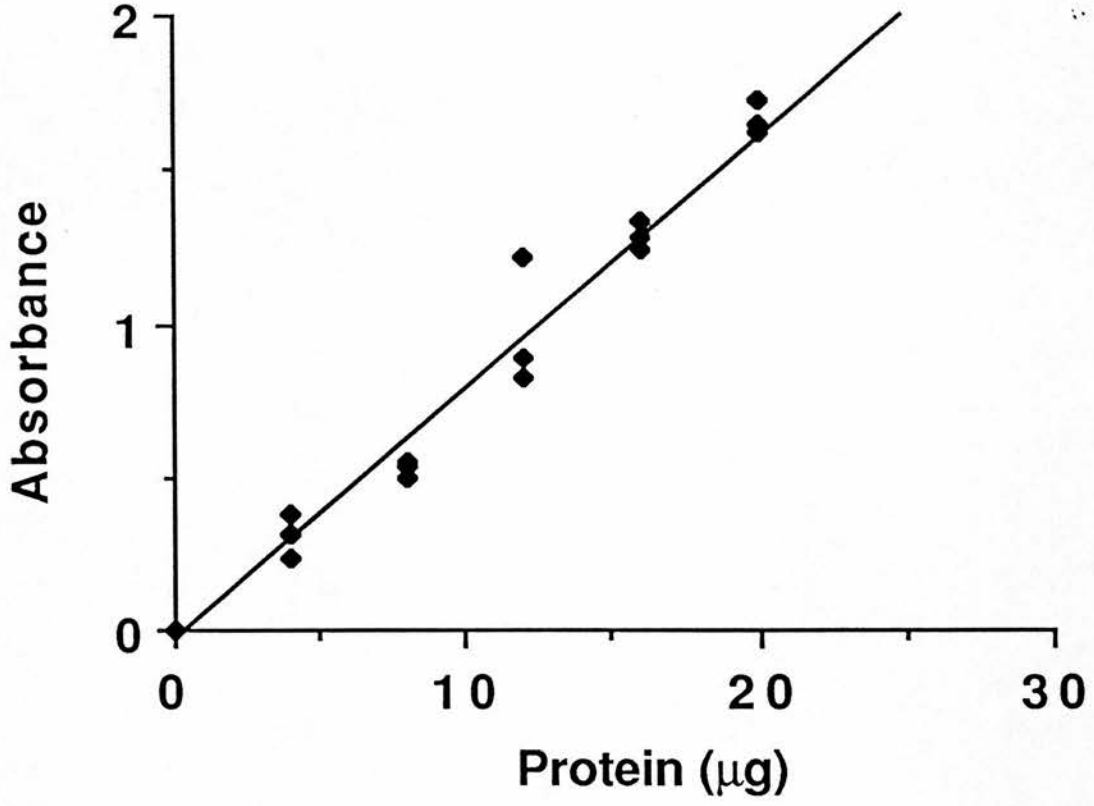


Figure 2.14

Representative protein assay standard curve. Aliquots (0-50μl) of a standard solution (BSA; 500μg/ml) made up to 200μl in 25mM Tris-HCl pH7.4 were precipitated using TCA and resuspended into 0.03M NaOH (50μl) and made up to 200μl with distilled water for use in the protein assay (see Appendix IV). Absorbance (at 725nm measured using a Gilson model 250 spectrophotometer) plotted against amount of protein revealed a linear standard curve.

Chapter 3

Characterisation of ligand binding to LHRH receptors in situ: relation to post-receptor events

3.1 Introduction

In common with other neurotransmitters and neuropeptides LHRH is thought to exert its actions on gonadotrophes by interacting with specific receptors (Vale, Rivier, Brown, Leppaluoto, Ling, Monahan and Rivier, 1976). Morphological studies using a ferritin conjugate of an LHRH analogue and electron microscopy (Hopkins and Gregory, 1977) and a fluorescent LHRH analogue with video-intensified fluorescence microscopy (Naor, Atlas, Clayton, Forman, Amsterdam and Catt, 1981) have localised these receptors to the plasma membrane. Initial ligand binding studies to investigate these receptors used radiolabelled native LHRH, either ^{3}H-labelled (Grant, Vale and Rivier, 1973; Theoleyre, Berault, Garnier and Jutisz, 1976) or ^{125}I-labelled (Spona, 1973; Marshall, Shakespear and Odell, 1976). These revealed two specific binding sites, one of high affinity (nM) but low capacity - only 30% of the total - and one low affinity (μM) high capacity site. Since the concentration of LHRH in portal blood is less than nM the physiological significance of the low affinity site is dubious. The possibility that it may represent interaction with the recognition site of an degradative enzyme has been widely suggested (Clayton, Shakespear, Duncan and Marshall, 1979(b); Clayton and Catt, 1981; Conn, Marian, McMillian, Stern, Rogers, Hamby, Penna and Grant, 1981). Saturation experiments using ^{125}I-LHRH revealed only one class of sites; that is high affinity receptors, whereas displacement of ^{125}I-LHRH by unlabelled LHRH showed only the low affinity site (Braumann and Kuhl, 1979; Wagner, Adams and Nett, 1979). This is in contrast to the multiple-binding sites seen using ^{3}H-LHRH (Clayton, Shakespear, Duncan and Marshall, 1979(b)). The inconsistency of

these results highlights the three major difficulties encountered by the early LHRH ligand binding experiments:-

1) Low specific binding of the radiolabels
2) Rapid dissociation of radiolabelled native LHRH (Clayton and Catt, 1981)
3) Susceptibility of the native LHRH (radiolabelled and unlabelled) to protease degradation (Kochman, Kerdelhue, Zor and Jutisz, 1975; Benuck and Marks, 1976; Clayton, Shakespear, Duncan and Marshall, 1979(b); Griffiths and Kelly, 1979).

As described previously (2.11.1), the principal degradation sites of native LHRH are said to be the Tyr^5-Gly^6 and Gly^6-Leu^7 bonds, (cleaved by an endopeptidase) and the Pro^9-$Gly^{10}NH_2$ bond (cleaved by a carboxyamide peptidase) (Marks, 1970; Koch, Baram, Chobsieng and Fridkin, 1974). The synthesis of LHRH analogues altered to a D-amino-acid at position 6 or desGly^{10} with an ethylamide at Pro^9 not only resulted in analogues resistant to degradation but also with increased potency (Arimura, Vilchez-Martinez, Coy, Hirotsu and Schally, 1974; Fujino, Fukuna, Shinagawa, Kobayashi, Yamazaki, Nakayama, Seely, White and Rippel, 1974; Kochman, Kerdelhue, Zopr and Jutisz, 1975; Schally, Kastin and Coy, 1976; Vale, Rivier, Brown, Lappaluoto, Ling, Monahan and Rivier, 1976; Koch, Baram, Hazum and Fridkin, 1977; Clayton and Shakespear, 1978; Clayton, Shakespear, Duncan and Marshall, 1979(b); Sandow, Kuhl and Krauss, 1979; Swift and Crighton, 1979; Perrin, Rivier and Vale, 1980). The increase in potency of the analogues has been suggested to be due not only to their resistance to degradation but also to the stabilisation of the peptide bond (6-7)

in the lowest energy conformation by the presence of a D-amino acid in position 6 (Monahan, Amass, Anderson and Vale, 1973; Fujino, Fukuna, Shinagawa, Kobayashi, Yamazaki, Nakayama, Seely, White and Rippel, 1974; Momany, 1976(b)) and the slower dissociation of the analogues from the LHRH receptor (Reeves, Tarnavsky, Becker, Coy and Schally, 1977; Clayton and Catt, 1981). Use of analogues with both an ethylamide in lieu of Gly^{10} and a D-amino-acid at position 6 as radioiodinated labels in binding studies has been a successful approach in experiments using dispersed anterior pituitary cells (Meidan and Koch, 1981), gonadotrophes in culture (Berault, Jansem de Almeida Catanho, Theoleyre and Jutisz, 1983) and membrane preparations (Conne, Aubert and Sizonenko, 1979). As regards tissue preparations, identical binding characteristics have been shown for sucrose density gradient-enriched pituitary plasma membranes, partially purified pituitary membrane particles and a crude rat anterior pituitary homogenate preparation (Clayton and Catt, 1981). In all cases whilst the degradation-resistant analogue displaced radiolabelled native LHRH from both the low and high affinity sites, the ^{125}I-analogue, which bound exclusively to a high affinity site, was completely displaceable by native LHRH and by various LHRH analogues.

[D-Ser(tBu)^{6},des Gly^{10}] LHRH ethylamide (buserelin) has been extensively used in ligand binding studies (Sandow and Konig, 1979; Reeves, Seguin, Lefebvre, Kelly and Labrie, 1980; Clayton and Catt, 1981; Hazum, 1981; Meidan and Koch, 1981; Berault, Jansem de Almeida Catanho, Theoleyre and Jutisz, 1983; Eidne, Hendricks and Millar, 1985). Buserelin is an ideal choice for use as an iodinated label for LHRH-receptor binding assays as it is easily iodinated to a high

specific activity of ~ 1100μCi/μg and the mono-iodinated buserelin readily purified (Chapter 2.3). ^{125}I-Buserelin has been found to retain its specific binding activity for 2-4 weeks when stored at 4°C (Clayton and Catt, 1981), the affinity constant shown to be the same as that of unlabelled buserelin, with up to 60% of the total binding to rat anterior pituitary membrane preparation being specific (Clayton, Shakespear, Duncan and Marshall, 1979(b)). Buserelin - generously donated by Hoechst A.G. (Frankfurt, F.R.G.) has been used in the majority of the ligand binding experiments in this Thesis as the radiolabel. ^{125}I-Buserelin binding has been shown to maximal at 4°C to both dispersed rat pituitary cells (Meidan and Koch, 1981) and to rat anterior pituitary membranes (Clayton and Catt, 1981), reaching an equilibrium after about 90 minutes (Chapter 2.4.1.2). During this time there is no significant degradation of either the radiolabelled or unlabelled buserelin (Clayton, Shakespear, Duncan and Marshall, 1979(b)). Since a variety of cations Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} (Clayton, Shakespear and Marshall, 1978; Marian and Conn, 1980; Mitchell, Ogier, Johnson, Cleland, Bennie and Fink, 1985) reduce the specific binding of ^{125}I-buserelin, assays here (as by other workers) were routinely carried out in 25mM Tris-HCl buffer, pH 7.4 with 0.1% BSA to reduce nonspecific binding of the peptides to the plastic tubes (Clayton, Shakespear, Duncan and Marshall, 1979(a)).

The second messenger systems mediating the action of LHRH on gonadotrophes have been extensively investigated. Early studies of the responsiveness of anterior pituitary tissue to crude hypothalamic extract showed a requirement for extracellular calcium for LH release from gonadotrophes (Samli and Geshwind, 1968;

Wakabayashi, Kamberi and McCann, 1969). A role for cyclic AMP was suggested but,whilst some groups found an increase in cyclic AMP levels after LHRH stimulation (Borgeat, Chevaney, Dupont, Labrie, Arimura and Schally, 1972; Labrie, Borgeat, Lemay, Lamarie, Barden, Drouin, Lemaire, Jolicoeur and Belanger, 1974; Adams, Wagner, Sawyer and Nett, 1979),there were inconsistencies. In some cases no changes in the levels of cyclic AMP were seen (Sundberg, Fawcett and McCann, 1976; Conn, Dufau and Catt, 1979; Conn, Morrell, Dufau and Catt, 1979) or no activation of adenylate cyclase was observed (Theoleyre, Berautt, Garnier and Justisz, 1976; Clayton, Shakespear and Marshall, 1978). Naor, Zor, Meidan and Koch (1978) found that whilst male anterior pituitaries showed an increase in cyclic AMP in response to LHRH, this was not seen in tissue from either female or castrated male. A possible role for cyclic GMP was also suggested (Naor, Clayton and Catt, 1980) with increases in guanylate cyclase activity demonstrated (Vesely, 1985). However both cyclic AMP and cyclic GMP changes can be experimentally dissociated from the LH response to LHRH (Cronin, Evans, Hewlett, Rogol and Thorner, 1983; Naor and Catt, 1980).

Whilst a direct role for the cyclic nucleotides in LHRH-induced LH release seems rather unlikely, the role of calcium appears to be more certain. Conn, Marian, McMillian and Rogers (1980) put forward three basic criteria to be fulfilled by calcium if its role in mediating LHRH stimulated LH release was to be correct. These were:

a) Blockade of LHRH-stimulated LH release in the absence of calcium
b) Release of LH in response to an increase in intracellular calcium levels
c) An increase in intracellular calcium levels in response to LHRH.

LH responses to LHRH are inhibited by the removal of calcium from the extracellular medium (Bourne and Baldwin, 1980; Stern and Conn, 1981; Borger, Scott, Kaiser, Evans and Thorner, 1983) or by the presence of calcium channel blockers (Pickering and Fink, 1979; Marian and Conn, 1979; Stern and Conn, 1981; Bates and Conn, 1984), and calcium ionophores give rise to a concentration-dependent LH release that is blocked by the presence of calcium chelators such as EGTA (Conn, Kilpatrick and Kirshner, 1980; Conn and Rogers, 1980). These results would seem to fulfil the first two criteria for the role of calcium in mediating the LHRH response. Experiments using the calcium fluorophore Quin 2 (Tsien, Pozzan and Rink, 1982) show an increase in fluorescence levels when pituitary cells (enriched for gonadotrophes) are exposed to LHRH (Clapper and Conn, 1985) indicating increases in free intracellular calcium. This would appear to fulfil the third criteron. Although many of these studies indicate that extracellular calcium is required for LHRH action, it is possible that other (cellular) stores of calcium may play a role. Some studies have shown that there is a delay (of a few minutes) between the removal or block of the extracellular calcium supply and inhibition of LHRH stimulated LH release (Borger, Scott, Kaiser, Evans and Thorner, 1983; Bates and Conn, 1984). Efflux of $^{45}Ca^{2+}$ in response to LHRH is inhibited but not abolished by the removal of extracellular calcium (Williams, 1976) and a calcium ionophore able to cross cell membranes, X537A, was able to cause LH release independent of extracellular calcium (Conn, Rogers and Sandhu, 1979). These initial studies, suggesting a component of LHRH induced LH release results from the liberation of intracellular calcium stores, have been substantiated by more recent work. Chang,

McCoy, Graeter, Tasaka and Catt (1986) showed that nitrendipine (a voltage-sensitive calcium channel blocker) could fully block the potentiation of submaximal responses to LHRH that was caused by BAY K8644 (a voltage-sensitive calcium channel activator) but it could only partially inhibit the LHRH-induced LH release or increase in Quin 2 fluorescence. This could be due to a role for intracellular calcium or alternatively, the presence of non-dihydropyridine-sensitive calcium channels in gonadotrophes, for which there is not yet evidence. In a calcium free, EGTA medium, LHRH gives rise to an initial spike of Quin 2 fluorescence, but is unable to maintain a plateau of increased fluorescence as seen in the presence of extracellular calcium (Limor, Ayalon, Capponi, Childs and Naor, 1987). Another (related) voltage-sensitive calcium channel blocker, nimodipine, was found to block LHRH induced $^{45}Ca^{2+}$ influx in anterior pituitary tissue whereas the transient efflux of $^{45}Ca^{2+}$ in response to LHRH was blocked by stabilisers or depletors of non-mitochondrial intracellular calcium stores (Mitchell, Johnson, Ogier and Fink, 1988). LHRH would therefore seem to utilise two sources of calcium in the mediation of its response in anterior pituitary cells:-

i) Intracellular calcium from non-mitochondrial stores.

ii) Extracellular calcium via voltage-sensitive calcium channels.

Electrophysiological studies on gonadotrophs confirm the idea that LHRH results in the activation of voltage-dependent calcium channels (Mason, Bicknell, Cobbett, Waring and Ingram, 1986). The activation of a large conductance calcium-dependent K^+ channel as a result of LHRH application to gonadotrophs was also seen, whilst action potentials did not occur even in response to depolarisation

of the cell membrane. Effects of LHRH on K^+ channels have also been seen using intracellular recording techniques in bullfrog sympathetic ganglia (Jan, Jan and Kuffler, 1979; Adams and Brown, 1980; Smith and Zidichouski, 1985). Here the channels involved appear to be those of the voltage-sensitive M-current. The activation by acetylcholine, histamine and dopamine of similar K^+ channels in Aplysia ganglion cells (recorded using voltage clamp techniques) was shown to be blocked by intracellular injections of Pertussis toxin. Furthermore, GTPγS caused a slow and irreversible opening of the channel at a rate dependent on the level of agonist activation (Sasaki and Sato, 1987). These results suggest a receptor modulation of the K^+ channel mediated by the direct interaction of a G-protein. In embryonic atrial cells, using a whole cell voltage clamp method, activation by cholinergic agonists of inward rectifying, voltage sensitive K^+ channels was shown to be independent of a diffusible second messenger, have a delay of 30-100 msecs, require GTP and be blocked by Pertussis toxin (Pfaffinger, Martin, Hunter, Nathanson and Hille, 1985). Perfusion of purified G-protein subunits revealed that the α_i subunit was the direct activator of the K^+ channel (Yatani, Cordina, Brown and Birnbaumer, 1987). Studies showing the $\beta\gamma$ complex of G-protein subunits to be the activators (Logothetis, Kurachi, Galper, Neer and Clapham, 1987) have been questioned and suggested to be a result of α subunit contamination (Rosenthal and Schultz, 1987).

In addition to an apparent direct role in the receptor mediated activation of K^+ channels in some tissues, results of work by Gomperts (1983) suggest a role for guanine nucleotides in the mechanism of action of calcium-mobilizing receptors. Introduction

of non-hydrolysable GTP analogues (such as GTPγS) into permeabilized mast cells and increasing extracellular calcium concentrations resulted in exocytosis dependent upon GTP analogue concentration. This GTP-specific exocytosis was not observed when the mast cells were metabolically inhibited. Evidence for a role of guanine nucleotides in LHRH actions has been found in primary cultures of enriched gonadotrophes where GTP analogues result in a time and concentration-

dependent stimulation of LH release and inositol phosphate accumulation. Since this can be blocked by LHRH antagonists, a close association between the LHRH receptor and a G-protein has been suggested (Andrews, Staley, Huckle and Conn, 1986). However no effect of guanine nucleotide on agonist binding affinity (as characterises receptors linked via a G-protein to adenylate cyclase; Blume, 1978; Glossman and Presek, 1979; Tsai and Lefkowitz, 1979; Shane, Gammon and Bilzeikian, 1981) has been demonstrated to confirm the interaction between LHRH receptors and G-proteins (Hazum, 1981(a); Perrin, Haas, Rivier and Vale 1983(a)).

As well as anterior pituitary gonadotrophes, early studies using ^{125}I-LHRH suggested that a low affinity receptor could be found in other tissues such as liver, spleen, lung and gonads (Heber, Marshall and Odell, 1978). Using the more reliable LHRH analogues however, high affinity binding sites could be detected only in the gonads (Reeves, Seguin, Lefebvre, Kelly and Labrie, 1980; Clayton and Catt, 1981; Pieper, Richards and Marshall, 1981). Binding of LHRH analogues suggested that these sites were similar to those found in anterior pituitary (Reeves, Seguin, Lefebvre, Kelly and Labrie, 1980) and physiological actions of LHRH at the gonads have been shown (Sharpe, 1980).

In the brain immunohistochemical techniques have been used to detect LHRH containing cell bodies and axons (Barry, 1979; Moss, 1979; McCann, 1982). Most LHRH immunoreactivity is found in the hypophysiotropic area - that is the medial basal hypothalamus and preoptic area - with rather small amounts seen in some extrahypothalamic tracts such as the hypothalamo-mesencephalic, preopticosupraoptic and rostral limbic tracts (see Krey and Silverman, 1983, for review) . The projections are largely to regions thought to be involved in inducing mating behaviour (McCann and Moss, 1975; Samson, McCann, Chud, Dudley and Moss, 1980). Both behavioural (McCann and Moss, 1975; Moss, 1977; Moss, 1979) and electrophysiological (Moss 1977; Moss and Dudley, 1978; Sakuma and Pfaff, 1979; Samson, McCann, Chud, Dudley and Moss, 1980) data suggest a physiological role for LHRH in the brain in determining sexual responsiveness.

Autoradiography using ^{125}I-[DAla^{6}, N_{α} Me Leu^{7}, des Gly^{10}]-LHRH ethylamide has revealed high levels of specific LHRH receptors in CA_1, CA_2 and CA_3 of rat hippocampus (Reubi and Maurer, 1984). These receptors were shown to have a similar rank order of affinity for several LHRH analogues to that seen in anterior pituitary tissue (Reubi, Palacios and Maurer, 1987) whereas those in the preoptic and mesencephalic grey areas show potency ratios for analogues that are at variance with those in anterior pituitary (Moss and Dudley, 1978; Sakuma and Pfaff, 1983). These results may suggest that whilst the receptors seen in hippocampal areas are similar to those found in the anterior pituitary, those involved in mediating the role of LHRH in sexual responsiveness in forebrain areas are different.

In situ hybridization has revealed LHRH-like mRNA in cell bodies in rat forebrain which contain LHRH immunoreactivity (Shivers, Harlan, Jentmancik, Conn and Pfaff, 1986). The pre-pro-LHRH protein (encoded for by the LHRH mRNA) has been found using recombinant DNA techniques to be 92 amino-acids in length (Seeburg and Adelman, 1984; Fig 3.13). The presence in the sequence of one signal peptide and two possible enzymatic cleavage sites is said to be indicative of a poly-protein precursor (Douglass, Civelli and Herbert, 1984). That is the 56-amino-acid C-terminal extension of the pre-pro-LHRH following the LHRH sequence and its cleavage site (gonadotrophin-associated-peptide, GAP) may have a functional role. This has been said to be further implicated by the finding that immunoreactive GAP and LHRH coexist in secretory granules (Nikolics, Mason, Szonyi, Ramachandran and Seeburg, 1985). The same group have demonstrated an LH- and FSH-releasing and prolactin release-inhibiting action of GAP (1-56) on cultured rat anterior pituitary cells (Nikolics, Mason, Szonyi, Ramachandran and Seeburg 1985). Whilst the dose-response relationship for LH and FSH release by GAP was similar to that of LHRH and inclusion of both GAP and LHRH did not result in additive responses (suggesting that the same post-receptor mechanisms are used by both peptides),the effect of an LHRH antagonist on GAP actions was not shown. Whether or not GAP is acting through specific LHRH receptors has therefore not been demonstrated.

In this Thesis, several aspects of the membrane bound LHRH receptor have been investigated using ligand binding techniques. Initial studies on analogue binding have been used to validate the binding assay used. A possible involvement of potassium channels

and G-proteins in mediating gonadotrophe responses to LHRH binding and the interaction between the receptor and second messenger has been investigated. Extrapituitary binding of LHRH in hippocampal tissue was examined along with the binding of a fragment of GAP (GAP 27-41, prepared by Dr. C.M. Bladon for use in immunological studies and kindly made available for these experiments). In particular the possibility that GAP may be binding to LHRH receptors was investigated. Finally, as the number of high affinity, specific LHRH receptors found in rat anterior pituitary tissue is low, 206fmol/mg protein (Conne, Aubert and Sizonenko, 1979) or about 90fmol per gland, anterior pituitary tissue from other species have been examined as a potential source of larger quantities of the receptor protein.

3.2 Materials and Methods

3.2.1 Materials

Buserelin ([D-Ser (Bu^t)^6 des Gly^{10}]-LHRH-ethylamide) was a generous gift from Hoechst A.G. (Frankfurt, F.R.G.). LHRH and its analogues [des $pGlu^1$]-LHRH and [D-$pGlu^1$,D-Phe^2, D-$Trp^{3,6}$]-LHRH were obtained from Sigma Chemical Company Ltd (Dorset, England) 4-AP., (4-aminopyridine) and 3,4-DAP (3,4-diaminopyridine) were also from Sigma, as were GTPγS (guanosine 5'-O-(3-thiotriphosphate)), ATP (adenosine triphosphate), EGTA (ethylene glycol bis (β-aminoethylether)N,N,N',N'-tetraacetic acid), EDTA (ethylenediaminetetraacetic acid), diamide and dithiothreitol (DTT). The LHRH antagonist ([$DpGlu^1$,$DPhe^2$,$DTrp^{3,6}$,$desGly^{10}$] -LHRH-ethylamide) and the GAP (27-41) fragment (gonadotropin-associated peptide, H-Cys-Thr-Thr-His-Glu-Pro-Arg-Ser-Pro-Lev-Arg-Asp-Lev-Lys-Gly-OH) were synthesised by Dr C M Bladon (MRC Brain Metabolism Unit, Edinburgh).

3.2.2 Methods

3.2.2.1 LHRH analogue binding to rat anterior pituitary membrane preparations.

Details of the rat anterior pituitary dissection and equilibrium binding assay are given in Chapter 2 (2.2 and 2.4). Briefly male Wistar rats(200-250g) were stunned, decapitated and their anterior pituitary glands rapidly removed. These were placed in 100 volumes ice-cold 25mM Tris-HCl pH 7.4 and sonicated, followed by centrifugation at 60,000g for 15 minutes at 4°C. The washed membranes were then resuspended into 20 volumes of 25mM Tris-HCl pH 7.4, 0.1% bovine serum albumin (Tris/BSA).

Assays were carried out in a total volume of 500μl containing

10-60μg protein, ~ 50,000 cpm (approx 25pM) ^{125}I-buserelin (iodinated by the chloramine-T method, see Chapter 2.3 for details) and LHRH analogues as appropriate in Tris/BSA. Non-specific binding was determined in the presence of 1μM LHRH. Incubation for 90 minutes at 4°C was followed by PEG precipitation (Chapter 2.4.1.3). The resultant pellet containing bound ligand was counted. Displacement of specific ^{125}I-buserelin binding by LHRH and buserelin (agonists), [des pGlu^{1}]-LHRH (partial agonist) and [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH (antagonist) was determined.

3.2.2.2 Effect on LHRH receptor affinity of K^+ channel manipulation

To determine whether or not the LHRH receptor interacted with potassium channels directly, the effect of K^+ ions and several blockers of K^+ channels on the affinity of the LHRH receptor for buserelin was investigated.

Any close relationship between the LHRH receptor and K^+ channels may possibly be revealed by a change in the affinity state of the receptor in the presence of high concentrations of K^+ ions. Hazum (1981(a)) showed that for female rat anterior pituitary tissue, K^+ ion concentrations (> 50mM) reduced agonist specific binding whilst the effect on antagonist binding was much less. This suggests that there may be an allosteric interaction between K^+ channels and LHRH receptors. As such the affinity of the agonist buserelin was here determined by displacement of ^{125}I-buserelin as described above (3.2.2.1) but with the addition of a high concentration of K^+ ions to the assay buffer. The assay was carried out in triplicate or presence of various concentrations of either 4-AP or 3,4-DAP.

3.2.2.3 The effect of guanine nucleotides on LHRH receptor affinity

In order to try and reveal an effect on LHRH agonist binding affinity by activation of G-proteins, the binding of LHRH analogues in the absence and presence of a guanine nucleotide analogue, $GTP\gamma S$, was investigated. An effect might be expected if the LHRH receptor and a G-protein are in close association, as was suggested by the inhibition of guanine nucleotide-stimulated LH release by an LHRH antagonist (Andrews, Staley, Huckle and Conn, 1986). Receptors linked to adenylate cyclase via a G-protein show just such a modulation of agonist affinity by guanine nucleotides (Blume 1978; Glossman and Presek, 1979; Tsai and Lefkowitz, 1979; Shane, Gammon and Bilzeikian, 1981) and conditions revealing this interaction have been used to try to reveal it in the case of the LHRH receptor. Consistently, experiments to show guanine nucleotide effects on binding affinity to different receptors have been carried out at room temperature (Blume, 1978; Tsai and Lefkowitz, 1979) 30°C (Glossman and Presek, 1979; Hulme, Berrie, Birdsall, Jameson and Stockton, 1983) or 37°C (Shane, Gammon and Bilzeikian, 1981). This series of experiments were therefore carried out at room temperature (typically found to be 25°C). Equilibrium at this temperature has been shown (Clayton and Catt, 1981) for LHRH analogue binding, to be reached by 30 minutes, and this was the time course chosen for the binding assays here. Peptidase inhibitors (soybean trypsin inhibitor, 50mg/ml; aprotinin, 400KIU/ml) were included in the Tris/BSA assay buffer to prevent enzymatic degradation of either the LHRH receptor or peptide analogues. Otherwise the binding assays were carried as described above (3.2.2.1), and free ^{125}I-buserelin was separated from the membranes and bound ^{125}I-buserelin by a PEG

precipitation (2.4.1.3), in the presence or absence of a nonhydrolysable GTP analogue, GTPγS. This particular analogue was chosen as it has been found to be the most potent in the activation of G-proteins linked to calcium-mobilizing receptors in mast cells (Gomperts, 1983).

Experiments on the nucleotide effects on adenylate cyclase linked receptors had shown that Mg^{2+} (> 1mM) was essential for GTP analogue induced changes in agonist binding affinity (Tsai and Lefkowitz, 1979; Glossmann and Presek, 1979). Concentrations of Mg^{2+} of 1mM have been shown to reduce LHRH receptor binding by ~ 30% (Marian and Conn, 1980) but have no effect on LHRH-stimulated LH release. Higher concentrations do also inhibit LHRH-stimulated LH release (Conn, Marian, McMillian, Stern, Rogers, Hamby, Penna and Grant 1981). For these reasons 1mM Mg^{2+} was the highest concentration included in the binding assays. The effect of Mg^{2+} on nucleotide actions has been shown to be further enhanced by the presence of calcium chelators (Glossmann and Presek, 1979), so the additional effect of 1mM EGTA was investigated. The GTP-activated phospholipid breakdown and LH release were only seen in gonadotrophs permeabilized with ATP (Andrews, Staley, Huckle and Conn 1986). Whilst permeabilization is required to give intracellular access to the GTP analogues, ATP permeabilization is thought to be mediated by a specific receptor peculiar to mast cells, on which the technique was developed (S. Cockcroft, personal communication to Dr. R. Mitchell). Therefore the possibility exists that ATP may be involved in helping to mediate the GTP effect in gonadotrophes. To test this hypothesis ATP (10μM) was included in one set of binding assays.

The susceptibility of muscarinic receptor sites to guanine nucleotide actions is apparently increased by pretreatment of the membrane preparation with high concentrations of the calcium-chelator EDTA (Hulme, Berrie, Birdsall, Jameson and Stockton, 1983). A similar EDTA pretreatment has been used in some experiments here. Washed male rat anterior pituitary membranes were resuspended into 200 volumes of 25mM Tris/HCl pH 7.4 containing 50μg/ml soybean trypsin inhibitor, 400 KIU/ml aprotinin and 10mM EDTA (Tris/10mM EDTA). After 15 minutes at 37°C the membranes were centrifuged (15 minutes at 60,000g, 4°C) and washed once in Tris/0.1mM EDTA (200 volumes), then resuspended into 60 volumes Tris/0.1mM EDTA/1mM Mg^{2+}. Specific ^{125}I-buserelin binding was then determined in the presence of 0.1mM EDTA/1mM Mg^{2+} with or without GTPγS as described above. Parallel controls (membranes similarly treated in the absence of EDTA and Mg^{2+}) were also assayed.

However, for α_2 and β adrenergic receptors, GTP modulation of affinity has been best demonstrated by changes in full agonist displacement of labelled antagonist (Tsai and Lefkowitz, 1979; De Lean, Stadel and Lefkowitz, 1980). The effect of GTPγS on LHRH displacement of an iodinated LHRH-antagonist (^{125}I-LHRH-ant) from EDTA pretreated membranes was therefore examined. Again binding was carried out in the EDTA/Mg^{2+} medium, both with and without GTPγS, at room temperature for 30 minutes. Various concentrations of LHRH were added to reveal any shift in its binding affinity as a result of the inclusion of GTPγS. The LHRH-antagonist was iodinated by the chloramine-T method (2.3 and Appendix 1).

3.2.2.4 The effect of sulphydryl group-oxidising (diamide) and reducing (dithiothreitol, DTT) agents on buserelin binding to anterior pituitary membranes

Gurwitz, Baron and Sokolovsky (1984) have shown that the presence of sulphydryl oxidising agents (such as cupric ions or diamide) abolishes the sensitivity of muscarinic agonist binding to guanine nucleotides. Subsequent exposure to a reducing agent (DTT) was found to restore the guanine nucleotide-sensitivity (in the case of the diamide treated membranes). They concluded from these studies that sulphydryls (in a reduced state) are involved in the G-protein and muscarinic receptor interaction. In the case of the LHRH receptor Hazum (1981(a)) has shown that, on female rat anterior pituitary membranes, DTT has no effect on either agonist or antagonist specific binding. However, sulphydryl reagents were found to increase agonist binding but have no effects on antagonist binding. As it seems likely that the LHRH receptor interacts with a G-protein (3.1 and 3.2.2.3) the effect of oxidising and reducing agents (diamide and DTT respectively) have been examined using membranes from male rat anterior pituitaries.

Washed membranes were resuspended into 50 volumes of 25mM Tris HCl pH 7.4 containing either 4mM diamide or 100mM DTT. After incubating at 22°C for 30 minutes the membranes were used in a buserelin displacement of ^{125}I-buserelin binding assay. The binding assay was carried out as previously described (2.4) at 4°C with ~ 50,000 cpm ^{125}I-buserelin in a total volume of 500µl, except that either diamide (2.4mM) or DTT (60mM) was present. In some cases GTPγS was added to see if the specific binding of ^{125}I-buserelin in the presence of diamide or DTT was affected by

guanine nucleotides. Non-specific binding was determined in the presence of 1μM LHRH. Parallel controls (membranes similarly treated but in the absence of either DTT or diamide) were included and the assay was carried out in triplicate. After 90 minutes at 4°C the unbound and bound ^{125}I-buserelin were separated by a PEG precipitation step (2.4.1.3) and the aspirated pellet counted by γ spectrometry.

3.2.2.5 ^{125}I-Buserelin binding to rat hippocampal tissue

Whilst autoradiographic studies have shown specific LHRH binding sites in the male rat hippocampus which were similar to those in the anterior pituitary (Reubi and Maurer, 1984; Reubi, Palacios and Maurer, 1987), no studies have been reported on the characterisation of these binding sites using conventional ligand binding techniques. Here the binding of ^{125}I-buserelin to hippocampal tissue from both male and female rats has been examined. Electrophysiological and behavioural responses to LHRH in rat forebrain have been shown to be affected by the hormonal status of female rats (McCann and Moss, 1975; Moss, 1977; Moss and Dudley, 1978; Moss, 1979). Therefore female rats at different stages of the oestrous cycle were used in case the hormonal status influenced the LHRH receptor in hippocampus.

Hippocampal tissue, dissected as in Chapter 2.2 from male or female Wistar rats, was prepared and used in ^{125}I-buserelin ligand binding assays as described (Chapter 2.4.1). Total and non-specific levels of binding were determined.

3.2.2.6 GAP (gonadotropin associated peptide) binding to rat anterior pituitary and hippocampal tissue

The 56 amino-acid peptide GAP has been suggested to be a

prolactin-inhibitory factor (Nikolics, Mason, Szonyi, Ramachandran and Seeburg, 1985). However it was also shown to have a stimulatory action on LH and FSH release. The site of this action was not fully elucidated, although it was suggested that the same post-receptor mechanisms may be utilised by the two peptides. Here we have investigated the binding of a fragment of GAP (GAP(27-41)), both radioiodinated and unlabelled to rat anterior pituitary and hippocampal membranes. Displacement of specific ^{125}I-buserelin by unlabelled GAP (27-41) was examined to determine whether or not LHRH and GAP (27-41) bind to the same receptor site in either the anterior pituitary or hippocampus.

Binding assays were carried out using ^{125}I-buserelin or ^{125}I-GAP(27-41) on rat anterior pituitary and hippocampal membranes as previously described (3.2.2.1 and 3.2.2.5). Non-specific binding was determined in the presence of 1μM LHRH or 1μM GAP (27-41). GAP(27-41) was iodinated by the chloramine-T method (2.3 and Appendix I).

3.2.2.7 Assay of LHRH receptor binding in anterior pituitary tissue of other species

Current methods of protein microsequencing (the ultimate goal of the receptor purification programme) require relatively large amounts of protein (10-100pmol: Hunkapiller, Strickler and Wilson, 1984). The rat anterior pituitary gland contains only approximately 90fmol of LHRH receptors. A rather more abundant source of receptors would therefore be desirable. Anterior pituitary glands from other (larger) animal species have therefore been examined as possible sources of high affinity LHRH receptors.

3.2.2.7.1 ^{125}I-Buserelin binding to bovine anterior pituitary LHRH receptors

As described in Chapter 2.4.2 bovine anterior pituitary tissue was used in ^{125}I-buserelin displacement by unlabelled buserelin. Incubations were for 16 hrs. at 4°C. Separation of non-bound label was by centrifugation for 15 minutes at 1,600 g 6°C and the resultant pellet was counted after aspiration of the supernatant.

3.2.2.7.2 ^{125}I-Buserelin binding to porcine anterior pituitary LHRH receptors

Porcine anterior pituitary tissue was prepared as described for bovine (Chapter 2.4.2.). ^{125}I-buserelin ligand binding assays were set up as described above for both rat and bovine tissue, with total binding, non-specific (1μM LHRH) and various concentrations of unlabelled buserelin (0.01nM - 10nM). The assays were incubated for either 90 minutes, 150 minutes or 16 hrs at 4°C. After this the membranes were centrifuged (15 minutes at 1,600 g, 6°C) to separate non-bound ^{125}I-buserelin from bound, and the resultant pellet was counted.

3.3. Results

3.3.1 Analogue binding to rat anterior pituitary membrane preparations

All four of the LHRH analogues, i.e. native LHRH, buserelin (a superagonist), [des pGlu^1]-LHRH (a partial agonist) and [DpGlu^1, DPhe^2, DTrp^{3,6}]-LHRH (an antagonist), displaced specific ^{125}I-buserelin binding in a concentration dependent manner (Fig. 3.1). Analysis of the displacement data by an error weighted computer programme (Zivin and Waud, 1982; 2.5) revealed their apparent dissociation values (Table 3.1; Fig. 3.2). This suggests that the methods of membrane preparation, peptide iodination and ligand binding assay used here are valid for the investigation of LHRH receptor binding characteristics.

3.3.2 Effects of K^+ and K^+ channel modulators on LHRH receptor binding.

Inclusion of 150mM K^+ in a ^{125}I-buserelin displacement assay apparently did not affect the affinity of buserelin for the LHRH-receptor (Fig.3.3). Total specific binding was 3655 ± 298 cpm and 3512 ± 128 cpm in the absence or presence of 150mM K^+ respectively (58% of total binding being specific in both cases). Calculated K_i values for this experiment were 1.05 ± 0.23nM (± SEM, n = 3) in control displacements and 1.5 ± 0.6nM (± SEM, n = 3) in the presence of 150mM K^+. Whilst the trend was for a higher K_i in the presence of 150mM K^+ there was no significant difference in these two values (Mann Whitney U-test). Previous studies, using 50mM K^+ on total ^{125}I-buserelin binding to male and female anterior pituitary membranes (Mitchell, Ogier, Johnson, Cleland, Bennie and Fink, 1986) had shown a reduction of 44 ± 6% of

Figure 3.1

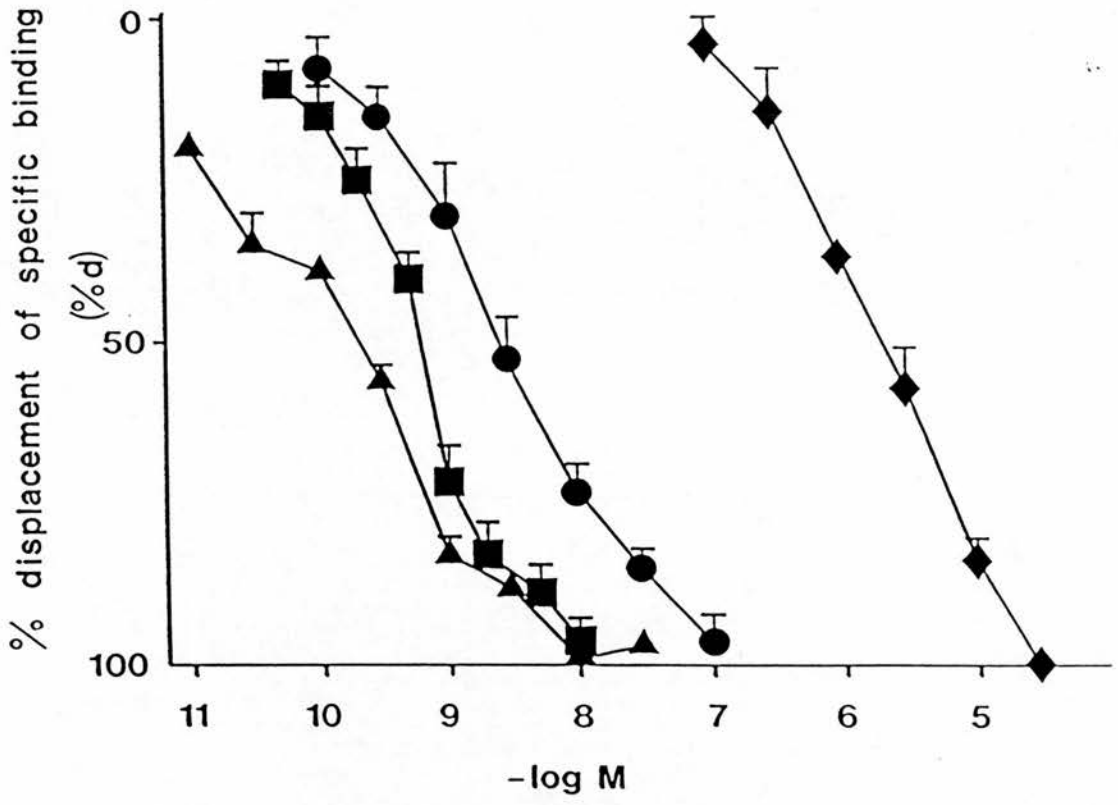


Figure 3.1

Displacement of specific ^{125}I-buserelin binding to rat anterior pituitary gland membranes by LHRH analogues -

(●) LHRH

(■) Buserelin

(◆) [des-pGlu^{3}]-LHRH

(▲) [DpGlu^{1},DPhe^{2},DTrp^{3,6}]-LHRH

Each point is the mean ± SEM from 3-10 separate determinations. Assays were carried out as described (2.4.1.2); non-specific binding being determined in the presence of 1μM LHRH and free ^{125}I-buserelin separated from bound by a PEG precipitation step.

Table 3.1

Analogue	DISSOCIATION (K_i) constant
LHRH	2.20 + 0.72 nM
Buserelin	0.47 + 0.14 nM
[D-pGlu^1,D-Phe^2,D-Trp^{3,6}] LHRH	0.15 + 0.01 nM
[Des-pGlu^1] LHRH	1.52 + 0.26 μM

Table 3.1

Dissociation constants of LHRH analogues for the rat anterior pituitary membrane preparation. ^{125}I-Buserelin displacement data was analysed according to the Eadie-Hofstee method by an error-weighted programme (see 2.5). Values are given as mean ± SEM of 3-6 separate determinations.

Figure 3.2

Hofstee analysis of LHRH displacement of ^{125}I-buserelin from rat anterior pituitary gland membranes. Points are mean values from four separate determinations. Data was analysed using an error-weighted programme (see 2.5). K_i = 2.2 ± 0.72nM (mean ± SEM, n = 4).

Figure 3.2

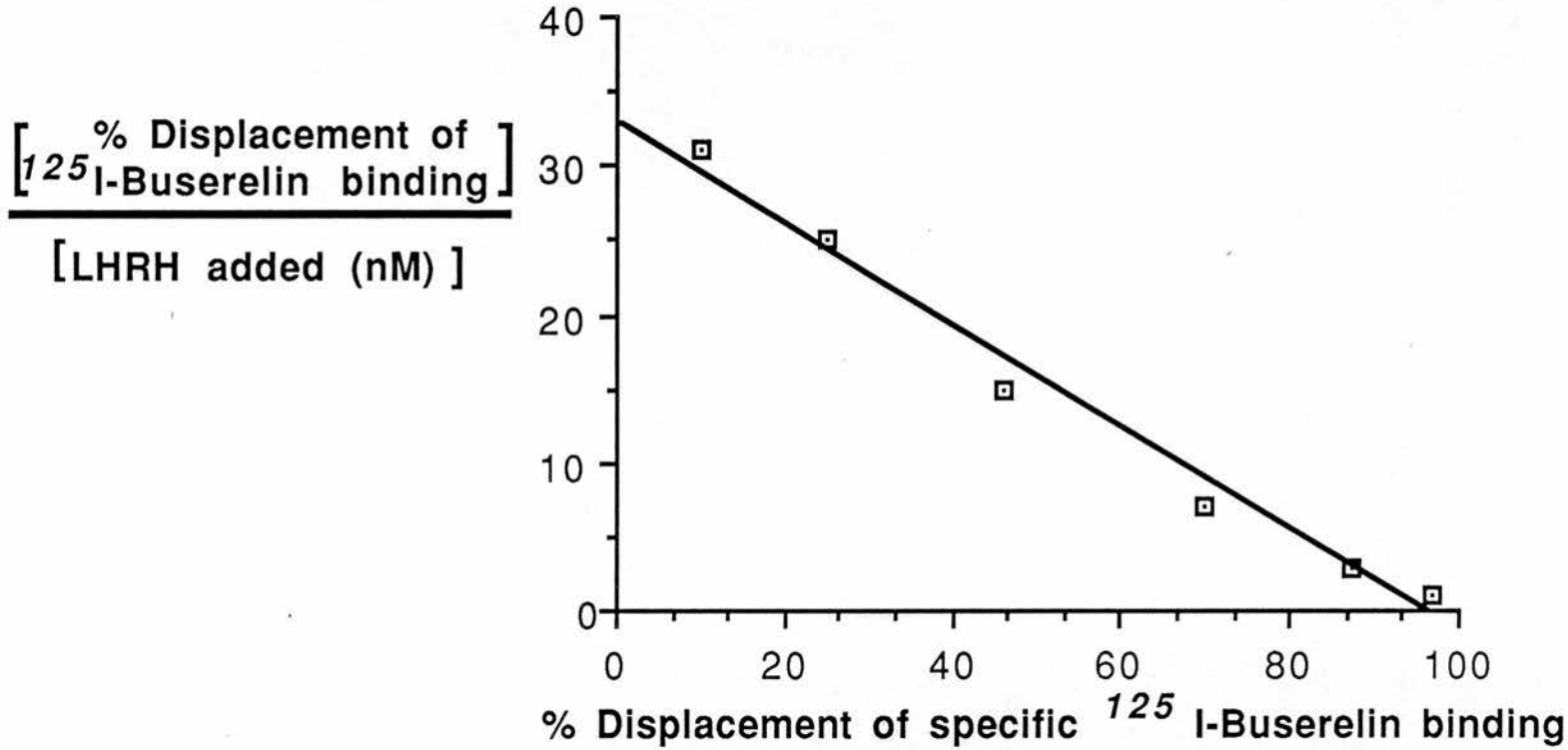
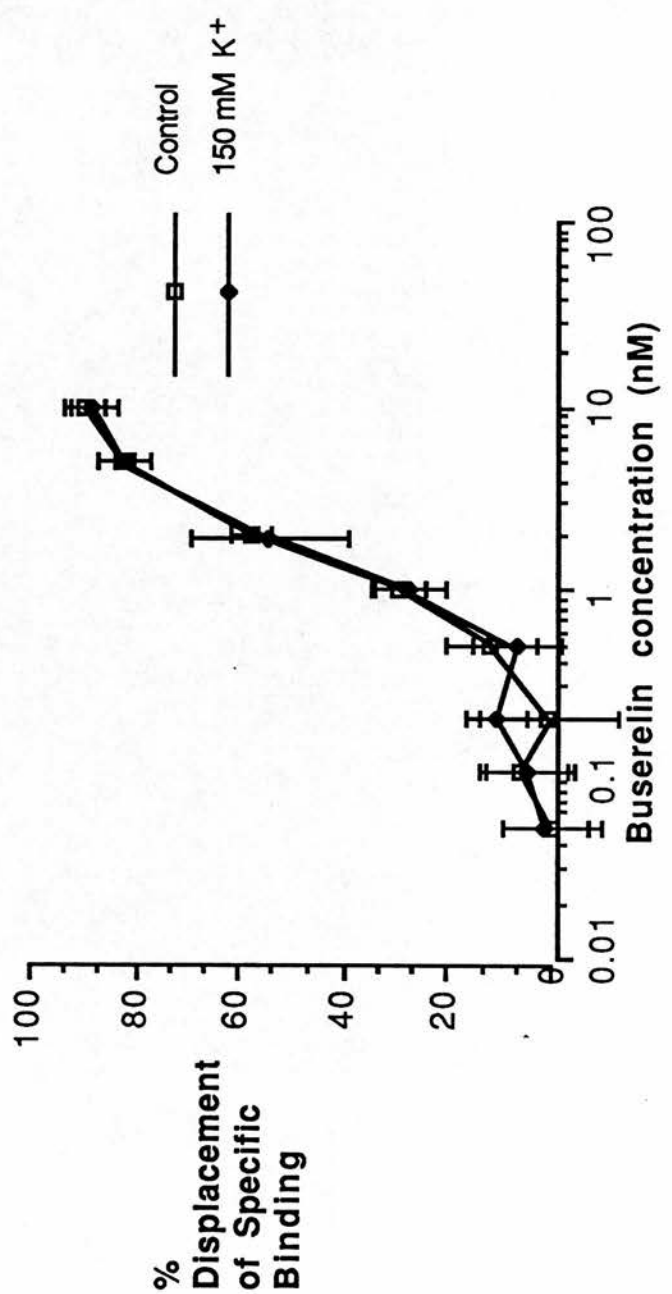

[% Displacement of 125I-Buserelin binding]
[LHRH added (nM)]
40
30
20
10
0
0
20
40
60
80
100
% Displacement of specific 125 I-Buserelin binding

Figure 3.3

Buserelin displacement of ^{125}I-buserelin binding from rat anterior pituitary gland membranes in the absence and presence of 150mM K^+. Points are means ± SEM of triplicate samples. Analysis by an error-weighted programme (see 2.5) revealed K_i values of 1.05 ± 0.23nM for control conditions and 1.5 ± 0.6nM in the presence of 150mM K^+ (mean ± SEM n = 3).

Figure 3.3


% Displacement of Specific Binding
100
80
60
40
20
0
0.01
0.1
1
10
100
Buserelin concentration (nM)
Control
150 mM K+

total specific binding for male anterior pituitary membranes, whereas specific binding in membranes from female rats had been largely unaffected. In a separate study by Hazum (Hazum 1981(a)) concentrations of K^+ ions above 10mM were found to reduce specific ^{125}I-buserelin binding in female anterior membranes. The results from the present experiments, whilst not confirming the clear inhibition of LHRH agonist binding by high concentrations of K^+ ions, do follow the trend for a reduced affinity under such conditions.

Total specific ^{125}I-buserelin binding to rat anterior pituitary membranes was also found to be unaffected by the inclusion of 0.01 - 1mM 3,4-DAP (Fig. 3.4). The channel blocker 4-AP had no effect on the binding except at the highest concentration used, 10mM. 4-AP has previously been shown to block K^+ A-current channels effectively with an IC_{50} of about 1-2mM (Thompson, 1977; Yen, Oxford, Wu and Narahashi, 1976). The 3,4-DAP analogue has been suggested to be a more selective and potent K^+ channel blocker, with a μM IC_{50} (Kirsch and Toshino, 1978). The apparent lack of effect by the more potent of the two blockers and the inability of 4-AP to affect ^{125}I-buserelin specific binding except at 10mM concentrations, suggests that the block of A-current K^+ channels does not have any effect on the affinity of the male rat anterior pituitary LHRH receptor.

3.3.3. Effect of G-protein activation on LHRH receptor affinity.

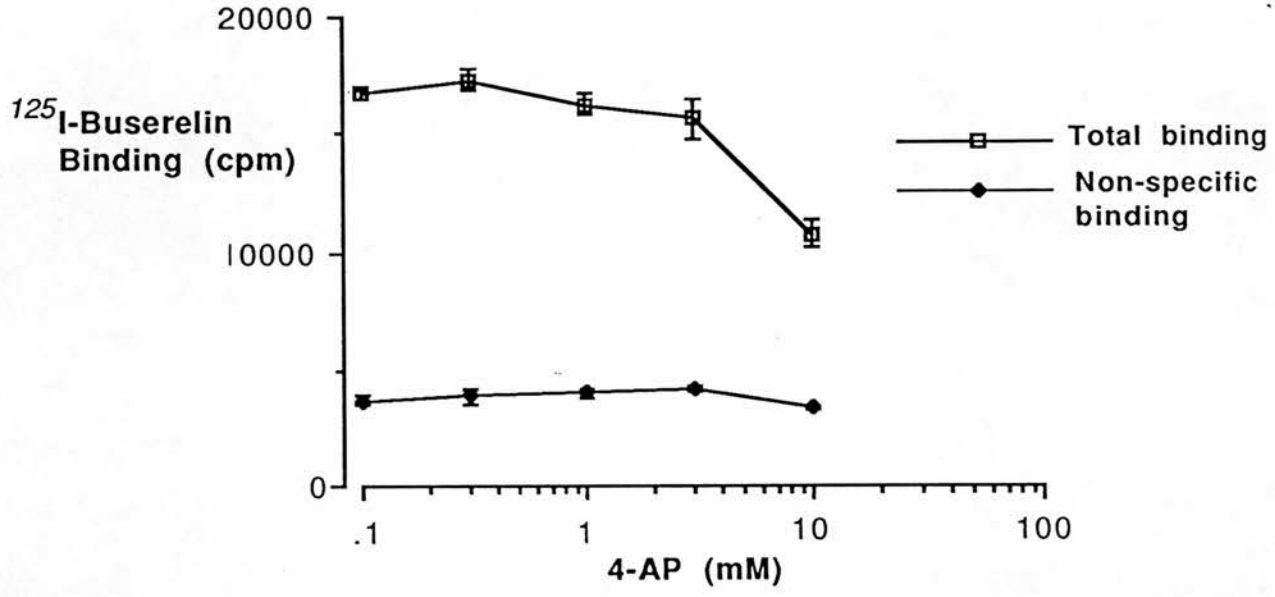
Specific ^{125}I-buserelin binding to rat anterior pituitary membranes was not affected by GTPγS (at concentrations in the range (3-300μM) shown previously to result in polyphosphoinositide phosphodiesterase activation: Cockcroft and Gomperts, 1985; and

Figure 3.4

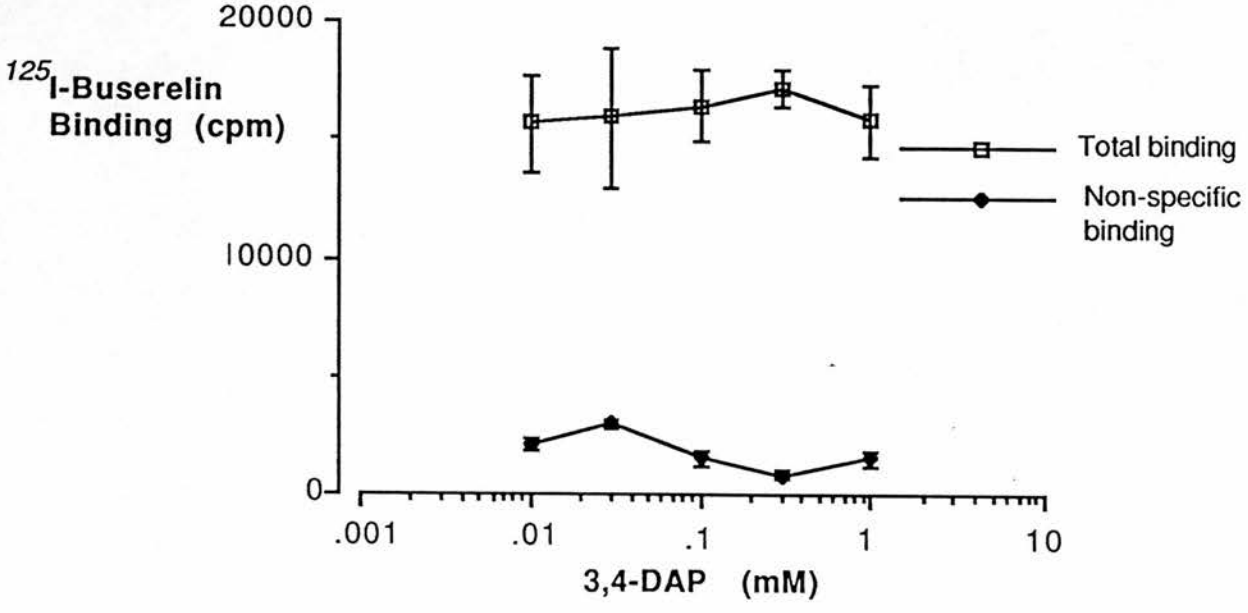
^{125}I-Buserelin binding to rat anterior pituitary gland membranes in the presence of various concentrations of the K^+ A-current channel blockers 4-AP and 3,4-DAP. Points are means ± SEM of four determinations, non-specific binding was determined in the presence of 1μM LHRH.

Figure 3.4

a. 4-AP



b. 3,4-DAP



activation of K^+ channels linked directly to a G-protein: Sasaki and Sato, 1987) under any of the three conditions tested. That is:

i) In the presence of 1mM Mg^{2+} (Fig. 3.5(a))

ii) 1mM Mg^{2+} and 20μM ATP (Fig. 3.5(b))

iii) 1mM Mg^{2+} and 1mM EGTA (Fig. 3.5.(c))

In view of this lack of effect and since in other receptor systems G-protein-receptor interactions had been observed (as a change in agonist affinity) more clearly after pretreatment of the membrane preparation to EDTA (Hulme, Berrie, Birdsall, Jameson and Stockton, 1983), the effect of EDTA pretreatment was examined. It was found that total specific ^{125}I-buserelin binding, whilst reduced both by the preincubation treatment and the exposure to EDTA (Fig. 3.6), was not further changed by the presence of 300μM GTPγS.

It is possible that any change in agonist binding affinity by guanine nucleotides would be revealed more clearly in agonist displacement of a radiolabelled antagonist rather than agonist. This has been previously demonstrated in the case of adenylate cyclase linked receptors and may relate to receptor uncoupling by high affinity agonist ligands (Tsai and Lefkowitz, 1979; De Lean, Stadel and Lefkowitz, 1980). This possibility was investigated.

LHRH displacement of the ^{125}I-LHRH-antagonist was affected by the presence of GTPγS (300μM). The displacement curve was shifted to the right (Fig. 3.7.(a)), suggesting that the affinity of the LHRH-receptor for LHRH was reduced (about 10 fold) by the presence of GTPγS. Unfortunately the binding of the ^{125}I-LHRH antagonist prepared was only about 10% specific (Fig. 3.7.(b)). The large amount of background "noise" that this entailed meant that the LHRH displacement values were not easily calculated. So whilst

Figure 3.5

^{125}I-Buserelin binding to rat anterior pituitary gland membranes in the presence of various concentrations of GTP$_\gamma$S and under different conditions. All binding assays were carried out at 25°C in 500µl Tris-HCl 25mM, pH7.4/0.1% BSA/50µg/ml soybean trypsin inhibitor/400KIU aprotinin/ml, non-specific binding was determined in the presence of 1µM LHRH. All points are means ± SEM of 3-6 determinations.

a) ^{125}I-buserelin binding in the presence of 1mM Mg^{2+}

b) ^{125}I-buserelin binding in the presence of 1mM Mg^{2+} and 20µM ATP.

c) ^{125}I-buserelin binding in the presence of 1mM Mg^{2+} and 1mM EGTA.

Figure 3.5

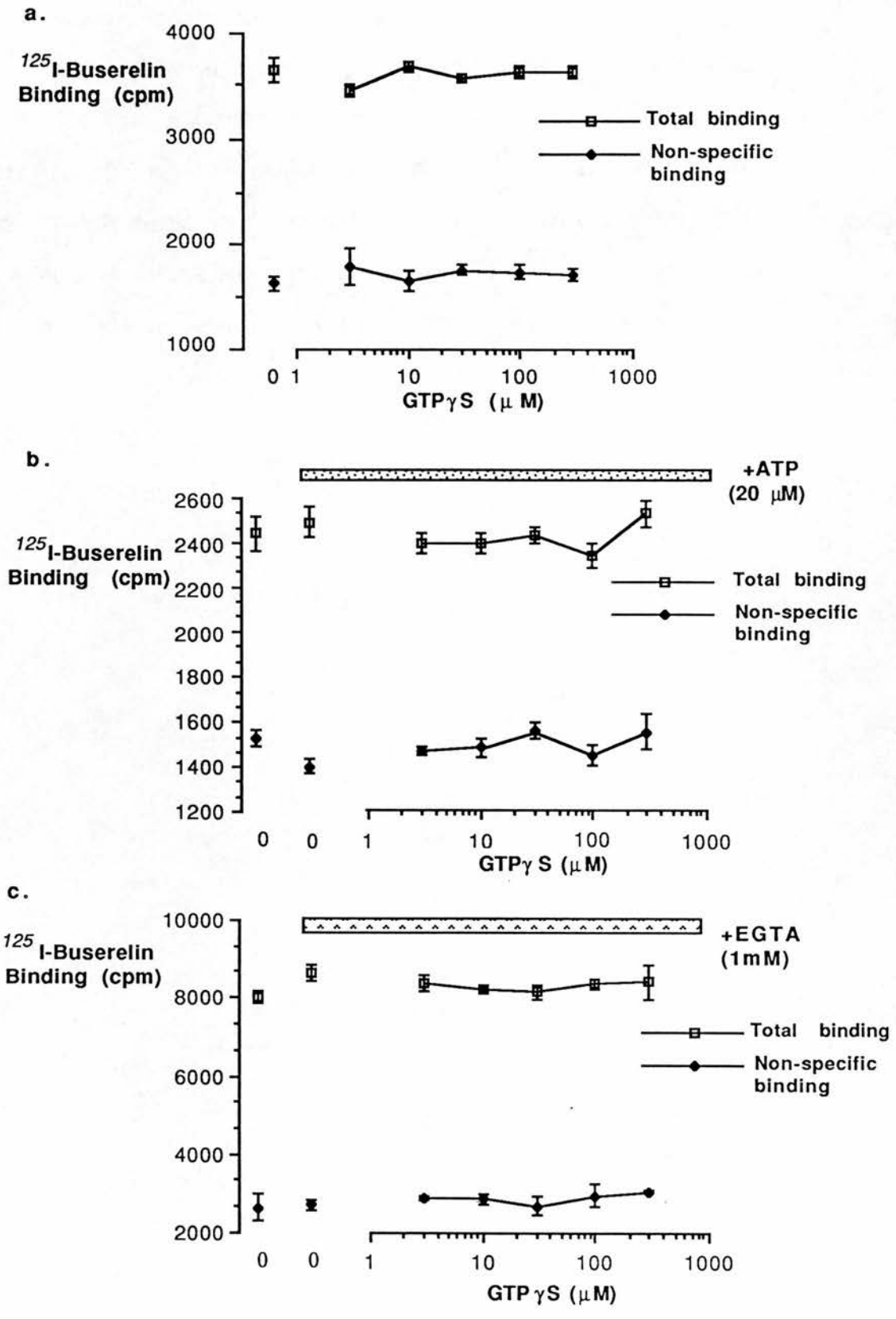

a.
125I-Buserelin Binding (cpm)
4000
3000
2000
1000
Total binding
Non-specific binding
0 1 10 100 1000
GTPγS (μM)
b.
125I-Buserelin Binding (cpm)
2600
2400
2200
2000
1800
1600
1400
1200
+ATP (20 μM)
Total binding
Non-specific binding
0 0 1 10 100 1000
GTPγS (μM)
c.
125I-Buserelin Binding (cpm)
10000
8000
6000
4000
2000
+EGTA (1mM)
Total binding
Non-specific binding
0 0 1 10 100 1000
GTPγS (μM)

Figure 3.6

^{125}I-Buserelin binding to rat anterior pituitary gland membranes at 25°C under control conditions or after a preincubation (15' at 37°C) either in the absence (control) or presence of 10mM EDTA, which was then present at a concentration of 0.1mM with 1mM Mg^{2+} during the binding assay. Inclusion of 300μM GTP$_\gamma$S had no effect on ^{125}I-buserelin binding after EDTA preincubation. All values are mean ± SEM of three determinations, non-specific binding was determined in the presence of 1μM LHRH.

Figure 3.6

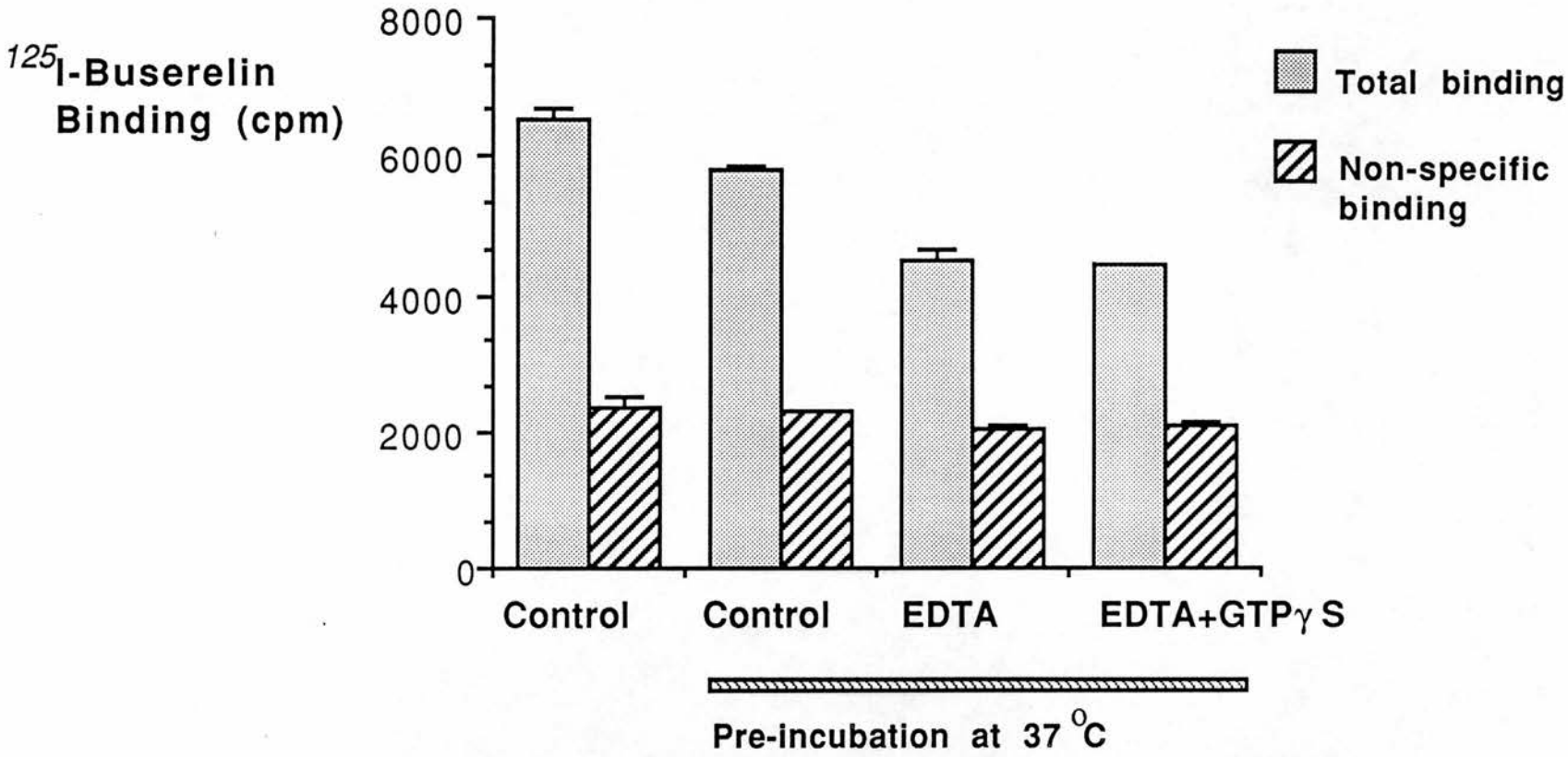

^{125}I-Buserelin Binding (cpm)
8000
6000
4000
2000
0
Total binding
Non-specific binding
Control
Control
EDTA
EDTA+GTPγ S
Pre-incubation at 37 °C

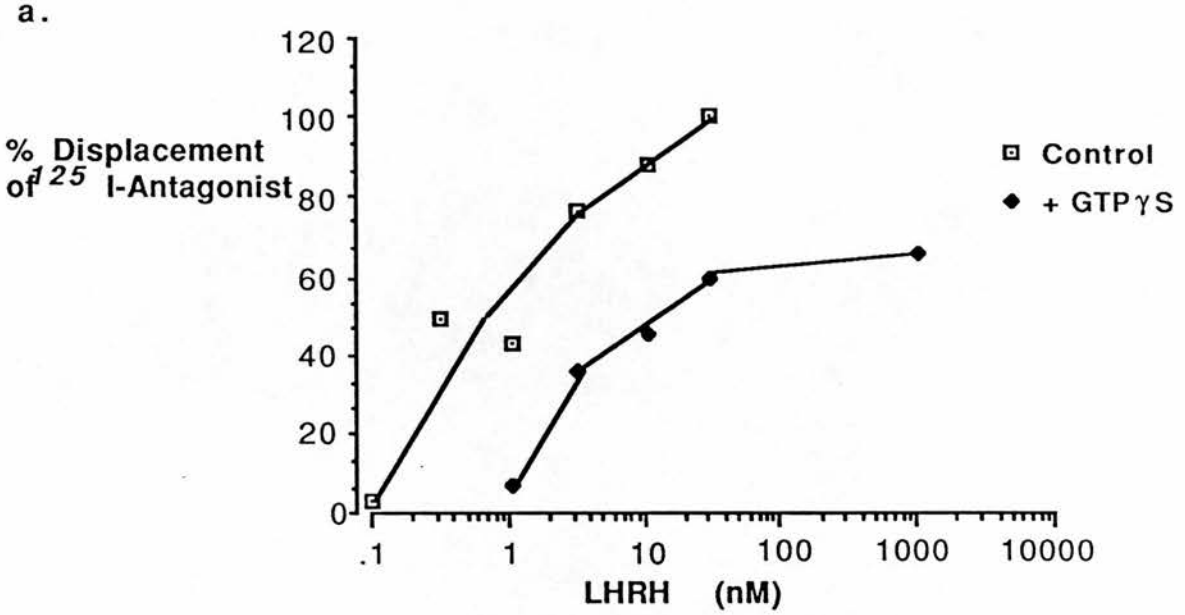
Figure 3.7

a) Displacement of specific ^{125}I-LHRH Antagonist binding to rat anterior pituitary gland membranes by LHRH, at 25°C, in the absence or presence of 300μM GTP$_\gamma$S. Points are mean values of triplicate samples.

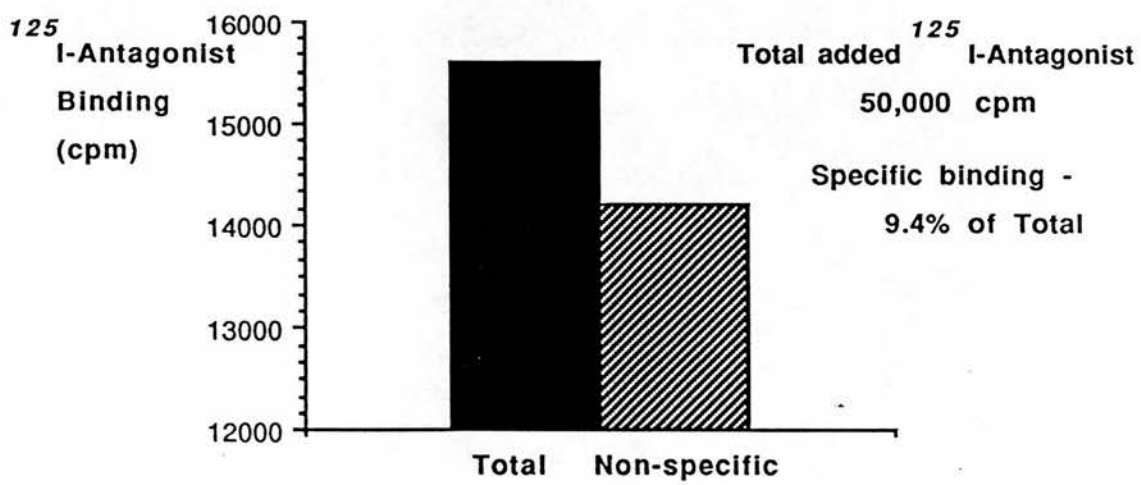
b) Total and non-specific binding of ^{125}I-LHRH Antagonist to rat anterior pituitary at 25°C. Values are means of triplicate samples. Non-specific binding was determined in the presence of 1μM LHRH.

Figure 3.7

a.


% Displacement of 125 I-Antagonist
120
100
80
60
40
20
0
.1
1
10
100
1000
10000
LHRH (nM)
Control
+ GTPγS

b.


125 I-Antagonist Binding (cpm)
16000
15000
14000
13000
12000
Total
Non-specific
Total added 125 I-Antagonist 50,000 cpm
Specific binding - 9.4% of Total

indications of the reduced affinity of the LHRH receptor in the presence of 300μM GTPγS have been observed, reliable K_i values for the LHRH displacement of ^{125}I-LHRH-antagonist could not be determined. The results are however consistent with the hypothesis that the LHRH receptor in male rat anterior pituitary interacts with a G-protein on activation by LHRH agonists.

3.3.4 The effects of oxidising (diamide) and reducing (DTT) agents on buserelin binding to anterior pituitary membranes.

Assay of buserelin displacement of ^{125}I-buserelin specific binding in the presence of 2.6mM diamide on membranes pretreated with 4mM diamide (concentrations found to be effective in previous studies: Gurwitz, Baron and Sokolovsky, 1984) showed a small reduction in total specific binding (89 ± 5% of control values). This was seen as a reduction in both the total binding value (7177 ± 292 cpm compared to 8203 ± 178 cpm for controls) and also a small reduction in non-specific binding (1750 ± 16 cpm compared to 2093 ± 72 cpm, mean ± SEM n = 3 for all values). IC_{50} values for both controls and diamide treated membranes were very similar, 0.4mM and 0.5mM respectively.

Pretreatment of the membrane preparation with DTT resulted in an abolition of virtually all specific ^{125}I-buserelin binding. Total binding value using DTT was 746 ± 28 cpm and the non-specific binding value 644 ± 42 cpm (other conditions were identical to those in the diamide treated and control membrane assays). This is in contrast to the results of Hazum (1981(a)) who found DTT had no effect on specific ^{125}I-buserelin binding. However, the concentrations of DTT used in that study were lower than those used here (0.1 and 2mM compared to 100mM pretreatment and 60mM in the

binding assay in these experiments). It is possible that the high concentrations used in this study (concentrations 10 fold higher than those that had no effect alone but reversed diamide effects on ^{3}H-acetylcholine binding to muscarinic receptors: Gurwitz, Baron and Sokolovsky, 1984) have resulted in a denaturing of the LHRH receptor protein, or buserelin, or both.

The diamide results suggest a slight trend to a lower affinity of the LHRH receptor than controls. Diamide treatment of cerebral cortex membranes has been shown to increase specific ^{3}H-acetylcholine binding and abolish its sensitivity to guanine nucleotides. Whilst 300μM GTPγS had no effect on ^{125}I-buserelin binding to diamide-treated membranes here, no effect on control membrane binding was seen either. The conditions used for the binding assay were very different to those that have revealed an effect on LHRH agonist binding of guanine nucleotides, so this result is not unexpected.

The results of this study neither confirm or deny conclusively the involvement of sulphydryl groups in the interaction between the LHRH receptor and its second messenger system (possibly a G-protein) maintaining the LHRH-receptors' high affinity conformation.

3.3.5. ^{125}I-Buserelin binding to rat hippocampal tissue

No specific ^{125}I-buserelin binding could be detected in rat hippocampal membranes (Fig. 3.8) either in male or female (either pro-oestrous or met/dioestrous rats). Rat anterior pituitary tissue in the same experiment bound ^{125}I-buserelin with 58% specificity (Mean total binding 7667 cpm, mean non-specific binding 3235 cpm). Compared to the anterior pituitary tissue, hippocampal membranes

Figure 3.8

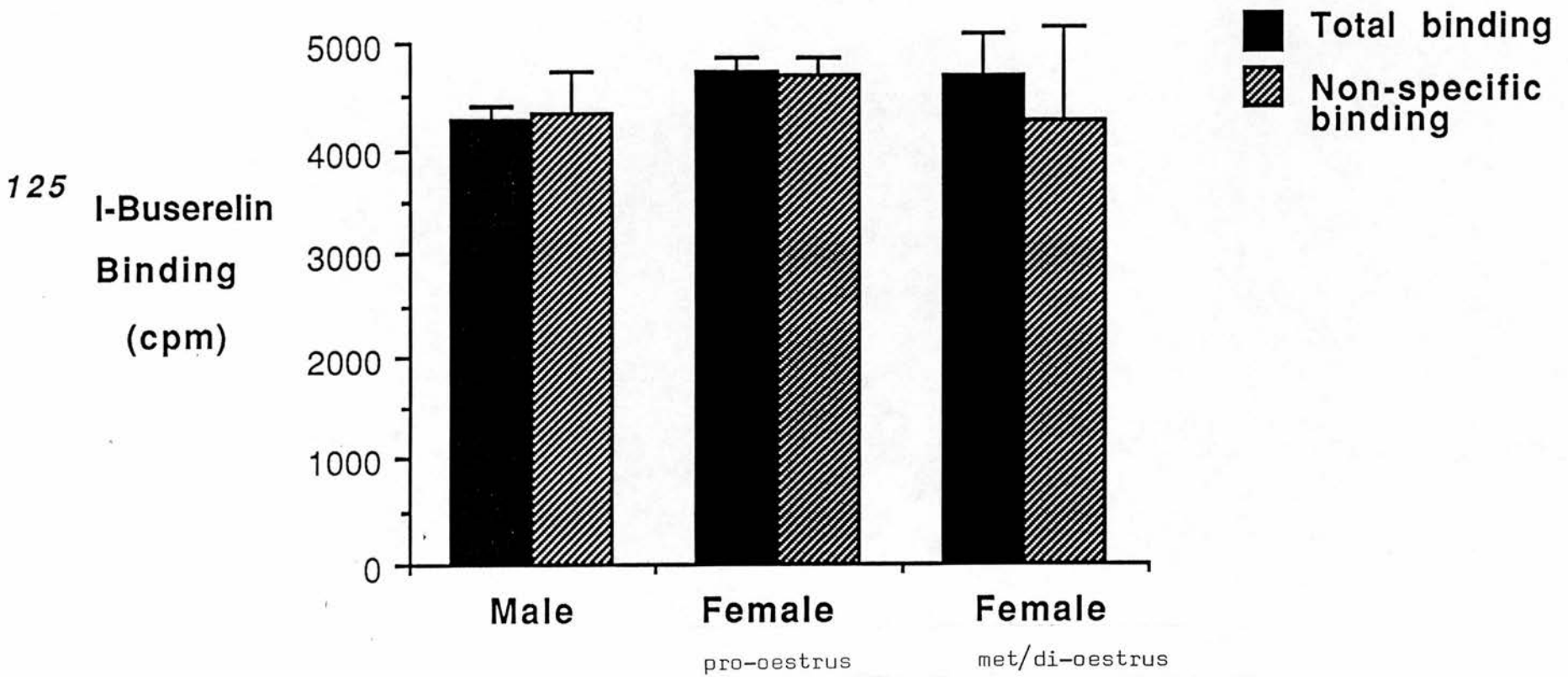


Figure 3.8

^{125}I-Buserelin binding to rat hippocampal membranes. Values are mean ± SEM of four determinations. Non-specific binding was determined in the presence of 1μM LHRH.

showed rather higher levels of non-specific binding (mean value of 4446cpm). The amount of tissue (wet weight) used in the hippocampal binding assay was three times that in the anterior pituitary binding assay. Therefore a higher level of non-specific binding would be expected. More tissue had been used in the hippocampal assay as the autoradiographic study had shown specific LHRH binding sites to be less dense in the hippocampus than the pituitary. ^{125}I-buserelin ligand binding in this study has failed to confirm the presence of specific LHRH receptors in hippocampal tissue.

3.3.6 GAP (27-41) binding to rat anterior pituitary and hippocampal tissue

In neither anterior pituitary or hippocampal tissue was the peptide GAP (27-41) able to displace specific ^{125}I-buserelin binding (Fig. 3.9). This suggests that any specific binding of this peptide is not to LHRH receptors. This result was confirmed by the inability of 1μM LHRH to displace ^{125}I-GAP (27-41) from either tissue preparation. ^{125}I-GAP (27-41) binding was not displaced by unlabelled GAP (27-41) either. This suggests that specific binding sites for this peptide (or at least the fragment investigated) are not found in either male rat anterior pituitary tissue or male or female (prooestrous) rat hippocampal tissue.

3.3.7 Assay of LHRH receptor binding in anterior pituitary tissue of other species.

3.3.7.1 ^{125}I-Buserelin binding to bovine anterior pituitary tissue

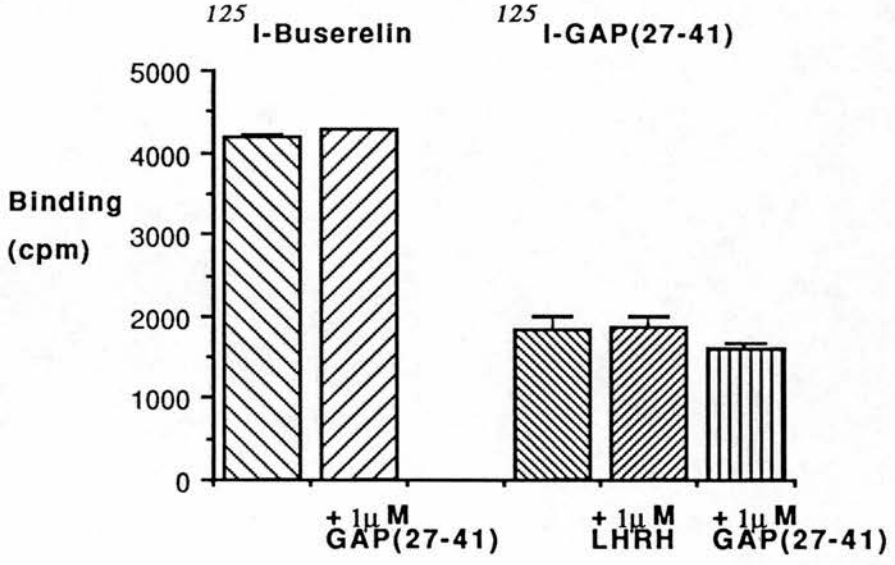
Displacement of ^{125}I-buserelin by unlabelled buserelin from bovine anterior pituitary membranes was found to be dose-dependent (Fig. 3.10). Binding was 22% specific under standard conditions.

Figure 3.9

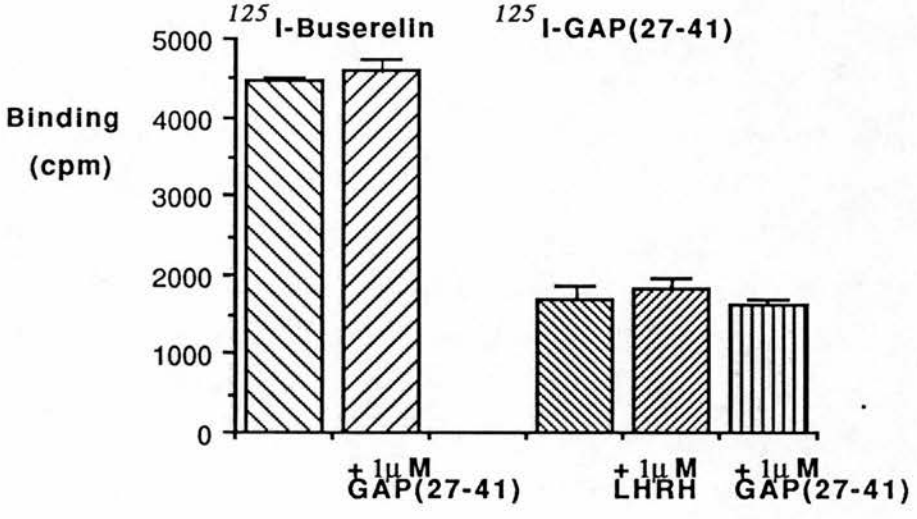
GAP(27-41) binding to rat hippocampal and anterior pituitary gland membranes. 1μM GAP(27-41) was used to try to displace total ^{125}I-buserelin binding to these tissues and 1μM LHRH and 1μM GAP(27-41) to displace ^{125}I-GAP(27-41) total binding to these tissues. Values are means ± SEM for four determinations.

Figure 3.9

a) Male hippocampus



b) Female hippocampus



c) Male pituitary

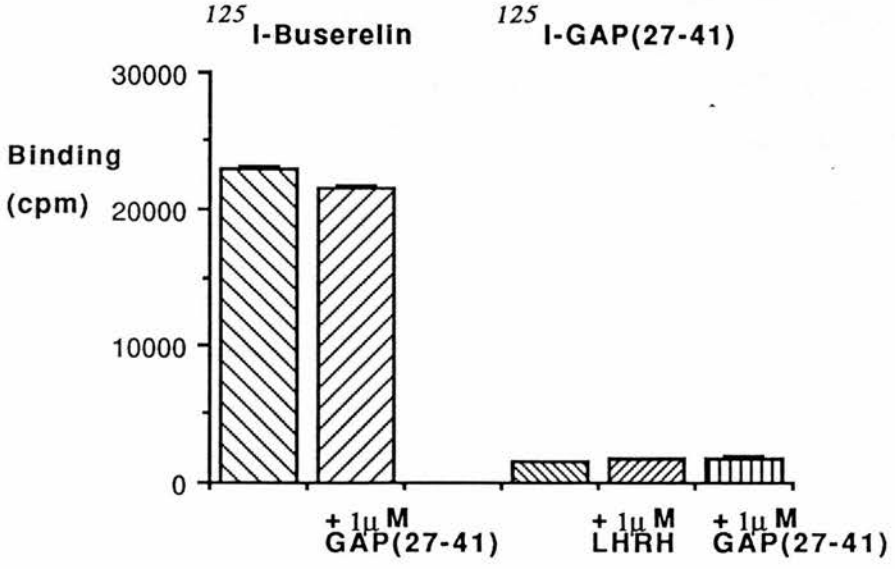


Figure 3.10

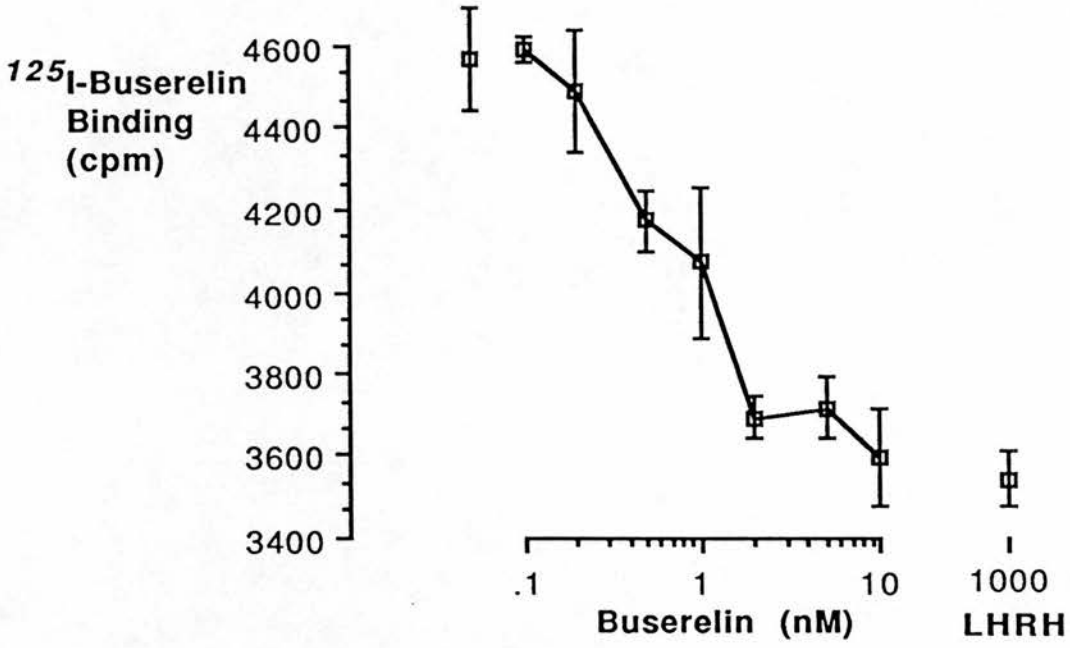


Figure 3.10

Displacement of ^{125}I-buserelin binding to bovine anterior pituitary gland tissue by buserelin and 1μM LHRH. Binding assays were carried out as described (2.4.2) with 50,000cpm ^{125}I-buserelin and ~2.5mg of tissue in 500μl of 25mM Tris-HCl pH7.4; 50μg/ml soybean trypsin inhibitor; 400 KIU/ml aprotinin, reaching binding equilibrium after 16hrs at 4°C. Values are means ± SEM (n = 4).

Analysis of the data (Fig. 3.11) revealed a K_i for buserelin of 0.84 ± 0.17 nM (± SEM, n = 3), similar to that seen in rat anterior pituitary tissue. Tissue (wet weight) equivalent to half a male rat anterior pituitary gland was used in each assay tube. Average specific binding for this amount of tissue was 1024cpm, compared to 5025cpm for anterior pituitary tissue, which was equivalent to binding by a quarter of a male rat gland (2.4.1). That is the density of specific ^{125}I-buserelin binding sites in bovine anterior pituitary is approximately one-tenth of that found in rat anterior pituitary tissue. The calculated total number of specific ^{125}I-buserelin binding sites (cpm equivalent) in a bovine anterior pituitary gland is approximately 350,000 cpm, compared to 20,100 for a rat gland. Despite the reduced density of ^{125}I-buserelin binding sites, bovine anterior pituitaries do contain nearly 18 times the number of sites, which have a similar affinity for ^{125}I-buserelin as the LHRH receptors found in rat anterior pituitary tissue.

3.3.7.2 ^{125}I-Buserelin binding to porcine anterior pituitary tissue.

No specific ^{125}I-buserelin binding could be detected after 90 minutes incubation with porcine anterior pituitary membranes. After 150 minutes, 14% of the total ^{125}I-buserelin binding was specific and could be displaced by unlabelled buserelin in a concentration dependent manner (Fig. 3.12). 50% displacement occurred at a concentration of unlabelled buserelin between 10^{-10} and 10^{-9} M, giving an IC_{50} value of approximately 0.4nM. Increasing the incubation time to 16 hours increased the level of specific ^{125}I-buserelin binding to 26%, and displacement by unlabelled buserelin was similar to that seen after 150 minutes with an

Figure 3.11

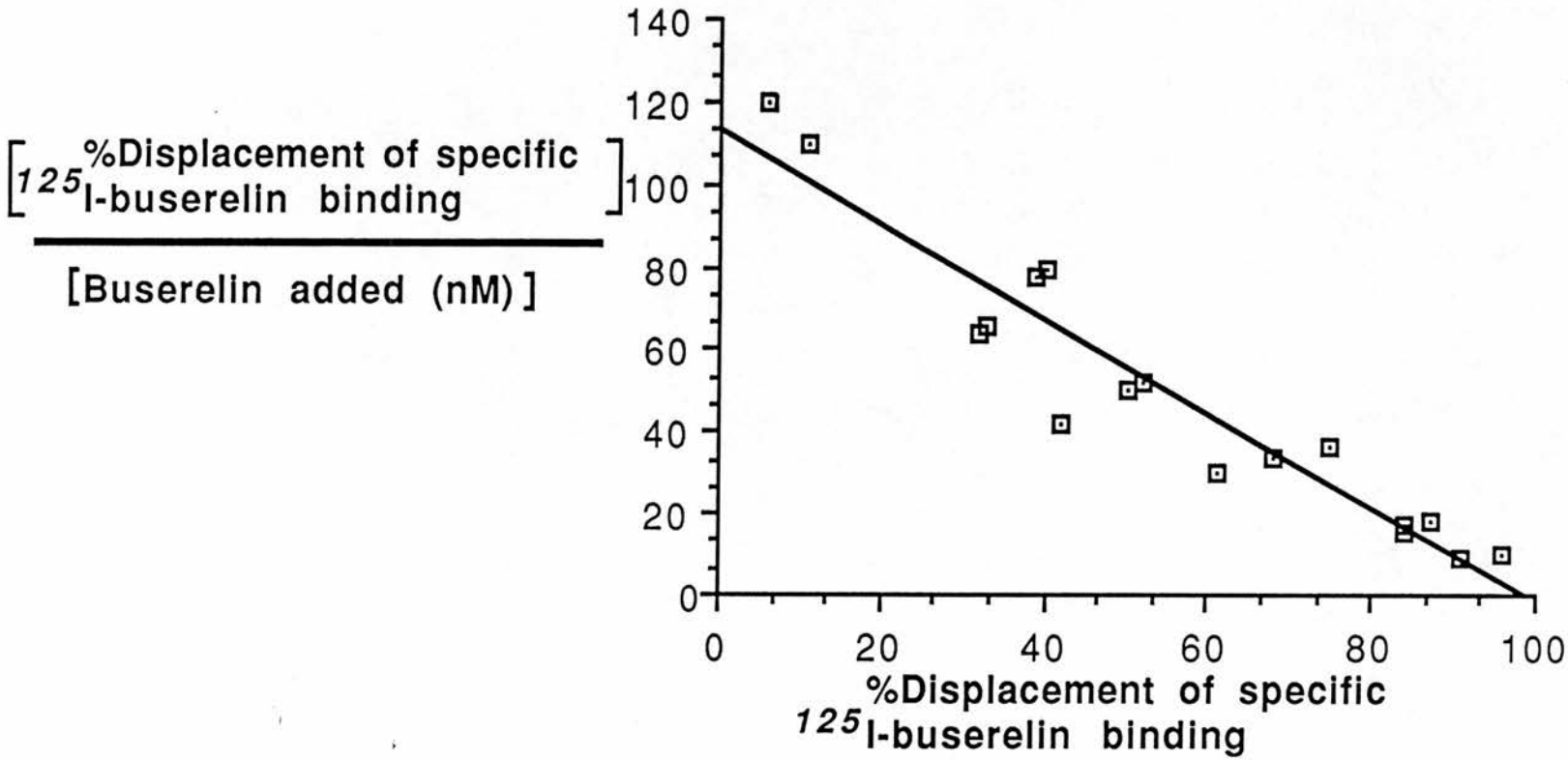


Figure 3.11

Hofstee analysis of buserelin displacement of ^{125}I-buserelin binding to bovine anterior pituitary gland tissue. Non-specific binding was determined in the presence of 1μM LHRH. Displacement data were analysed by an error-weighted programme (see 2.5), points are means of triplicate samples. K_i = 0.84 ± 0.17nM (mean ± SEM, n = 3).

Figure 3.12

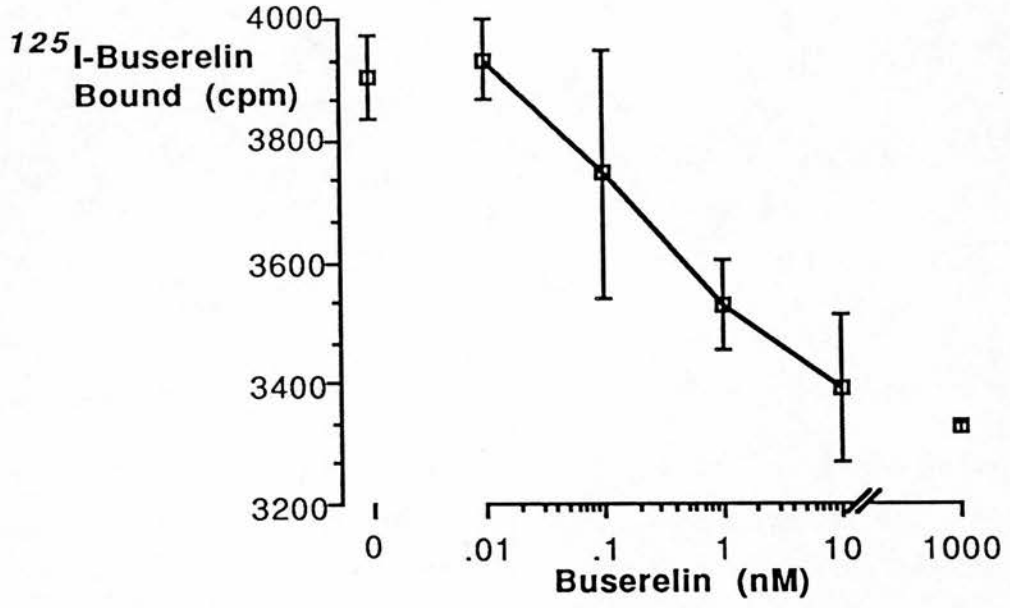


Figure 3.12

Buserelin displacement of ^{125}I-buserelin binding to porcine anterior pituitary gland tissue. Ligand binding was carried out with 50,000cpm ^{125}I-buserelin and ~2.5mg tissue in a total volume of 500µl 25mM Tris-HCl pH7.4; 50µg/ml soybean trypsin ibhibitor; 400 KIU aprotinin; 0.1% BSA with various concentrations of unlabelled buserelin. After 150' at 4°C free ^{125}I-buserelin was separated from that bound to the tissue preparation by centrifugation (15' at 1,600g, 6°C).

apparent IC_{50} value of 0.6nM. The average specific binding (after an incubation period of 150 minutes) was 570 cpm per (rat anterior pituitary gland) tissue equivalent. Porcine anterior pituitary glands appear therefore to contain specific ^{125}I-buserelin binding sites at only one-fortieth the density of male rat glands. Total ^{125}I-buserelin specific binding (cpm) expected under these conditions for a whole porcine gland would be 15,200 cpm, or rather less than that found in one male rat anterior pituitary gland.

3.4 Discussion

3.4.1 LHRH analogue binding to LHRH receptors on rat anterior pituitary membranes

Binding of the analogues to the rat anterior pituitary membranes and displacement of ^{125}I-buserelin gave K_i values very similar in all cases (native LHRH, superagonist, antagonist and partial agonist) to those obtained by other workers (Clayton and Catt, 1980). These results not only validate the techniques used in this Thesis, but also provide reference values for the affinity of the LHRH receptor *in situ* in the rat anterior pituitary membrane preparation. A series of further experiments to investigate whether any allosteric influences on these binding characteristics of the LHRH receptor could be revealed, were undertaken. Any influences may reflect receptor interactions with its transducing (second messenger) system (De Lean, Stadel and Lefkowitz, 1980).

Potassium channels have been implicated as being involved in the transducing system of LHRH receptors (Mitchell, Ogier, Johnson, Cleland, Bennie and Fink, 1986). In experiments using high K^+ concentrations and K^+ channel blockers (A-current) no obvious effect on the LHRH receptor's affinity could be observed. However the highest concentration of 4-AP (10mM, far higher than its published K_D for A-current channels of 1.5mM) did result in a 43% reduction in specific ^{125}I-buserelin binding, and the trend of the binding affinity in the presence of 150mM K^+ was for a reduction. As well as blocking K^+ A-currents, 4-AP has been found to promote calcium-dependent transmitter release from neurons by a direct facilitation of calcium entry through voltage sensitive channels (4-AP at 1mM; Rogawski and Barker, 1983). It has also been

suggested to block calcium activated K^+ channels (Zhand and Krnjevic, 1986) although higher concentrations (above 5mM) are found to facilitate these channels (Hermann and Gorman, 1981). The implication from these results is that LHRH receptors do not interact directly with A-current channels. The role of K^+ channel activation in response to LHRH is not however disproven, since indirect influences would not be demonstrable in the present experiments. Previous results had suggested that Ca^{2+} activated K^+ channels were not involved in LHRH-stimulation of LH release (Mitchell, Ogier, Johnson, Cleland, Bennie and Fink, 1986). However, the finding that 4-AP at concentrations higher than those effective at blocking K^+ A-current channels or activating voltage-sensitive Ca^{2+} channels, but in the range found to facilitate Ca^{2+} activated K^+ channels, reduce specific ^{125}I-buserelin binding (by 43%), might be consistent with involvement of these channels with the LHRH receptor protein. This would be in agreement with electrophysiological studies which have revealed such channels (of the large conductance type, sensitive to TEA and quinine; Cook, 1988) to be activated by LHRH (Mason, Bicknell, Cobbett, Waring and Ingram, 1986). Non-specific effects of high 4-AP concentrations cannot of course be excluded. However, since the results from electrophysiological and superfusion experiments are at variance, it would be interesting to investigate the effect of other Ca^{2+} activated K^+ channel blockers TEA (large conductance channel) and apamin (low conductance, voltage insensitive channel: Cook, 1988), to resolve whether there is really any direct association between such K^+ channels and the LHRH receptor.

Some K^+ channels have been suggested to be directly activated by subunits of a G-protein (Pfaffinger, Martin, Hunter, Nathanson and Hille, 1985; Sasaki and Sato, 1987) in particular the α subunit of the G-protein (Yatani, Cordina, Brown and Birnbaumer, 1987). Results of a series of experiments to try to reveal the involvement of a G-protein in LHRH receptor activation and a consequential effect of guanine nucleotides on LHRH receptor affinity suggest that indeed G-protein activation probably results from LHRH agonist binding to the receptor. However, there is no evidence that the LHRH activated G-protein interacts with a K^+ channel. It appears that LHRH (Andrews, Staley, Huckle and Conn, 1986) and other calcium activiting receptors (Gomperts, 1983; Cockcroft and Gomperts, 1985) interact with a G-protein which in turn stimulates the activity of a polyphosphoinositide phosphodiesterase. Previous attempts to use ligand binding techniques to show a guanosine nucleotide effect on LHRH receptor have not been successful. In order to show it here, LHRH displacement of an iodinated antagonist from membranes, pretreated with high concentrations of EDTA, in the presence of EDTA and Mg^{2+} at room temperature was required. These were conditions that have been shown previously to optimise the effects of guanine nucleotides on receptors linked to adenylate cyclase via a G-protein (3.2.2.3). The attempt by Hazum (1981(a)) to show an effect of GTP on LHRH receptor affinity used conditions similar to the normal binding conditions used for rat membranes in this Thesis, that is incubation for 90 minutes at 4°C in 10mM Tris-HCl pH 7.4/0.1% BSA. Similarly, the experiments by Perrin, Haas, Rivier and Vale (1983) used a binding assay in 10mM Hepes-KOH pH 7.6, 0.2% BSA and 0°C for 120 minutes.

Whereas the binding of agonists to receptors linked to adenylate cyclase via a G-protein have been widely reported to be inhibited by guanosine nucleotides, for example α_2-receptors (Glossman and Presek, 1979; Tsai and Lefkowitz, 1979), β adrenergic receptors (Shane, Gammon and Bilezikian, 1981), opiate receptors (Blume, 1978) and muscarinic receptors (Hulme, Berrie, Birdsall, Jameson and Stockton, 1983), this does not appear to be the case for calcium-activating receptors, although the evidence does suggest that they interact with a G-protein (Graziano and Gilman, 1987; Putney, 1987). The demonstration here of a reduction in affinity of LHRH in the presence of GTPγS appears to be the first such report for a receptor linked to polyphosphoinositide phosphodiesterase activation. A several-fold right shift in the displacement curve was seen, but the change in affinity could not be accurately measured. This may have been due to several factors, including the fact that the binding assay was carried out at room temperature. At room temperature total specific LHRH receptor binding is greatly reduced (Clayton and Catt, 1981). Another factor is the very low specific/non-specific binding ratio seen with the iodinated antagonist used, a problem not helped by the high temperature used. Clearer changes in LHRH receptor affinity may be seen if the assay is repeated using improved LHRH antagonists as the iodinated ligand. Highly potent antagonists have been described (Folkers, Bowers, Shao-bo, Tang and Kubota, 1986) and further antagonist analogues are being prepared (by Dr. C.M. Bladon) in an attempt to optimise specific binding and high affinity for the LHRH receptor site.

Sulphydryl groups have been implicated in the interaction of G-proteins and some muscarinic receptors (Gurwitz, Baron and Sokolovsky, 1984). They have also been found to be important in conformational transformations of nicotinic acetylcholine receptors from Torpedo californica, responsible for the appearance of different affinity states of this receptor (Moore and Raftery, 1979), and in the modulation of opiate receptor function (Marzullo and Hine, 1980). Treatment of rat anterior pituitary membranes with the sulphydryl oxidising agent diamide resulted in an 11% reduction of specific ^{125}I-buserelin binding. The effect of diamide on the GTPγS mediated change in LHRH receptor affinity would be interesting to investigate, as it might reveal the involvement of disulphide bonds in the interaction between the LHRH receptor and G-protein. Use of lower concentrations (1-10mM) of DTT may also be informative.

In summary, these experiments have revealed several aspects of the LHRH receptor protein. It does not appear to interact directly with A-current K^+ channels, but may possisbly have an effect on Ca^{2+}-activated K^+ channels. Overall however, any evidence for direct association with K^+ channels is tenuous. An effect of GTPγS on the LHRH receptors' affinity for agonists has been shown under carefully controlled conditions, suggesting that the LHRH receptor protein may interact with a G-protein. No conclusive evidence of a role for sulphydryl residues in either the maintenance of the LHRH receptor conformation or its interaction with its second messenger system was found.

3.4.2. Studies on LHRH receptors in rat hippocampal tissue and on the binding of GAP(27-41) in rat anterior pituitary and hippocampal tissue

It had been reported in autoradiography studies that LHRH analogues displaced the specific ^{125}I-[DAla^{6}, NαMeLeu^{7}, des Gly^{10}]-LHRH labelling of CA_1, CA_2 and CA_3 areas of male rat hippocampus with the same rank order of potency as their binding affinities to anterior pituitary tissue (Reubi, Palacios and Maurer, 1987). Whilst there does not appear to be any evidence of a physiological role for LHRH in the hippocampus,these findings suggested that it would be interesting to examine the binding of ^{125}I-buserelin to this tissue. Receptors for LHRH in other brain areas, in particular the preoptic and mesencephalic grey areas, appear (from both electrophysiological and behavioural responses) to have different characteristics to those found in the anterior pituitary (Moss and Dudley, 1978; Sakuma and Pfaff, 1983). The discrepancies in localisation of immunoreactive LHRH and LHRH specific binding sites found between immunohistochemical studies and autoradiography (Barry, 1979; Moss, 1979; McCann, 1982 and Reubi and Maurer, 1984) are not unique. The so-called 'mismatch' of receptors and neurotransmitter has been observed for other systems in the brain (Kuhar, 1985). However autoradiography has been suggested to be a less sensitive technique than immunohistochemistry (Kuhar, 1987) so the inability to find neurons immunoreactive to LHRH in the hippocampus where autoradiography reveals LHRH receptors is puzzling. Barry (1979) has suggested that such discrepancies may be explained by LHRH reaching its receptors via local vascular systems or the cerebrospinal fluid. It therefore seemed reasonable to look, using ligand binding techniques, for LHRH binding sites in rat hippocampal tissue.

in which no receptors for LHRH were demonstrated in the hippocampus

The results of these studies do not substantiate the findings of Reubi and co-workers (1984 and 1987). One possible reason for that is the different susceptibility of the ligands used in the two studies to enzymatic degradation. ^{125}I-buserelin, containing a D-amino acid at position 6 and C-terminal alteration to des Gly^{10} ethylamide, is a degradation-resistant LHRH analogue (Koch, Boram, Hazum and Fridkin, 1977). However, the analogue used in the autoradiographical study ^{125}I-[DAla^{6}, N$_{\alpha}$MeLeu^{7}, des Gly^{10}]-LHRH ethylamide has been found to be even more resistant to enzymatic degradation (Koch, Baram, Hazum and Fridkin, 1977). If the hippocampal tissue preparation has a higher enzyme activity than anterior pituitary tissue preparations under the binding conditions used (90 minutes at 4°C, pH 7.4) then degradation of the ^{125}I-buserelin may be responsible for the lack of specific binding seen. However, the enzymatic activity of the hippocampal preparation would have to be much higher than that of anterior pituitary gland tissue, as in this preparation under the binding conditions used, no significant degradation of LHRH analogues such as buserelin has been observed (Clayton, Shakespear, Duncan and Marshall, 1979(b)).

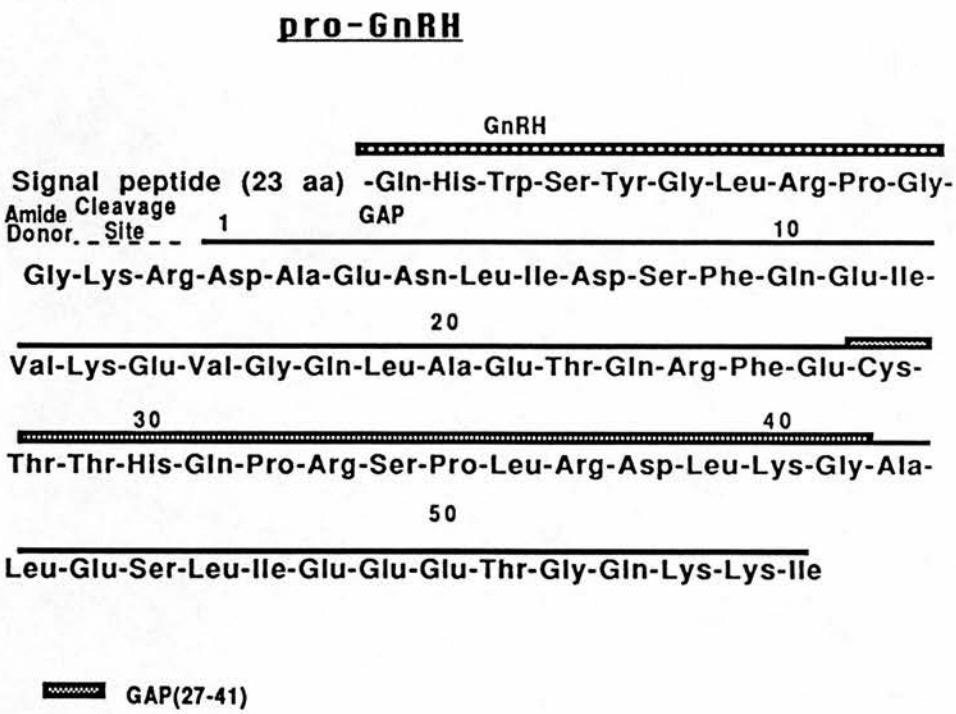
It is also possible that the binding sites detected in hippocampal tissue are not LHRH receptors, but receptors for a related peptide that show some affinity for the LHRH analogues used in the autoradiography studies. Such a peptide may be GAP. GAP has been shown to act in a similar manner to LHRH on anterior pituitary cells to release LH and FSH, but also to inhibit prolactin release (Nicolics, Mason, Szonyi, Ramachandran and Seeburg, 1985). In order to investigate the possibility of GAP binding specifically to LHRH

receptors in the anterior pituitary, or to specific receptors in hippocampal tissue, the peptide GAP (27-41) was used in ligand binding assays (Fig 3.13). Unfortunately no specific binding, to either LHRH receptors or specific GAP receptors could be detected using this fragment. Subsequent to the present experiments, Milton, Wormald, Brandt and Millar (1986) have isolated the LH and FSH releasing activity of the peptide GAP to a fragment consisting of the amino-acid-residues 4-13. This has been shown to be a calcium-dependent effect independent of LHRH receptors. No effect of GAP (4-13) on prolactin release was observed. The binding of this peptide to brain tissue was not reported, and it would be interesting to see if any specific binding sites for GAP (4-13) could be demonstrated either in hippocampus (where no physiological role for LHRH but specific binding sites have been demonstrated) or forebrain areas (where LHRH does appear to have a physiological role, Sakuma and Pfaff, 1983).

3.4.3 Binding affinities and abundance of LHRH receptors in anterior pituitary glands of other species.

As the rat anterior pituitary gland is only approximately 5mg (wet weight) of tissue, despite the high density of receptors in this tissue, the total number per gland is very low, about 90fmol (3.1). Other anterior pituitary glands have therefore been investigated as possible sources of larger quantities of high affinity LHRH receptors. Other pituitary glands were chosen in preference to other organs, as extrapituitary LHRH receptors (with the exception of those found in the gonads) have been shown to be only low affinity binding sites (Reeves, Segiun, Lefebvre, Kelly and Labrie, 1980). For example, that in placental tissue exhibits only

Figure 3.13



(Seeburg and Adelman, 1984)

Figure 3.13

Pre-pro-GnRH showing the amino-acid sequence of pro-GnRH and the positions of GnRH and GAP. The fragment GAP(27-41) is delineated.

μMolar affinity for analogues of LHRH that have nMolar affinities for the pituitary binding site (Iwashita, Evans and Catt, 1986). Another possible source of larger quantities of the LHRH receptor protein are cell lines. Unfortunately no stable cell lines expressing high affinity specific LHRH receptors exist. MCF-7 cells do express a low affinity LHRH binding site (Miller, Scott, Morris, Fraser and Sharpe, 1985) but its relationship to functional LHRH receptors is unclear.

Of the tissues tested bovine anterior pituitary glands would appear to be the most suitable source of LHRH receptors in relatively large numbers. Previously, purified bovine pituitary plasma membranes have been used in LHRH ligand binding assays (Clayton, Shakespear and Marshall, 1978; Conne, Aubert and Sizonenko, 1979). In the present study ligand binding was successfully shown in a crude membrane fraction of the tissue. The affinity of the site for ^{125}I-buserelin is very similar to that found in anterior pituitary tissue from male rats (0.84nM compared to 0.47nM). Calculations suggest that each bovine gland contains an equivalent number of receptors to 18 male rat glands. Therefore bovine anterior pituitary tissue was used in subsequent studies in attempts to isolate large amounts of LHRH receptors, sufficient for protein microsequencing.

Chapter 4

Solubilisation of the LHRH binding protein

4.1. Introduction

As the results of Chapter 3 show, membrane bound receptors can be characterised *in situ* by ligand binding methods. Extensive studies with analogues of native ligands can supply information about the structural requirements of the ligands (Momany, 1976(b)) and pharmacological manipulations may reveal components on the receptor (or adjacent to it) important in maintaining its conformation (and therefore its affinity for specific ligands, see Chapter 3). For example, the reduction in binding of LHRH analogues seen after treatment of rat pituitary membranes with neuroaminidase and wheatgerm agglutinin has led to the suggestion that the LHRH receptor is a glycoprotein, containing sialic acid residues (Hazum, 1982). Comparison of information from such experiments and the structural requirements of ligands for the receptor site can lead to an hypothesised model of the ligand binding site (Keinan and Hazum, 1985). These methods however are rather imprecise and can provide very little detailed information about the structure of the receptor in terms of its amino-acid sequence and their tertiary (or 3-dimensional) structure. One suggestion that has been put forward is that the binding site of peptide receptors is encoded by the complementary DNA to that of its ligands (Bost, Smith and Blalock, 1985). Antibodies to peptides derived from RNA complementary to LHRH mRNA have been shown to bind to gonadotrophes in culture (Mulchahey, Neill, Dion, Bost and Blalock, 1986) suggesting that these peptides do resemble a protein found in gonadotrophe cell membranes. However as peptides derived from complementary DNA transcribed in either the 5' to 3' or 3' to 5' direction are equally

as good at binding to their native peptide ligands (Blalock and Bost, 1986) it seems most likely that this is a fortuitous, non-specific finding. In fact studies by Ramussen and Hesch (1987) have shown that random sequences of amino-acids are just as good as "complementary-peptides" at providing possible binding sites for the native peptides. In the ultimate test of the hypothesis, cloning of the receptor for the peptide substance K, was unable to reveal the binding site predicted from the complementary DNA to the ligand (Hanley and Jackson, 1987).

More information about the structure of the receptor molecule can be obtained by characterising it as a molecular entity, isolated from its membrane environment. This can be achieved on two levels:-

1. its physical characteristics, such as its molecular weight, the number of subunits making up the receptor complex etc. These are aspects of its secondary and tertiary structure.
2. Its primary structure, that is the sequence of amino-acids that make up the receptor protein (its molecular characteristics).

The essential initial step for physical characterisation and the common route in determining molecular characteristics (Strosberg, 1987) is the removal of the receptor from its membrane environment. Ideally the method used to remove the receptor from the membrane would be non-denaturing, so that the unique characteristics (such as specific ligand binding) can still be used to identify the receptor protein.

Membrane proteins, such as receptors, have been recognised as containing both hydrophilic and hydrophobic regions. This has led to the hypothesis that they are positioned (partly) within the membrane lipid bilayer (ie they are integral membrane proteins), and

that the hydrophilic regions on the surface of the bilayer can interact with other components of the aqueous phase, such as adjacent proteins and lipid head groups (Lenard and Singer, 1966; Wallach and Zahler, 1966). Disruption of the lipid matrix is therefore required to dissociate the receptor protein from the membrane. Several approaches have been described. One is the use of organic solvents (Boyan and Clement-Cornier, 1984). This method however has been found to extract complexes of membrane proteins and phospholipids (Boyan and Clement-Cornier, 1984) and tends to result in some degree of protein denaturation. As such, the method has been suggested to be useful where the retention of a membrane protein's biological activity is not required (Van Renswoude and Kempf, 1984). A more widely used method of extraction for integral membrane proteins is detergent solubilisation (Helenius and Simons, 1975).

Detergents, being amphiphilic, are able to disrupt the hydrophobic interactions between the membrane phospholipids around the protein and so remove it from the membrane and into solution. In order to maintain the protein in an active form, the detergent should simulate its membrane environment but not interfere with the molecular interactions maintaining the protein's tertiary structure. Many different detergents have been used in membrane protein solubilisation. Ionic detergents such as sodium dodecyl sulphate have been found to bind extensively to proteins tending to denature them (Reynolds and Tanford, 1970). Other classes (such as the nonionic detergents, the bile salts and their derivatives) have generally less deleterious effects on a protein's tertiary structure, but their solubilising ability is often lower (Helenius and Simons, 1975).

The way in which detergents act to solubilise cell membranes has previously been investigated using the detergent Triton X-100 and the Semliki Forest virus (Helenius and Soderlund, 1973). It was found that at very low concentrations the Triton X-100 molecules appear to become incorporated into the membrane, binding to it and resulting in a reduction of its density. This occurs well below its critical micellar concentration (cmc, the concentration above which detergent molecules aggregate in an ordered manner to form micelles) suggesting that it was the detergent monomer that was binding rather than micelles. Increasing the detergent concentration results in the disruption of the virus membrane, and this is apparently enhanced by further increases in the detergent concentration, resulting eventually in a disintegration of the membranes into protein:lipid:detergent complexes. Finally by further increasing the detergent concentration,homogeneous protein:detergent and lipid:detergent complexes are said to be formed. All these stages (appearing as a continuum with increasing detergent concentration) have been observed whilst the free Triton X-100 concentration is below the its cmc. The suggestion therefore was that micelles do not play an important role in membrane protein solubilisation. Tanford (1972) has shown by both theoretical and experimental means that as long as the change in negative free energy was greater for the binding of amphipathic molecules (in that case alkylsulphates and sulphonates) to proteins and membranes than for forming micelles, then micelles were unlikely to be formed. Again the role of micelle formation as a prerequisite for detergent solubilisation of membrane proteins was questioned.

Lipophilic proteins - such as membrane proteins - have been

shown on delipidation to bind large quantities of detergent (Simons, Helenius and Garoff, 1973; Meunier, Olsen and Changeux, 1972), up to 60% of the protein's weight. In the case of cytochrome b_5 (an integral protein of mitochondrial membranes) detergent binding is highly cooperative at detergent concentrations near their cmc (Robinson, Nozaki and Tanford, 1974). No detergent however, appears to bind to the hydrophilic domain of the protein which has been said to be conformationally unchanged by the presence of the detergent (Robinson, Nozaki and Tanford, 1974). It was suggested that the lipophilic region of the protein acts as non-specific nucleus for the formation of a micelle-like complex of detergent molecules (Helenius and Simons, 1975). This may act to protect the lipophilic region of the protein from the ionic forces of the aqueous solution. Solubilisation of membrane proteins by mild detergents would appear from these results to occur by a process whereby the detergent molecules, first become incorporated into the lipid membrane. The lipophilic portion of an integral protein may act as a nucleus for the binding of detergent molecules resulting eventually in its release into solution. Finding a detergent that will solubilise a particular protein without denaturing it may be difficult to predict as it would appear to be dependent on the (as yet unknown) physicochemical properties of that protein.

The leading detergents currently in use for receptor solubilisation are synthetic and often derived from natural compounds. One such series is the sulphobetaine derivatives of the bile salts -sodium cholate and sodium deoxycholate (Hjelmeland, Nebert and Osborne, 1983). These have been designed specifically for non-denaturing solubilisation of membrane constituents. One of

these, 3-((3-cholamidopropyl)-dimethylammino)-1-propanesulphonate (CHAPS) has previously been used in studies of LHRH-receptor solubilisation. Generally however, only low yields have been achieved, for example 8% of available sites from bovine pituitaries (Perrin, Haas, Rivier and Vale, 1983) and 20% from rat ovaries (Capponi, Aubert and Clayton, 1984).

Increasing the ionic strength of a deoxycholate solution has previously been shown to increase the efficiency of protein solubilisation (Meissner, Connor and Fleischer, 1973). Similar results were seen using the related detergent cholate (Carson, 1982; Hall, Frankham and Strange, 1983; Hooper, 1986) in the solubilisation of bovine striatum dopamine D_2 receptor. As a cholate derivative, a CHAPS solution of high ionic strength might also be expected to give higher yields of membrane protein solubilisation. Alone, CHAPS has been found to solubilise either no D_2 receptors (Wheatley, Hall, Frankham and Strange, 1984) or only a small proportion (14%) of available D_2 receptors (Hooper, 1986). The addition of NaCl to a concentration of 0.72M increased the solubilisation yield to 26% (Kuno, Saijoh and Tanaka, 1983), or in the case of 1.5M NaCl to 47% (Hooper, 1986). Results from studies on the solubilisation of rat brain serotonin S_2 receptor also show a dramatic increase in the amount of receptor protein solubilised on addition of high NaCl concentrations (1-1.5M NaCl; Wouters, van Dun, Leysen and Laduron, 1985). In all cases CHAPS has been used at or about its cmc, 8mM (Hjelmeland, Nebert and Osborne, 1983). In fact Womack, Kendall and MacDonald, (1983) have suggested that for maximum solubilisation with minimum damage to proteins (the aim of most receptor protein solubilisation

studies) all detergents should be used at concentrations equal to or slightly below their cmc values. Increasing the concentration of CHAPS much above its cmc has not been shown to increase yields and apparently may even decrease them (Hooper, 1986; Perrin, Haas, Rivier and Vale, 1983; Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert, 1983; Hjelmeland and Chramback, 1984).

Once solubilised, some receptor proteins have been found to rapidly loose their binding activity, for example FSH receptors (Dias, Huston and Reichert, 1981), gonadotrophin (LH/hCG) receptors (Ascoli, 1983) and TSH receptors (Iida, Amin and Ingbar, 1987). The presence of 30% glycerol during solubilisation (Dias, Huston and Reichert, 1981; Ascoli, 1983) or 40-50% either during or immediately after solubilisation (Iida, Amin and Ingbar, 1987) appears to retain the binding activity of the solubilised preparation, so enabling further characterisation of the receptors. Hazum, Schwartz, Waksman and Keinan, (1986) have used a protocol that includes the addition of 10% glycerol to a preparation of solubilised LHRH receptors before attempting to purify them. Although there does not appear to be any direct evidence that this stabilises or improves the binding activity of the solubilised LHRH receptors, this possibility does exist.

In all the studies described above, and most other attempts to solubilise receptor proteins, the solubilised membrane components have been separated from the remaining unsolubilised portion by centrifugation. The rigorous definition of criteria for solubilised material is of major importance in such studies. Razin (1972) has suggested three criteria to use in the definition of solubilised membrane components:

1) Solubilised material is not sedimentable after 1 hr centrifugation at 100,000g,

2) Solubilised material should not be excluded from the void volume of a sepharose 4B column.

3) No membranous structures should be detectable under electron microscopy.

The criterion used in most studies has been the centrifugation one. Some caution may be required when only this one criterion is used as it is said to be dependent on both the temperature and density of the solution (Hjelmeland and Chramback, 1984).

The series of experiments described here have been carried out in order to try and optimise the solubilisation of LHRH receptors from rat anterior pituitaries. Initial experiments have been carried out using CHAPS, as this has previously been shown to successfully solubilised LHRH receptors (Perrin, Haas, Rivier and Vale, 1983; Capponi, Aubert and Clayton, 1984; Hazum, Schwartz, Waksman and Keinan, 1986). Other detergents have also been tried, as well as various additions to the solubilisation solution which have been reported to improve receptor solubilisation or stability in solution. In all cases the ability not only to solubilise the LHRH receptor protein but also to retain its affinity for LHRH analogues has been determined. The aim was not only to optimise solubilisation yields but to also find conditions under which the LHRH receptor was held in solution and retained affinity for its specific ligands.

4.2. Materials and Methods

4.2.1. Materials

Digitonin, sodium deoxycholate, polyoxyethylene sorbitan mono-oleate (Tween 80), (iso-octyl phenoxy)deca-ethoxyethanol (Triton X-100) and CHAPS were purchased from Sigma Chemical Co Ltd, Poole, as were the peptidase inhibitors aprotinin and soybean trypsin inhibitor. Other detergents were obtained from the following sources:
N,N,-di-(propylamido(2,3,4,5,6-pentahydroxyhexanoyl)) cholamide (deoxyBIGCHAP; Pierce Chemical Co., Chester,), nonanoyl-N-methylglucamide (MEGA-9; Cambridge Research Biochemicals, Cambridge), (N-dodecyl)-sulphobetaine (Zwittergent 3-12; Calbiochem Brand Biochemicals, Cambridge) Lauryl maltoside (Boehringer Mannheim, Lewes.)

4.2.2. Methods

4.2.2.1. General solubilsation method for rat anterior pituitary membranes.

Rat anterior pituitary membranes were prepared as described (2.4.1.1.). The washed membranes were resuspended into the solubilisation buffer (detergent; ± 1.5M NaCl; soybean trypsin inhibitor 50µg/ml; aprotinin 400KIU/ml, in 25mM Tris/HCl pH 7.4). All detergents were used at concentrations approximately equal to their published critical micellar concentrations and in some cases at various other concentrations as well. Samples were then agitated for 90 minutes at 4°C using a Stuart Flask Shaker (speed 5). Centrifugation for 2 hours at 60,000g at 4°C (MSE Superspeed 65) was used to remove all non-solubilised membrane constituents from those solubilised. Aliquots (typically 50µl) of the solubilised

preparation (the supernatant) were then subjected to a PEG precipitation (2.4.1.3.) after increasing their volume to 500µl with 25mM Tris-HCl, pH 7.4/0/1% BSA (Tris/BSA) and used in ^{125}I-buserelin binding assays (2.4.1.1.). In some cases the pellet was also resuspended into Tris/BSA (20 volumes) for ligand binding. The PEG precipitation step was included for the solubilised preparation to remove the solubilised proteins from the detergent solution. Detergent solutions have previously been shown to inhibit the binding of LHRH analogues to their receptor, (Perrin, Haas, Rivier and Vale, 1983).

4.2.2.2. Investigation into the effect of glycerol on the solubilisation of ^{125}I-buserelin binding sites with CHAPS.

As the addition of glycerol to the solubilisation solution had been shown to improve the stability of FSH, LH and TSH receptors, (Dias, Huston and Reichert, 1981; Ascoli, 1983; Iida, Amir and Ingvar, 1987) the possibility existed that it might also improve the amount of LHRH binding activity detectable in the solubilised preparation from rat anterior pituitaries. Inclusion of glycerol in the solubilisation step for pancreatic somatostatin receptors was found to improve the solubilisation yield of receptor complexes (Zeggari, Viguerie, Susini, Garnier, Esteve and Ribet, 1987). This possibility has been investigated here for the LHRH receptor. Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert (1983) have reported a high yielding method of LHRH receptor solubilisation from bovine anterior pituitaries using a CHAPS solution containing 30% glycerol, 0.32M sucrose. Solubilisation was determined by centrifugation for 1 hour at 45,000g, rather slower than the usual centrifugation criterion for solubilisation (Razin, 1972; Hjelmeland

and Chrambach, 1984). The viscous solubilisation solution may have given rise to artifactual solubilisation and this possibility has been examined here.

Washed rat anterior pituitary membranes were resuspended into 5mM CHAPS with various concentrations of glycerol (0-30%), or the solubilisation solution used by Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert (1983; 10mM CHAPS, 0.32M sucrose, 30% glycerol, 1mM phenylmethylsulphonyl fluoride (PMSF), 0.01% dimethylsulphoxide (DMSO), 50µg/ml soybean trypsin inhibitor in 50mM Tris/HCl pH 7.5). In all cases the membranes were shaken (90' at 4°C, speed 5) and then centrifuged to separate solubilised from non-solubilised constituents. As an alternative to 2 hrs at 60,000 g, samples of membranes solubilised using the 30% glycerol/0.32M sucrose solution were centrifuged at 45,000g for 1hr. Membranes suspended in the 30% glycerol/0.32M sucrose solution in the absence of 10mM CHAPS were also centrifuged at 45,000 g for 1 hr.

4.2.2.3 ^{125}I-Buserelin binding to rat anterior pituitary membranes in the presence of detergent solutions.

The aim of this series of experiments was to not only optimise solubilisation conditions for LHRH receptors but also to find conditions under which the solubilised receptor retains its affinity for LHRH ligands. Previous studies have shown that CHAPS reduces specific binding of LHRH analogues to solubilised receptors (Perrin, Haas, Rivier and Vale, 1983). In order to try and find conditions where the deleterious effects of CHAPS on LHRH analogue binding was minimal, initial experiments were carried out on rat anterior pituitary membrane preparations.

Washed membranes resuspended into detergent solutions (CHAPS ±

NaCl ± glycerol) were subjected to ^{125}I-buserelin binding (2.4.1.1.) in the presence of these various detergent solutions. Bound and non-bound ligand were separated by a PEG precipitation (2.4.1.3.) so that both membrane bound receptors and any solubilised as a result of the 90 minute incubation with the detergent solution could be assayed.

4.2.2.4. The effect on solubilised ^{125}I-buserelin binding sites of reducing the detergent solution concentration after solubilisation.

Hazum, Schwartz, Waksman and Keinan (1986) reduced the detergent concentration and added glycerol to the solubilised preparation after it has been separated from the non-solubilised membrane constituents. No evidence of what happens to the solubilised proteins as a result of this strategy was presented. This, and the following set of experiments were designed to reveal whether or not reducing the detergent concentration after solubilisation retains the ^{125}I-buserelin binding sites in solution. Also the ability of ^{125}I-buserelin to bind to the solubilised sites in the presence of the reduced detergent concentration has been investigated. In the first set of experiments the only change in conditions was to reduce the detergent solution concentration. In the following set, as well as reducing the detergent solution concentration, glycerol has been added to the solubilised preparation.

Aliquots of supernatant from membranes solubilised using 20 volumes of either 5mM CHAPS/1.5M NaCl or 5mM CHAPS alone were diluted 10 fold with CHAPS or CHAPS/NaCl solutions of various concentrations.

Samples from each condition were then either:

a) subjected to ^{125}I buserelin binding in an assay adjusted to the appropriate CHAPS/NaCl concentration to determine the effect of the various detergent solution concentrations on specific ^{125}I-buserelin binding. PEG precipitation was used to separated bound from non-bound ligand after binding had reached equilibrium.

b) subjected to a PEG precipitation step followed by a) in the absence of CHAPS/NaCl. This allowed the total number of specific ^{125}I-buserelin binding sites to be determined and would reveal any direct effects on the ^{125}I-buserelin binding site by the reduction in detergent solution concentration.

c) centrifuged (2 hrs at 60,000g, 4°C) and then subjected to a PEG precipitation (for the supernatant) or resuspended directly into Tris/BSA (the pellet) followed by a) in the absence of CHAPS/NaCl. The proportion of available specific ^{125}I-buserelin binding sites retained in solution after reducing the detergent concentration could therefore be determined (by comparison with the results from (b)).

4.2.2.5. <u>The effect on solubilised ^{125}I-buserelin binding sites of reducing the detergent concentration and adding glycerol after solubilisation</u>

Aliquots of supernatant from membranes solubilised with 30 volumes of 5mM CHAPS/1.5M NaCl were diluted 10 fold into a glycerol-containing Tris-HCl solution (25mM, pH 7.4 containing peptidase inhibitors as present in the solubilisation solution). Samples were then treated as above (a,b, or c) except that the CHAPS/NaCl solution with anappropriate concentration of glycerol was used.

Whilst these experiments were designed to examine the effect on the solubilised ^{125}I-buserelin binding site of a protocol described by Hazum, Schwartz, Waksman and Keinan (1986), small differences between their methods and those used here do exist. In case these had a great effect on the result obtained, the protocol described by Hazum and colleagues (1986) was also followed. Solubilisation was carried out in 20 volumes of 25mM Tris-HCl pH 7.4, 5mM CHAPS by shaking for 60 minutes at 4°C. Centrifugation (1 hr at 100,000 g) was followed by a 5 fold dilution into a Tris-HCl/glycerol solution, giving final concentrations of 10% glycerol, 1mM CHAPS, 1mM PMSF. Aliquots of this solution were then treated as above (a, b, c).

4.2.2.6 The effect on solubilised ^{125}I-buserelin binding sites of using other detergents to replace the CHAPS/NaCl solution after solubilisation.

It has been suggested (Tanford and Reynolds, 1976) that whilst one detergent solution may be most efficient at solubilising a particular membrane protein, another may be better able to keep it in solution in a non-denatured state. This has been investigated for ^{125}I-buserelin binding sites, solubilised with 5mM CHAPS, 1.5M NaCl, in an attempt to find conditions under which the sites are both retained in solution and show affinity for ^{125}I-buserelin.

Anterior pituitary membranes were solubilised in 5mM CHAPS/1.5M NaCl as above (4.2.2.1). Aliquots subjected to a PEG precipitation were then resuspended into 25mM Tris-HCl pH 7.4 containing various detergents (at concentrations approximately equal to their cmc) with or without 1.5M NaCl (and 50µg/ml soybean trypsin inhibitor and 400 KIU/ml aprotinin). After a further centrifugation (2 hrs at

60,000 g, 40°C) the pellets and supernatants were separated. After PEG precipitation of the supernatants or resuspension of the pellets into Tris/BSA, ^{125}I-buserelin binding assays were carried out. The proportion of total available (supernatant plus pellet) specific ^{125}I-buserelin binding sites retained in solution (ie found in the supernatant) could therefore be determined.

4.3. Results

In all cases results are expressed ± SEM, with values being the mean of 3-6 separate determinations except where otherwise stated.

4.3.1 Optimisation of conditions for solubilisation of ^{125}I-buserelin binding sites from rat anterior pituitaries

4.3.1.1 Determination of optimal NaCl concentrations

The absence of NaCl and detergent from the solubilisation solution resulted in the retention of all specific ^{125}I-buserelin binding in the pellet and none being found in the supernatant of the solubilised preparation (Fig. 4.1). Addition of 5mM CHAPS led to the appearance of a small amount (19 ± 3%) of the specific binding in the supernatant. This was marginally increased by the addition of 0.2M or 0.6M NaCl to the 5mM CHAPS. The combination of 5mM CHAPS/1.5M NaCl however, extracted 73 ± 5% of the specific binding sites for ^{125}I-buserelin into the supernatant. These results were obtained using 30 volumes (compared to wet tissue weight) of the solubilisation solution. The routine method of separating solubilised from non-solubilised membrane constituents was centrifugation for 2 hrs at 60,000g, 4°C. However, centrifugation of the anterior pituitary membrane preparation solubilised with 5mM CHAPS/1.5M NaCl for 1 hr at over 230,000 g, 4°C gave identical results. This confirms that the specific ^{125}I-buserelin binding sites seen in the supernatant after 2 hrs at 60,000 g are truly solubilised.

4.3.1.2 Determination of ^{125}I-buserelin binding site solubilisation by various detergents in the presence or absence of 1.5M NaCl.

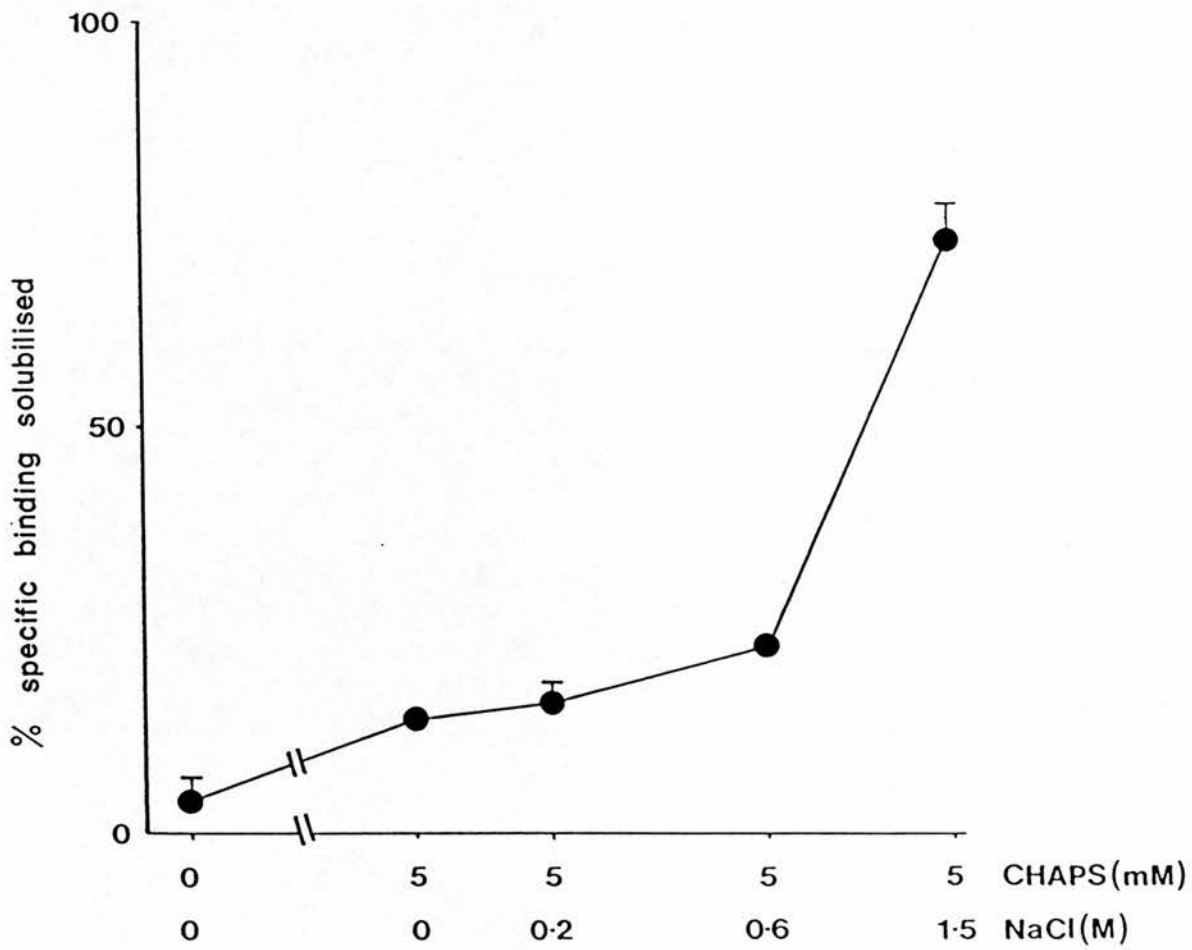
Various detergents (as listed in 4.2.1), in the presence and

Figure 4.1

Effects of increasing concentrations of NaCl on solubilisation of specific ^{125}I-buserelin binding sites from rat anterior pituitary gland membranes. Membranes were solubilised as described (4.2.2.1) in 30 volumes of solubilisation solution containing 5mM CHAPS and various concentrations of NaCl. After separation of non-solubilised components by centrifugation (2hrs at 60,000g, 4°C) aliquots of the solubilised supernatant (equivalent to the volume of suspension for one quarter pituitary gland before solubilisation) were subjected to a PEG precipitation step before ^{125}I-buserelin ligand binding. Non-specific binding was determined in the presence of 1μM LHRH. Comparison of specific ^{125}I-buserelin binding to the PEG-precipitated solubilised samples with that to equivalent amounts of membrane tissue (one quarter anterior pituitary gland) gave values for % specific ^{125}I-buserelin binding solubilised.

Specific ^{125}I-buserelin binding to PEG precipitated, 5mM CHAPS, 1.5M NaCl-solubilised preparation was around 6,000cpm, with ~50% of total binding being specific. Values are mean ± SEM for 3 separate determinations.

Figure 4.1


100
50
0
% specific binding solubilised
0
0
5
0
5
0·2
5
0·6
5
1·5
CHAPS(mM)
NaCl(M)

absence of 1.5M NaCl (shown above, 4.3.1.1., to be a concentration of NaCl giving optimal yields of solubilised ^{125}I-buserelin binding sites with 5mM CHAPS), were screened for their ability to solubilise the LHRH receptor in an undenatured form (as indicated by the ability of the supernatant, after PEG precipitation, to bind ^{125}I-buserelin). Twenty volumes (compared to wet tissue weight) of the solubilisation solutions were used. The results are shown in Table 4.1.

In the absence of 1.5M NaCl, Zwittergent 3-12 and sodium deoxycholate were as efficient as CHAPS in protein solubilisation (protein assayed according to the method of Geiger and Bessmann (1972), detailed in Appendix IV). However, no specific ^{125}I-buserelin binding was detectable in the supernatant, whilst that seen in the pellet was much reduced (< 10% of that seen in control pellets not treated with detergent). This suggests that the LHRH receptor was being denatured in some way. Similar results of minimal recovery of functional binding sites and only small fraction of these being found in the supernatant were obtained using digitonin, lauryl maltoside (1.8mM), Triton X-100 (1%) and MEGA-9 (5%). Other conditions, 0.18mM lauryl maltoside, 0.16% Triton X-100, 0.5% MEGA-9, 0.13% and 1.3% Tween-80, appear to result in no deleterious effects on binding site recovery but little solubilisation of the LHRH receptor. Only CHAPS produced a marked solubilisation together with only modest losses in recovery. Whilst this efficiency of solubilisation was also seen with deoxyBIGCHAP, the recovery of ^{125}I-buserelin binding sites with this detergent was further reduced, suggesting it was in some way denaturing them.

The addition of 1.5M NaCl to the detergent solutions was

Table 4.1

Rat anterior pituitary membranes were solubilised as described (4.2.2.1) in 20 volumes of the solubilisation solution containing detergent ± NaCl (1.5M) as shown. After centrifugation (2hrs at 60,000g, 4°C) to separate non-solubilised components, the supernatant was subjected to a PEG precipitation step before determination of ^{125}I-buserelin specific binding. Values for the recovery of binding sites are the binding recovered (in the supernatant plus pellet) as a % of initial membrane binding. All values are mean ± SEM for 3 separate determinations.

Table 4.1

Solubilisation conditions		Recovery of binding sites (% of membrane binding)	Solubilised binding sites (% of total recovered)	Solubilised protein (% of total)
CHAPS 5mM	alone	46 ± 1	19 ± 3	53 ± 6
	+ NaCl 1.5M	58 ± 3	56 ± 1	52 ± 2
deoxyBIGCHAP 1.4mM	alone	36 ± 1	24 ± 1	20 ± 2
	+ NaCl 1.5M	34 ± 4	24 ± 1	35 ± 4
Mega-9 0.5%	alone	103 ± 1	5 ± 1	31 ± 4
	+ NaCl 1.5M	1 ± 1	1 ± 1	40 ± 4
5%	alone	3 ± 1	1 ± 1	74 ± 9
Zwittergent 3-12 4mM	alone	1 ± 1	N.D.	62 ± 5
	+ NaCl 1.5M	3 ± 2	N.D.	67 ± 6
digitonin 0.5%	alone	1 ± 0.5	N.D.	28 ± 2
	+ NaCl 1.5M	1 ± 1	N.D.	31 ± 18
sodium deoxycholate4mM	alone	11 ± 1	N.D.	46 ± 6
	+ NaCl 1.5M	2 ± 1	N.D.	68 ± 10
Triton X100 0.16%	alone	92 ± 1	7 ± 1	24 ± 7
	+ NaCl 1.5M	18 ± 1	4 ± 1	33 ± 8
1%	alone	10 ± 1	4 ± 1	64 ± 6
Tween 80 0.13%	alone	88 ± 1	7 ± 13	24 ± 6
	+ NaCl 1.5M	81 ± 2	10 ± 1	43 ± 12
1.3%	alone	108 ± 3	4 ± 1	25 ± 8
lauryl maltoside 0.18mM	alone	100 ± 1	13 ± 1	24 ± 5
	+ NaCl 1.5M	67 ± 2	14 ± 1	21 ± 2
1.8mM	alone	12 ± 1	3 ± 1	22 ± 6

generally seen to cause a small increase in the amount of protein solubilised (Table 4.1). Percentage recovery of binding sites (supernatant and pellet combined) was found to be rather less than corresponding values without NaCl present for most of the detergents. Prominent reductions in recovery were seen with Triton X-100 (0.16%), digitonin, sodium deoxycholate and MEGA-9 (0.5%). These results suggest that, for the majority of the detergents tested, the presence of 1.5M NaCl increases their denaturing action on the ^{125}I-buserelin binding site. The efficiency of solubilisation of this protein was however generally similar to that of the detergents in the absence of NaCl. Only in the case of CHAPS did the presence of 1.5M NaCl effect a two- to four-fold increase in solubilisation of the receptor.

4.3.1.3. Investigatiion of the effects of the detergent: protein ratio on the efficiency of solubilisation using 5mM CHAPS, 1.5M NaCl

It had been observed in the previous two studies that both the percentage recovery of binding sites and the percentage of binding sites solubilised by 5mM CHAPS, 1.5M NaCl were dependent on the detergent: protein ratio used for solubilisation. Further investigations varying the amount of solubilisation solution used per mg of tissue wet weight were carried out. Optimal conditions for solubilisation were found at a ratio of 30 volumes solubilising solution to tissue weight (Fig 4.2). Under these conditions there was full recovery of binding sites and maximal solubilisation of about 70% of these sites. This corresponds to a detergent: protein ratio of 1.6 (w/w).

This survey of solubilisation conditions therefore revealed that

Figure 4.2

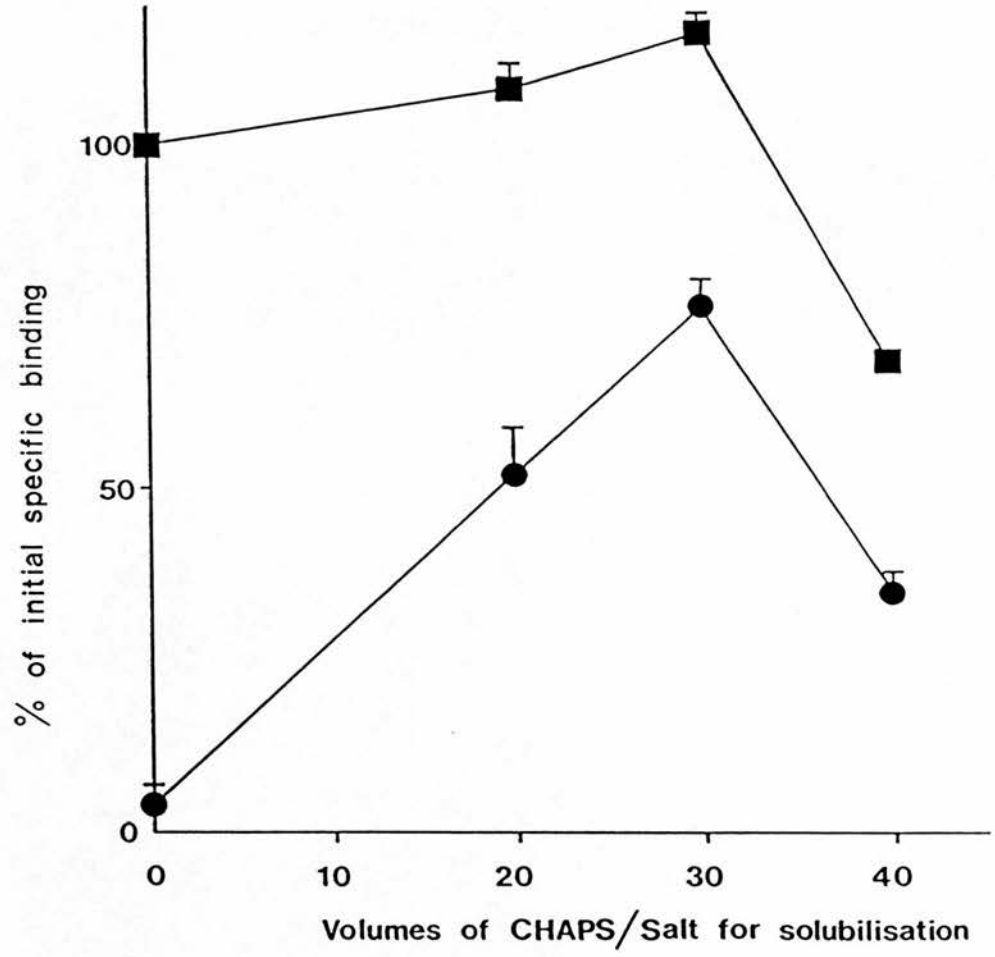


Figure 4.2

Effect of detergent/protein ratio on receptor recovery and efficiency of solubilisation: Receptor solubilisation was carried out as described (4.2.2.1) using CHAPS 5mM, NaCl 1.5M, but at a varying ratio of volumes of solubilising solution to initial tissue volume (as indicated). Solubilised and residual sites were again separated by centrifugation prior to polyethylene glycol precipitation and ^{125}I-labelled buserelin binding.

■ total recovery of binding (values for supernatant plus pellet as a percentage of initial membrane binding).

● solubilised binding (values for supernatant as a percentage of initial membrane binding).

Values are the mean ± SEM for three separate determination.

an optimal yield of solubilised ^{125}I-buserelin binding sites can be obtained using the detergent CHAPS at a concentration of 5mM, with 1.5M NaCl at a ratio of 30 volumes to tissue wet weight. These conditions result in no apparent denaturing of the binding site and over 70% solubilisation.

4.3.2. Investigation into the effect of glycerol on the solubilisation of ^{125}I-buserelin binding sites with CHAPS

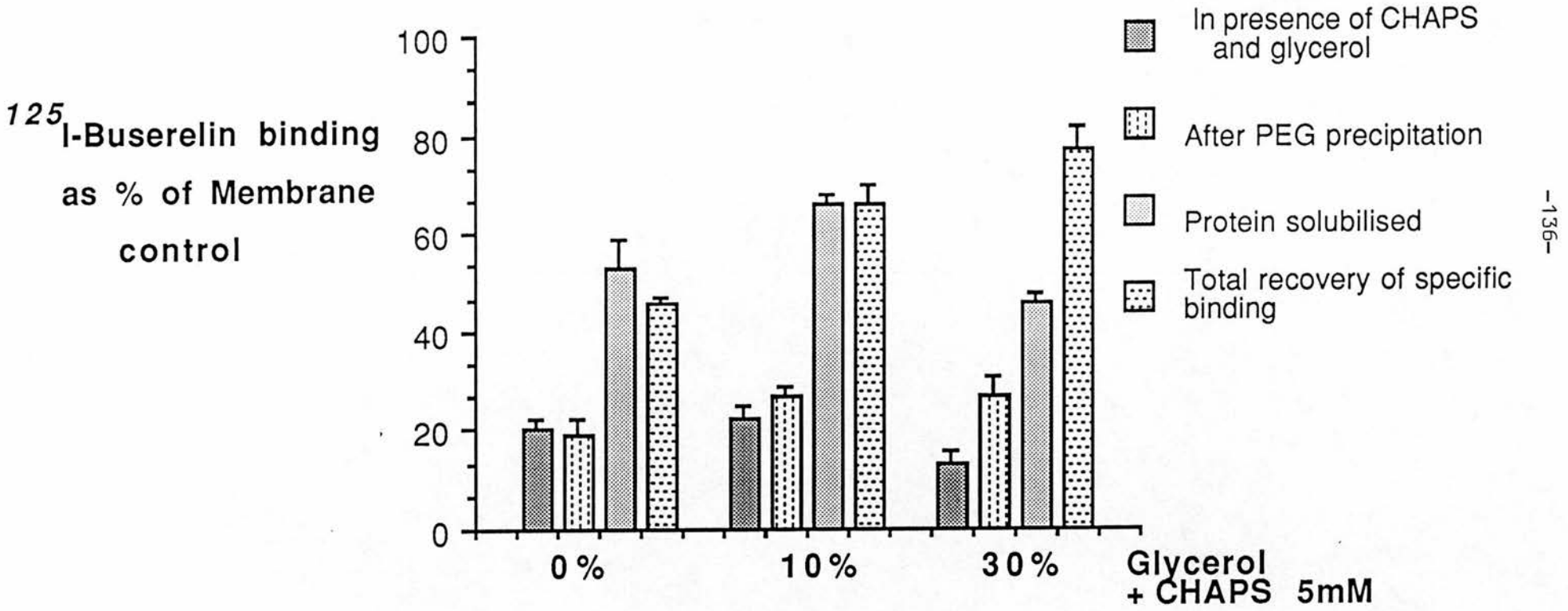
Using a solubilisation volume of 20, the effect of adding various concentrations of glycerol to the 5mM CHAPS solution was investigated. 10% glycerol resulted in a slight increase in the percentage of ^{125}I-buserelin binding sites solubilised compared to that found using 5mM CHAPS alone (19 ± 3% to 27 ± 2%: Fig 4.3). The amount of binding detectable in the solubilised preparation (assayed in the presence of 5mM CHAPS, 10% glycerol and without a previous PEG step) was no different from that seen in the absence of glycerol (22 ± 3% compared to 20 ± 2% of control membrane binding). Both the amount of protein solubilised and the % binding site recovery were higher than that for CHAPS alone (66 ± 2% and 66 ± 4% respectively compared to 53 ± 6% and 46 ± 1% for 5mM CHAPS alone under similar conditions). These results suggest that whilst 10% glycerol may protect the ^{125}I-binding site from denaturation by 5mM CHAPS under the conditions used (20 volumes solubilisation solution) it does not increase the solubilisation yield greatly, or enable all the solubilised sites to exhibit specific ^{125}I-buserelin binding in the presence of the solubilisation solution.

Increasing the glycerol concentration to 30% resulted in a further increase in % binding site recovery (77 ± 5%) but had no effect on the amount of ^{125}I-buserelin binding solubilised, whilst

Figure 4.3

Effects of increasing concentrations of glycerol on solubilisation by 5mM CHAPS of ^{125}I-buserelin specific binding sites from rat anterior pituitary gland membranes. Solubilisation was carried out as described (4.2.2.1) in 20 volumes of the 5mM CHAPS, glycerol solution. ^{125}I-Buserelin specific binding was determined on the supernatant (after centrifugation) both with or without the inclusion of a PEG precipitation step before the binding assay. The binding assay in the absence of a PEG precipitation step was carried out in the presence of 5mM CHAPS and the appropriate glycerol concentrations. Total recovery of specific binding sites was assessed from the binding found in supernatant (after a previous PEG precipitation) plus that in the pellet. Values for protein solubilised are given as a % of total membrane protein and determined by protein assay (2.10). All values are mean ± SEM of four separate determinations.

Figure 4.3


125I-Buserelin binding as % of Membrane control
100
80
60
40
20
0
0 %
10 %
30 %
Glycerol + CHAPS 5mM
In presence of CHAPS and glycerol
After PEG precipitation
Protein solubilised
Total recovery of specific binding

that detectable in the presence of the solubilisation solution was reduced (13 ± 3%). These apparently contradictory results can be explained by the hypothesis that 30% glycerol acts to protect the membrane bound receptor (^{125}I-buserelin binding site) from any denaturing influence of the detergent (5mM CHAPS) but seems itself to inhibit specific ^{125}I-buserelin binding to the solubilised protein. Unlike the pancreatic somatostatin receptor (Zeggari, Viguerie, Susini, Garnier, Esteve and Ribet, 1987) the yield of solubilised ^{125}I-buserelin binding sites was not increased by the presence of either 10% or 30% glycerol.

Using the 30% glycerol/0.32M sucrose solution described by Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert (1983) and centrifugation at 45,000g for 1hr, artifactually high results for the percentage solubilisation were found. That is, after solubilisation in the presence of 10mM CHAPS and the low speed centrifugation, 42 ± 3% of the ^{125}I-buserelin binding sites were found in the supernatant. However, centrifugation of an anterior pituitary membrane for 1hr under the same conditions, that is at 45,000g, 4°C in the 0.32M sucrose/30% glycerol but in the absence of 10mM CHAPS, an apparent solubilisation of 19 ± 2% of control ^{125}I-buserelin binding sites was seen. These solubilisation criteria (as used by Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert, 1983) would appear not to be stringent enough. The viscous solubilisation medium has been shown here to be able to maintain small membrane particles in solution in the absence of detergent treatment. It is unlikely that these are truly solubilised ^{125}I-buserelin binding sites as no time interval between membrane resuspension and centrifugation was allowed for solubilisation to occur.

4.3.3. ^{125}I-Buserelin binding to Membranes in the presence of detergent solutions

The addition of 5mM CHAPS to the ^{125}I-buserelin binding assay on rat anterior pituitary membranes resulted in a 37 ± 4% reduction in the specific binding seen. A further dramatic reduction - to only 31 ± 4% of control membrane specific binding was seen when 0.2M NaCl was included in the assay. This was not significantly altered on increasing the NaCl concentration up to 1.5M (Fig 4.4). It would appear therefore that whilst 5mM CHAPS has a deleterious effect on specific ^{125}I-buserelin binding, of the two solubilisation solution constituents, CHAPS and NaCl, the NaCl has the greatest effect in reducing ^{125}I-buserelin binding.

When glycerol (10%) alone was included in the binding assay 84 ± 3% control binding to membrane was detectable (Fig 4.5). Inclusion of an additional 0.5mM CHAPS (one-tenth of the concentration used in solubilisation) only slightly reduced this value, but it was decreased to 56 ± 1% of control on increasing the CHAPS to 1mM. Inclusion of an additional 150mM NaCl (again one-tenth of the concentrations found in the solubilisation solution) with the 0.5mM CHAPS/10% glycerol greatly decreased the amount of specific ^{125}I-buserelin binding seen to 29 ± 2% of control values. In the absence of detergent, 150mM NaCl and 10% glycerol together resulted in an intermediate reduction of control specific binding (to 46 ± 1%). Again the greatest deleterious effects on specific ^{125}I-buserelin binding to the membrane preparation occur in the presence of NaCl. Glycerol (at 10%) did not appear to improve the specific ^{125}I-buserelin binding and may even reduce it to some small extent.

Figure 4.4

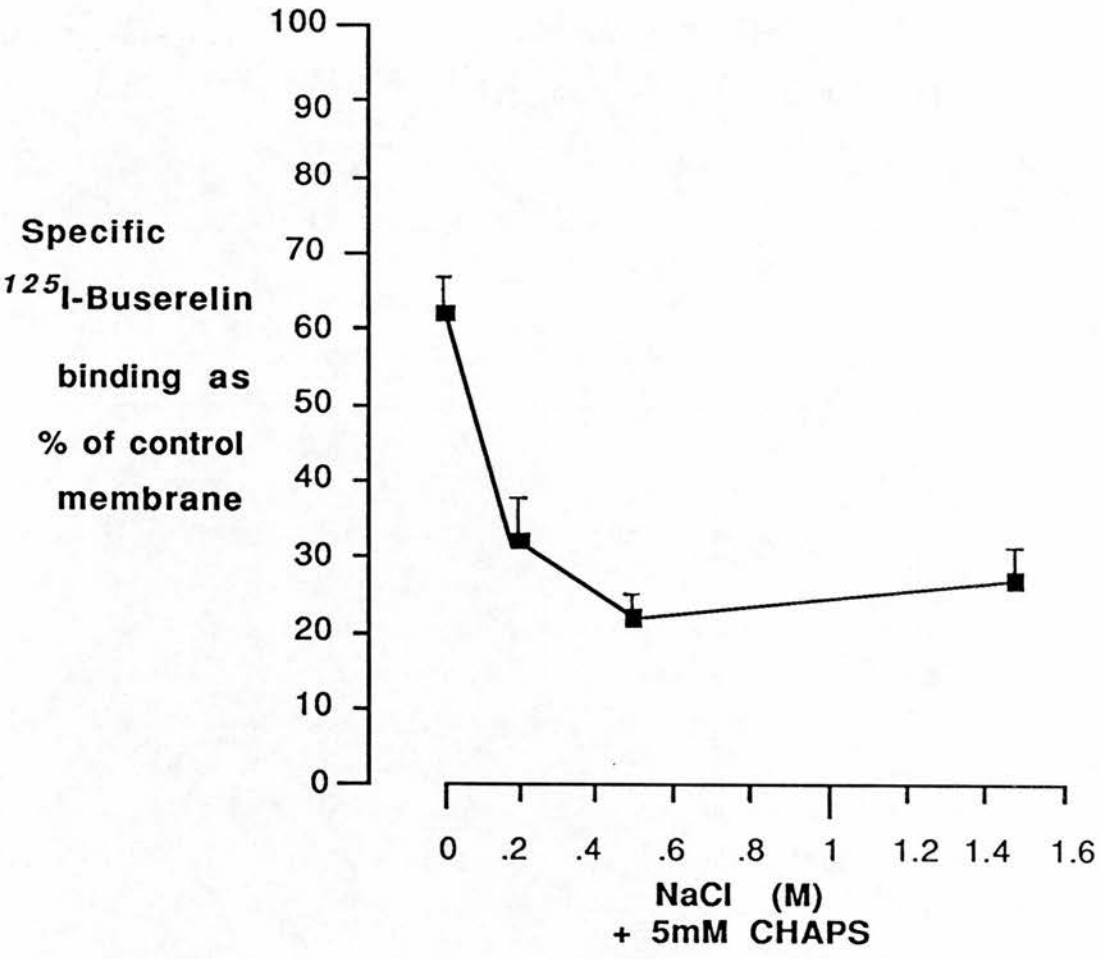


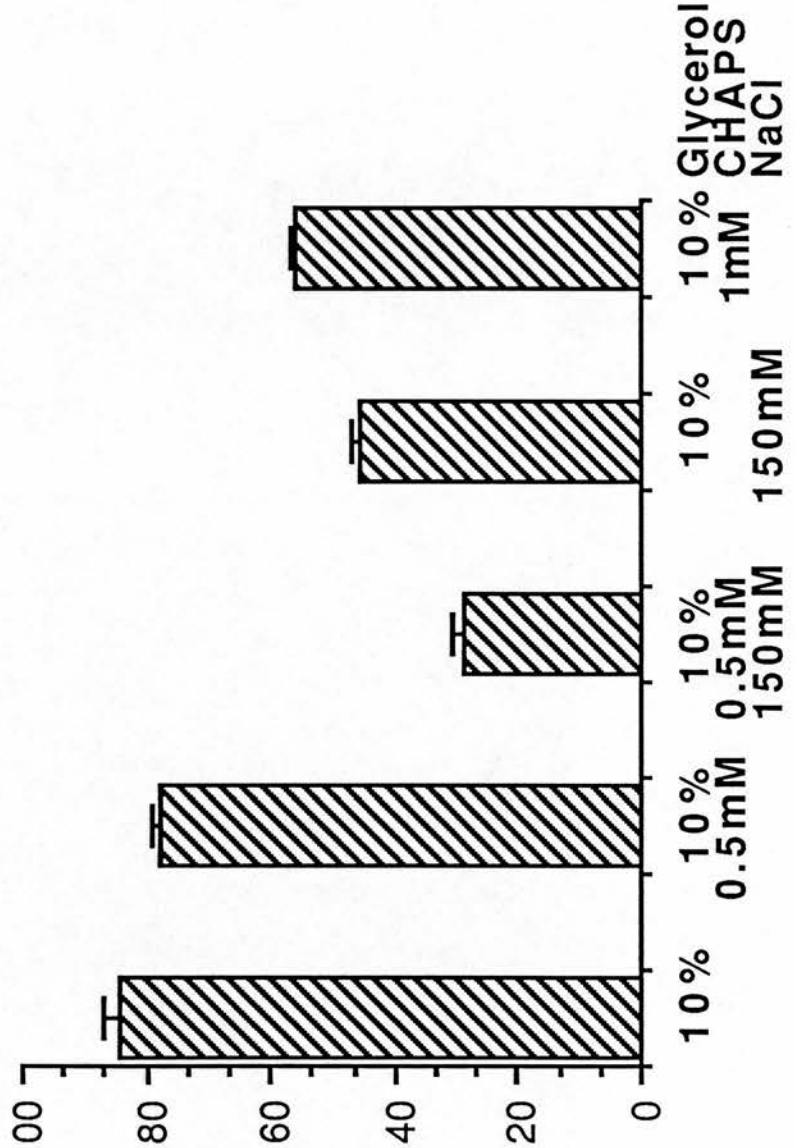
Figure 4.4

Effects of the presence of detergent/salt on the ligand binding assay. % of control membrane specific ^{125}I-buserelin binding detectable in the presence of 5mM CHAPS and various concentration of NaCl. Points are mean ± SEM of four determinations.

Figure 4.5

Effects of detergent/salt on the ligand binding assay in the presence of glycerol. % of control membrane specific ^{125}I-buserelin binding detectable in the presence of 10% glycerol and various CHAPS and NaCl concentrations, as indicated. Values are means ± SEM of four determinations.

Figure 4.5


Specific
125
I-Buserelin
binding as %
of control
membrane
binding
100
80
60
40
20
0
10%
10%
0.5mM
10%
0.5mM
150mM
10%
150mM
10%
1mM
Glycerol
CHAPS
NaCl

4.3.4. The effect on solubilised ^{125}I-buserelin binding sites of reducing the detergent solution concentration after solubilisation.

These experiments were carried out in order to try to find conditions under which the solubilised preparation is maintained in a solubilised state and also retains its affinity for ^{125}I-buserelin. In this and the following set of experiments ^{125}I-buserelin specific binding has been determined both in the presence of the detergent solution (a) and after its removal by a PEG precipitation step (b). The binding detectable in the presence of the detergent solution has therefore been expressed as a percentage of that seen after its removal. The amount of specific binding detectable (after a PEG precipitation step) in the supernatant following a second centrifugation (c) (in the appropriate detergent solution) has also been assayed. The expression of this (c) as a percentage of the initial specific binding found in the initial supernatant PEG precipitate (b), indicates the degree to which the LHRH receptor is still maintained in solution under conditions of reduced detergent concentration.

In the presence of 5mM CHAPS alone only 16 ± 0.5% of the total specific ^{125}I-buserelin binding sites found in the membrane were solubilised. ^{125}I-buserelin binding in this 5mM CHAPS solution revealed only 20 ± 2% of these sites (Fig 4.6). However, following the reduction of the CHAPS concentration to 1.5mM, the specific binding sites detectable in the presence of CHAPS were increased to 62 ± 10%, and to 75 ± 7% on further reducing the CHAPS concentration to 0.5M. On centrifugation all the CHAPS concentrations retained at least 50% of these solubilised sites in the supernatant (Fig 4.7).

Figure 4.6

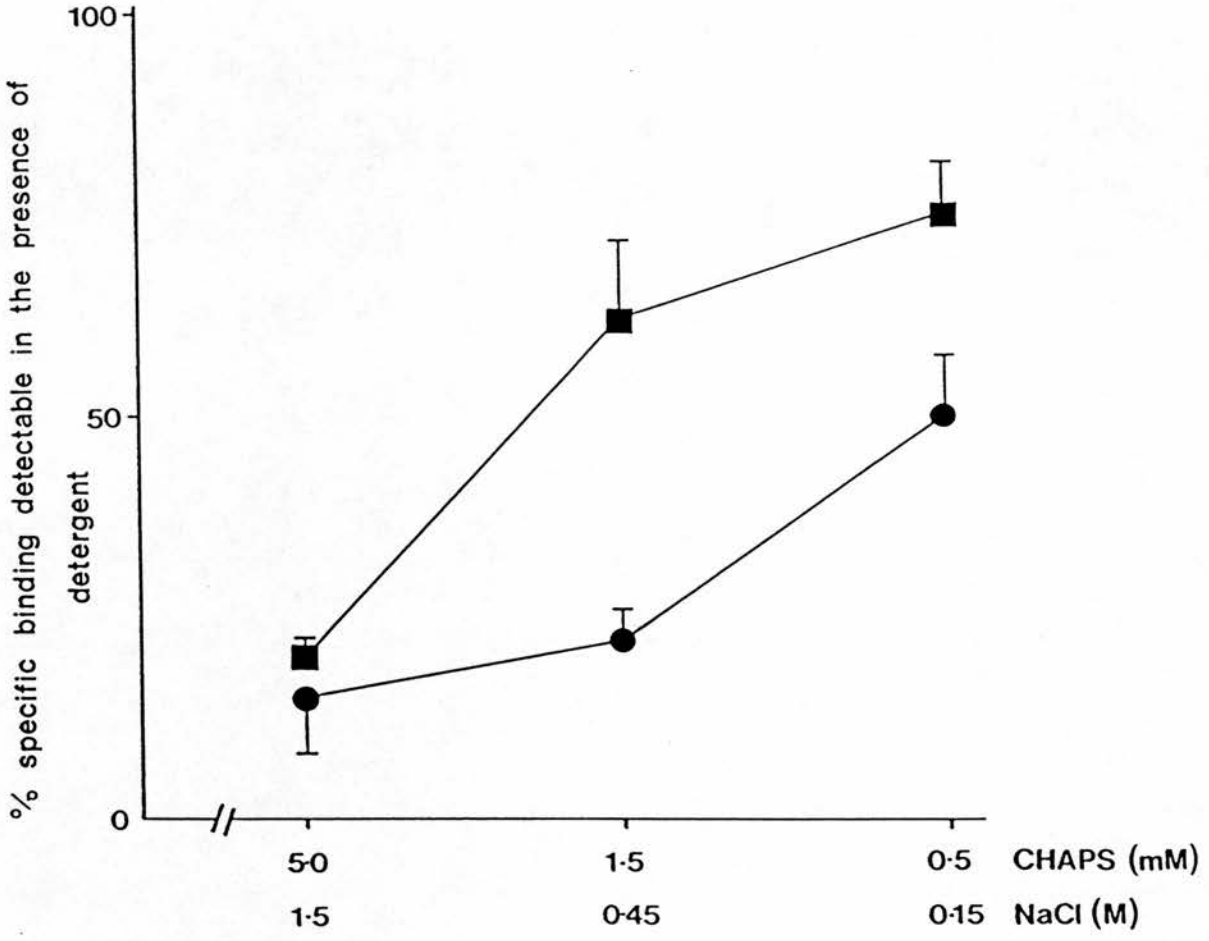


Figure 4.6

Effects of various concentrations of CHAPS and NaCl on specific ^{125}I-buserelin binding to solubilised rat anterior pituitary gland. After solubilisation as described (4.2.2.1) in 20 volumes of 5mM CHAPS (■), of CHAPS 5mM, NaCl 1.5M (●), and centrifugation, supernatant samples were assayed for binding of ^{125}I-buserelin. Assays were adjusted to the detergent and salt concentrations indicated and parallel determinations of identical aliquots after PEG precipitation, were always carried out. The binding detectable in the presence of CHAPS/NaCl is expressed as percentage of that detected after removal of CHAPS/NaCl by PEG precipitation. Values are mean ± SEM for three separate determinations.

Figure 4.7

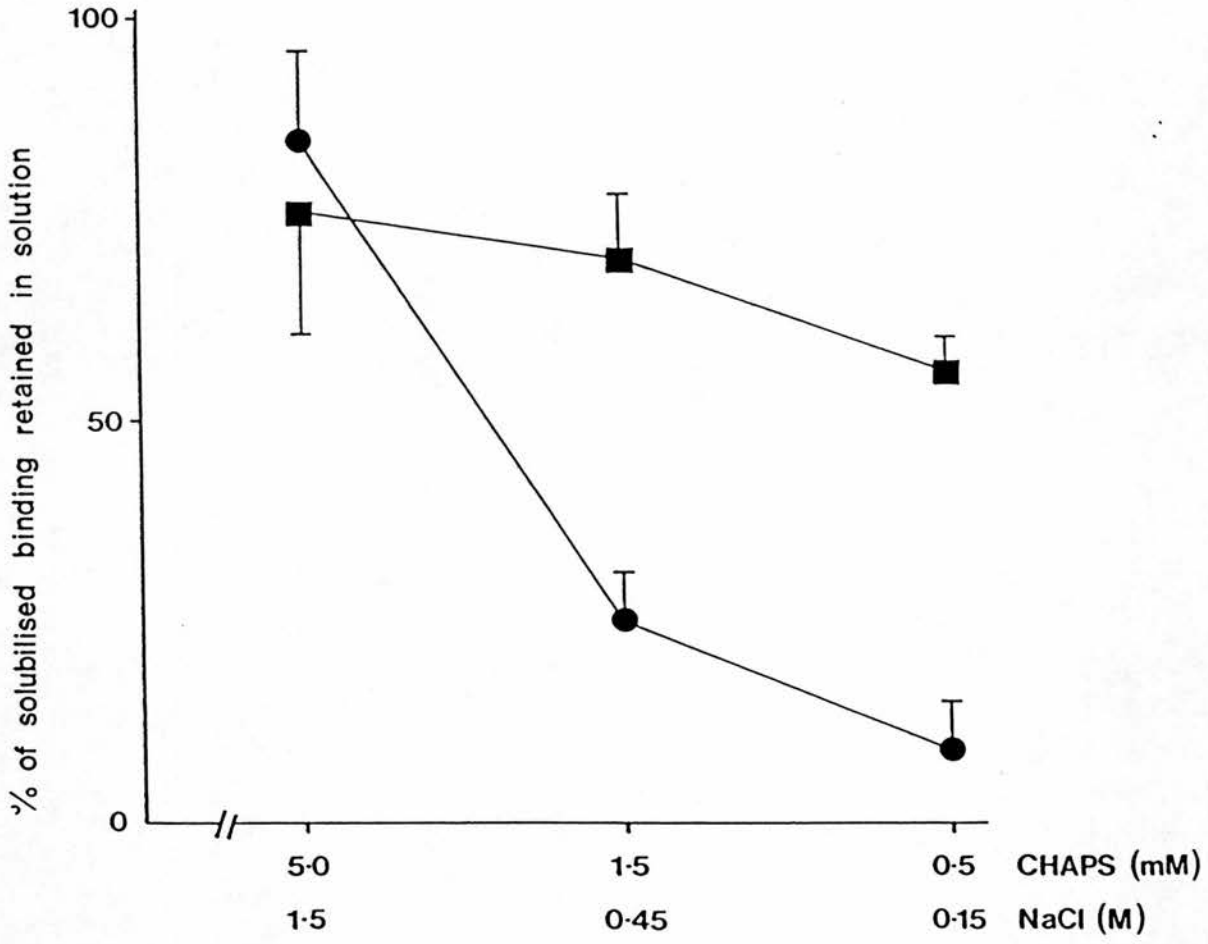


Figure 4.7

Ability of reduced CHAPS/NaCl concentrations to retain solubilised receptors in solution: Receptors were solubilised in 20 volumes of CHAPS 5mM or CHAPS 5mM, NaCl 1.5M solubilisation solution as described (4.2.2.1). The supernatant fraction was adjusted to the CHAPS/NaCl concentration indicated, vortexed, then subjected to further centrifugation (2hrs at 60,000, 4°C) to pellet any proteins that had precipitated under the new conditions. The specific ^{125}I-buserelin binding in PEG precipitates of the supernatant from such a second centrifugation was determined and expressed as a percentage of that in PEG precipitates of the first supernatant. This value indicates what proportion of the sites remain in solution after reduction of the CHAPS/NaCl concentrations. (●) CHAPS/NaCl, (■) CHAPS alone. Values are mean ± SEM for three separate determinations.

Solubilisation in 20 volumes of 5mM CHAPS, 1.5M NaCl resulted in 54 ± 1% of the specific ^{125}I-buserelin binding sites found originally in the preparation appearing in the supernatant (lower than the maximum ~70% as the amount of detergent solution used was 20 volumes rather than the ideal 30, see 4.3.1.3). Binding of ^{125}I-buserelin in the presence of 5mM CHAPS, 1.5M NaCl (Fig 4.6) revealed only 15 ± 7% of these solubilised binding sites, which was increased slightly to 22 ± 4% in the presence of 1.5mM CHAPS, 0.45M NaCl. Further reducing the detergent solution concentration to 0.5mM CHAPS, 0.15M NaCl increased the amount of binding detectable in the CHAPS/NaCl solution to 50 ± 8%. Centrifugation in the 0.5mM CHAPS, 0.15M NaCl solution however resulted in all but 9 ± 6% of the specific binding sites being precipitated (Fig 4.7). That is, in order to detect appreciable amounts of the solubilised ^{125}I-buserelin binding sites by specific binding, the concentration of the CHAPS/NaCl solution has to be reduced to levels where it is no longer capable of maintaining these proteins in solution.

4.3.5. <u>The effect on solubilised ^{125}I-buserelin binding sites of reducing the detergent solution concentration and adding glycerol after solubilisation</u>.

In an attempt to create conditions where solubilised receptors were maintained in solution and able to specifically bind ^{125}I-buserelin, glycerol was added to the solubilised preparation. As above the concentration of the detergent solution was also reduced, to give final concentrations of 30% glycerol, 0.5mM CHAPS, 150mM NaCl. In this case the initial solubilisation (in 30 volumes) by 5mM CHAPS/1.5M NaCl resulted in 75 ± 8% of

Figure 4.8

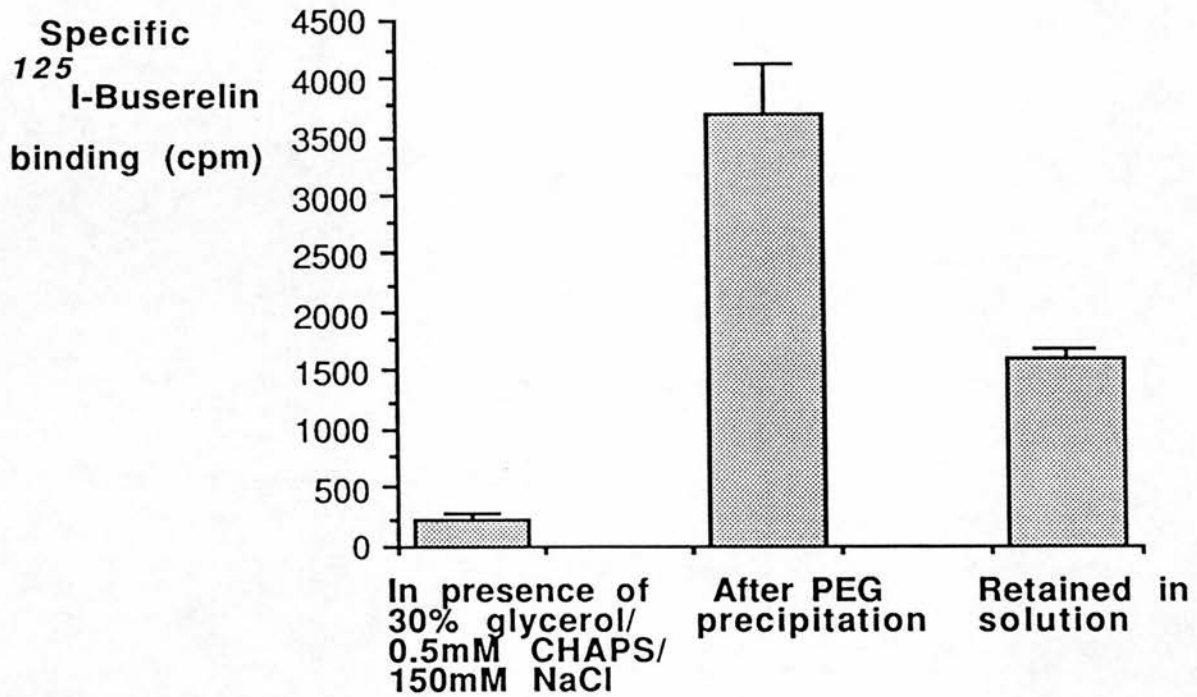


Figure 4.8

Specific ^{125}I-buserelin binding to rat anterior pitutary membranes solubilised in 30 volumes of 5mM CHAPS, 1.5M NaCl solubilisation solution. Binding was assayed both in the presence of 30% glycerol, 0.5mM CHAPS, 150mM NaCl and after a PEG precipitation step. Subjecting the 5mM CHAPS, 1.5M NaCl-solubilised supernatant to a second centrifugation (2hrs at 60,000g) in the 30% glycerol, 0.5mM CHAPS, 150mM NaCl and subsequently binding to this second supernatant (after a PEG precipitation step) reveals the amount of specific ^{125}I-buserelin binding sites retained in solution. Control membrane specific ^{125}I-buserelin binding was 4930 ± 260cpm. All values are mean ± SEM for three separate determinations.

control membrane binding appearing in the supernatant. Whilst 43 ± 2% of the binding in the 30% glycerol, 0.5mM CHAPS, 150mM NaCl dilution was found in the supernatant after a second high speed centrifugation (c), only 6 ± 1% was detectable by ^{125}I-buserelin binding in the presence of the glycerol/CHAPS/NaCl solution (Fig 4.8). Compared to a similar assay in the presence of 0.5mM CHAPS, 150mM NaCl alone (4.3.4.) the detectable specific binding had been greatly reduced by the inclusion of 30% glycerol.

Using the protocol described by Hazum, Schwartz, Waksman and Keinan (1986), 20 ± 1% of the initial membrane specific binding appeared in the solubilised preparation. A five fold reduction of detergent concentration and addition of glycerol to give 1mM CHAPS, 10% glycerol resulted in an apparent loss of 19 ± 3% of this specific binding. That is the amount of specific binding detectable after a PEG precipitation of the solubilised preparation from the glycerol/low CHAPS solution was only 81 ± 3% of that found in the solubilised preparation before adding glycerol and reducing the CHAPS concentration. 55 ± 6% of this remaining specific binding was detectable in the presence of 1mM CHAPS,10% glycerol (Fig 4.9) and 67 ± 7% of it was retained in solution after a second high speed centrifugation. This is a higher percentage value for retention in solution than the previous case, but the lower initial solubilisation (20 ± 1% of initial membrane specific binding compared with 75 ± 4%) means that the actual amount of ^{125}I-buserelin specific binding retained in solution after reducing the CHAPS concentration and adding glycerol is lower (493 ± 49cpm compared to 796 ± 49cpm for a tissue equivalent of

Figure 4.9

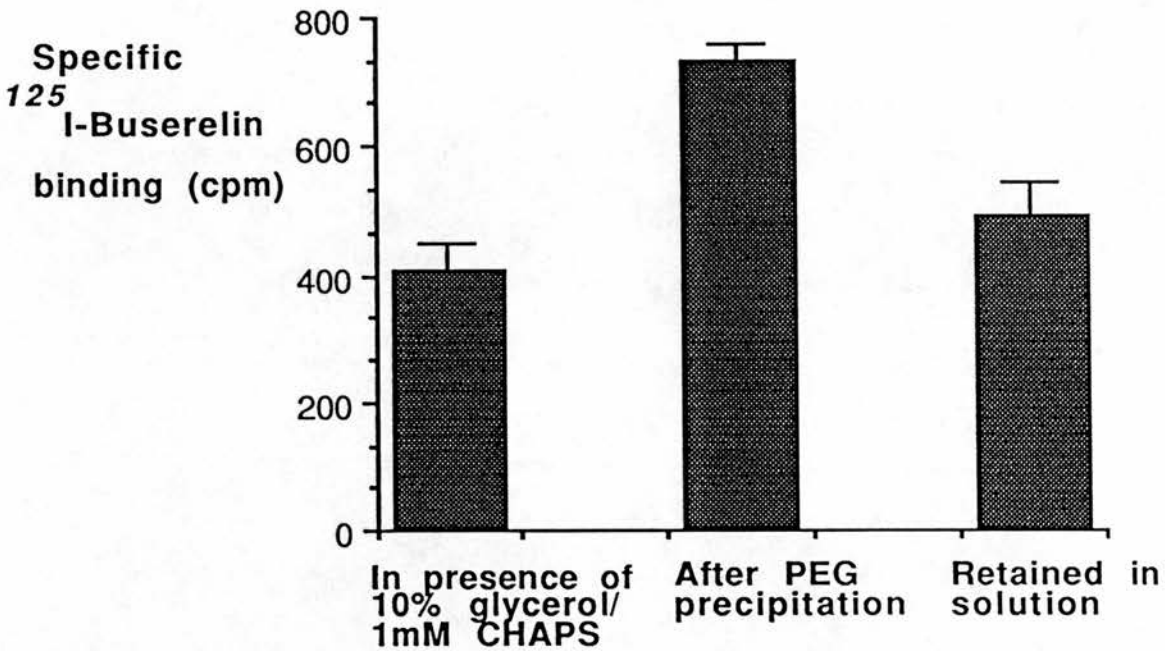


Figure 4.9

Specific ^{125}I-buserelin binding detectable in rat anterior pituitary gland preparation solubilised with 20 volumes of 5mM CHAPS as described (4.2.2.5) and diluted in Tris-glycerol to give 10% glycerol, 1mM CHAPS, 1mM PMSF. Binding was assayed both in the presence of this 1mM CHAPS, 10% glycerol solution and after removal of the solution by a PEG precipitation step. The amount of specific ^{125}I-buserelin binding in the initial solubilised supernatant (detected after a PEG precipitation step) was 905 ± 10cpm. After a second centrifugation (2hrs at 60,000g) of the solubilised preparation, this time in the 10% glycerol, 1mM CHAPS solution, and following a PEG precipitation of the second supernatant obtained, the amount of specific ^{125}I-buserelin binding retained in a solubilised state in the 10% glycerol, 1mM CHAPS solution was determined. All values are mean ± SEM of six determinations.

one-eighth of a rat anterior pituitary). The presence of 10% glycerol did however appear to have a far less deleterious effect on the specific binding sites than glycerol at a concentration of 30%. This was similar to the result found on the membrane preparation where 10% glycerol gave only a slight (~ 16%) reduction in the amount of specific ^{125}I-buserelin binding detectable.

4.3.6. The effect on solubilised ^{125}I-buserelin binding sites of using other detergents to replace the CHAPS/NaCl solution after solubilisation.

Further investigations, to try to find conditions under which the solubilised ^{125}I-buserelin binding site from rat anterior pituitary membranes both retain their affinity for ^{125}I-buserelin and are maintained to a large degree in solution, have been carried out using a protocol that enables the solubilising detergent solution to be replaced with another detergent solution.

The detergents chosen for the resuspension in these experiments were those which gave a high recovery of binding sites in the initial experiments on detergent solubilisation of rat anterior pituitary membranes (4.3.1.2, Table 4.1). That is, those that appeared not to denature the binding site to any great extent. These included lauryl maltoside (0.18mM) Tween-80 (0.13%) and MEGA-9 (0.5%). The proportion of the solubilised specific ^{125}I-buserelin binding sites retained in solution by these detergents (in both the absence and presence of 1.5M NaCl) was determined.

The initial solubilisation (in 20 volumes of 5mM CHAPS, 1.5M NaCl) resulted in 62 ± 5% of the initial specific binding of membrane preparation appearing in the supernatant. After a PEG precipitation and resuspension into the Tris-HCl, peptidase

inhibitor solution, 72 ± 12% of this specific binding was still detectable on assaying the precipitate from a second PEG step. That is, subjecting the solubilised preparation to the protocol of a PEG precipitation, resuspension,centrifugation and a second PEG precipitation appears to result in a loss of 28 ± 12% of the solubilised specific binding activity(Table 4.2). Recovery from the detergent resuspensions was found to be either similar or further reduced. Lauryl maltoside (0.18mM) both with or without 1.5M NaCl gave approximately 72% recovery; the presence of lauryl maltoside either with or without NaCl did not have any further denaturing effect on the binding sites. However, on subjecting the lauryl maltoside solutions of CHAPS/NaCl solubilised binding sites to a centrifugation (2 hours at 60,000g) none of the specific ^{125}I-buserelin binding could be detected in the supernatant. There was no apparent retention of the binding sites in solution. MEGA-9 (5%) alone and Tween-80 (0.13%, with or without 1.5M NaCl) were also unable to retain the binding sites in solution. MEGA-9 (5%) with 1.5M NaCl resulted in some retention in solution up to 52 ± 22%, but this was found to be very variable. Recovery of the binding sites was also found to be variable, ranging from 45% to 63% suggesting that some denaturing of the receptor site was taking place.

Table 4.2

Specific ^{125}I-buserelin binding found in the supernatant (snt) or pellet (plt) after resuspension of the PEG precipitate from the supernatant of a preparation solubilised in 20 volumes of 5mM CHAPS, 1.5M NaCl. The specific ^{125}I-buserelin binding initially solubilised was 533 ± 46cpm (assayed in an aliquot equivalent to one tenth of an anterior pituitary gland). All supernatant preparations were PEG-precipitated prior to ligand binding. Pellet preparations were resuspended into 25mM Tris-HCl pH7.4/0.1% BSA for binding assay. Concentrations of detergents used for resuspension were lauryl maltoside 0.18mM, MEGA-9 5% and Tween-80 0.13%. The snt/plt ratio indicates the efficiency of "re-solubilisation" for each of the conditions used for the resuspension step. All values are mean ± SEM of four determinations.

Table 4.2

Detergent for resuspension		Specific ^{125}I-buserelin binding (cpm) (±SEM)	
		No NaCl	1.5 M NaCl
None	Plt	366 ± 86	225 ± 65
	Snt	58 ± 64	118 ± 77
Lauryl maltoside	Plt	374 ± 63	345 ± 82
	Snt	49 ± 93	-6 ± 71
MEGA -9	Plt	207 ± 110	163 ± 52
	Snt	-12 ± 57	249 ± 106
Tween-80	Plt	215 ± 53	98 ± 54
	Snt	-19 ± 70	79 ± 49

4.4 Discussion

The detergent chosen for the first attempts to solubilise the LHRH-receptor in this study was CHAPS. A synthetic detergent designed specifically to be of use in membrane biochemistry (Hjelmeland, Nebert and Osborne, 1983), CHAPS has been used to successfully solubilise several membrane receptors in previous studies, including the LHRH receptor, (Perrin, Haas, Rivier and Vale, 1983; Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert, 1983; Capponi, Aubert and Clayton, 1984; Capponi, Birabeau and Vallotton, 1983; Kuno, Saijoh and Tanaka, 1983; Katoh, Djiane and Kelly, 1985; Wouters, Van Dun, Leysen and Laduron, 1985).

Results, both of these studies and others involving detergent-solubilisation of membrane protein (Womack, Kendall and MacDonald, 1983), suggested that best yields of non-denatured solubilised proteins would be achieved using the detergent at or just below its critical micellar concentration (cmc). Therefore CHAPS was used in all the experiments at 5mM, just below its reported cmc of 8mM (Hjelmland, Nebert and Osborne, 1983).

As in previously reported experiments using CHAPS (Wouters, van Dun, Leysen and Laduron, 1985) and cholate (Hooper, 1986) the addition of NaCl to the 5mM CHAPS solution was found to dramatically increase the yield of solubilised receptor. As in the above two studies, maximum solubilisation was achieved using 1.5M NaCl with the 5mM CHAPS. This increase in yield was not seen with any of the other detergents tested (Table 4.1). The other sulphobetaine derivative tested, deoxyBIGCHAP gave the next best yield for detergents alone but this was not improved by the inclusion of 1.5M NaCl. Sodium deoxycholate, although efficient at protein

solubilisation appeared to have a denaturing effect on the ^{125}I-buserelin binding site. The other detergent that had been reported previously as successfully solubilising the LHRH receptor - Triton X-100 (Jansen de Almeida Catanho, Berault, Theoleyre and Jutisz, 1983) at 1% was found under our solubilisation conditions to denature the LHRH receptor without appearing to achieve its solubilisation.

How was the increase in ionic strength of the detergent (5mM CHAPS) solution by the addition of 1.5mM NaCl able to improve the efficiency of membrane protein solubilisation? From the literature it appears that this high ionic strength may have been having an effect in two ways:

1) A reduction in the detergent cmc.
2) Interacting with the hydrophilic part of the integral protein.

Ray and Nemethy (1971) showed that for non-ionic detergents, increasing the salt concentration leads to a linear reduction in the critical micellar concentration. Similar results were seen with zwitterionic detergents (Ray and Nemethy, 1971). In terms of membrane protein solubilisation this may mean that at a given detergent concentration (below the normal cmc) the protein-detergent complex is more readily formed in a solution of high ionic strength.

It also has been suggested (Hjelmeland and Chramback, 1985) that the external portion of integral proteins may interact (via ionic or hydrogen bonds) with the polar heads of the lipids making up the membrane bilayer. Increasing the ionic concentration of the aqueous solution containing a membrane preparation may to some extent act to disrupt these van der Waals interactions and so help in the removal of the protein from the lipid environment.

One condition that was found from these experiments to be important in obtaining maximum yields of LHRH receptors from the solubilisation procedure was the ratio of detergent:protein. Optimum conditions were found to occur when 30 volumes (c.f. tissue weight) of the 5mM CHAPS, 1.5M NaCl solution were used. This corresponds to an optimal detergent:protein ratio of 1.6(w/w), very similar to the value of 2 reported by Hazum, Schwartz, Waksman and Keinan (1986). Tanford and Reynolds (1976) also suggested that a detergent:protein ratio of about 2 would reduce the probability of protein molecules in the detergent solution aggregating and therefore reduce the probability of artifactual protein-protein complexes occuring. Regarding the membrane phospholipid component Womack, Kendall and MacDonald (1983) have suggested that,for efficient solubilisation,the detergent concentration should not be exceeded by the concentration of the lipids.

The efficiency of solubilisation of the ^{125}I-buserelin binding protein found using the optimal solubilising conditions as determined in this study (that is 30 volumes of 5mM CHAPS, 1.5M NaCl solubilisation solution per mg of tissue initial wet weight, agitating for 90 minutes at 4°C) suggest that the criteria for both membrane phospholipid and membrane protein solubilisation have been fulfilled.

In some cases membrane receptors have been shown to be stabilised in solution by the presence of > 30% glycerol either in the solubilisation solution, or added after solubilisation (Dias, Huston and Reichert, 1981; Ascoli, 1983; Iida, Amir and Ingbar, 1987). The way in which this occurs has been proposed to be by enhancing hydrophobic interactions within the solubilised proteins

possibly resulting in an increased association between adjacent protein subunits (Timasheff, Lee, Pittz and Tweedy, 1976). In the case of the ^{125}I-buserelin binding site glycerol (at 10% and 30%) appears to protect to some extent the membrane bound site from any denaturing influence of the detergent solution (4.3.2) but was not found to increase the proportion of binding sites solubilised. The presence of glycerol during ^{125}I-buserelin binding assays of either the rat anterior pituitary membrane or solubilised preparation apparently reduced the amount of specific binding detectable, this effect being far greater for 30% glycerol than the lower concentration of 10% tried (4.3.5). These results suggest that in terms of the retention of the specific affinity of the solubilised ^{125}I-buserelin binding site no benefit would be gained by the inclusion of glycerol either in the solubilisation step or subsequently. This may imply that hydrophobic interactions are not important in the maintenance of the conformation of the ^{125}I-buserelin binding sites.

Retention of the high affinity, specific interaction between ^{125}I-buserelin and the solubilised binding site in the presence of the solubilising solution was an important factor if affinity chromatography was to be used for subsequent receptor protein purification (Chapter 6). In the case of the anterior pituitary membrane preparation, 63% of control specific ^{125}I-buserelin binding was detectable in the presence of 5mM CHAPS. Addition of NaCl (0.2-1.5M) dramatically reduced this to around 30% (4.3.3.1). That is, NaCl appeared to have a far greater deleterious effect on ^{125}I-buserelin specific binding than the detergent CHAPS. This may be due to disruption, by the high ionic strength of the NaCl

solution, of polar and ionic interactions important in maintaining the active conformation of the ^{125}I-buserelin binding site. Experiments to investigate the effect of reducing the concentration of detergent (CHAPS) and NaCl (when used in the solubilisation step) after solubilisation were carried out to try to find conditions suitable for both specific ^{125}I-buserelin binding and the retention of these binding sites in solution. It was found that both for CHAPS alone and CHAPS/NaCl, at various reductions of the solubilisation solution (either 5mM CHAPS or 5mM CHAPS/1.5NaCl) concentrations, binding was significantly reduced in the presence of the detergent, compared to that seen after removal of the detergent by the inclusion of a PEG precipitation step (Fig. 4.6). Whilst the % of available specific binding seen in the presence of the detergent solution was consistently higher in the presence of CHAPS alone, the initial low yield on solubilisation by 5mM CHAPS in the absence of NaCl means that the actual amounts of specific binding detected were less than for the equivalent reduced detergent concentration sample solubilised in CHAPS/NaCl. The effect of the reduction in concentration of the solubilisation solution on the retention of the ^{125}I-buserelin binding site in solution can be seen in Fig. 4.7. For all the conditions it would appear that the receptor can no longer effectively be held in solution. Again whilst the % of available sites retained in solution by reduced concentrations of CHAPS alone were higher than for the equivalent reduction of CHAPS/NaCl concentrations, the actual numbers of binding sites still found in the supernatant (in terms of specific cpm of ^{125}I-buserelin bound) were lower because of the reduced efficiency of solubilisation. At 0.5mM CHAPS, 0.15M NaCl (a

ten-fold reduction of solubilisation concentrations), where almost 50% of available specific binding sites could be seen in the presence of the detergent solution, less than 20% of these ^{125}I-buserelin binding sites were found to remain in the supernatant. Whilst the addition of 30% glycerol (Fig. 4.8) was found to improve the retention in solution of the binding sites, it also resulted in a dramatic reduction (to 6 ± 1% of available sites) of the binding seen in presence of the detergent solution.

Reductions in the concentration of the solubilisation solution, with or without the addition of glycerol seem unlikely to create conditions under which the ^{125}I-buserelin binding protein would be both maintained in solution and retain its specific affinity for ^{125}I-buserelin.

The procedure described by Hazum, Schvartz, Waksman and Keinan (1986) of anterior pituitary membrane solubilisation in 5mM CHAPS followed by a 5-fold dilution with a glycerol solution to give concentrations of 1mM CHAPS, 10% glycerol for use in an affinity chromatography method has also been investigated (4.3.5). The initial solubilisation yields of the LHRH receptor were found to be low, as expected, only 20 ± 1%. As a result of this the amount of specific ^{125}I-buserelin binding found to be retained in solution by the CHAPS/glycerol was equivalent to only 10. ± 1% of the initial membrane specific binding. Of this 55 ± 6% were detectable in the presence of the CHAPS/glycerol solution. So in effect only ~5% of the membrane LHRH receptors were detectable in the 1mM CHAPS, 10% glycerol solution. The protocol described by Hazum, Schwartz, Waksman and Keinan (1986) would appear therefore to result in only a very small % (~ 5%) of total membrane ^{125}I-buserelin being

available for interaction with specific ligands. The yield on affinity chromatography of the sites might therefore be expected to be very low.

Although none of the detergents screened for LHRH receptor solubilisation showed an improvement over the solubilisation yields obtained using 5mM CHAPS, 1.5M NaCl, some of them resulted in very little denaturing of the receptor site (Table 4.1). The possibility of replacing the LHRH receptors solubilised in 5mM CHAPS, 1.5M NaCl into these non-denaturing detergents so enabling specific ^{125}I-buserelin binding to occur on the sites whilst solubilised, was therefore investigated. Specific binding of ^{125}I-buserelin was monitored both in the supernatant (after a PEG precipitation step) and the pellet, after the resuspended preparation had been subjected to a centrifugation at 60,000 g, 4°C for 2 hrs. Controls in the absence of detergent gave a recovery of ~76% of the solubilised binding sites (Table 4.2). PEG precipitation had previously been shown to have no deleterious effects on the binding of LHRH analogues to either membrane bound or solubilised receptor sites (2.4.1.3.). The loss in binding activity seen here was unlikely to be a direct effect of the PEG precipitation, but may be in part due to this. Another possibility would be the loss of the binding protein as a result of absorption onto the assay tubes. If this was a contributory factor, the inclusion of 0.1% BSA in the solutions may have reduced this loss. Whilst a small proportion of these solubilised binding sites did appear in the supernatant (more in the presence of 1.5M NaCl than without), the actual amounts were very variable. Lauryl maltoside gave similar recoveries (total amount of binding detectable in both the pellet and supernatant, 72%

in the presence or absence of 1.5M NaCl) but none appeared to be retained in solution. Replacement of the CHAPS/NaCl solubilisation solution would not appear to be a successful method for both maintaining the ^{125}I-buserelin binding site in solution and retaining its specific affinity for ^{125}I-buserelin.

The aims of experiments carried out in this Chapter had been two fold:

1. the optimisation of conditions for solubilisation of the LHRH receptor.
2. to find conditions suitable for LHRH receptor affinity chromatography, that is conditions allowing specific, high affinity binding to the LHRH receptor in a solubilised state.

Throughout these investigations the LHRH receptor has been assayed in terms of specific ^{125}I-buserelin binding. Successful solubilisation of this binding site was achieved using 5mM CHAPS and 1.5M NaCl, giving a yield of over 70% under optimal conditions. In order to confirm that this ^{125}I-buserelin binding site is in fact the LHRH receptor further studies to characterise the solubilised site would be required. These are described in Chapter 5.

Molecular characterisation (as described in 4.1) of the LHRH receptor protein would be the ultimate aim of this study to try to characterise the LHRH receptor. Solubilisation and purification of the receptor protein are the initial steps of a strategy that has been successfully used to characterise several membrane proteins (Strosberg, 1987). Having optimised solubilisation conditions the next step for the LHRH receptor protein would therefore be its purification.

The general method of choice for receptor purification is affinity chromatography (Strosberg, 1984). Using an immobilised ligand specific for the receptor, this method (by relying on unique characteristics of the receptor protein) is highly selective. Several receptor types have been purified using this technique, including the $GABA_A$ (Sigel and Barnard, 1984); muscarinic acetylcholine receptor (Haga and Haga, 1985); glycine (Pfeiffer, Graham and Betz, 1982); B_2 adrenergic (Homcy, Rockson, Countaway and Egan, 1983) and insulin (Cuatrecasas, 1972). The two criteria found to be essential for receptor affinity purification are the retention of the receptor in a solubilised state, together with the retention of its affinity for the ligand utilised (Strosberg, 1984). Extensive surveying of different conditions has failed to reveal any under which both criteria are fulfilled for the solubilised ^{125}I-buserelin binding site. Investigation of the conditions used by Hazum, Schwartz, Waksman and Keinan (1986) suggest that only approximately 5% of the total ^{125}I-buserelin binding sites in anterior pituitary membranes would be available for affinity chromatography purification step. By examining the effect of the detergent CHAPS and NaCl on the specific binding of ^{125}I-buserelin to membrane preparations it would appear that the inhibition of binding is a direct effect of the presence of CHAPS and, to a greater extent, NaCl. This implies that the possibility that the inhibition of specific binding is due to the removal of essential elements (such as phospholipids or other proteins) from the binding site is unlikely. This was further implied by the finding (along with other groups, see 2.4.1.3) that full specific binding activity returned after the removal of the CHAPS/NaCl solution.

From these results it can be concluded that an alternative method to direct receptor affinity chromatography, would be necessary to give an efficient purification of the solubilised ^{125}I-buserelin binding site. The selection of this method, its development and application are the subject of Chapter 6.

Chapter 5

Characterisation of the solubilised receptor.

5.1 Introduction

It has been suggested that integral membrane proteins solubilised by mild detergents under optimal conditions are very likely to retain their native confirmation (Tanford and Reynolds, 1986). One way of testing this directly would be to investigate the spectral properties of the solubilised proteins, as the refractive index and circular dichronism of proteins are dependent on the relative positions of a protein's amino-acid side chains (Tanford, Nozaki, Reynolds and Makino, 1974). However, for detergent solubilised proteins this is not always easily interpretable as the presence of the amphipathic detergent molecules around the protein molecule may mask these spectral changes (Tanford, Nozaki, Reynolds and Makino, 1974). Another way of determining whether a protein's native conformation has been retained is to test for biological activity (Tanford and Reynolds, 1976). For many solubilised membrane receptors, such as the myocardial muscarinic acetylcholine receptor (Berrie, Birdsall, Hulme, Keen and Stockton, 1984), the angiotensin II receptor (Sen, Him and Soffer, 1983), the atrial natriuretic receptor (Kuno, Andersen, Kawisaki, Waldman, Chang, Saheki, Lertman, Nakane and Murand, 1986; Hamade, Rondon, Frohlich and Cole, 1987), the LH/hCG receptor (Wimalasena, Moore, Wiebe, Abel and Chen, 1985), the prolactin receptor (Shiu and Friesen, 1974; Church and Abner, 1982; Rae-Venter and Daod, 1983; Mitani and Dufau, 1986; Sakai, Ike, Kohomoto and Johke, 1986; Ashkenazi, Mador and Gertler, 1987) and the vasoactive intestinal peptide (VIP) receptor (Paul and Said, 1987), this has been assayed for in terms of specific ligand binding, although ligand activated enzymatic activity (such as the adenylate cyclase activation by FSH-bound solubilised FSH receptors: Dattatreyamurty, Figgs and Reichert, 1987) has also been used.

Whereas in some cases, for example the atrial natriuretic factor receptor (Kuno, Andresen, Kamisaki, Waldman, Chang, Saheki, Leitman, Nakane and Murad, 1986; Hamada, Rondon, Frohlich and Cole, 1987) and the LH/hCG receptor (Wimalasena, Moore, Wiebe, Abel and Chen, 1985) the binding affinities have been found to be very similar in both the solubilised and membrane preparations, others have been found to exhibit specific binding in the solubilised state but of a different affinity to that seen for the membrane bound receptor, for example the angiotensin II receptor (Sen, Jim and Soffer, 1983) and the prolactin receptor (Shiu and Friesen, 1974; Rae-Venter and Daod, 1983; Ashkenazi, Madar and Gertler, 1987). In many cases the susceptibility of high affinity specific binding to external influences (such as guanine nucleotides; Berry, Birdsall, Hulme, Keen and Stockton, 1984; Dattatreyamurty, Figgs and Reichert, 1987; Paul and Said, 1987) has been demonstrated to exist in the solubilised preparation in a similar manner to that found in the membrane preparation. It would appear, from the literature, that the determination of the binding characteristics of a solubilised membrane receptor provides a convenient method of assaying for the retention of native conformation. This method would also enable the identity of the solubilised protein to be confirmed as the receptor site (if similar binding affinities and profiles could be shown).

Further, physical characterisation of solubilised membrane proteins has also been achieved. For example the finding that guanine nucleotides regulate agonist binding to a solubilised receptor, as found for the myocardial muscarinic acetylcholine receptor (Berry, Birdsall, Hulme, Keen and Stockton, 1984), the

cholecystokinin (CCK) receptor (Lambeert, Svoboda, Furnelle and Christophe, 1985), the FSH receptor (Dattatreyamurty, Figgs and Reichert, 1987) and the VIP receptor (Paul and Said, 1987), might suggest that a G-protein and the receptor binding site have been solubilised as one unit. In the case of the CCK receptor, the G-proteins N_i (the G-protein coupled to adenylate cyclase inhibition) and N_s (stimulates adenylate cyclase) have both been found (after ADP-ribosylation using cholera toxin and pertussis toxin respectively) to coelute from a gel filtration column with the solubilised CCK binding sites, suggesting that the receptor exists as a complex containing the CCK binding site and N_i and N_s (Lambert, Svoboda, Furnelle and Christophe, 1985). Gel filtration techniques have also been used to reveal the existence of other receptor complexes. For example, the prolactin binding activity solubilised from mammary tissue was found to appear in two peaks on ion-exchange chromatography, the first peak of lower binding affinity than the second (Sakai, Ike, Kohmoto and Johke, 1986). Gel filtration in 7.5mM CHAPS of the lower affinity peak revealed an apparent molecular weight of 37,000 whereas in 5mM CHAPS the prolactin binding activity eluted as a volume suggesting a molecular weight of 74,000. This was suggested to reveal the existence, under condition of low detergent concentration, of either binding subunit dimers or the existence of a complex between a binding subunit and another similar molecular weight protein.

The presence of detergent during a gel filtration column calibration with soluble proteins of known molecular weights has been shown not to affect the calculated calibration curve (Fish, Reynolds and Tanford, 1970). This suggests that the technique is

valid to give estimates of molecular weights of detergent solubilised proteins (subject to the usual limitations of the technique, see 2.8). The ability to detect the presence of solubilised membrane receptor proteins in the eluate from a gel filtration column by the specific binding of radiolabelled ligands has led to the wide use of gel filtration techniques to provide estimates of their apparent molecular weights. For example the somatostatin (Zeggari, Viguerie, Susini, Garnier, Esteve and Ribet, 1987), VIP (Paul and Said, 1987) and benzodiazepine (Ray, Mernoff, Saugames-Warah and de Blas 1985) receptors' apparent molecular weights have been estimated using calibrated gel filtration columns. Strictly, gel filtration will give an apparent Stokes radius value, not a molecular weight. The Stokes radius of a molecule is a manifestation of its overall three-dimensional shape (Tanford, Nazaki, Reynolds and Makino, 1974). A more accurate determination of molecular weight requires the use of sedimentation centrifugation techniques (Tanford, Nazaki, Reynolds and Makino, 1974). In combination with gel filtration techniques, sedimentation centrifugation has been used to provide estimates of molecular weight for the GABA/benzodiazepine receptor complex (Stephenson, Watkins and Olsen, 1982), the platelet vasopressin receptor (Thiobonnier, 1987),the prolactin receptor (Rae-Venter and Daod, 1983; Haldosen and Gustafsson, 1987),and the muscarinic acetylcholine receptor from cardiac tissue (Peterson, Rosenbaum, Broderick and Schimerlik, 1986) and brain tissue (Berrie, Birdsall, Haga, Haga and Hulme, 1984) amongst others. Unlike gel filtration however, the sedimentation centrifugation techniques require relatively pure preparations of the solubilised proteins (Tanford, Nazaki, Reynolds and Makino, 1974). When only unpurified

solubilised preparations are available,the best method of obtaining an estimate of an apparent molecular weight would appear to be gel filtration.

For some solubilised membrane receptors, such as that for insulin (Aiyer, 1983), LH/hCG (Wimalasena, Moore, Wiebe, Abel and Chen, 1985) prolactin,(from ovarian tissue: Mitani and Fufai, 1986), VIP (Paul and Said, 1987) and angiotensin II (Laribi, Allard, Vincent and Simonnet, 1987), gel filtration reveals the existence of specific binding to more than one molecular species. In these cases and in some where only one peak of specific binding has been found on gel filtration (for example the hepatic prolactin receptor: Yamada and Donner, 1985), SDS PAGE may reveal lower molecular weight species that make up the undenatured receptor complex. That is, the polypeptide components of the receptor macromolecule can be determined. By carrying out the SDS PAGE under both non-reducing and reducing conditions the presence or absence of disulphide bonds between the polypeptide components has been shown (with for example the prolactin receptor :Mamada and Donner,1985). In the case of the hepatic lactogen receptor identical results have been found under both conditions, suggesting disulphide bonds are not involved in the tertiary structure of the receptor complex (Haldosen and Gustafsson, 1987). One of the most convenient and widely used methods of identifying the receptor proteins after SDS PAGE is to pre-label them covalently with an iodinated ligand (as has been done with angiotensin II receptors: Laribi, Allard, Vincent and Simonnet, 1987). For this reason the analysis of solubilised ^{125}I-buserelin binding sites by SDS PAGE was not attempted in this Thesis until a method of covalently radiolabelling them was achieved (Chapter 6.)

Limited proteolysis is another technique that has been used to

investigate the gross structure of proteins (Noelken, Nelson, Buckley and Tanford, 1965; Lowry, Slayter, Weeds and Baker, 1969; Spatz and Stittmatter, 1971). As in the case of the membrane protein cytochrome b_5, the presence of exposed or unstructured links between two domains in the proteins tertiary structure can be revealed (Robinson and Tanford, 1975). However, as for the sedimentation centrifugation techniques, partial proteolysis appears to require purified preparations of the solubilised protein. Prior to developing an efficient purification technique for the solubilised ^{125}I-buserelin binding protein only crude, unpurified solubilised preparations are available, making partial proteolysis investigations unsuitable.

From the literature it would appear that the ^{125}I-buserelin binding site, solubilised with an optimum yield of 73 ± 5% from rat anterior pituitary membranes, would be best characterised initially using ligand binding techniques. This would also enable the solubilised site to be positively identified as an LHRH receptor. Further characterisation may be possible using gel filtration techniques, to give an estimate of the apparent molecular weight of the solubilised protein.

Unfortunately it was clear that the presence of the CHAPS/NaCl solution,that uniquely provided high solubilisation yields,severely hampers ligand binding to the LHRH receptor site, both in situ in the membrane preparation and once solubilised (4.3.3. and 4.3.4.). Precipitation of solubilised proteins by PEG has previously been shown to have no effect on LHRH binding site characteristics (Perrin, Haas, Rivier and Vale, 1983; Winiger, Birabeau, Lang, Capponi, Sizonenko and Aubert, 1983; and 2.4.1.3). So inclusion of

a PEG precipitation step to remove the CHAPS/NaCl solution from the solubilised proteins should allow the binding characteristics of the formerly-solubilised ^{125}I-buserelin binding site to be determined, confirming whether or not these sites represent undamaged, high affinity LHRH receptors.

5.2 Materials and Methods

5.2.1 Materials

LHRH and its analogues [des pGlu^{1}]-LHRH and [DpGlu^{1}, DPhe^{2}, DTrp^{3,6}]-LHRH were obtained from Sigma Chemical Company. Sepharose 6B and high and low molecular weight calibration kits were from Pharmacia Fine Chemicals.

5.2.2 Methods

5.2.2.1 Characterisation using ligand binding techniques of the solubilised ^{125}I-buserelin binding protein

Rat anterior pituitaries solubilised as in Chapter 4.2.2.1 using 20 volumes of 1.5M NaCl, 5mM CHAPS (in 25mM Tris-HCl pH 7.4 with 50mg/ml soybean trypsin inhibitor and 400KIU/ml approtinin, 4.2.2.1) were used. Aliquots (50µl) of the supernatant were first subjected to a PEG precipitation (Chapter 2,4.1.3) after increasing the volume to 500µl using 25mM Tris-HCl pH 7.4/0.1% BSA (Tris/BSA).

The resultant pellets were resuspended using Tris/BSA to a total volume of 500µl containing ~50,000 cpm ^{125}I-buserelin and unlabelled LHRH analogue as appropriate. Non-specific binding was determined in the presence of 1µM LHRH (Chapter 2.4.1.2). After incubation for 90 minutes at 4°C, equilibrium had been reached (Chapter 2.4.1.2) and bound and non-bound ^{125}I-buserelin were separated by a second PEG precipitation. Displacement of ^{125}I-buserelin by LHRH, buserelin (a superagonist), [des pGlu^{1}]-LHRH (a partial agonist) and [DpGlu^{1}, DPhe^{2}, DTrp^{3,6}]-LHRH (antagonist) was determined. In all cases binding data were analysed according to the Eadie-Hofstee method (Chapter 2.5).

5.2.2.2 Estimation of the apparent molecular weight of the solubilised ^{125}I-buserelin binding protein by gel filtration.

SDS-PAGE techniques have been used previously to give estimates of the molecular weight of an LHRH binding site protein. Values obtained have been in the range of 60,000 (Hazum, 1981; Jansem de Almeida Catanho, Berault, Theoleyre and Justisz, 1983; Edne, Hendricks and Millar, 1985; Iwashita and Catt, 1985). These results obtained under conditions of protein denaturation, contrast with the estimate of a molecular weight of 135,000 for in situ LHRH receptors (Conn and Venter, 1985). The matrix chosen here for gel filtration of the solubilised ^{125}I-buserelin binding site under mild detergent conditions was therefore Sepharose 6B, with a molecular weight fractionation range of 10,000-2,000,000. The column (see 2.7.1 for details) was calibrated using proteins from both the high and low molecular weight calibration kits, to construct a calibration curve covering the molecular weight range 13,700 to 235,000 (Fig. 2.9). Before every gel filtration of the solubilised anterior pituitary preparation, the void volume (V_o) of the column was determined using dextran 2,000 (Pharmacia Fine Chemicals).

The density of the solubilised preparation was first increased by the addition of sucrose (50mg/ml), the preparation was then applied to the column (using a 5ml capacity sample applicator, Pharmacia Ltd). This facilitated the entry of the sample onto the gel bed as a well-defined band which would optimise the resolution of the sample into its different molecular weight constituents. As described in 2.7.1, the sample was eluted using the 5mM CHAPS/1.5M NaCl solubilisation buffer, at a flow rate of 19.5ml/hr. Fifteen minute

fractions were collected and samples (1.3ml) were subjected to a PEG precipitation (2.4.1.3). This was scaled up appropriately to a total volume of 35mls, and after standing on ice for 20 minutes the precipitated proteins were separated by centrifugation for 30 minutes at 15,000g. Following resuspension into 1.8ml of 25mM Tris-HCl pH 7.4, 300µl aliquots were used in ^{125}I-buserelin binding assays (as previously described, 2.4.1). A plot of specific binding against elution volume enabled the elution volume (V_e) of the peak(s) of specific binding to be determined. This in turn could be used to calculate a K_{av} value -

$$Kav = \frac{Vt - Vo}{Vt - Ve}$$

where Vt is the total volumn volume.

The apparent molecular weight of the protein making up the peak(s) of specific binding could then be estimated using the calibration curve.

5.3 Results

5.3.1 Analogue Binding to the Solubilised Preparation

^{125}I-Buserelin binding to the PEG precipitated solubilised preparation under the conditions described was 52 ± 4% specific compared to 69 ± 3% for an equivalent amount of rat anterior pituitary membrane preparation (calculated from initial tissue weight wet: ± SEM, n = 4 in both cases) and was displaced in a concentration-dependent manner by all the LHRH-analogues tested (Fig. 5.1). Hofstee analysis (Fig. 5.2) of the displacement data gave $K_{dissociation}$ constants very similar to those found on the anterior pituitary membrane preparation (Table 5.1). The finding of similar rank order of analogue affinity and actual K_i values in both the membrane and solubilised preparations would strongly suggest that the solubilised ^{125}I-buserelin binding protein represents solubilised, functional LHRH receptors. This could be confirmed by showing the same influences of cations and guanine nucleotides on ligand binding to the solubilised LHRH receptor site as characterise the in situ receptor (Chapter 3). However optimal solubilisation requires the presence of 1.5M NaCl (4.3.1). The probability of a single PEG precipitation step removing all NaCl might be expected to be relatively low. Therefore the effect on ligand binding by the addition of other cations could possibly be obscured. In the case of the effect of guanine nucleotides on agonist binding to the LHRH receptor in a soluble state, lengthy pretreatments and the presence of Mg^{2+} ions are required to reveal it in the membrane preparation (3.3.3). Until improved antagonist radioligands are available (see Chapter 3) it is unlikely that this effect would be observed using a solubilised preparation.

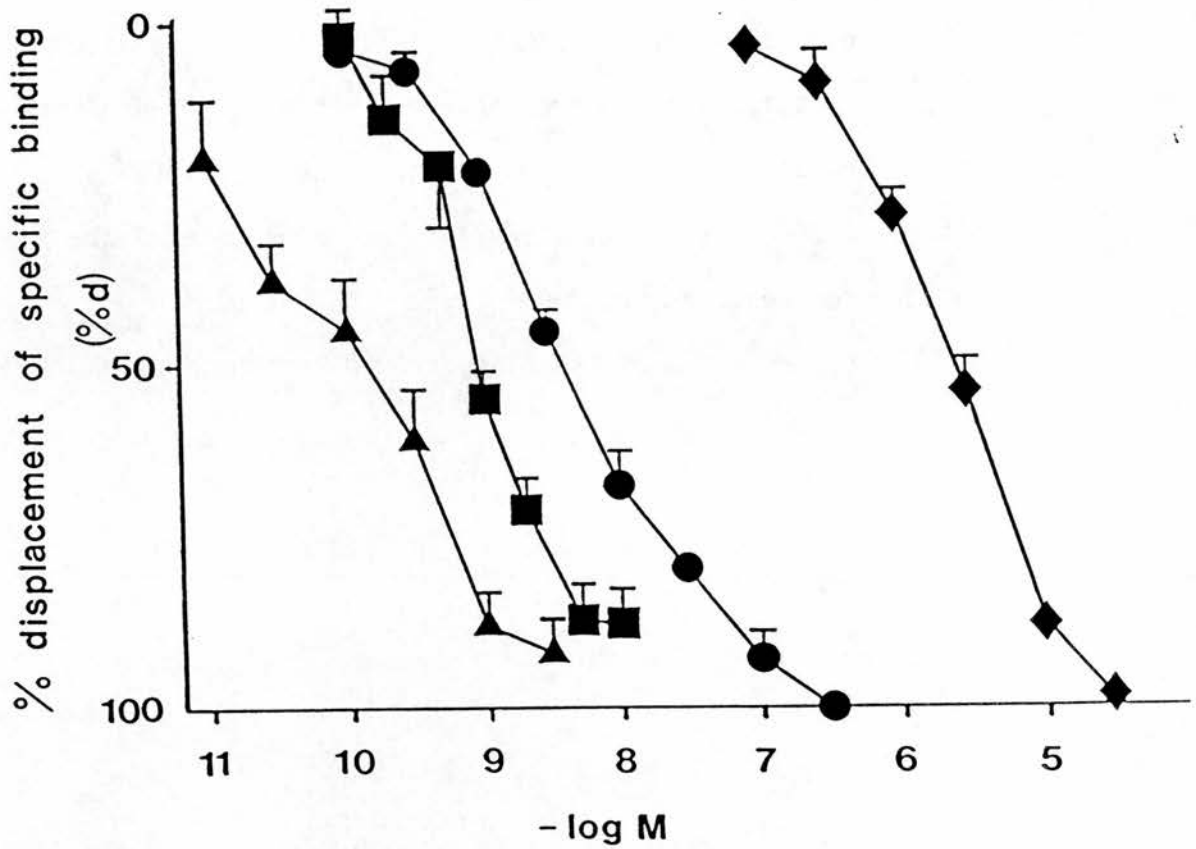


Figure 5.1

Displacement of specific ^{125}I-buserelin binding to the 5mM CHAPS, 1.5M NaCl solubilised rat anterior pituitary gland preparation by various LHRH analogues. Membranes were solubilised in 20 volumes of solubilisation solution as described (4.2.2.1) and the supernatant subjected to a PEG precipitation before ligand binding. Binding assays were carried out as described (2.4.1.2) with 50,000cpm ^{125}I-buserelin, supernatant aliquots equivalent to one sixth of an anterior pituitary, and various concentrations of unlabelled analogue in a total volume of 500µl 25mM Tris-HCl pH7.4, 0.1% BSA. Non-specific binding was determined using 1µM LHRH.

(●) LHRH

(■) Buserelin

(◆) [des-pGlu^{1}]-LHRH

(▲) [DpGlu^{1},DPhe^{2},DTrp^{3,6}]-LHRH

Each point is the mean ± SEM of 3-8 separate determinations.

Figure 5.2

Hofstee analysis of LHRH displacement of specific ^{125}I-buserelin binding to 5mM CHAPS, 1.5M NaCl-solubilised rat anterior pituitary gland preparation after a PEG precipitation step. Solubilisation and ligand binding were carried out as described (4.2.2.1 and 2.4.1.2) and the displacement data analysed by an error-weighted programme (2.5). K_i = 2.91 ± 0.4nM.

Points are means of three separate determinations.

Figure 5.2

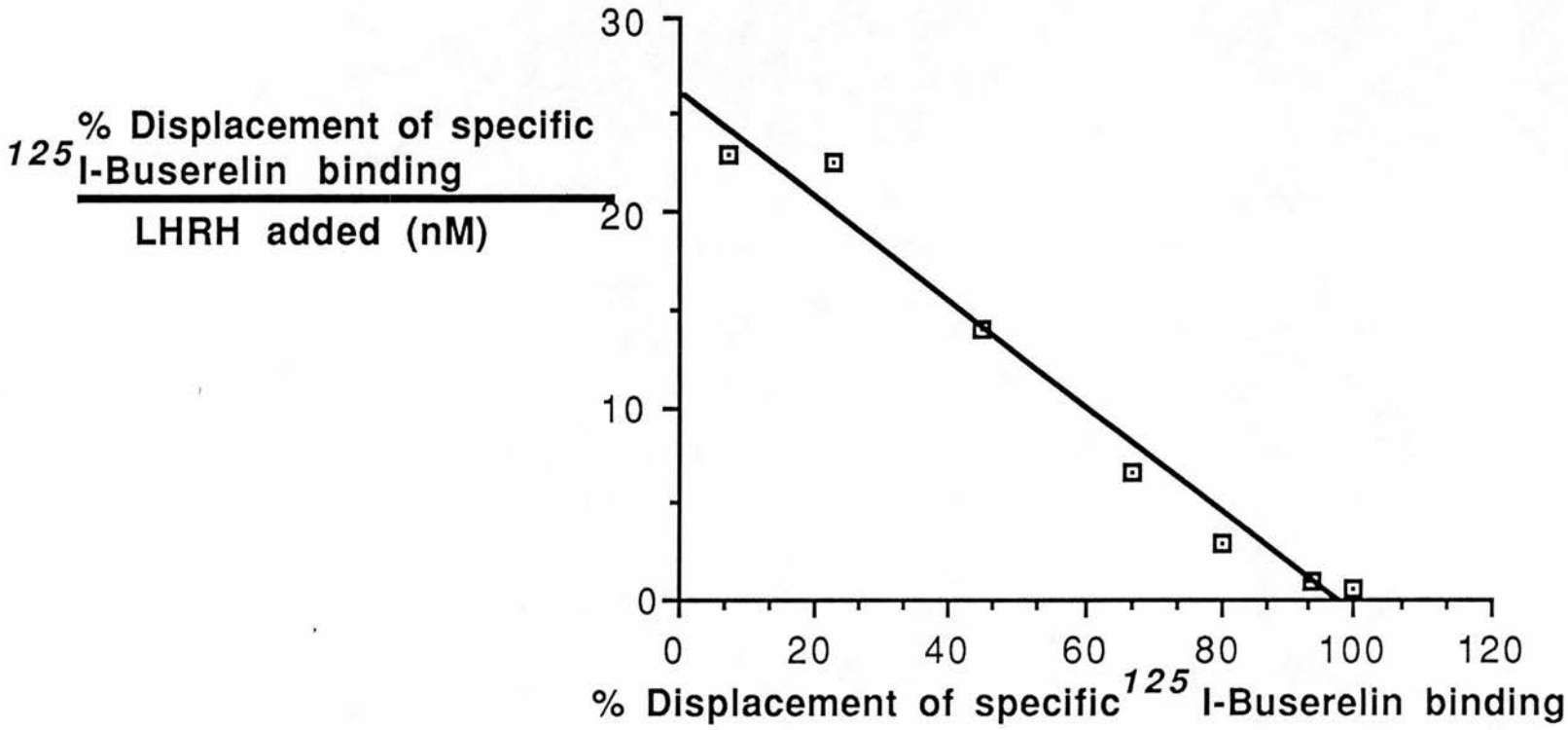

% Displacement of specific 125 I-Buserelin binding
LHRH added (nM)
30
20
10
0
0
20
40
60
80
100
120
% Displacement of specific 125 I-Buserelin binding

Table 5.1

Analogue	DISSOCIATION (K_i) constant	
	Membranes	Solubilised
LHRH	2.20 ±0.72nM	2.91 ± 0.4nM
Buserelin	0.47 ±0.14nM	0.66 ± 0.3nM
[D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}] LHRH	0.15 ±0.01nM	0.11 ± 0.02nM
[Des-pGlu^{1}] LHRH	1.52 ± 0.26 μM	2.53 ± 0.48 μM

Table 5.1

Dissociation constants of LHRH analogues for rat anterior pituitary gland membranes and solubilised preparations. ^{125}I-Buserelin displacement data were analysed according to the Eadie-Hofstee method by an error-weighted programme (2.5). Values are given as mean ± SEM of 3-6 separate determinations.

5.3.2 Gel filtration of the solubilised preparation

^{125}I-Buserelin binding to the PEG precipitated samples of fractions eluted from the sepharose 6B column revealed a peak of specific binding in fractions 45-48. This corresponds to an apparent molecular weight of between 100,000 and 160,000 (Fig. 5.3). Four separate gel elutions (each assayed in duplicate) consistently revealed the high molecular weight peak of specific binding. A second much smaller peak was found in fractions 54-56, corresponding to a molecular weight of 46,000 to 53,000. The specific ^{125}I-buserelin binding in the peak fractions represented approximately 35% of the total binding, compared to 52 ± 4% for the solubilised preparation before applying it to the gel filtration column. Subjecting a sample of the solubilised preparation to a large scale PEG precipitation, as described for the column eluate, resulted in less specific ^{125}I-buserelin binding being found than after a small scale PEG precipitation (1494 ± 32 cpm compared to 2727 ± 123 cpm: ± SEM, n = 4). This was accompanied by a large increase in non-specific binding (from 1787 ± 50 to 4108 ± 64), which could also explain to some extent the lower % specific binding seen in the eluted peak. Despite this reduction in detectable specific binding, protein assay (2.10) on the peak tubes revealed an increase in specific binding activity of seven fold over that in the membrane preparation. The non-specific binding (and total in the fractions which contained no specific ^{125}I-buserelin binding) was not constant for the entire elution. This presumably reflected the different proteins, eluted according to size, at different volumes from the column. The absence of specific binding in the void volume (V_o) fulfils another of the solubilisation criteria suggested by

Figure 5.3

Estimation of apparent molecular weight of the solubilised LHRH receptors using gel chromatography. After solubilisation in 30 volumes of 5mM CHAPS, 1.5M NaCl, the supernatant fraction (with 50mg/ml sucrose added) was loaded onto a Sepharose-6B column using a sample applicator (SA-5, Pharmacia Ltd). The column was equilibrated, calibrated and eluted using the CHAPS (5mM)NaCl (1.5M) solution.

The molecular weight standards and their weights (x 10^{3}) are as indicated: Vo, Blue Dextran 2000; T 669, thyroglobulin; C 232, catalase; Ald 158, aldolase; Alb 63, albumin; Ov 43, ovalbumin; R 13.7, ribonuclease. Fractions of the eluate were PEG precipitated and aliquots used in the ^{125}I-buserelin binding assay for duplicate determinations of specific and non-specific binding. The specific binding in the peak fractions (45-48) represented approximately 35% of total ^{125}I-buserelin binding. Solubilisation of 30 pituitaries gave some 24,000cpm of specific binding in the peak fraction of eluate, which when corrected for protein values (assayed for as in 2.10) indicated at least a 7-fold purification of the receptor over membrane values. The elution profile shows means ± SEM of specific binding (cpm) in samples of fractions from four separate gel elutions.

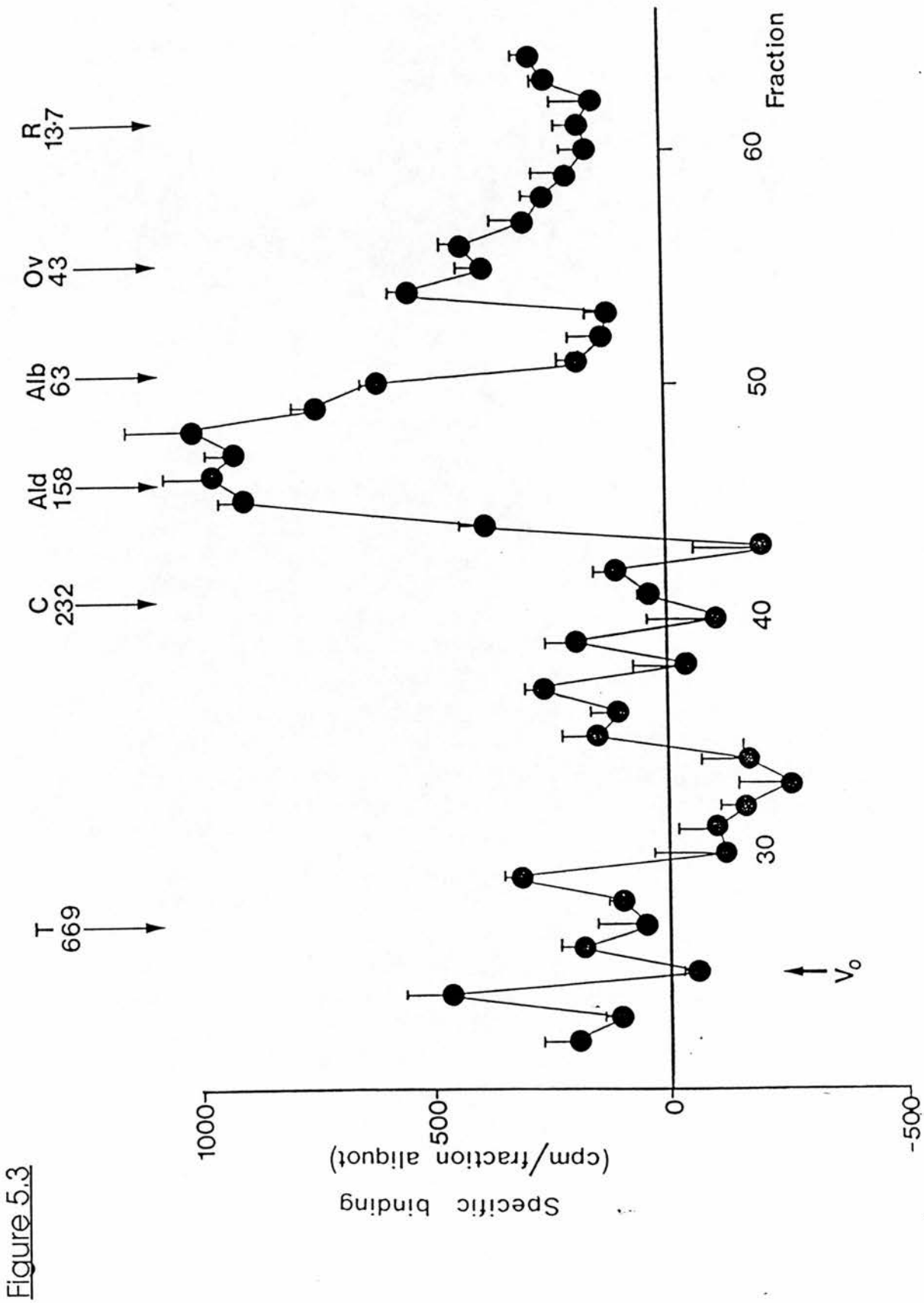

T
669
C
232
Ald
158
Alb
63
Ov
43
R
13·7
Specific binding
(cpm/fraction aliquot)
1000
500
0
-500
30
40
50
60
Fraction
V_o

Figure 5.3

Razin (1972) and confirms that the ^{125}I-buserelin binding protein (LHRH receptor binding site) is definitely in solution.

5.4 Discussion

Displacement of ^{125}I-buserelin from solubilised receptors by the four LHRH-analogues (native LHRH, a super-agonist, a partial agonist and an antagonist) has shown the solubilised receptor protein to have very similar binding characteristics to that of the in situ receptor. Any small difference in the calculated binding constants may be due to the removal of the receptor from its membrane environment and therefore the removal of the influence of adjacent membrane constituents. For example, the solubilised and membrane affinity constants for buserelin displacement of ^{125}I-buserelin were found to be 0.66 ± 0.3nM and 0.47 ± 0.14nM, and those seen for the antagonist in the two preparations were 0.11 ± 0.02nM and 0.15 ± 0.01nM, respectively. Whilst the differences in affinity constants seen for the two analogues are not significant, the trend was for the antagonist to have a higher affinity for the solubilised LHRH receptor, whereas buserelin (along with LHRH and the partial agonist [DpGlu^{1}]-LHRH) had a higher affinity for the membrane preparation. This may indicate that membrane constituents mediating the response to LHRH agonist binding are no longer present to exert their influence on agonist affinity in the solubilised preparation. As antagonist binding should be independent of receptor activation any influence seen might be expected to smaller, or in some other way different from that observed for agonists.

Hazum, Garritsen and Keinan (1982) have studied the effect of membrane lipid hydrolysis on the binding of LHRH analogues to female rat anterior pituitary membranes. They suggest that the reduction in affinity observed, apparently more pronounced in the case of agonists than antagonists, suggests a role for membrane phospholipids

in the interaction of LHRH and its receptor. These results are apparently in agreement with those of this investigation and the supposition of a role for membrane components in the binding of LHRH analogues (in particular agonists) to the anterior pituitary LHRH receptor. However further studies would be required to confirm these findings. Possibly the re-addition of membrane phospholipids to the solubilised receptor preparation would reverse the changes in dissociation constants found. If this were found to be the case, then it would strongly suggest a direct role for phospholipids in supporting the LHRH-binding conformation of the receptor. Such experiments may reveal different effects on adding different phospholipids. Feasibly, but not necessarily, this may reflect the second messenger system involved in LHRH receptor activation (e.g. a specific effect of polyphosphoinositides may suggest the involvement of phospholipase C activation and inositol trisphosphate production). It would also be interesting to investigate the role of other membrane proteins in LHRH receptor binding. For example, as shown in Chapter 3, activation of G-proteins can exert an influence on LHRH agonist binding. Addition of purified G-protein subunits to the solubilised membrane preparation (or if possible purified, solubilised LHRH receptors) may be found to affect LHRH analogue binding.

Gel filtration of the solubilised preparation gave an estimate of apparent molecular weight for the solubilised LHRH receptor of 100,000-160,000, or an approximate Stokes radius of 46 Å (Tanford, Nazaki, Reynolds and Makino, 1974). Determination of the molecular weight and size by this method relies on a good correlation between molecular radius and molecular weight (Ackers 1975). This may apply

to proteins in a denatured state or the globular soluble proteins used to calibrate the column but solubilised intrinsic membrane proteins have not been shown to exist as compact globular molecules (Tanford and Reynolds, 1976). This value must therefore be regarded only as an estimated apparent molecular weight or size of the solubilised LHRH receptor.

Despite this caveat the result is in good agreement for that of the molecular weight of the in situ LHRH receptor (135,000, Conn and Venter, 1985). The technique used by Conn & Venter to obtain the estimate of the in situ molecular weight of the LHRH receptor was radiation inactivation. This method depends on the relationship between the dose of radiation required to inactivate a macromolecule or multisubunit macromolecule and its molecular weight, and it has been suggested that by calibrating the system with proteins of known molecular weights that of an unknown protein can be found (Lo, Barnard and Dolly, 1982). As the molecular weight determined is that of any structure in a compact enough arrangement for significant energy transfer to occur within all parts of it, the technique in principle would be expected to detect any functional complex that exists. The finding of an apparently high molecular weight LHRH receptor by gel filtration and the binding data (showing affinities very similar to those found in the membrane preparation) suggest that the LHRH receptor solubilised by 5mM CHAPS/1.5M NaCl is in very much the same state as the membrane bound molecule detected by Conn and Venter (1985). The smaller peak of specific ^{125}I-buserelin binding eluting from the column at a volume suggestive of a molecular weight of 46,000 - 53,000 (a Stokes radius of ~30Å (Tanford, Nazaki, Reynolds and Makino, 1974)) is more

consistent with the molecular weight for LHRH receptors found under the denaturing conditions of SDS PAGE (Hazum 1982; Jansen de Almeida Catanho, Berault, Theoleyre and Justisz, 1983; Eidne, Hendricks and Millar, 1985; Iwashita and Catt, 1985). Whereas an LHRH immunoblotting technique visualised a single 60K band on SDS PAGE corresponding to the adrenal cortex LHRH receptor (Eidne, Hendricks and Millar, 1985),and photolabelled LHRH receptor sites from placenta revealed a single 53,700 molecular weight band (Iwashita, Evans and Catt, 1986),pituitary photolabelled LHRH binding sites have been found (using SDS PAGE) to reveal two molecular weight components (Iwashita and Catt, 1985) or a single 60,000 component (Hazum, 1981(b)) Two protein bands (60,000 and 54,000) have also been specifically photo-labelled with an LHRH agonist in ovarian granulosa cells (Hazum and Nimrod, 1982). In the rat, the two components specifically labelled in pituitary tissue appear to have molecular weights of about 59,000 and 40,000, the larger molecular weight component possibly being two (one of 63,000 and a smaller one of 52,000: Iwashita and Catt, 1985). This may suggest that the apparently high molecular weight species detected in this study using gel filtration is in fact a dimer of two identical or possibly non-identical binding subunits. Alternatively it may represent a single binding subunit in a complex with non-binding protein.

The existence <u>in situ</u> of the LHRH receptor as a dimer could be construed as consistent with evidence for a role of microaggregation in LHRH agonist action. Conn, Rogers, Stewart, Niedel and Sheffield (1982) demonstrated that an LHRH-antagonist could be converted into an agonist using a divalent antibody, suggesting dimenisation of LHRH binding sites as a requirement for activation. The solubilised

preparation used in these gel filtration had not been exposed to LHRH prior to membrane disruption. Following the agonist-induced dimerisation hypothesis, therefore, the detection of large receptor complexes here implied that at least part of the LHRH receptor population exists normally in the dimerised form.

As mentioned above, an alternative explanation is that the LHRH receptor has been solubilised as a complex of one binding site component and other subunit(s) involved in modulation of LHRH analogue binding or the mediation of the cells response to agonist binding. The ratio of detergent to membrane protein used here in the solubilisation step has been suggested to be sufficiently high to greatly reduce the probability of artifactual protein adhesion (Tanford and Reynolds, 1976). Any complex of molecules found should therefore exist due to definite attractive forces. These may however be disrupted by the major conformational changes associated with denaturation of the proteins, explaining why a much smaller apparent molecular weight of the receptor has been consistently observed in SDS PAGE experiments.

Interestingly many receptor types whose responses are mediated via a G-protein (Miller, 1988) have been solubilised both as isolated receptor binding sites and in a complex with a G-protein, both adenylate cyclase linked complexes (Limbird and Lefkowitz, 1978; Limbird, Gill and Lefkowitz, 1980; Smith and Limbird, 1981; Kirkpatrick and Caron, 1983; Nissenson, Mann, Winer, Teitelbaum and Arnaud, 1986; Couvineau, Amiranoff and Laburthe, 1986; Fitzgerald. Uhing and Exton, 1986; Watanabe, Umegaki and Smith, 1986) and phosphotidyl inositol linked receptors (Berri, Birdsall, Hulme, Keen and Stockton, 1984; Bojanic and Fain, 1986). In many cases it was

necessary to prelabel the membrane preparation with an agonist prior to solubilisation in order to obtain the receptor-G-protein complex. Agonists binding to the solubilised complex were then susceptible to modulation by GTP analogues. The presence of the G-protein resulted in an increase in apparent molecular weight of about 100,000. This is similar to the size difference between the denatured molecular weight of the LHRH receptor found on SDS PAGE and that found under non-denaturing gel filtration conditions (approximately 80,000).

Another possible explanation for the apparent high molecular weight of the LHRH receptor seen here is the binding of detergent and/or phospholipids to the protein. Binding of mild detergents to solubilised intrinsic membrane proteins was first shown for rhodopsin, where 180-200 molecules of digitonin can bind to one rhodopsin molecule (Hubbard, 1954). Many other membrane proteins have been shown to bind varying amounts of detergents from 20% of the apparent molecular weight (Meunier, Olsen and Changeux, 1973; Rubin and Tzagoloff, 1973) to over 50% (Sargent and Lampen, 1970; Simons, Helenius and Garoff, 1973). As soluble, globular proteins such as those used to calibrate the gel filtration column do not tend to bind mild detergents in a similar way (Tanford and Reynolds 1976) no compensation for this is included in the column calibration. Fish, Reynolds and Tanford (1970) showed that Kav values for such soluble proteins are independent of the detergent present. The binding of some detergents, such as triton X-100, to the solubilised protein can be seen as a change in the expected partial specific volume (calculated using centrifugation sedimentation techniques). This has been done for triton X-405

solubilised cardiac muscarinic receptors (Peterson, Rosenbaum, Broderick and Schimerlik, 1986), where the difference between the calculated partial specific volume and that measured gave an estimate of 1.011g of triton X-405 bound per g of receptor. For the prolactin receptor solubilised from rat hepatic membranes using triton X-100, 35 molecules of detergent have been estimated to be bound to every receptor molecule (Rae-Venter and Daod, 1983). However the partial specific volume of CHAPS is very similar to that of proteins (0.81 (Hjelmand, Nevert and Osborne, 1983) compared to 0.71-0.76). Binding of CHAPS even in very large amounts would not be expected to have a detectable effect on the measured partial specific volume of the solubilised receptor. Therefore the amount of detergent bound could not be measured using this technique. Other methods, such as equilibrium dialysis (Steinhardt and Reynolds, 1969) which require pure protein preparations, are necessary. Before purification techniques have been developed for the CHAPS/NaCl solubilised LHRH receptor, the measurement of detergent binding could not easily be achieved.

The presence of phospholipid in detergent-solubilised membrane proteins has also been shown (Awasthi, Chuang, Keenan and Crane, 1971; Chareau, Defau and Catt 1974). In these two examples (cytochrome oxidase and the testis gonadotropin receptor) activity of the protein is dependent on the presence of the phospholipid. In some cases phospholipids have to be re-added to solubilised proteins to restore activity (Cori, Garland and Chang, 1973). In the case of the LHRH receptor it is possible that removal of the CHAPS/NaCl by PEG precipitation allows for protein-phospholipid reaggregation in order to restore binding affinity. However as PEG precipitation

after gel filtration of the solubilised preparation also results in a return of binding affinity, this is unlikely as phospholipids are likely to be separated from proteins by gel filtration (Helenius and Simons, 1975). The presence of tightly bound phospholipids in the solubilised LHRH-receptor complex cannot however be ruled out, although serotonin receptors solubilised by a similar CHAPS/NaCl solution have been shown to be free of bound phospholipid (Wouters, van Dun, Leysen and Laduron, 1985).

In conclusion the solubilised ^{125}I-buserelin binding site has been shown to have very similar binding characteristics to the in situ LHRH receptor. This suggests that it is in fact the LHRH receptor in solution. Estimates of the apparent molecular weight of the solubilised LHRH receptor by gel filtration are of a major component at 100-150,000 and a small amount at 46-53,000. Whilst the lower molecular weight component is in good agreement with the LHRH receptor molecular weight obtained using SDS PAGE techniques, the higher is in agreement with estimates of the receptor size made in situ by radiation inactivation techniques. This high molecular weight receptor may represent a dimer of identical or non-identical binding sites, a single binding site associated with accessory or transducing elements (possibly a G-protein), or a binding site in a complex with bound detergent and/or phospholipid. Present techniques do not allow for these alternatives to be distinguished. The absence of any specific ^{125}I-buserelin binding from the void volume eluate confirms the truly solubilised state of the rat anterior pituitary LHRH receptor protein.

Chapter 6

Strategies for LHRH receptor purification

6.1 Introduction

Characterisation of the proteins making up receptor macromolecules, as mentioned before (4.1) can be achieved at two levels - characterisation of their secondary and tertiary structure (that is physical characterisation - such as molecular weight, subunit composition and so on) and the characterisation of their primary structure or amino-acid sequence (or molecular characterisation). The approach used in this Thesis has been initially to solubilise the receptor protein using a mild detergent (Chapter 4). Whilst this allows for the characterisation of the LHRH receptor in the crude solubilised extract by ligand binding and gel filtration techniques, further more detailed characterisation requires that the receptor protein is purified. Purification of solubilised receptor proteins has been a widely used as an initial step in the strategy for their molecular characterisation (Strosberg, 1987). Extensive purification is required prior to attempting the amino-acid sequencing of any protein (Koningsberg and Steinman, 1977). The more complex the protein, the more crucial the purity of the sample is for precise sequencing, up to 97% purity has been a suggested requirement (Koningsbert and Steinman, 1977). For very large complex proteins such as receptors a common strategy is to sequence highly purified portions (such as the amino-terminal or enzymatic proteolysis fragments) of the whole (Strosberg, 1987). For example, for the Electrophorus electricus sodium channel (Noda et al., 1984), growth hormone receptor and serum binding protein (Leung, Spencer, Cachlenes, Hammonds, Collins, Henzel, Barnard, Waters and Wood, 1987), and the dihydropyridine calcium antagonist

receptor (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirase and Numa, 1987), primary structures were determined by cloning and sequencing complementary DNA screened with oligonucleotides prepared on the basis of partial amino-acid sequences of the purified proteins.

Since the LHRH receptor (in common with other peptide hormone receptors) occurs in only low abundance in anterior pituitary tissue (90fmol per gland in male rat anterior pituitary tissue) a highly selective method of purification is required. One such method is affinity chromatography (Strosberg, 1984; Wilchek, Moron and Kohn, 1984). Purification is achieved by the specific interaction of the protein of interest with immobilised ligands that are highly selective for the protein. Unrelated proteins are removed by washing through the affinity column to elute non-bound molecules. Selective removal of the required protein can be achieved by the addition of excess free ligand, alternatively by the use of non-selective means such as the manipulation of pH, temperature or ionic conditions (Strosberg, 1984; Wilchek, Miron and Kohn, 1984). The method of affinity chromatography was first described for the isolation of antibodies (Lerman, 1953) and with the introduction of improved, inert gels for ligand immobilisation, has been adapted for the purification of other proteins (Cuatrecasas, Wilchek and Anfinsen, 1968). Many different membrane receptors have been purified using the technique, for example the insulin receptor (Jacobs, Shechter, Bissell and Cuatrecasas, 1977), β_1 adrenergic (Shorr, Lefkowitz and Caron, 1981; Shorr, Strohsacker, Lavin, Lefkowitz and Caron, 1981), α_1 adrenergic (Graham, Hess and Homay,

1982), glycine receptor (Pfeiffer, Graham and Betz, 1982), β_2 adrenergic receptor (Homcy, Rockson, Countoway and Egan, 1983), $GABA_A$-benzodiazepine receptor (Sigel and Barnard, 1984) and a muscarinic acetylcholine receptor (Haga and Haga, 1985). In all cases the receptor protein was first solubilised from a membrane preparation, and the solubilised preparation applied to the ligand affinity column. The retention of receptor protein affinity for the ligand utilised in the presence of the detergent solution as used for the affintiy chromatography step was therefore essential. Unfortunately in the case of the LHRH receptor, extensive investigations suggested that these two conditions - retention of the protein in solution and retention of its affinity for LHRH analogues - were mutually incompatible (Chapter 4). If the principle of affinity chromatography was to be used for LHRH receptor purification, some alternative strategy would be required.

A method of affinity chromatography based on the biotin-avidin/streptavidin interaction has been described (Hofmann and Finn, 1985; Kohanski and Lane, 1985). Incorporation of a biotin molecule into a specific ligand for a receptor enables that receptor to be purified on an avidin/streptavidin-gel. This method has been used to purify insulin receptors (Kohanski and Lane, 1985(b)), growth hormone receptors (Haruptle, Aubert, Djiane and Kraehenbuhl, 1983) and parathyroid hormone receptors (Brennan and Levine, 1987). Biotin analogues of many specific receptor ligands have been synthesised - including insulin (May, Williams and de Haen, 1978), Leu^5-enkephalin (Koman and Terenius, 1980), glucagon (Flanders, Hung Mar, Folz, England, Coolican, Harris, Floyd and Gurd, 1982),

adrenocorticotropic hormone (Romovacek, Finn and Hofmann, 1983) and β nerve growth factor (Rosenbert, Hawrot and Breakefield, 1986). [Biotinyl D-Lys^6]-LHRH has been used to localise LHRH receptors on anterior pituitary cell cultures using avidin and a biotin-labelled peroxidase to visualise the binding sites (Childs, Baor, Hazum, Tibolt, Westland and Hancock, 1983). The same analogue has been used in an attempt to purify LHRH receptors by affinity chromatography utilising its interaction with an avidin affinity column (Hazum, Schvartz, Waksman and Keinan, 1986). In that case the initial solubilisation of LHRH receptors was carried out in 5mM CHAPS, which has been found to give only 20% solubilisation of the rat anterior pituitary LHRH receptor sites (Chapter 4). The affinity chromatography step was performed in 1mM CHAPS, 10% glycerol, conditions which were found earlier (4.3.5) to result in an apparent loss of 19% of solubilised binding sites, retention in solution of only 67% of sites and detectable binding to only 55% of the total sites available. Under these conditions, Hazum, Schvartz, Waksman and Keinan estimate a recovery yield of 4-10% of the solubilised receptor from the affinity chromatography step.

It has been suggested (Finn, Stehle and Hofmann, 1985) that the ligand-biotin, avidin affinity chromatography method could be adapted for receptor purification by covalently labelling receptors with the biotin analogue. Covalent labelling of a biotin-ligand to the LHRH receptor site in the membrane preparation would by-pass the problem of detergent suppression of LHRH analogue binding.

Covalent labelling of receptor proteins has been commonly achieved in two ways -

1) Photoaffinity labelling (Bayley and Knowles, 1977)

2) Affinity crosslinking (Pilch and Czech, 1984)

Both methods have been investigated here in conjunction with various iodinated and biotinylated LHRH ligands to assess optimal conditions for the covalent affinity labelling of LHRH receptors for their subsequent purification.

6.2 Materials and Methods

6.2.1 Materials

[DLys^6]-LHRH was obtained from Peninsula Laboratories Europe Ltd (Merseyside) and [^{125}I]-Denny-Jaffe reagent (N-[4-(p-azido-m-[^{125}I]iodophenylazo)-benzoyl]-3-amindopropyl-N'-oxysuccinimide ester) from NEN Research Products (Du Pont (UK) Ltd, Herts). The divalent crosslinkers BS^3 (bis(sulphosuccinimidyl) suberate), DSS (disuccinimidyl suberate), DST (disuccinidyl tartarate), EGS (ethylene glycolbis(succinimidylsuccinate) and sulpho-SANPAH (sulphosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate) were purchased from Pierce and Warriner (UK) Ltd (Chester). LHRH analogues PBL ([biotinyl-p-azidophenylalanyl-DLys^6]-LHRH),[DLys^6, des Gly^{10}]-LHRH ethylamide, PBAL ([biotinyl-p-azido phenylalanyl-DLys^6, des Gly^{10}]-LHRH ethylamide) and XBAL ([biotinyl-aminoethylglycyl-DLys^6, des Gly^{10}]-LHRH ethylamide) were synthesised by Dr C.M. Bladon (2.11 and Appendix III). All other chemicals and peptides, unless otherwise stated, were obtained from Sigma Chemical Company Ltd (Dorset).

6.2.2 Methods

6.2.2.1 Label transfer crosslinking of the LHRH-receptor site using the [^{125}I]-Denny-Jaffe reagent

The commercially available [^{125}I]-Denny-Jaffe reagent is a photoactivatable, cleavable crosslinking reagent. Its structure is based on the N-[4-(p-azido-m-[^{125}I] iodophenylazo)-benzoyl]- 3-aminopropyl-N'-oxysulphosuccinimide ester used by Denny and Blobel (1984) in the affinity radiolabelling of human serum IgG. The [^{125}I]-Denny-Jaffe reagent is a heterobifunctional molecule with both an N-hydroxysuccinimide ester group and a photoreactive aryl

azido group. This enables it to be linked first with a peptide via an amino group and subsequently photoactivated to give rise to a highly reactive aryl nitrene of non-selective reactivity (Ji, 1976). It should therefore be possible to react the reagent with a specific ligand, equilibrate the derivatised ligand with its specific receptor or binding site and, as a result of photoactivating the reagent, covalently label the receptor or binding site with the [^{125}I]-Denny Jaffe reagent. Subsequent cleavage of the reagent by dithionite splits the reagent molecule between the iodinated portion crosslinked to the receptor site and that derivatised with the specific ligand (Denny and Blobel, 1984) raising the possibility of ligand dissociation and hypothetically allowing for the subsequent binding of other specific ligands.

The LHRH-analogue chosen for conjugation with the [^{125}I]-Denny-Jaffe reagent was [DLys^{6}]-LHRH which contains only one free amino-group, on the DLys^{6} side chain. As the reagent is supplied as a solution in benzene and the reaction with the peptide was to occur in aqueous conditions, the benzene was first evaporated, immediately prior to use, with dry nitrogen. Either an excess of [DLys^{6}]-LHRH (approximately 24,000 fold molar excess over the [^{125}I]-Denny-Jaffe reagent) or an equimolar amount of [DLys^{6}]-LHRH was added in a total of 100µl phosphate buffer, pH 8. After being left to react for an hour at room temperature, excess (0.5ml of 0.2M) glycine was added to quench the reaction, and the mixture was shaken and left at room temperature for a further 4 minutes. The reaction mixture was then diluted 1:10 into 25mM Tris-HCl pH 7.4, 0.1% bovine serum albumin (Tris/BSA).

Binding of the conjugated [DLys^{6}]-LHRH-[^{125}I]-Denny-Jaffe

was investigated on anterior pituitary membranes. Rat anterior pituitary membranes were prepared as previously described (2.4.1.1). Two anterior pituitary gland membrane equivalents were used in a total volume of 1ml of Tris/BSA with 10µl of the diluted [DLys^6]-LHRH-[^{125}I]-Denny Jaffe conjugate added. Non-specific binding was determined in the presence of 1µM LHRH. Binding was allowed to reach equilibrium (90 minutes at 4°C as previously described: 2.4.1.) before photoactivation of the reagent occurred. All previous steps had been carried out under conditions of subdued lighting to prevent the photoactivation of the reagent (Denny and Blobel, 1984). Photolysis was carried out using a 12 watt Hg lamp (366nm) at a distance of 5cm for 10 minutes (Jaffe, Lis and Sharon, 1980). Subsequent cleavage of the reagent [DLys^6]-LHRH conjugate (to leave specifically radioiodinated LHRH receptor sites) was achieved by two successive additions of 100µl sodium dithionite (2M, pH 7.6 in 25mM Tris-HCl) followed by a 15 minute reaction period.

Separation of the [^{125}I]-labelled receptors from free [^{125}I]-reagent was carried out by diluting the reaction mixture five-fold with Tris/BSA followed by centrifugation (1,600g for 20 minutes at 6°C). Specific labelling could be determined by comparing total [^{125}I]-binding with that carried out in the presence of 1µM LHRH. Bound radiolabel was counted using a γ-counter.

6.2.2.2 Affinity labelling of the LHRH-receptor with biotinylated LHRH-analogues.

Development of the method of receptor affinity chromatography by biotin-avidin interaction (Hoffman and Finn, 1985; Kohanski and Lane, 1985) for use with solubilised LHRH receptor proteins requires

the crosslinking of a biotin molecule onto the receptor site. One way of achieving this would be by covalently labelling the receptor site with specific, biotinylated LHRH analogues (Finn, Stehle and Hoffmann, 1985).

The problem of covalent binding of a biotinylated-LHRH analogue was approached in two ways - using photoaffinity biotinyl-LHRH analogues (6.2.2.2.1) and a non-photoreactive biotinyl-LHRH analogue in conjunction with bifunctional chemical crosslinkers (6.2.2.2.3).

6.2.2.2.1 Use of photoreactive, biotinyl-LHRH analogues as affinity ligands for the LHRH-receptor.

As previously described (2.9) the LHRH molecule is thought to be in the form ofahairpin, with a β turn at position 6 forming the bend (Momany, 1976(a)) and the two extremities of the amino-acid sequence being included in receptor interaction (Momany, 1976(b)). Substitutions at position 6, as long as they did not disrupt the backbone shape of native LHRH, have been shown to have no deleterious effects on its affinity for the receptor (Momany, 1976(b)). Positioning of the biotin and photoreactive groups on the side chain of a suitable amino acid at position 6 therefore seemed least likely to disrupt specific binding of the analogue to the LHRH receptor site. The most suitable photoreactive group would appear to be an aryl azide, a group said to be chemically inert in an aqueous medium but with a low activation energy and being activated at relatively long wavelengths (see 2.11.2.1).

Two photoreactive, biotinyl-LHRH analogues were synthesised (2.9.4 and Appendix III), PBL and its des Gly^{10}-ethylamide derivative PBAL.

In order to confirm that these two analogues retained their

specific affinity for the LHRH-receptor site the binding characteristics on anterior pituitary membranes of PBL and the amino-acid backbone of PBAL, (that is, [DLys^{6}, des Gly^{10}]-LHRH ethylamide) were investigated. Insufficient PBAL was available to allow similar ligand binding assays to be performed with it. Displacement of ^{125}I-buserelin from anterior pituitary membranes by the unlabelled peptides was determined as previously described (2.4.1) but under conditions of subdued light for PBL, and the data analysed to give $K_{dissociation}$ constants (2.5). Iodination of PBL and PBAL by the chloramine-T method (2.3 and Appendix I) allowed the specificity of their binding to be determined (non-specific binding levels determined in the presence of 1μM LHRH). Again ligand binding incubations using the photoreactive analogues were carried out in subdued light.

Photoactivation of PBL and PBAL was initiated on membrane preparations that had reached binding equilibrium with the iodinated analogues (as described previously: 2.4). For all the crosslinking studies (photoaffinity and chemical crosslinkers) Hepes-KOH (25mM, pH 7.4) replaced Tris/BSA in the ligand binding assay (as both Tris and BSA contain primary amino groups which would compete for the crosslinking reaction; Pilch and Czech, 1984). For photoreactive analogues the assays were carried out in subdued light. Non-specific binding and crosslinking were determined in the presence of 1μM LHRH. Exposure to UV light (366nm) at a distance of 20cm from a 12 watt Hg lamp for 0-10 minutes, on ice, was used to activate the aryl azide moiety. The membrane preparation was then centrifuged (45 minutes at 1,600g, 6°C, subdued light) to remove excess unbound label.

A period of dissociation and displacement by 1μM LHRH at room temperature was used to assess the efficiency of the crosslinking reaction. The photo-activated membrane preparations (total and non-specific determinations of ^{125}I-PBL and ^{125}I-PBAL photoaffinity labelling, in triplicate) were resuspended into 0.75ml Tris/BSA (1.5 x equilibrium binding reaction volume) containing 1μM LHRH and the peptidase inhibitors soyabean trypsin inhibitor (50μg/ml) and aprotinin (400KIU/ml). The peptidase inhibitors were included to try to minimise any enzymatic degradation of both ligand and receptor. Radioactivity present initially was determined by γ-spectrometry whilst the preparations were left for 4 hours at room temperature in the dark. After this time the membranes were again centrifuged (to separate any dissociated ^{125}I-ligand) and recounted. Comparison of the difference between total and non-specific binding-samples both before and after the period of dissociation was used to estimate the fraction of the specific binding that had been covalently attached.

6.2.2.2.2 Binding of a non-photoreactive biotinyl-LHRH ligand to anterior pituitary membranes.

The non-photoaffinity biotinyl-LHRH analogue synthesised for use with chemical crosslinkers was XBAL. The amino-acid backbone of the ligand [DLys^{6}, des Gly^{10}]-LHRH ethylamide binds to the LHRH receptor site with high affinity (6.3.2, Fig. 6) and would be expected to have high specificity for the site (2.9.1). In addition to the biotin molecule, the side chain contains a free amino group with which N-hydroxysuccinimide ester containing bivalent chemical crosslinkers can react (2.9.2.2).

XBAL binding to rat anterior pituitary membranes was

investigated by its displacement of both ^{125}I-buserelin and ^{125}I-XBAL from the membrane preparation. The peptides were iodinated by the chloramine-T method (as described in 2.3 and Appendix I) and the equilibrium binding assays were carried out as in 2.4. Displacement data were analysed by a computer programme (Zivin and Waud 1982) to yield dissociation constants (Ki) as previously described (2.5). For the self-displacement assay, washed membranes in Tris/BSA (as used in ligand binding assays, 2.4) were incubated with increasing concentrations of ^{125}I-buserelin together with 50,000 cpm of ^{125}I-buserelin. For each condition non-specific binding was determined in the presence of 1μM LHRH. After graphical transformation of the data according to Scatchard (1949), it was analysed by linear regression to yield a dissociation constant (K_D) and a value for the total number of binding sites (B_{max}) for ^{125}I-XBAL.

6.2.2.2.3 Optimisation of LHRH-receptor affinity labelling using bifunctional chemical crosslinkers and ^{125}I-XBAL

Two types of bifunctional chemical crosslinkers were used in these studies, a heterobifunctional photoreactive, N-hydroxysuccinimide ester (sulpho-SANPAH) and homobifunctional N-hydroxysuccinimide esters (DSS, DST, BS^3 and EGS). Of the homobifunctional crosslinkers only BS^3 was soluble in aqueous solution, all the others required to be dissolved initially in an organic solvent, DMSO (dimethysulphoxide). All of the chemicals were kept desiccated and only dissolved immediately prior to use to prevent the hydrolysis of the N-hydroxysuccinimide esters to free acid (Pilch and Czech, 1984). ^{125}I-XBAL iodinated by the chloramine-T method and purified by HPLC (Appendix I) was used in

these studies. This avoided the inclusion of additional protein (in the form of BSA) in the binding and crosslinking steps of the procedure.

The heterobifunctional sulpho-SANPAH was crosslinked with ^{125}I-XBAL before it was used in membrane equilibrium binding. Excess sulpho-SANPAH (1.5mg) was left to react for 40 minutes with ~0.03µg ^{125}I-XBAL in Hepes-KOH (200µl) at room temperature in the dark. An equal volume (200µl) of 0.2M glycine was then added to quench the reaction, and the ^{125}I-XBAL-sulpho-SANPAH conjugate diluted in Hepes-KOH for use in membrane equilibrium binding (2.4) under subdued lighting conditions.

After binding equilibrium had been reached, the photo-reactive ^{125}I-XBAL-sulpho-SANPAH was photo-activated and the resulting covalent specific labelling of the LHRH-receptor site was determined as described for the photo-reactive analogues PBL and PBAL (6.2.2.2.1).

For the homobifunctional crosslinkers, ^{125}I-XBAL was first allowed to come to equilibrium (total binding and non-specific conditions) with anterior pituitary membranes (2.4, except that Hepes-KOH replaced the Tris/BSA buffer). After dissolving the crosslinkers into DMSO, or Hepes-KOH in the case of BS^3, they (or vehicle) were added to the membrane preparation at 1:100 dilutions. These were then vortexed and left for 30 minutes either at room temperature (BS^3 and DST) or on ice (EGS and DSS), after which excess glycine (to 67mM) was added to quench the reactions. Excess unbound ^{125}I-XBAL and crosslinker were then separated by centrifugation (45 minutes at 1,600g, 6°C) and the labelled membranes resuspended into Tris/BSA, 1µM LHRH, peptidase inhibitors

and assayed for specific LHRH-receptor site covalent labelling as previous described (6.2.2.2.1).

6.2.2.3 Biotin affinity chromatography of the covalently labelled and solubilised LHRH receptors.

Successful purification of biotin-labelled LHRH-receptors using biotin-streptavidin affinity chromatography is dependent on a number of factors.

1. Retention of the biotin-streptavidin affinity under the detergent conditions used,
2. Accessibility of the biotin molecule crosslinked to the LHRH receptor for interaction with the streptavidin and vice-versa
3. The specificity of the biotin labelling of the LHRH-receptor.

Under the 5mM CHAPS/1.5M NaCl conditions used here to maintain the LHRH-receptor in solution, it has been shown that the ^{3}H-biotin-avidin-sepharose interaction is largely unchanged (2.6). Optimisation of the specific crosslinking of biotinyl-LHRH analogues to rat anterior pituitary membranes (6.2.2.2.1 and 6.2.2.2.3) would theoretically result in a suitable level of specificity of biotin labelling of the LHRH receptors. The remaining unknown factor is the accessibility of the biotin and streptavidin molecules for each other. In order to increase the probability of the two being accessible for interaction, carbon spacers were used in linking the streptavidin to the agarose (a six carbon spacer). PBL displacement of ^{3}H-biotin from avidin-sepharose in the presence of 5mM CHAPS/1.5M NaCl was assayed as previously described (2.8.1) to determine whether or not the LHRH-backbone interfered with the biotin-avidin interaction.

Rat anterior pituitary membranes were covalently labelled with

either ^{125}I-PBL or ^{125}I-XBAL and 0.5mM EGS as described above (6.2.2.2.1 and 6.2.2.2.3; for both ligands total and non-specific conditions were assayed in parallel). The labelled sites were then solubilised using 5mM CHAPS/1.5M NaCl (4.2.1). The resultant labelled supernatant was used for biotin affinity chromatography (2.6). The eluate was collected (both before and in the presence of 2mM biotin) and counted by γ-spectrometry to follow the progress of the biotin-^{125}I-labelled components.

6.2.2.4 Estimation of the molecular weight of the covalently labelled LHRH receptor using SDS PAGE

Previous studies using photoaffinity analogues of LHRH have shown specific labelling of one or two proteins of ~ 60,000 mwt to be revealed using SDS PAGE techniques (Hazum, 1981(b); Iwashita and Catt, 1985). The same techniques (described in full in 2.8) have been used here to try to identify the protein specifically labelled by ^{125}I-XBAL and EGS in rat anterior pituitary membranes.

6.2.2.5 The use of bovine anterior pituitary tissue for the covalent labelling of LHRH receptors using biotinylated-LHRH analogues.

Being far larger than rat anterior pituitaries, bovine anterior pituitaries are potentially a better source of LHRH receptors for large scale preparative purposes (see 3.3.7). Conditions for optimal covalent labelling of LHRH-receptors using the biotinylated LHRH ligand XBAL were therefore tested on bovine tissue.

Initial displacement assays of ^{125}I-buserelin with XBAL on bovine anterior pituitary membranes (as described in 2.4.2) were carried out in order to confirm that XBAL bound to the bovine LHRH receptor. The specificity of the ^{125}I-XBAL binding sites, its

displacement constant (K_D) and the number of binding sites (B_{max}) were determined by Scatchard analysis of self-displacement data. This was carried out as described for rat anterior pituitary membranes (6.2.2.2.2) except that bovine anterior pituitary membranes were used in a Tris/BSA peptidase inhibitor medium (2.4.2.1) and the samples were left at 4°C for 16 hours to come to equilibrium (Jansem de Almeida Catanho, Berault, Theoleyre and Justisz, 1983; 2.4.2).

Covalent crosslinking of ^{125}I-XBAL on to bovine anterior pituitary membranes was attempted using the bifunctional chemical crosslinker EGS. This was carried out as described for rat anterior pituitary membranes (6.2.2.2.3) except that peptidase inhibitors (soybean trypsin inhibitor 50mg/ml and aprotinin 400KIU/ml) were included in the equilibrium binding assay and the incubation period was 16 hours at 4°C. Efficiency of the crosslinking step was determined by dissociation as described for the rat anterior pituitaries (6.2.2.2.1).

6.3 Results

6.3.1 Label transfer crosslinking of the LHRH-receptor site using the [^{125}I]-Denny-Jaffe reagent

In order to try and optimise both the derivatisation of [DLys^6]-LHRH, and the specific binding of the conjugate ([^{125}I]-Denny-Jaffe reagent - [DLys^6]-LHRH) to membrane preparations, two concentrations of [DLys^6]-LHRH were used in the conjugation step. The presence of excess peptide in the reaction with the [^{125}I]-Denny-Jaffe reagent theoretically would result in maximal derivatisation. On binding to the membrane preparation however only low levels of radiolabel were found to be bound (1239 ± 76cpm, ± SEM, n = 5) probably due to the low specific activity of the conjugate. Higher levels of bound radiolabel were found when equimolar [DLys^6]-LHRH was used in the derivation step (18405 ± 670cpm, ± SEM, n = 5, under similar conditions of membrane concentration and total counts added). No specific binding was detectable using either preparation. One possible reason for this could be that the [DLys^6]-LHRH peptide binds with only a low specificity for the LHRH receptor site, as has been reported using native LHRH as a ligand. In the light of further studies with LHRH analogues altered at their C-terminal to desGly^{10} ethylamide, better specific binding may have been seen using such analogues for the Denny-Jaffe reagent conjugate. Another possibility is that the presence of the [^{125}I]-Denny-Jaffe reagent on the LHRH analogue affects the shape of the peptide in such a way as to destroy its specific binding to the LHRH receptor site. Unfortunately it was not possible to test for this using the Denny-Jaffe conjugate.

Another possibility is that specific binding present before the

photoactivation of the reagent was masked by the activation and non-specific crosslinking of a relatively high concentration of free reagent. The high concentration of free reagent was a result of the low specific activity of the conjugate. This could have been overcome in two ways - the inclusion of scavenger molecules (such as BSA or Tris: Bayley and Knowles, 1977; Das and Fox, 1979) in the photoactivation step to provide excess, non-membrane sites to react with the excess reagent, or the inclusion of a wash step between the equilibrium binding and photoactivation to remove excess reagent. This second option may however have resulted in a re-equilibration of bound peptide conjugate taking place, resulting in a reduction in the number of LHRH receptor sites specifically bound. A third possibility would have been to increase the specific activity of the conjugate by separating it from unreacted peptide and reagent. However, this and the inclusion of a wash step would have been difficult without exposing the conjugate to some light. It was therefore decided that some other means of specifically labelling the LHRH receptor site should be attempted. Use of different LHRH backbones may have resulted in increased specific binding, and other methods would allow for the inclusion of a biotin molecule in the LHRH.

6.3.2 Affinity labelling of the LHRH-receptor with biotinylated-LHRH analogues

6.3.2.1 The use of photoreactive, biotinyl-LHRH analogues as affinity ligands for the LHRH receptor

Displacement of ^{125}I-buserelin by PBL and [DLys^{6}, desGly^{10}]-LHRH ethylamide, the peptide backbone of PBAL, showed that they both bound to the LHRH-receptor site with high affinity in

a concentration-dependent manner (Fig 6.1). Analysis of the data revealed dissociation constants (K_i) of 0.92nM ± 0.16 (± SEM, n = 4) for PBL (Fig 6.2.a). This is similar to that reported for the parent peptide [DLys^6]-LHRH (Clayton and Catt, 1980) suggesting that addition of the photo-biotin group in the DLys^6 position does not impede binding to the LHRH receptor. It is a reasonable contention therefore that the presence of the same photo-biotin group on the [DLys^6, desGly^{10}]-LHRH ethylamide backbone will have little effect on the binding of this analogue. Analysis of the displacement by [DLys^6, desGly^{10}]-LHRH ethylamide of ^{125}I-buserelin showed it to have an apparent K_i of 0.098nM ± 0.01 (± SEM, n = 3; Fig 6.2(b)).

Iodination of PBL and PBAL allowed for the specificity of their binding on rat anterior pituitary membranes to be assessed. ^{125}I-PBL binding (at a concentration of ~29pM) could only be partially displaced by excess LHRH with 12% of the total binding being specific for the LHRH receptor (compared to 50-60% of ^{125}I-buserelin binding under similar conditions). A similar level (10%) of specific binding was seen with ^{125}I-PBAL under the same assay conditions. These results suggest that neither photoreactive LHRH analogue binds with a high enough specificity for the LHRH receptor site to make them suitable affinity ligands. Whereas the alteration of the C-terminal of LHRH analogues to desGly^{10} ethylamide has previously been shown to enhance the specificity of binding, (2.11), this does not appear to be the case for the photoreactive analogues used here.

Photoactivation (0-10 minutes) of ^{125}I-PBL at binding equilibrium with the membrane preparation resulted in no detectable

Figure 6.1

Displacement of specific ^{125}I-buserelin binding to rat anterior pituitary gland membranes by [DLys^{6},desGly^{10}]-LHRH ethylamide and PBL. Binding assays were carried out as described (2.4.1.2) with 50,000cpm ^{125}I-buserelin and various concentrations of unlabelled LHRH analogue. Non-specific binding was determined in the presence of 1μM LHRH. Points are mean ± SEM of 3-4 separate determinations.

Figure 6.1

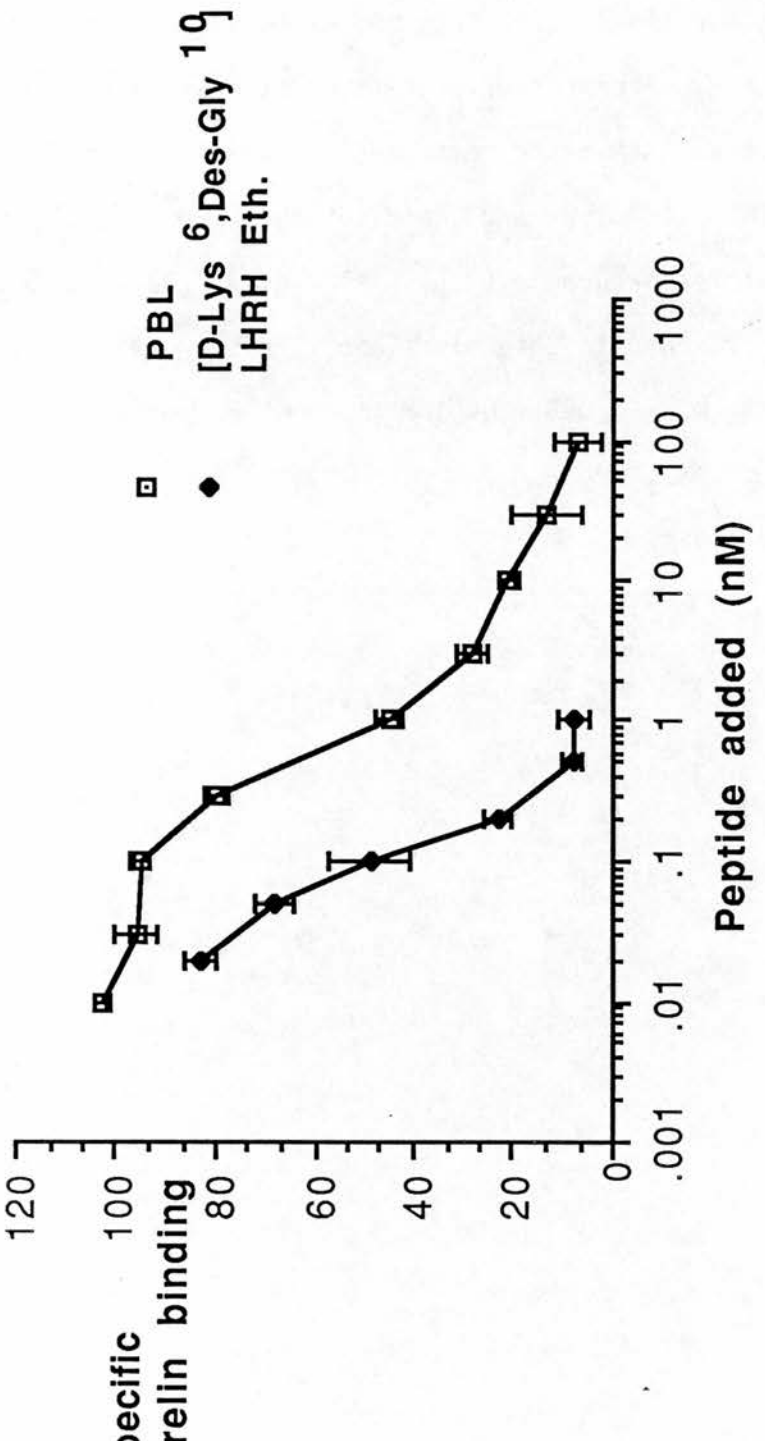
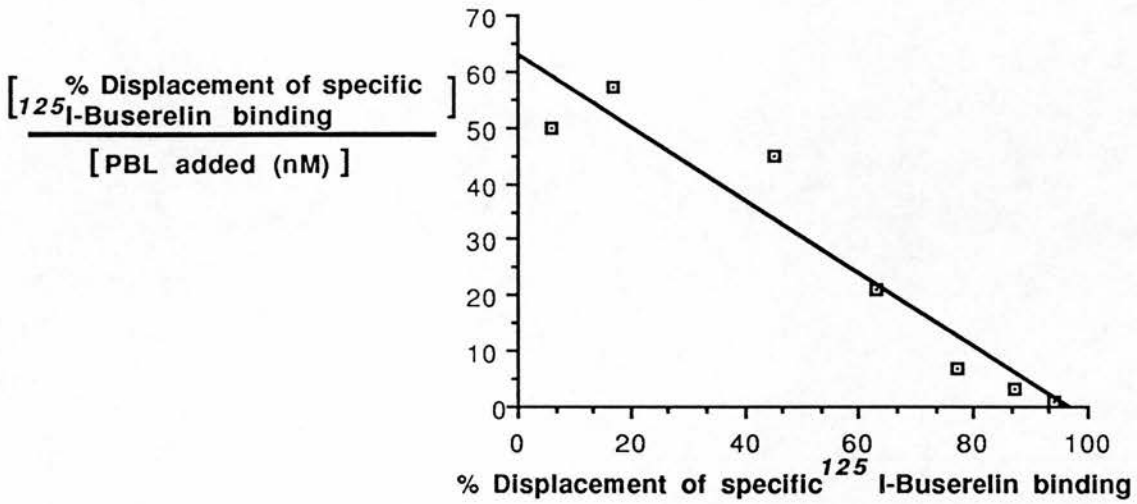

%Of specific
125I-Buserelin binding
120
100
80
60
40
20
0
.001
.01
.1
1
10
100
1000
Peptide added (nM)
PBL
[D-Lys 6,Des-Gly 10]
LHRH Eth.

Figure 6.2

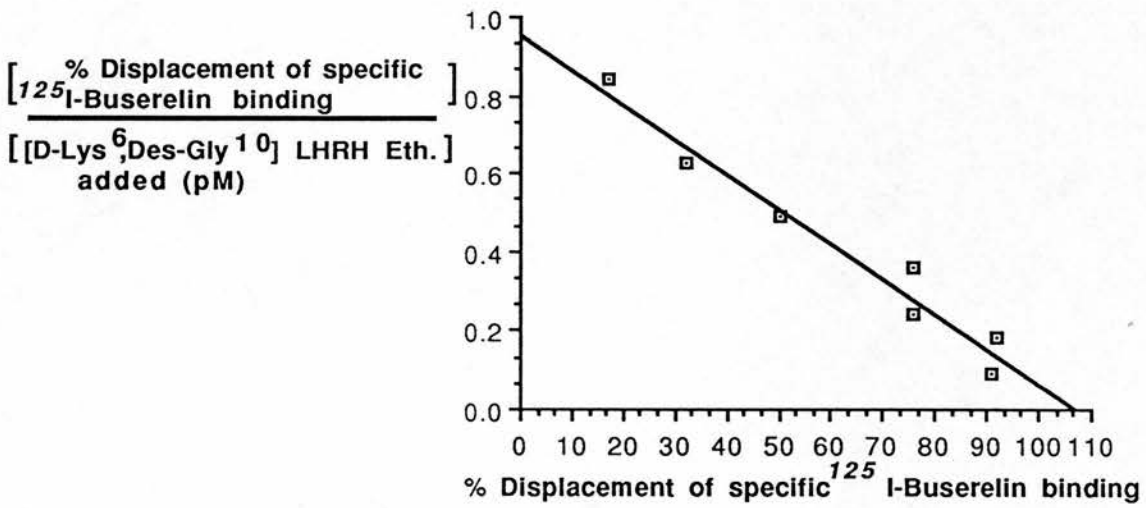
Hofstee analysis of a) PBL and b) [DLys^{6},desGly^{10}]-LHRH ethylamide displacement of specific ^{125}I-buserelin binding to rat anterior pituitary gland membranes. Binding assays were carried out as described (2.4.1.2) and the displacement data analysed by an error-weighted programme (2.5). PBL, K_i = 0.92 ± 0.16nM (mean ± SEM of four separate determinations). [DLys^{6},desGly^{10}]-LHRH ethylamide, K_i = 0.098 ± 0.01nM (mean ± SEM, of three separate determinations). Points shown are means of 3-4 separate determinations.

Figure 6.2

a.


[% Displacement of specific 125I-Buserelin binding]
[PBL added (nM)]
70
60
50
40
30
20
10
0
0
20
40
60
80
100
% Displacement of specific 125 I-Buserelin binding

b.


[% Displacement of specific 125I-Buserelin binding]
[[D-Lys 6,Des-Gly 10] LHRH Eth.] added (pM)
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100 110
% Displacement of specific 125 I-Buserelin binding

specific labelling of the LHRH receptor site. That is the amount of binding remaining, after 4 hours dissociation in the presence of 1μM LHRH, was no different in samples labelled with ^{125}I-PBAL either in the absence or presence of non-specific levels (1μM) of LHRH. Photoactivation of ^{125}I-PBAL resulted a small amount of specific labelling being detected, reaching a maximum, after 2 minutes activation, of 28% ± 5 (± SEM, n = 3) of the initial specific binding (10% of total binding) (Fig 6.3). The method used to analyse the efficiency of covalent labelling of the LHRH-receptor site and the usefulness of the covalent labelling for receptor purification is dependent on the specific binding of the iodinated analogues used. In the case of both ^{125}I-PBL and ^{125}I-PBAL the low specificity of binding and the low level of covalent labelling meant that they were unlikely to be the ligands of choice for this method.

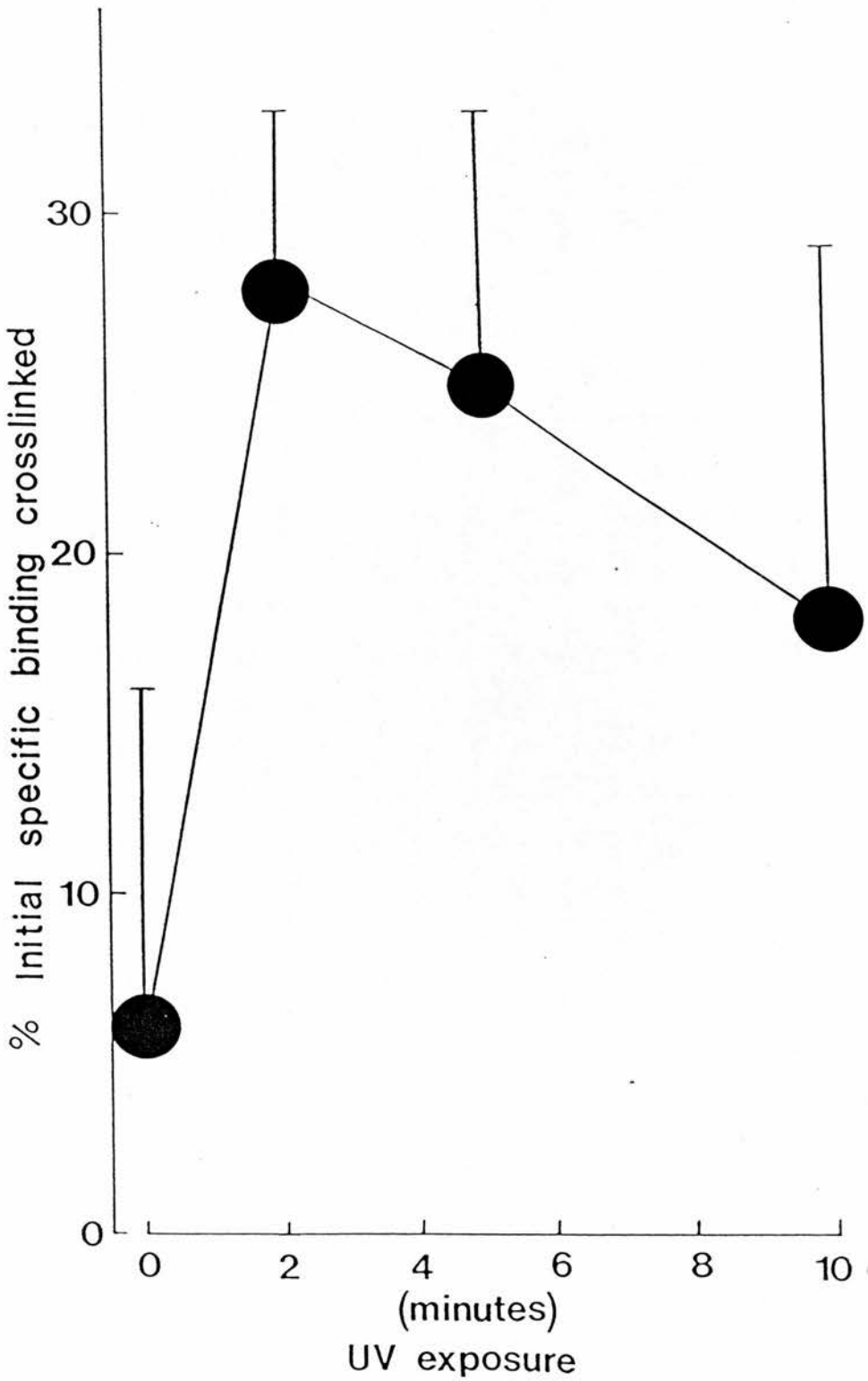
6.3.2.2 Binding of a non-photoreactive biotinyl-LHRH ligand to anterior pituitary membranes

The non-photoreactive biotinyl-LHRH ligand synthesised was XBAL, based on [DLys^{6}, desGly^{10}]-LHRH ethylamide. This LHRH backbone might be expected (from the literature) to have a high specific binding (2.9), and displacement of ^{125}I-buserelin from rat anterior pituitaries revealed a $k_{dissociation}$ constant of 0.098nM ± 0.01 (± SEM, n = 3) (Fig 6.1 and 6.2(b)). Addition of the amino-biotinyl group at position 6 (to form XBAL) did not seem to inhibit but apparently to enhance binding affinity (K_i of 13.6pM ± 1.2; ± SEM, n = 6; Fig 6.4.a,b). Hofstee analysis of XBAL displacement of ^{125}I-XBAL binding (Fig 6.5) to the membrane preparation gave a K_i of 57pM ± 14 (± SEM, n = 3) and Scatchard analysis using increasing

Figure 6.3

Photoaffinity labelling with ^{125}I-PBAL. % of initial membrane specific ^{125}I-PBAL binding covalently bound after exposure to UV light (12 Watt Hg lamp, 366nm at 20cm) for various periods of time. Initial specific ^{125}I-PBAL binding was 10% of the total ^{125}I-PBAL bound. Amount of specific binding crosslinked was determined by comparing ^{125}I-PBAL binding remaining in total and non-specifically bound ^{125}I-PBAL membrane preparations (after photoactivation) after a period of four hours displacement by 1μM LHRH at room temperature in subdued light. All points are mean ± SEM of three separate determinations.

Figure 6.3


% Initial specific binding crosslinked
30
20
10
0
0
2
4
6
8
10
(minutes)
UV exposure

concentrations of ^{125}I-XBAL gave a K_D of 131pM ± 16 (± SEM, n = 3: Fig 6.6). The specific activity of the iodinated XBAL was approximately assessed from the iodination data to be 2.65 x 10^5cpm/pmol (Appendix IV). The apparent Bmax value for ^{125}I-XBAL binding on male rat anterior pituitary tissue was 63fmol/gland, comparable to that for buserelin of 92fmol/gland. The specificity of ^{125}I-XBAL binding under the conditions used (~50,000cpm added in 500µl) was consistently 40-50%. The high affinity of its binding and its relatively high specificity for the LHRH receptor site indicate that ^{125}I-XBAL was a suitable ligand for the covalent labelling of this site.

6.3.2.3 Optimisation of LHRH-receptor affinity labelling using bifunctional chemical crosslinkers and ^{125}I-XBAL

In theory photoaffinity ligands might be expected to give a more specific labelling of receptor sites than non-specific divalent chemical crosslinkers. This is because covalent linking is initiated at a molecule bound specifically to the receptor site. For this reason the heterobifunctional crosslinker sulpho-SANPAH was tested for covalent labelling of LHRH receptor sites using ^{125}I-XBAL. After reaction of the N-hydroxysuccimide ester moiety of sulpho-SANPAH with the free amino side chain of ^{125}I-XBAL the conjugate, ^{125}I-XBAL-sulpho-SANPAH, gave 15% specific binding to rat anterior pituitaries. This is much less than that seen with ^{125}I-XBAL (6.3.2.2) and possibly could be the result of one, or a combination of two, factors. Either addition of the sulpho-SANPAH onto the biotin-containing side chain of ^{125}I-XBAL reduces the affinity of the analogue for the LHRH receptor site, or it confers extra non-specific binding qualities to the molecule. In fact the

Figure 6.4

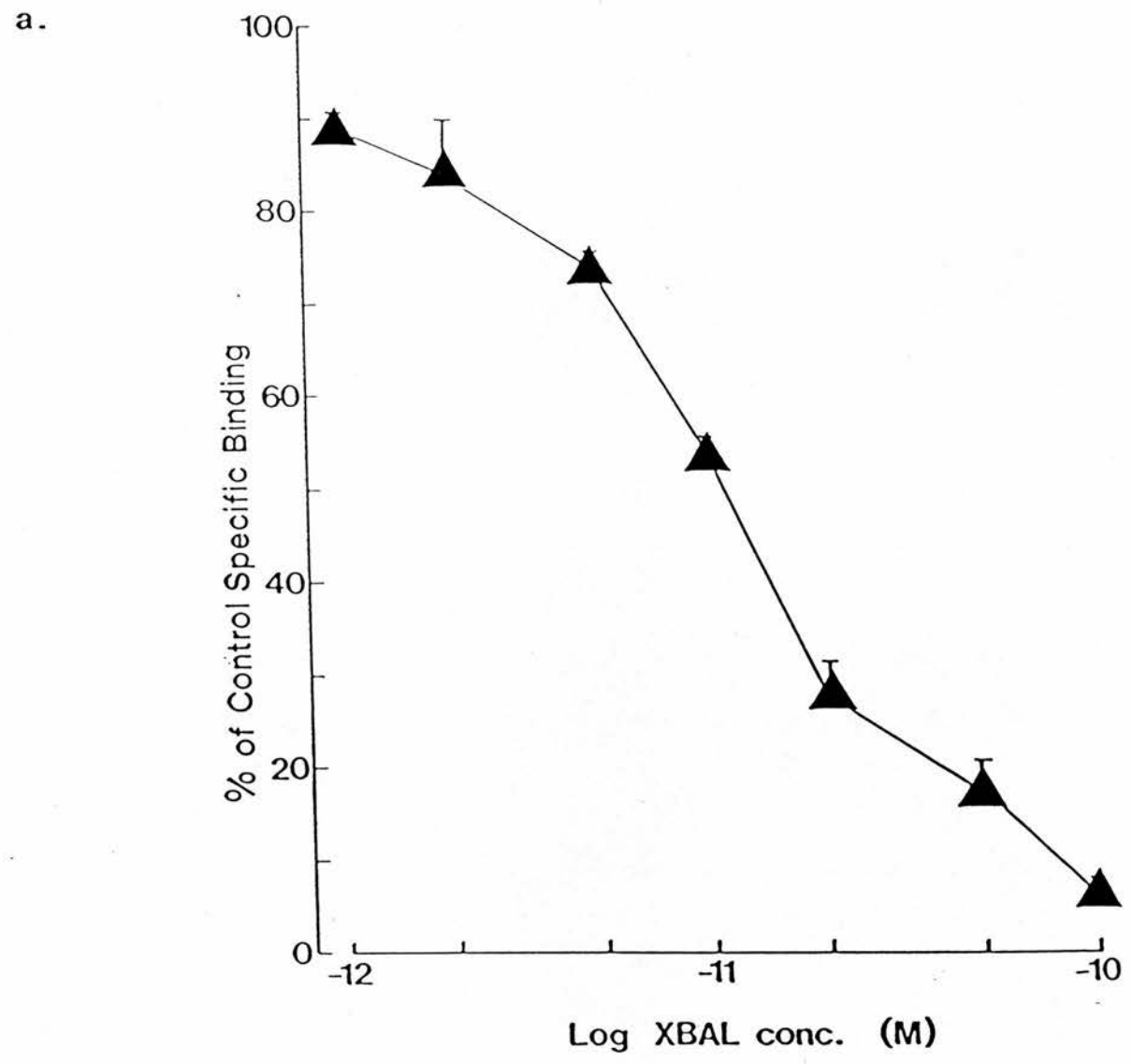


Figure 6.4

a. Displacement of specific ^{125}I-buserelin binding to rat anterior pituitary membranes by XBAL. Binding assays were carried out as described (2.4.1.2) with ~50,000cpm ^{125}I-buserelin, tissue equivalent to one eighth anterior pituitary gland and various concentrations of XBAL in a total volume of 500µl 25mM Tris-HCl pH7.4/0.1% BSA. Non-specific binding was determined in the presence of 1µM LHRH. Total ^{125}I-buserelin binding was 6475 ± 58cpm, with specific binding 72 ± 2% of total binding. Points are mean ± SEM of six separate determinations.

Figure 6.4

b.

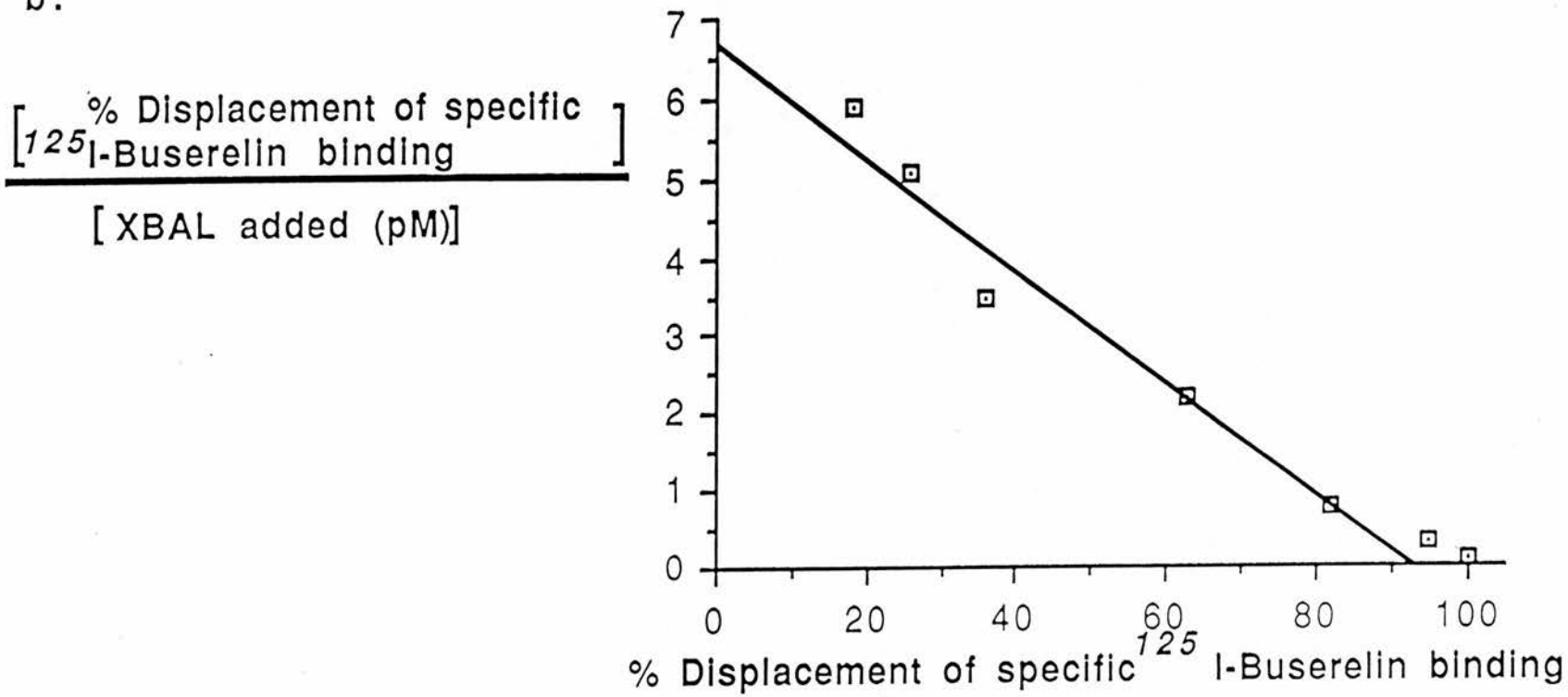


Figure 6.4

b. Hofstee analysis of XBAL displacement of specific ^{125}I-buserelin binding to rat anterior pituitary gland membranes. Ligand binding was carried out as described (2.4.1.2) and displacement data analysed by an error-weighted programme (2.5). Points are mean values of six separate determination. K_i = 13.6 ± 1.2pM (mean ± SEM, n = 6).

Figure 6.5

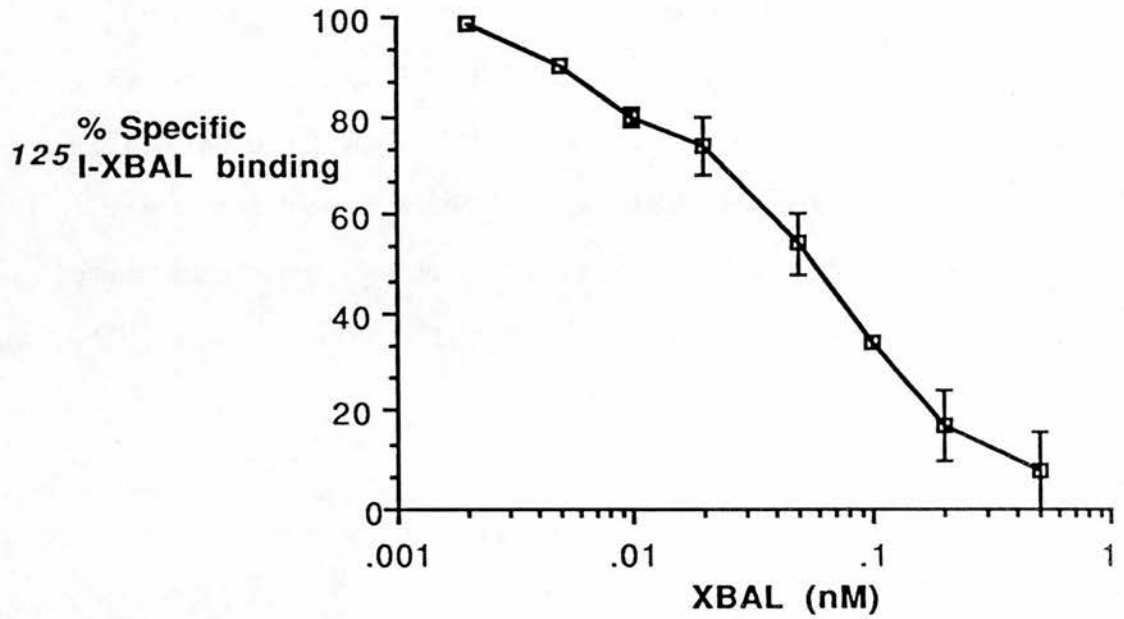


Figure 6.5

Displacement of specific ^{125}I-XBAL binding to rat anterior pituitary gland membranes by unlabelled XBAL. Ligand binding was carried out as previously described (2.4.1.2) with 50,000cpm ^{125}I-XBAL, tissue equivalent to one eighth of an anterior pituitary gland and various concentrations of unlabelled XBAL in a total volume of 500µl 25mM Tris-HCl pH7.4/0.1% BSA. Total binding of ^{125}I-XBAL was 5232 ± 89cpm with 43 ± 3% of total binding being specific. Analysis of the data by an error-weighted programme revealed a K_i = 57 ± 14pM. All values and points are means ± SEM for six separate determinations.

Figure 6.6

Scatchard analysis of ^{125}I-XBAL binding to rat anterior pituitary gland membranes. Ligand binding was carried out in 500µl 25mM Tris-HCl pH7.4 containing 50,000cpm plus various concentrations of ^{125}I-XBAL with tissue equivalent to one eighth of an anterior pituitary gland. Non-specific binding was determined in the presence of 1µM LHRH. After graphical transformation of the binding data, they were analysed by linear regression. K_D = 131± 16pM, (mean ± SEM, n = 3) B_{max} = 63fmol/gland.

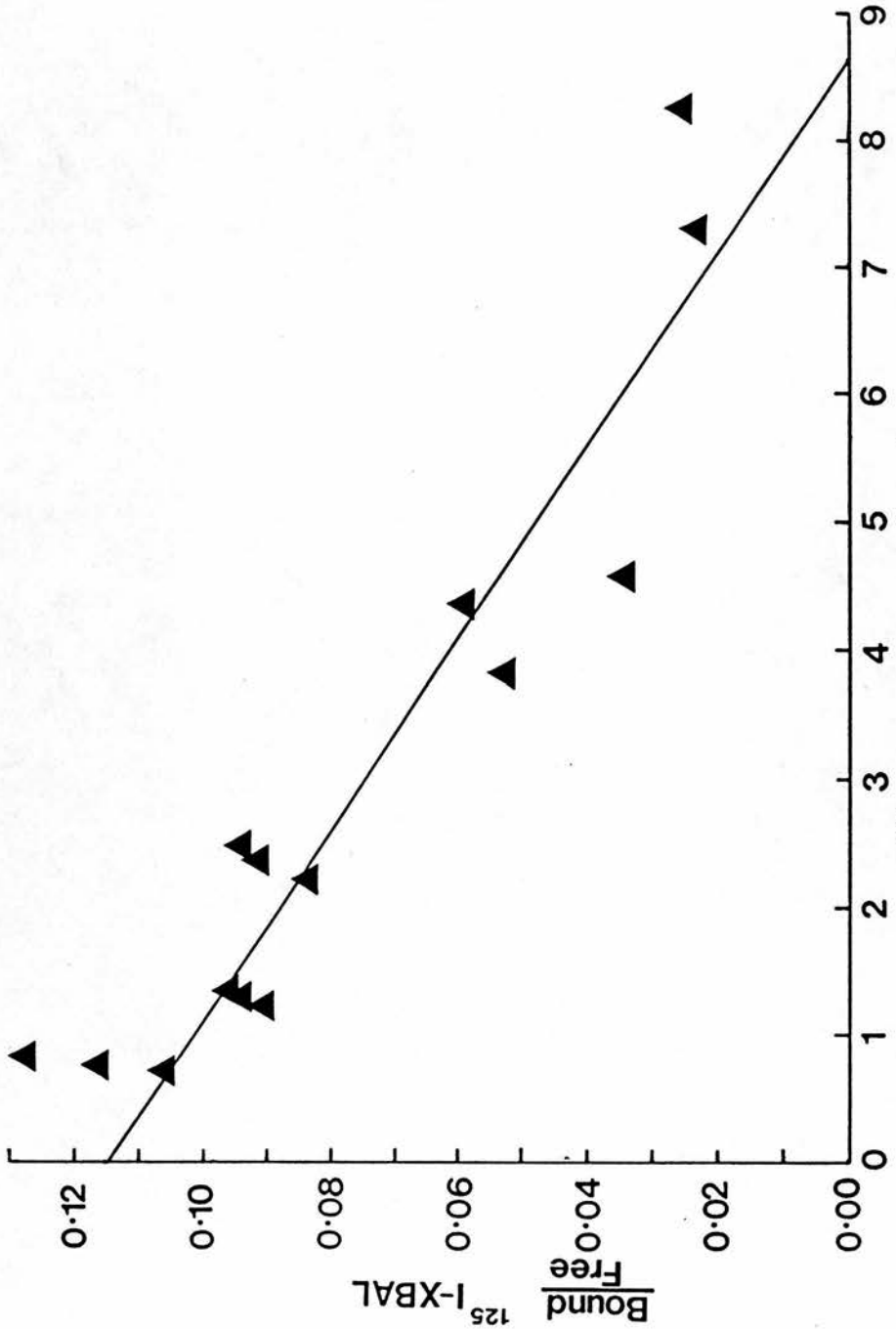

Bound / Free ^{125}I-XBAL
0·00
0·02
0·04
0·06
0·08
0·10
0·12
0
1
2
3
4
5
6
7
8
9
^{125}I-XBAL bound (fmol)

Figure 6.6

total binding of ^{125}I-XBAL-sulpho-SANPAH was 6417 ± 115cpm compared to 8470 ± 77cpm for ^{125}I-XBAL under similar conditions, whilst non-specific binding levels were relatively similar (5454 ± 104cpm for ^{125}I-XBAL-sulpho-SANPAH compared to 5039 ± 57cpm for ^{125}I-XBAL; ± SEM, n = 4). These results suggest that it is the sulpho-SANPAH moeity interfering with the affinity of the LHRH analogue and reducing the level of specific binding seen, rather than an increase in non-specific binding.

Photoactivation of the ^{125}I-XBAL-sulpho-SANPAH (0-10 mins, 20cm from a 12 watt Hg lamp, 366nm) resulted in no detectable specific labelling of the LHRH receptor site, assessed, as for PBL and PBAL, by comparing the amount of binding remaining in total and non-specifically labelled samples after a period of 4 hours dissociation in the presence of 1μM LHRH. As for the photoreactive LHRH-analogues (PBL, PBAL and the [D-Lys^6]-LHRH-Denny Jaffee reagent conjugate) the level of specific labelling may have been improved by the inclusion of a washing step to remove excess unbound ligand (6.3.2.1). However, as with ^{125}I-PBL and ^{125}I-PBAL, the low initial levels of specific binding seen here suggested that this strategy did not merit further study. The results of the binding with iodinated aryl-azido derivatives of LHRH suggest that this group has a detrimental effect on specific binding to the LHRH receptor site. For PBL, displacement studies (6.3.2.1) have shown that the affinity for the LHRH receptor site is retained by this analogue, but binding of the iodinated PBL revealed very high levels of non-specific binding. Increasing the hydrophobicity of a peptide can result in an increase in the portioning of the peptide in the hydrophobic environment of the membrane, rather than the aqueous

medium. This may be responsible for the increase in non-specific binding of this ligand. However, for the ^{125}I-XBAL-sulpho-SANPAH, no similar increase in non-specific binding was observed, only a reduction in total binding. This suggests that the affinity of the XBAL was adversely affected by the addition of the sulpho-SANPAH group.

The homobifunctional chemical crosslinkers that were tested had the advantage of being added only after the ^{125}I-XBAL binding had come to equilibrium. This might mean that they have less opportunity to affect the specificity of the ligand's binding than the heterobifunctional sulpho-SANPAH. The results (effect of the crosslinker on initial levels of specific binding and the percentage of initial specific binding successfully crosslinked) of the covalent crosslinking of ^{125}I-XBAL to the LHRH receptor site are presented in Table 6.1.

Despite not being present during equilibration of the ^{125}I-XBAL binding, both DSS and BS^3 had detrimental effects on the levels of initial specific binding seen. In the presence of 0.2mM DSS,values of specific binding were reduced to 58% of controls (that is control samples in the absence of crosslinker), and to 55% in the presece of 0.5mM BS^3. Whilst DST had less effect on initial specific binding at low concentrations (0.1 - 0.5mM) it, like DSS, did not result in any specific labelling of the LHRH-receptor site (again determined by comparing the amount of binding left, after 4 hours dissociation in the presence of 1μM LHRH, in total and non-specifically labelled samples). Only at the highest concentration tested (5mM) did EGS decrease initial specific binding (to 79% of control levels). Concentrations of EGS above 1mM

Table 6.1

Results of covalent crosslinking of specific ^{125}I-XBAL binding to rat anterior pituitary gland membranes with the chemical crosslinkers. The effect of the crosslinkers on specific ^{125}I-XBAL binding was determined by comparing initial specific ^{125}I-XBAL binding in the presence and absence of various concentrations of the crosslinkers. Specific binding crosslinked was determined by ^{125}I-XBAL binding remaining in total and non-specifically bound ^{125}I-XBAL membrane preparations (after the crosslinking reaction) subsequent to a period of 4 hrs displacement by 1μM LHRH at room temperature. All values are mean ± SEM for 4-12 separate determinations.

Table 6.1

Crosslinker and reaction conditions	Concentration of crosslinker (mM)	Initial specific binding as % of control specific binding (n = 4 - 12 ± SEM)	% of Initial specific binding crosslinked (n = 4 - 12 ± SEM)
DSS	0.025	96 ± 12	-6 ± 2
1% DMSO,	0.2	58 ± 6	-3 ± 2
30 minutes on ice	0.3	35 ± 3	5 ± 5
	0.5	29 ± 8	1 ± 5
	1	23 ± 8	5 ± 11
	2	21 ± 10	4 ± 6
BS^3	0.5	55 ± 9	19 ± 6
No DMSO	1	56 ± 2	17 ± 4
30 minutes at	2	33 ± 1	24 ± 4
room temperature	5	42 ± 8	16 ± 5
DST	0.1	78 ± 9	-6 ± 4
1% DMSO	0.2	78 ± 13	-9 ± 3
30 minutes at	0.5	72 ± 14	17 ± 10
room temperature	1	54 ± 22	17 ± 7
	2	35 ± 8	30 ± 20
	5	23 ± 26	68 ± 25
EGS	0.1	99 ± 1	-1 ± 1
1% DMSO	0.2	100 ± 2	-2 ± 2
30 minutes on ice	0.5	93 ± 2	0 ± 4
	1	100 ± 4	3 ± 2
	2	94 ± 14	11 ± 3
	3	108 ± 4	17 ± 5
	5	79 ± 2	23 ± 3

were successful at crosslinking ^{125}I-XBAL in an apparently specific way (Fig 6.7). Up to 23% ± 3 (± SEM, n = 3) of the control level of initial specific binding was covalently linked after treatment with 5mM EGS. Whilst theoretical considerations suggest a maximum of 40% specific labelling with these classes of crosslinkers (Strosberg, 1984), a result of 23% seems an acceptable maximal value in practice.

6.3.3 Biotin-affinity chromatography of the covalently labelled and solubilised LHRH receptors

Displacement of ^{3}H-biotin from avidin-sepharose by PBL in the solubilisation solution (Fig 6.8) showed not only that the biotinyl-LHRH analogue had an affinity similar to biotin for avidin-sepharose, but also that this was not detrimentally affected by the presence of 5mM CHAPS/1.5M NaCl. This suggests that LHRH receptors labelled with our biotinylated ligands will bind to an avidin-sepharose (or streptavidin-agarose) column under solubilisation conditions. Of course, although the rest of the PBL ligand did not affect the biotin interaction with the avidin-agarose, it was not possible to be entirely certain that, on crosslinking the ligand to the receptor site, this would still be possible.

Application of solubilised ^{125}I-biotin labelled rat anterior pituitary membranes to a streptavidin-agarose column, and subsequent elution with 2mM biotin resulted in the appearance of a peak of ^{125}I-activity. As expected in the case of ^{125}I-PBL labelling, the difference between parallel total and non-specifically labelled samples was very small due to the low specificity of its binding (Fig. 6.9). Affinity labelling with 0.5mM EGS treatment of ^{125}I-XBAL had rather more apparent specificity. As a result a

Figure 6.7

Efficiency of covalent linking of ^{125}I-XBAL. % of initial specific ^{125}I-XBAL binding to rat anterior pituitary membrane preparation covalently linked to the membrane preparation by various concentrations of EGS. Crosslinking was carried out on membrane preparations at equilibrium with ^{125}I-XBAL (total and non-specific binding samples, non-specific binding being determined on the presence of 1μM LHRH) for 30 minutes on ice. The reaction was then quenched by the addition of excess glycine (6.2.2.2.3). Specific binding crosslinked was determined by comparing ^{125}I-XBAL binding remaining in total and non-specifically bound samples (after crosslinking) after a period of 4 hrs displacement by 1μM LHRH at room temperature. All points are mean ± SEM for three separate determinations.

Figure 6.7

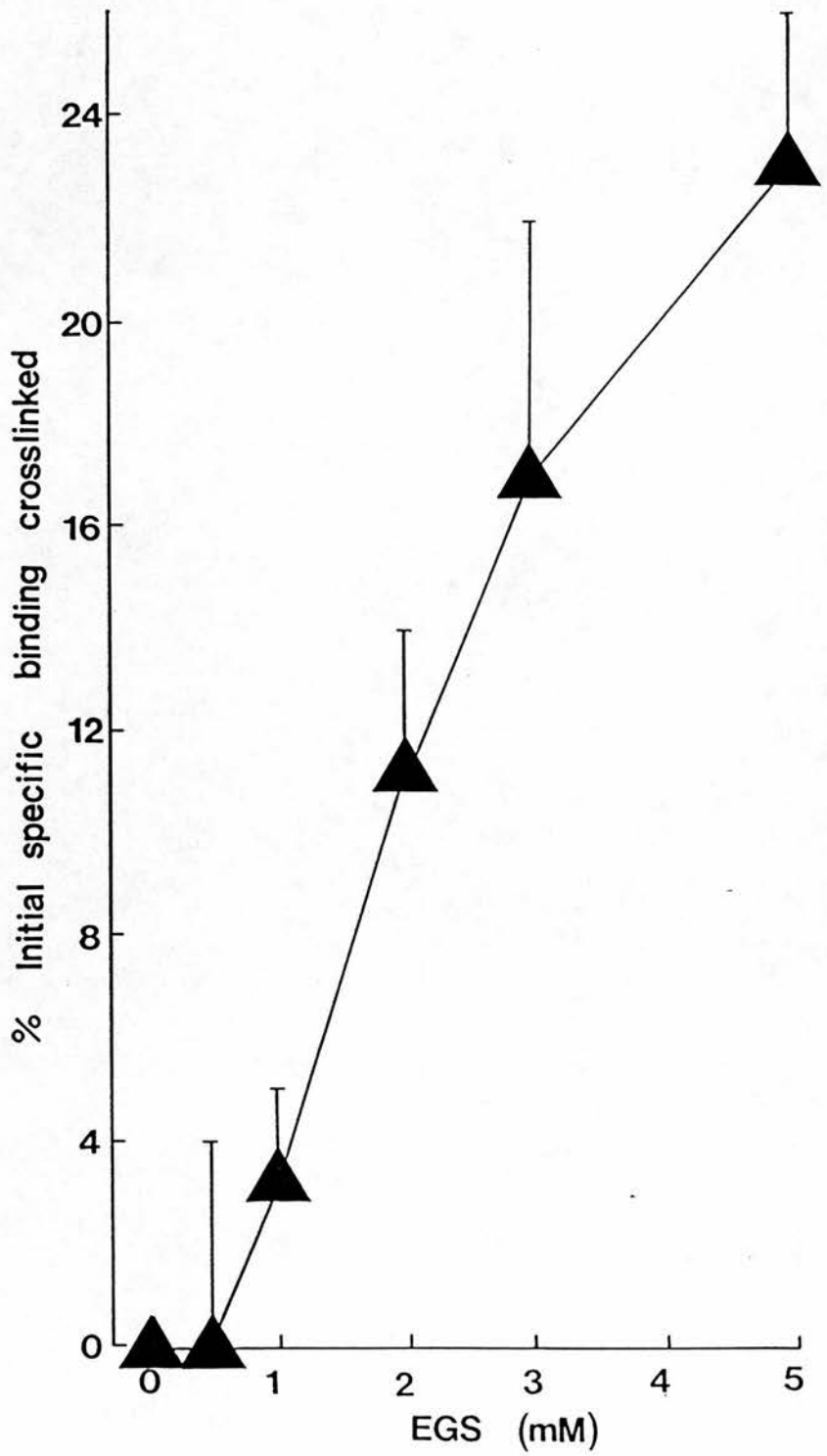

% Initial specific binding crosslinked
24
20
16
12
8
4
0
0
1
2
3
4
5
EGS (mM)

Figure 6.8

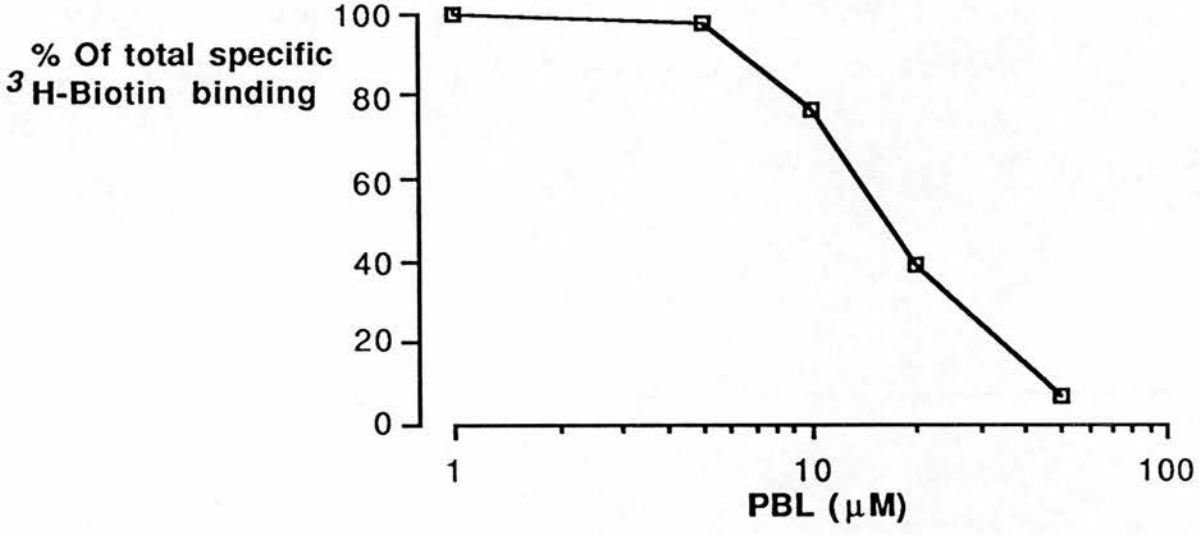


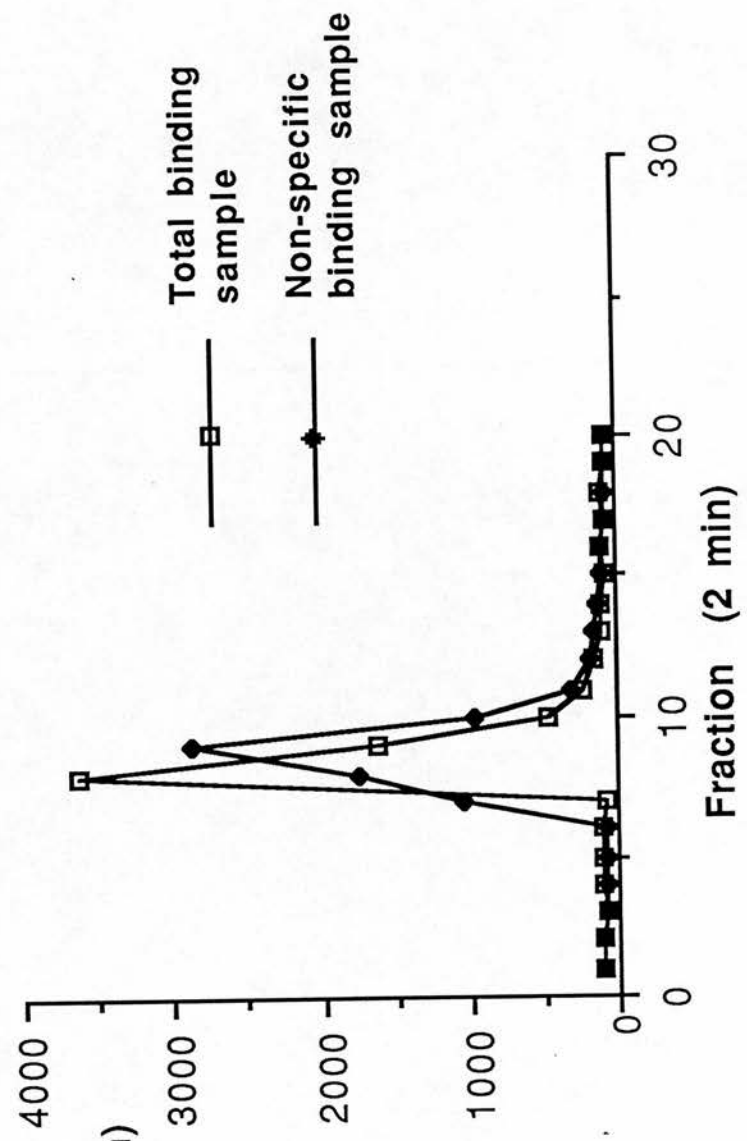
Figure 6.8

Displacement of ^{3}H-biotin from avidin-sepharose CL-4B by PBL. The binding assay was carried out as previously described (2.6.1) in the 5mM CHAPS, 1.5M NaCl solubilisation solution and revealed an IC_{50} of 11μM for PBL as a biotin derivative under these conditions. Points are means of triplicate samples.

Figure 6.9

^{125}I-PBL activity in aliquots of 2 min fractions eluted with 2mM biotin (in the 5mM CHAPS, 1.5M NaCl solubilisation solution) from the avidin-Sepharose CL-4B affinity column after application of a solubilised, ^{125}I-PBL-crosslinked rat anterior pituitary gland preparation (as described 2.6).

Figure 6.9


Total binding sample
Non-specific binding sample
^{125}I-PBL (cpm)
4000
3000
2000
1000
0
0
10
20
30
Fraction (2 min)

greater difference between total and non-specifically labelled preparations was seen, with 17% of the total radioactivity eluted by biotin apparently being specific (Fig 6.10). The recovery of radiolabel applied to the affinity column in the peak eluted by the 2mM biotin was 23% in the case of ^{125}I-PBL but only 5% for the EGS/^{125}I-XBAL preparation (both values calculated from the total binding conditions). One possibility is that different elution conditions are required for the two affinity matrices used, with those which used (2mM biotin) being more suitable for the avidin-agarose than the streptavidin-sepharose. Another possibility is that whilst the ^{125}I-XBAL crosslinked with 0.5mM EGS results in a greater amount of specific covalent labelling of the LHRH receptor site, a smaller proportion of this is available for binding with the streptavidin agarose. This may be a result of the non-selective action of EGS on membrane constituents. Unlike photoreactive affinity labels, homobifunctional chemical crosslinkers are capable of non-specifically crosslinking any two free amino groups. This may have resulted in protein crosslinking in the membranes that in some way hindered the biotin (covalently attached to the LHRH receptor site) from reacting with the streptavidin-agarose.

6.3.4 Estimation of the molecular weight of the specifically labelled LHRH receptor using SDS PAGE

The anterior pituitary membrane preparations used in SDS PAGE had been labelled using ^{125}I-XBAL and 5mM EGS as previously described (6.2.2.2.3). After exposure of the gel for four weeks, an autoradiograph was obtained (see Appendix II for details). Using a densitometer a standard curve was constructed of R(f) values against molecular weights (Fig 2.10) and this was used to provide an

Figure 6.10

^{125}I-XBAL activity in aliquots of 2 min fractions eluted with 2mM biotin (in the 5mM CHAPS, 1.5M NaCl solubilisation solution) from the streptavidin-agarose affinity column after application of a solubilised ^{125}I-XBAL-EGS-crosslinked rat anterior pituitary gland preparation (as described 2.6).

Figure 6.10

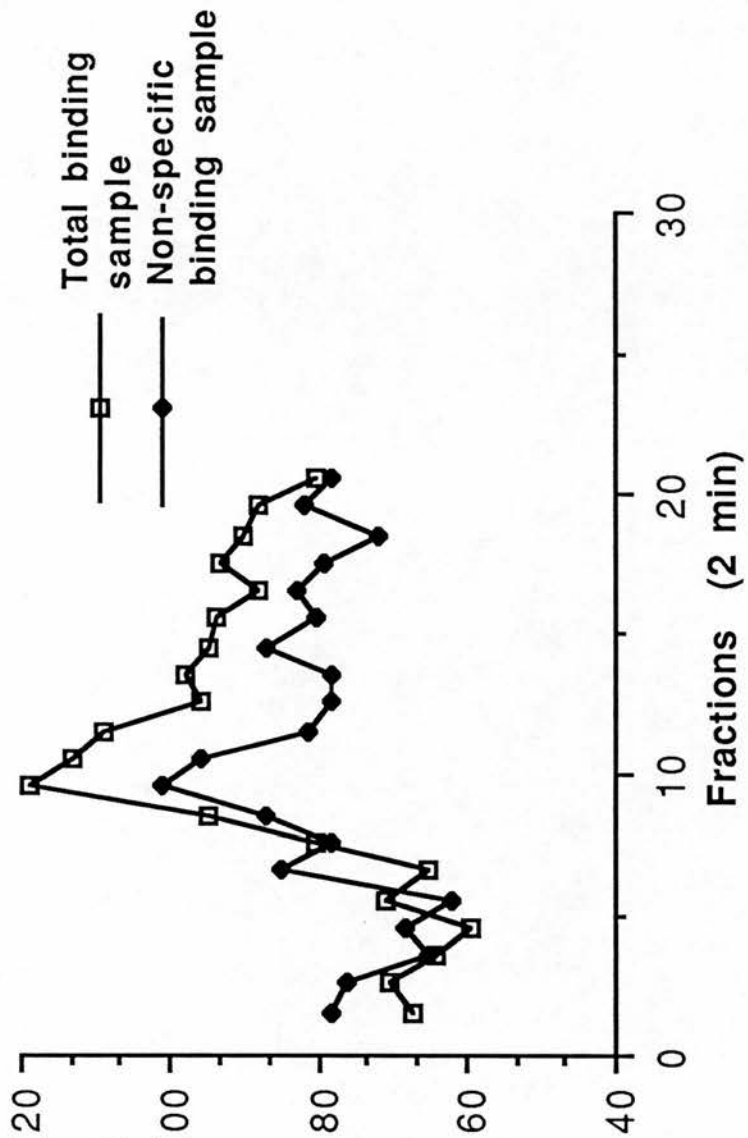

Total binding sample
Non-specific binding sample
^{125}I-XBAL (cpm)
120
100
80
60
40
0
10
20
30
Fractions (2 min)

Figure 6.11

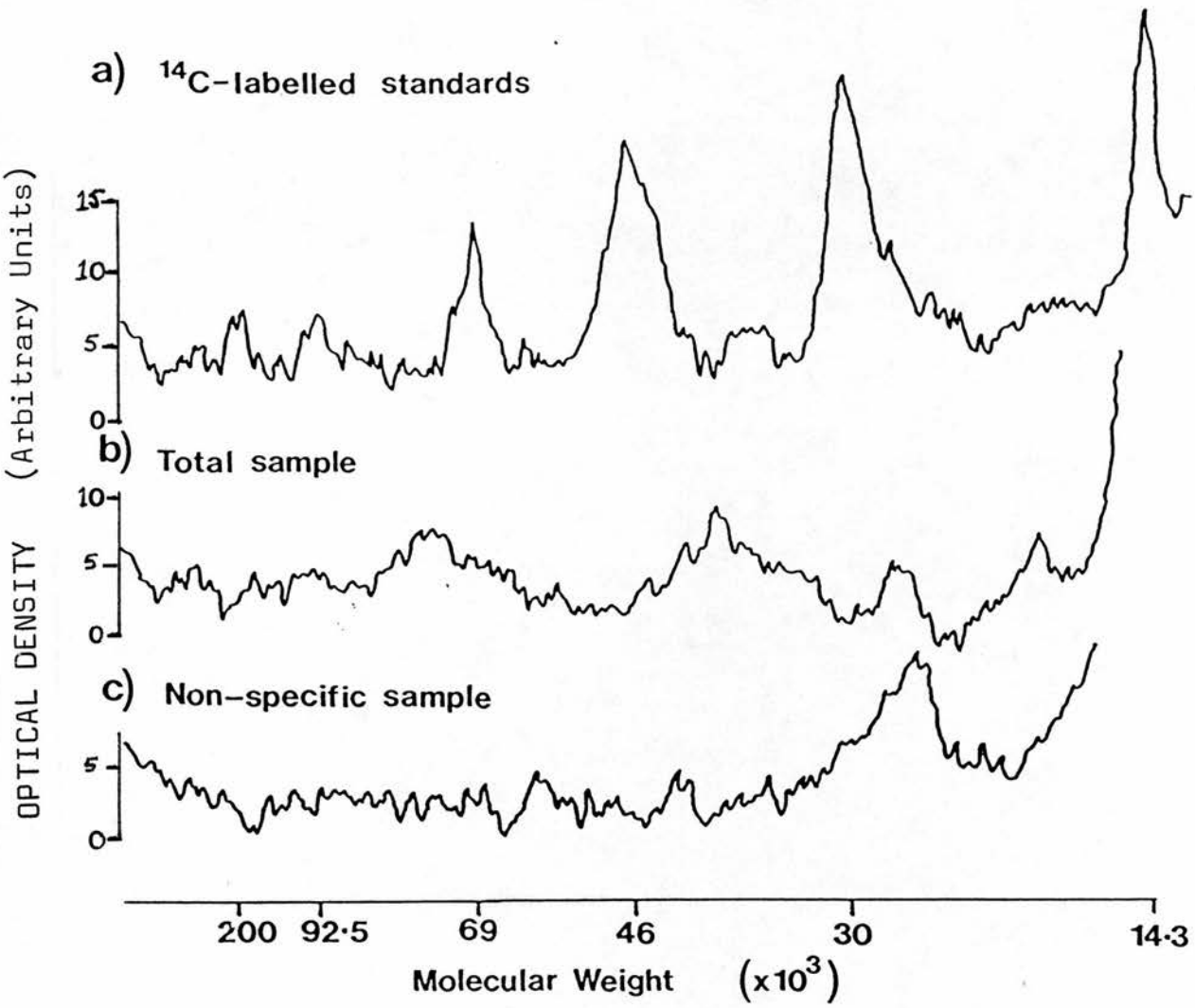


Figure 6.11

Densitometric traces of a representative SDS PAGE autoradiograph. A 7% acrylamide gel, in combination with a 4.75% acrylamide stacking gel was used (as described previously, 2.8.1).

Lane a) ^{14}C-labelled molecular weight standards.

Lane b) ^{125}I-XBAL-EGS crosslinked rat anterior pituitary gland sample. (Total labelling).

Lane c) ^{125}I-XBAL-EGS crosslinked rat anterior pituitary gland sample (Non-specific labelling), ^{125}I-XBAL binding to equilibrium and the covalent crosslinking reaction were both carried out in the presence of 1μM LHRH.

estimate of the molecular weights of radioactive bands (proteins covalently labelled with ^{125}I-XBAL) in the sample lanes (Fig 6.11). The sample crosslinked with EGS and ^{125}I-XBAL labelled proteins at approximate molecular weights of 72,000 (a broad band of between 60,000 and 82,000), 39,000 and 26,000. That labelled in the presence of 1μM LHRH (non-specifically labelled sample) showed a protein labelled with ^{125}I-XBAL of approximately 25,000 molecular weight.

In addition to the labelled proteins entering the gel, both total and non-specific samples had fairly large amounts of labelled proteins that did not enter the stacking gel. This suggests that either the proteins are of a very high molecular weight (over 500,000 to be excluded from the 7% stacking gel) or that the membrane sample was not completely dissolved by the sample buffer. Some aggregation of labelled proteins may be expected using 5mM EGS (a relatively high concentration of crosslinker). If this is the case, then more labelled protein may enter the gel if the amount of SDS used to dissolve the sample buffer is increased. The optimal ratio of SDS:protein (w/w) has previously been found to be 3:1 for complete protein solubilisation (Blackshear, 1984). Here a ratio of approximately 2:1 was used. An increase to 3:1 may prove fruitful in increasing the amount of labelled protein entering the gel.

6.3.5 The use of bovine anterior pituitary tissue for the covalent labelling of LHRH receptors using biotinylated LHRH analogues

Displacement of ^{125}I-buserelin from bovine anterior pituitary membranes by XBAL was concentration-dependent (Fig 6.12) and Hofstee analysis revealed a K_i of 0.096nM, of the same order of magnitude as that seen on rat anterior pituitary membranes. Scatchard

Figure 6.12

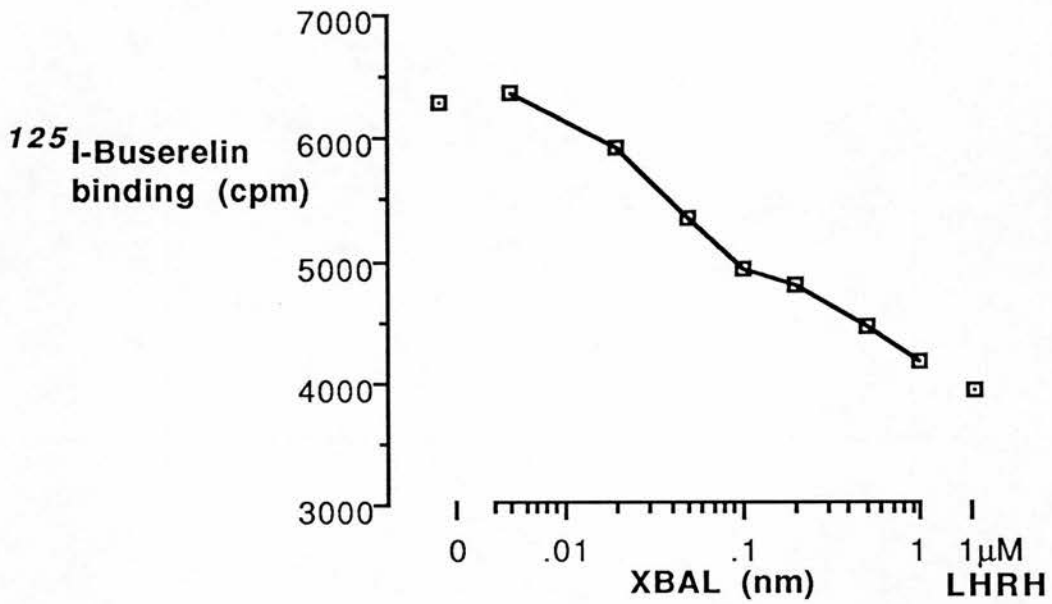


Figure 6.12

Displacement of specific ^{125}I-buserelin from bovine anterior pituitary gland membranes by unlabelled XBAL. Binding was carried out as previously described (2.4.2) with 50,000cpm ^{125}I-buserelin and ~2.5mg of tissue in 500μl of 25mM Tris-HCl pH 7.4; 50μg/ml soybean trypsin inhibitor; 400 KIU/ml aprotinin, reaching binding equilibrium after 16hrs at 4°C. Analysis of the data by an error-weighted programme revealed a K_i of 0.096nM. All points are means of triplicate determination.

analysis of ^{125}I-XBAL self-displacement data (Fig 6.13) gave a K_D value of 0.122nM and a B_{max} of 4.5fmol/mg initial tissue weight, or approximately 3.6pmol/bovine anterior pituitary gland. These results would suggest that the XBAL recognised the same site as ^{125}I-buserelin and was binding to a site of similar affinity to that seen in the rat anterior pituitary membrane, which was therefore likely to be the LHRH receptor site. The specific binding of ^{125}I-XBAL on the bovine anterior pituitary membrane preparation was between 40-50%, similar to that seen on rat tissue (6.3.2.2).

Because of the long equilibration period required (16 hrs) for LHRH-analogues binding to bovine anterior pituitary tissue (2.4.2), it was considered necessary to include protease inhibitors to prevent enzymatic degradation of both the LHRH analogue and the LHRH receptor protein. These were included in the equilibrium binding period prior to crosslinking, although non-selective crosslinking may have been increased as a result. However, the Tris/BSA used in equilibrium binding assays previously (2.4.2) was replaced by Hepes-KOH. This had the effect of drastically increasing the amount of non-specific binding of ^{125}I-buserelin seen (from 7976 cpm to 14328 cpm, under otherwise similar conditions) and concomitantly reducing the proportion of specific binding, to only 4 ± 9% (± SEM, n = 4) of total binding. Not surprisingly, crosslinking of ^{125}I-XBAL binding with 0.5mM EGS to bovine anterior pituitary tissue did not result in any detectable specific labelling of the LHRH receptor site (assayed as previously described (6.2.2.2.1) by comparing the amount of binding remaining after 4 hours dissociation in the presence of 1μM LHRH, in parallel total and non-specifically labelled samples). Bovine tissue would appear not to be suitable

Figure 6.13

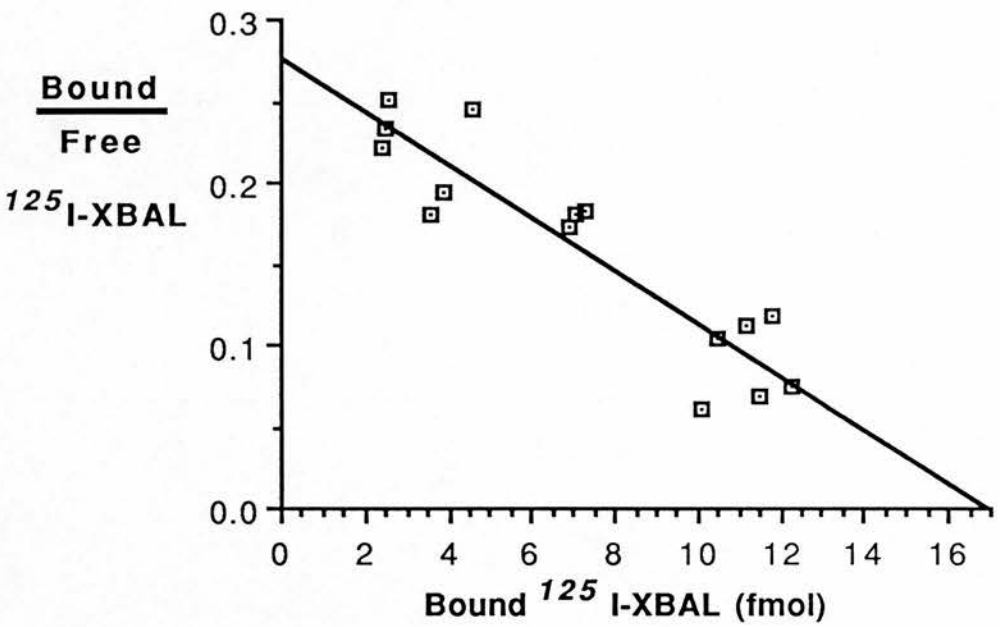


Figure 6.13

Scatchard analysis of ^{125}I-XBAL binding to bovine anterior pituitary gland membranes. Binding was carried out using 50,000cpm and various concentrations of ^{125}I-XBAL with tissue equivalent to ~2.5mg of initial tissue weight in a total volume of 500µl 25mM Tris-HCl pH 7.4; 50µg/ml soybean trypsin inhibitor; 400 KIU/ml aprotinin for 16 hrs at 4°C. Non-specific binding was determined in the presence of 1µM LHRH. After graphical transformation of the binding data it was analysed by linear regression (2.5). K_D = 0.122nM, Bmax = 3.6pmol/gland. All values are means of triplicate samples.

for use in the affinity labelling of LHRH receptor sites under these conditions which have been optimised for rat anterior pituitary tissue.

6.4 Discussion

Covalent affinity labelling of membrane proteins was first suggested to explain an irreversible blockade of adrenergic receptors seen after treatment with dibenamine ligands (Nickerson and Gump, 1949). The potential of such a technique for protein biochemistry was not realised until the early 1960's when both photoaffinity (Singh, Thornton and Westheimer, 1962) and chemical affinity crosslinking (Baker, Lee, Tong and Ross, 1961; Lawson and Schramm, 1962; Schowllmann and Shaw, 1962) were first used as tools for enzyme active site studies.

Affinity labelling of membrane receptors has been regarded as one way to provide positive identification of the protein after membrane disruption as an alternative to the retesting of ligand binding to show retention of receptor function (Zisapel and Sokolovsky, 1977; Finn, 1977). The success of the method (for protein identification, quantification and purification) is clearly dependent on the specificity of the labelling (Zisapel and Sokolovsky, 1977). Two steps are involved - the specific binding of the ligand and the covalent bond formation.

Of the two strategies for affinity labelling tried, from the literature photoaffinity labelling would appear to be the method of choice. Incorporation of an arylazide in a ligand analogue allows equilibrium binding conditions to be attained in an unreactive state (Ji, 1979). Photolysis results in the generation of a highly reactive species with a half-life in the order of 10^{-4}-10^{-2} seconds capable reacting with C-H bonds in the absence of more reactive sidechains (Bailey and Knowles, 1977; Das and Fox, 1979; Ji, 1979; Pilch and Czech, 1984). This would suggest that highly

specific labelling of the receptor site could be achieved (dependent on initial specificity of binding of the ligand).

Photoaffinity analogues of LHRH have previously been synthesised, both by the reaction of [DLys^6]-LHRH with the heterobifunctional chemical crosslinker, N-hydroxysuccinimidyl-4-azido-benzoate (Hazum, 1981(c); Iwashita and Catt, 1985), and by the incorporation in the amino-acid backbone of photosensitive groups (Mackiewicz, Belisle, Bellabarba, Gallo-Payet, Lehoux, Lagace and Escher, 1987). In all cases the affinity of the analogue was retained when the residues involved in incorporating the photoreactive group were not those involved in binding, that is usually residues 6 or 7 (see Chapter 3). The analogues used in these studies all involved substitution of the photoreactive group onto the amino-acid at position 6 (DLys^6). Both PBL and PBAL retained their affinity for the LHRH receptor site (as revealed by displacement of ^{125}I-buserelin), but in both cases the specificity of their binding was very low (6.3.2.1). Similarly, low specific binding was seen with the [DLys^6]-LHRH conjugated with ^{125}I-Denny-Jaffe reagent (6.3.1). In the case of the heterobifunctional crosslinker sulpho-SANPAH, the reduction in specific ^{125}I-XBAL binding seen appeared to be the result of a reduction in affinity of the ^{125}I-XBAL-sulpho-SANPAH conjugate rather than an increased level of non-specific binding (6.3.2.3).

Photo-activation of all the analogues and conjugates resulted in very low levels of specific covalent labelling. This could have been due to a number of factors, not least the low specificity of the initial ligand binding seen. Possibly the positioning of the photoreactive group at the part of the LHRH molecule not involved

with binding resulted in exposure of the photoreactive group to the bulk medium, or neighbouring macromolecules rather than the LHRH receptor protein itself, a possibility suggested by Bayley and Knowles (1977). It has also been suggested that ligand dissociation before reaction of the activated aryl nitrene may be contributory to low levels of photoaffinity labelling (Zisapel and Sokolovsky, 1977) although this would seem to be unlikely here in the case of the LHRH receptor. Dissociation of LHRH and its analogues at 4°C is slow (Clayton and Catt, 1981) relative to the expected half-life of aryl nitrenes of $\sim 10^{-4}$ seconds (Ji, 1979, Das and Fox, 1979). Another possible explanation put forward for the low level of photoaffinity labelling was that the electrophilic nature of the nitrenes results in a preferential reaction with water or buffer (Das and Fox, 1979). The low yield of specific photoaffinity labelling of membrane receptors is a result that has often been observed (Pilch and Czech, 1984). Possibly the use of scavenger molecules (such as Tris or p-amino benzoic acid, Bayley and Knowles, 1977; Das and Fox, 1979) to consume surplus photoactivated groups would have reduced the non-specific labelling and so increased the specific labelling seen, but concomitant reductions in yield may well occur.

The alternative strategy to photoaffinity labelling investigated here was that of chemical crosslinking using bifunctional reagents, the type chosen were the N-hydroxysuccinimide esters. These groups are said to be relatively stable to hydrolysis in aqueous solution, with the amide formation reaction proceeding at a higher rate at physiological pH and a temperature of 0-20°C (Ji, 1979; Pilch and Czech, 1984). Compared to photoaffinity labelling this method has three apparent disadvantages. Firstly, it is necessary to have

specific groups (usually free amino groups) occurring on both the ligand and receptor at an appropriate distance apart for crosslinking (Ji, 1979; Pilch and Czech, 1984). Secondly, the crosslinkers are likely to react with any suitably positioned free amino group, not just those on the receptor or ligand, resulting in considerably non-selective protein crosslinking (Ji, 1979; Pilch and Czech, 1984). Thirdly, the relatively long reaction time - about 10 minutes for reactions to go to completion (Ji, 1979) allows more time for receptor-ligand dissociation or the crosslinking of adjacent membrane constituents as a result of putative random collisions (Ji, 1979).

In order to overcome the first disadvantage,N-hydroxysuccinimide esters of varying chain lengths, ranging from 0.6nm (DST, Smith, Capaldi, Muchmore and Dahlquist, 1978) to 1.51nm (EGS, Baskin and Yang, 1980),were used. These N-hydroxysuccinimide esters are highly reactive for amide bond formation under mild conditions (Das and Fox, 1979; Ji, 1979; Pilch and Czech, 1984) and,although primarily they react with amino groups (and the crosslinking biotinyl-LHRH analogue X-BAL was constructed with a free amino group available, 2.11),they can also react with the imidazole group of histidine (Ji, 1979). (Possibly the apparent reduction in affinity of ^{125}I-XBAL seen after conjugation to excess sulpho-SANPAH could have been the result of substitution of the group onto the histidine, at residue 2, which is thought to be required for the biological activity of LHRH analogues (2.9)). One assumption made was the occurrence on the LHRH receptor of a suitable group for reaction with the esters. This is probably a valid assumption, as hydrophilic residues, such as lysine, which contains a free amino group on its side chain, tend

to be situated on the external surface of many proteins (Matthews, 1977).

Whilst the length of the crosslinker has been generally regarded as an important variable (Ji, 1979; Pilch and Czech, 1984), with those below 0.5nm being found to result in few or no crosslinks (Ji, 1979) and those over 1.1nm to crosslink proteins regarded as non-contiguous (Aizawa, Kurimoto and Yokono, 1977), in the case of ligand crosslinking to the insulin receptor it was not found to be an important variable (Pilch and Czech, 1984). The results we have obtained here for the LHRH receptor show that the longest crosslinker tried (EGS at 1.5nm) resulted in the highest level of specific binding. This may imply that there are few free amino groups at the receptor binding site, and those that do occur are at least 1.5nm from the free amino group of the ^{125}I-XBAL molecule as it occupies the binding site.

The high level of non-specific crosslinking seen, particularly with DSS (0.2-2mM) and the higher concentrations of BS^3 (2 and 5mM), DST (5mM) and to a lesser extent 5mM EGS, may well be a result of non-selective crosslinking of ^{125}I-XBAL (either free or bound) to membrane constituents - such as proteins or phospholipid head groups. Higher concentrations of crosslinkers have been shown to result in protein aggregation (Zysk, Cronin, Anderson and Thorner, 1986; Lewis and Williams, 1987). As a result, optimum affinity crosslinking is said to occur using the lowest effective concentration of crosslinker (Pilch and Czech, 1984) and a high receptor/ligand ratio (Das and Fox, 1979).

To try to optimise crosslinking with the bifunctional esters, reaction conditions were chosen to limit ^{125}I-XBAL dissociation

whilst (as far as possible) reproducing conditions under which crosslinking with that particular reagent had previously been shown to be effective. As such, EGS and DSS were left to react for 30 minutes on ice (Abdella, Smith and Royer, 1979. Massague, Guillete and Czech, 1981) and DST and BS^3 for 30 minutes at room temperature (Coggins, Hooper and Perham, 1976; Giedroc, Puett, Ling and Starod, 1983). The concentration ranges used had also previously been shown to give effective crosslinking of proteins (Smith, Capaldi, Muchmore and Dahlquist, 1978; Abdella, Smith and Royer, 1979; Massague, Guillette and Czech, 1981; Giedroc, Puett, Ling and Straros, 1983; Staros and Kakkad, 1983). Within this period of time, at pH7.4, ^{125}I-XBAL binding would not be expected to change significantly at 0°C, whilst at 30°C some re-equilibration may occur, but as dissociation of similar LHRH analogues is slow (Clayton and Catt, 1981), it would not be expected to have a large effect on the level of specific binding seen. As ^{125}I-XBAL is expected to be a degradation-resistant LHRH analogue (2.9), non-specific binding and affinity labelling, as a result of enzymatic breakdown of the ligand (Das and Fox, 1979), are not expected to be significant factors over these time periods (Clayton and Catt, 1981).

Optimal affinity labelling of the LHRH receptor site was found to occur using the bifunctional ester EGS (5mM for 30 minutes on ice) to crosslink ^{125}I-XBAL binding at equilibrium with the binding site. In attempts to purify the affinity-labelled receptor by affinity chromatography, a lower concentration of EGS (0.5mM) was used in an attempt to reduce non-selective protein crosslinking and increase the specificity of the ^{125}I-XBAL crosslinking (see above). Initial experiments had shown that the biotin-avidin

interaction was not supressed by the presence of 5mM CHAPS/1.5M NaCl, and that attaching an LHRH analogue to the biotin molecule did not affect its ability to bind to the avidin-agarose (2.6). The affinity of biotin for the avidin-agarose is in the order of 10^{-7}M, much lower than that for free avidin (about 10^{-15}M; Hoffman and Finn, 1985) making it easier to use specific elution of biotin from the avidin sites and therefore more suitable for use as an affinity chromatography method (Wilchek, Moron and Kohn, 1984). This practically advantageous reduction in affinity as a result of attaching a ligand to an inert gel has been seen for many different ligands (Strosberg, 1984).

Using a photoaffinity labelled, solubilised, rat anterior membrane preparation, the general strategy (of covalently attaching biotin to a membrane protein via an LHRH ligand and purifying it by use of an avidin-gel) was shown to be effective. However, the low level of specific labelling seen using ^{125}I-PBL meant that purification of proteins achieved was not selective for the LHRH receptor. The technique would be dependent on the specificity of the covalent labelling achieved. Having optimised crosslinking conditions for EGS and ^{125}I-XBAL, membranes labelled in this way were solubilised and applied to a streptavidin-agarose column. In this case, whilst the yield of labelled proteins was much reduced, the specificity (in terms of being blocked by the presence of excess LHRH) was much higher (6.3.3). The low yield of labelled proteins may be due to non-selective crosslinking of membrane constituents resulting in steric hinderance of biotin-streptavidin binding. One way to try to increase the yield would be to subject the solubilised preparation to an enzymatic (or non-enzymatic) digestion (Kasper,

1975). As long as the type of reaction chosen did not result in the cleavage of the biotin-receptor crosslink, the biotin would still be available as a "handle" with which to affinity purify the protein fragment relating to the LHRH receptor binding site. In principle, protein sequencing of such a fragment could in turn be used to construct oligonucleotides for cDNA screening and so provide a route to determining the amino-acid sequence of the LHRH receptor found (6.1).

Using SDS PAGE the proteins specifically labelled using ^{125}I-XBAL and 5mM EGS were found to be approximately 72,000 and 39,000 molecular weight. Previously Iwashita and Catt (1985) found pituitary LHRH binding proteins to have molecular weights of 59,000 and 40,000. As the binding to both these and those detected using EGS crosslinked membranes is displaceable by non-specific levels of LHRH (1μM), it is possible that the same proteins have been detected in both studies. This is possible as although the mean size of the higher molecular weight band was 72,000, it was a broad band spanning the 60,000-82,000 range of molecular weights. Another possibility is that the EGS has crosslinked the ^{125}I-XBAL not onto the LHRH receptor, but adjacent proteins. Whether or not this is the case would only be assessable using purified preparations of the LHRH receptor protein. However, the broad agreement with other estimates of the LHRH binding protein molecular weight by the same technique (see Chapter 5; Hazum, 1981(b); Iwashita and Catt, 1985; Eidne, Hendricks and Millar, 1985) suggests that it is this which has been specifically labelled by ^{125}I-XBAL using EGS.

No high molecular weight protein band appeared to be labelled on the SDS PAGE gel. This is in contrast to the estimate of the size

of the CHAPS/NaCl solubilised LHRH receptor obtained from gel filtration (5.3.2) of 100,000-160,000. Although as previously mentioned in Chapter 5, there are several reasons why a protein on gel filtration might artefactually appear to have a higher molecular weight, it is also possible that this does represent the receptor as it exists in the membrane. A dimer of binding proteins, or association of other proteins with the binding protein may be responsible for the higher molecular weight. It is interesting to note that Couvinear, Amiranoff and Laburthe (1986) found that crosslinking of ^{125}I-VIP to a membrane preparation revealed a 50,000 molecular weight component (on SDS PAGE), whereas crosslinking to a solubilised preparation revealed a 150,000 molecular weight protein. This might suggest that in situ covalent labelling of a receptor site does not enable the crosslinking of the binding protein to its adjacent proteins in the receptor complex. After solubilisation this would appear to become possible, so that the receptor macromolecule as it exists in the membrane (and in solution with mild detergents) is stabilised and can be detected on SDS PAGE. Similarly, crosslinking of ^{125}I-XBAL to CHAPS/NaCl solubilised LHRH receptors (after removal of the detergent solution by PEG precipitation) may provide confirmation of the existence of a high molecular weight complex under these conditions by stabilising it and allowing it to be detected using SDS PAGE. Although this had not been attempted in this study, it might be an illuminating experiment to carry out.

The total amount of LHRH receptor protein in rat anterior pituitary tissue is low (only about 90fmol of binding sites per rat anterior pituitary gland), therefore the intention was to transfer

the optimised affinity labelling procedure to a more abundant source of LHRH receptors. Such a source could possibly be bovine anterior pituitary tissue (3.7). Although receptors exist in the bovine gland at a rather lower density (10% of that found in male rat tissue 3.3.7.1), the total number per gland is much higher than that found in rats (about 3-4pmol). Unfortunately, removal of the Tris/BSA binding medium and replacing it with the Hepes-KOH buffer (which is free of primary amino-groups) resulted in a dramatic increase in non-specific and decrease in specific binding. It was clearly necessary to replace the Tris/BSA as this represented a major source of free amino-groups for non-selective crosslinking and may well have resulted in the sequestering of EGS activity. (Glycine at 67mM is used to quench the reaction normally, 25mM Tris/0.1% BSA may well have had a similar effect.) It is likely that, rather than the replacement of the Tris-HCl buffer with Hepes-KOH buffer, it was the removal of the BSA that resulted in the increased non-specific binding seen. As optimal conditions for specific ^{125}I-XBAL binding to bovine anterior pituitary tissue include the presence of BSA or a similar protein to reduce excessive non-specific binding, it is possible that photoaffinity labelling instead may be relatively more efficient and specific for this tissue, although very high yields and specificity would not be expected. Judging from most other photoaffinity labelling of receptor sites, the overall yield of labelled receptors may be low; a usual value given by Pilch and Czech (1984) is 4%, and judging from the work with the rat receptor (6.3.1 and 6.3.2.1) only poor levels of specificity would be predicted for the currently available ligands.

In conclusion, whilst conditions for the covalent labelling of rat anterior pituitary receptors have been optimised to give 23% specific labelling, these methods have not been found to be suitable for use on the more abundant source of LHRH receptors, bovine anterior pituitary tissue. Affinity chromatography of the biotin-labelled protein has been shown to be possible, but only low recoveries have been attained. Better yields may be found if the solubilised, crosslinked preparation is subjected to some method of protein fragmentation prior to affinity chromatography in an attempt to increase the accessibility of the biotin for the streptavidin-sepharose. Further development of such a strategy could result in the purification of a biotin labelled fragment of the LHRH receptor protein suitable for amino-acid sequencing. This in turn could be used to produce oligonucleotide probes to use in the determination of the molecular characteristics (cDNA and amino-acid sequence) of the LHRH receptor protein.

Chapter 7

Summary and General Discussion

In this Thesis the approach of measuring ligand-receptor interactions has been used extensively to investigate a wide variety of aspects of the LHRH receptor. Initial studies confirmed the validity of the method used (described in 2.4) showing it to reveal equilibrium binding to specific LHRH receptors that were pharmacologically similar to those described in the literature (Clayton and Catt, 1980). This approach proved useful in studies to try to further investigate the transducing systems used by the LHRH receptor of the pituitary gland and also in characterising the integrity of solubilised receptors.

7.1 Studies on the membrane-bound LHRH receptor with reference to LHRH action

In view of evidence that K^+ channel closure may participate in the mechanism of LHRH action and that agonist-activated G proteins may interact rather directly with certain K^+ channels (Chapter 3), experiments were carried out to assess any allosteric effects of K^+ or of K^+ channel blockers on LHRH receptor binding.

No support could be found for the hypothesis that potassium channels, particularly A-type channels, are closely associated with the LHRH receptor (Chapter 3). The pharmacology of potassium channels is, however, very complex, with many similar but not identical types having been identified (Cook, 1988). Recently the cDNA clones of the *Drosophilia Shaker* locus have been sequenced (Schwarz, Tempel, Papazian, Jan and Jan, 1988). This locus, absent from the *Shaker* mutation, was thought to encode the A-type K^+ channel (Salkoff and Wyman, 1981). It was found that by a mechanism of alternative splicing of transcripts at least four different proteins could be produced from the same locus (Schwarz, Tempel,

Papazian, Jan and Jan, 1986). Two of these, expressed separately in oocytes, resulted in functional K^+ channels similar to A-channels but each with slightly different kinetic properties (Timpe, Schwarz, Tempel, Papazian, Jan and Jan, 1988). This led the group to suggest that the multiplicity of K^+ channels observed could be the result of channel complexes (possibly each consisting of four gene products) of varying subunit composition, each subunit type derived from a differently spliced transcript. Possibly a similar mechanism may also be responsible for the complexity of other types of K^+ channels. This may in part explain the confusion existing in the identification of K^+ channels thought to be involved in LHRH actions, most electrophysiological studies suggesting the involvement of M-type and Ca^{2+} activated channels, but a minority of electrophysioligical together with secretion studies indicating the A-channel (see 3.3.4). It appears most likely that the LHRH-induced K^+ channel modulations observed by electrophysiological techniques are indirect effects involving diffusible second messengers and that the complexity of observations on secretion may result both from the great heterogeneity of K^+ channels and the simultaneous effects of LHRH on several different types. This is likely to become clear as the primary transducing systems of LHRH receptors are further elucidated.

Extensive investigations (under stringent conditions) revealed that the LHRH-receptor is indeed likely to have a direct interaction with a G-protein ~ as might be expected for receptors activating phosphoinositide metabolism (Graziano and Gilman, 1987; Putney, 1987). Although it was not possible to consistently quantify the reduction in affinity of LHRH in the presence of $GTP\gamma S$ (see 3.3.3),

the present report would appear to be the first describing such an effect. Alternative ligands (antagonists), in preparation, may facilitate these investigations. As it appears likely from molecular biological evidence that the sites of interaction of a receptor and G-protein are similar for all types of G-protein (Sullivan, Miller, Masters Beiderman, Heideman and Bourne, 1987), it is perhaps surprising that adenylate cyclase linked receptors appear to show a more obvious allosteric influence on G-protein activation (Blume, 1978; Glassman and Presek, 1979; Tsai and Lefkowitz, 1979; Shane, Gammon and Bilezikian, 1981; Hulme, Berrie, Birdsall, Jameson and Stockton, 1983). It may be that whilst all receptors that interact with G-proteins do have some structural similarities (in the same way as ion channels, both ligand and voltage gated, have been identified to have several structural features in common; Unwin, 1986; Stevens, 1987), they also possess features uniquely dependent on the type of G-protein they interact with. As such the relationship between the ligand binding site and the site of G-protein interaction in adenylate cyclase linked receptors may be closer than those linked to phosphoinositide phosphodiesterase, in the sense of displaying a greater allosteric interaction in ligand binding experiments. Recognition of such features (common to one type of receptor but not a related one) in cloned and sequenced receptor proteins or the use of site directed mutagenesis and expression vectors may help in assigning functional roles to the tertiary and quaternary structures of proteins.

7.1.1 Investigations of some extra-pituitary LHRH receptors

Despite the finding by autoradiography of high levels of specific LHRH binding sites in rat hippocampus (Reubi and Maurer,

1984), ligand binding to hippocampal membranes was unable to confirm this (3.3.5). Whilst it is possible that specific binding of ^{125}I-buserelin may be detectable using a purified plasma membrane preparation, its absence from the crude, washed homogenate preparation suggests that the level of specific binding may be very low. Immunological and electrophysiological techniques have also failed to reveal the presence of LHRH-like material in, or an action of LHRH on, hippocampus (see 3.4.2) suggesting that the sites detected by autoradiography may not be functional LHRH receptors. Improved knowledge of the LHRH receptor site in the pituitary may enable this putative hippocampal site to be clearly recognised as perhaps a true receptor or possibly an enzyme degradation site for an LHRH-like substance.

7.2 Studies on solubilisation and purification of the LHRH receptor

The series of experiments described in Chapters 4, 5 and 6 were designed to solubilise from a membrane preparation (male rat anterior pituitary) the ^{125}I-buserelin binding site, characterise it and so confirm its identity as the LHRH receptor and then to further develop a strategy for its isolation.

Results in the literature suggested that there was no clear pattern for predicting which detergent (or even which type of detergent, such as nonionic, or zwitterionic) would be most successful at solubilising a receptor protein in high yields without denaturing it (see Chapter 4). However, it did appear that increasing the ionic strength of a detergent solution may be effective (4.1). Initial studies were carried out with the zwitterionic detergent CHAPS - previously used in attempts to solubilise the LHRH receptor (Perrin, Haas, Rivier and Vale, 1983;

Capponi, Aubert and Clayton, 1984; Hazum, Schwartz, Waksman and Keinan, 1986). The results showed that highest solubilisation yields were obtained using 5mM CHAPS and 1.5M NaCl (4.3.1). A range of other detergents were also tested at concentrations approximately equal to their critical micellar concentration, both with and without the 1.5M NaCl found to be optimal with 5mM CHAPS. None of the detergents tested were capable of solubilising the ^{125}I-buserelin binding sites as efficiently as the combined 5mM CHAPS/1.5M NaCl (4.3.1.3). The other condition found to be important for optimal solubilisation of the binding sites was the ratio of detergent to protein, being optimal under the present conditions at a value of 1.6 (w/w) (4.3.1.3). Addition of glycerol (efficient at stabilising some other solubilised receptors; Dias, Huston and Reichert, 1981; Ascoli, 1983; Iida, Amir and Ingbar, 1987) did not appear to have a beneficial effect on solubilised LHRH receptors (4.3.1).

Although a high yielding solubilisation could be obtained using 5mM CHAPS/1.5M NaCl (over 70% of available sites under optimal conditions), the subsequent presence of specific binding of ^{125}I-buserelin appeared to be conditional on the removal of the CHAPS/NaCl solution by a PEG precipitation step. Binding to both membrane and solubilised preparations in the presence of various combinations of detergent, NaCl and glycerol suggests that whilst each has some detrimental effect on the amount of specific ^{125}I-buserelin binding detectable, by far the greatest effect is due to the presence of the high NaCl concentration (4.3.3; 4.3.4). In turn, the high NaCl concentration was essential for the high yielding solubilisation (4.3.1). The two conditions, high yielding

solubilisation and retention of specific ^{125}I-buserelin under solubilisation conditions did not appear to be compatible. Further experiments, altering the composition of the solubilisation solution after solubilisation had been achieved, were also unable to reveal conditions which would allow both properties to be retained (4.3.5; 4.3.6).

Despite this, it was apparent that the ^{125}I-buserelin binding site solubilised by 5mM CHAPS/1.5M NaCl could be fully recovered by subjecting the solubilised preparation to a PEG precipitation step. In this way ligand binding assays were used to characterise the ^{125}I-buserelin binding site as an undenatured LHRH receptor (Chapter 5). The binding affinities of native LHRH, a superagonist, partial agonist and antagonist were all very similar for both the solubilised and membrane bound site.

Having solubilised the LHRH receptor in an undenatured form, it was possible to try to further characterise it in this state. The apparent size of the LHRH receptor using SDS PAGE techniques has been shown previously to be approximately 60,000, whereas estimates obtained of the membrane bound receptor suggest a size of 135,000 (Conn and Venter, 1985). Gel filtration revealed that the CHAPS/NaCl-solubilised LHRH receptor eluted mainly in a peak corresponding to a molecular weight of between 100,000 and 160,000 (5.3.2). Whilst a number of factors, such as anomalous behaviour of the protein in the gel column (due to a non-uniform shape and/or the presence of carbohydrate moieties on the protein) or detergent or lipid binding to the protein, may contribute to the high molecular weight observed, it is possible that the solubilised form may be a complex of the 60,000 LHRH binding protein detected by SDS PAGE,

either as a dimer or associated with other non-binding subunits. In order to determine if that is in fact the case, further purification of the solubilised LHRH receptor would be necessary.

Initially, it was hoped that the solubilised receptor could be purified using LHRH ligand affinity chromatography. The finding that solubilisation and ligand binding conditions were incompatible meant that some other approach was required. As the binding of biotin to avidin (or streptavidin) is largely unaffected by the presence of detergent solutions, and the affinity of biotin for avidin-agarose is of suitable order for use in affinity chromatography (IC_{50} of about 10^{-7}M : 2.6),this interaction seemed to provide a suitable method for receptor purification. However, in order to be of any use in this case, the biotin had to be covalently attached to the LHRH receptor in a highly specific manner. To achieve this, several biotin-containing LHRH analogues were designed (2.9) and their properties characterised (6.3.2). These analogues were bound to the membrane preparation and either photoaffinity or covalent crosslinkers were used to try to link them to the receptor protein. Of those tried, the greatest values for irreversible, specific binding were obtained with the LHRH analogue XBAL and 5mM EGS; (6.3.2.3) up to 23% of the specific ^{125}I-XBAL binding being covalently linked of the LHRH receptor protein or closely related structures.

Both the photoaffinity and covalent crosslinking methods of biotin-labelling the anterior pituitary membrane preparations were used to test the feasability of the biotin-avidin affinity chromatography step. The results suggested that the biotin-avidin (or streptavidin) interaction retained its reversibility despite the

bulky receptor-ligand complex being conjugated to the biotin. In the case of the photoaffinity labelled preparation, using the ligand PBL (6.3.3.1), the specificity of the receptor labelling was too low to be of practical use. For the preparation labelled using covalent crosslinkers, although much greater specificity and efficiency of receptor labelling was seen, there appeared to be some steric hindrance of the biotin-streptavidin interaction. Multiple inter-protein links caused by the non-selective nature of the crosslinkers may be responsible, so it may be possible to overcome this problem by incorporating a partial protease degradation of the biotin-labelled membrane preparation prior to solubilisation. It will be necessary to optimise this step before the full potential of this method in the purification of the LHRH receptor (or a sequenceable fragment) can be realised.

7.3 Future perspectives for studies of the LHRH receptor

Although these studies have largely been carried out on rat anterior pituitary tissue, further studies on LHRH receptor isolation and molecular characterisation would be greatly facilitated by some more abundant source of LHRH receptors. With this in mind, other possible sources of the receptor protein were investigated (3.3.7), and the bovine anterior pituitary was found to be the most promising. Unfortunately, largely due to differences in the kinetics and specificity of LHRH binding to bovine and rat anterior pituitaries, the methods of covalent labelling optimised on rat tissue did not appear to be useful on bovine tissue. It would be necessary to repeat the optimisation process in order to use this larger supply of LHRH receptor sites in the biotin-avidin affinity chromatography strategy devised here.

An alternative, potentially more abundant source of LHRH receptors, would be from a cell line. Unfortunately no present cell line is known to express high affinity LHRH receptors. A recent brief report of electrophysiological responses to LHRH in the GH_3 rat somatomammotrophic cell line provides an interesting possibility, but the specificity and reproducibilty of these observations remain unsubstantiated as yet (Rosenthal, Hescheler, Hinsch, Spicher, Trautwein and Schultz, 1988). One method that has been used to create novel neural cell lines has been by the introduction of oncogenes into primary cells (Cepko, 1988). Using retrovirus-mediated transduction this can produce stable cell lines and such a technique may be useful in producing a gonadotrophic cell line expressing high affinity LHRH receptors. Such a preparation would greatly facilitate the study of the receptor, both investigations of its mechanism of action and molecular characterisation, by providing an abundant and uncontaminated source of gonadotrophes.

The detergent solubilisation and purification strategy for molecular characterisation of receptor proteins is only one of several that may be successful (Strosberg,1987). One that has proved fruitful for the cloning of two low abundance neurotransmitter receptors is that of functional expression of mRNA (Levitan, 1988). Expression of both $5\text{-}HT_{IC}$ receptor and substance K receptor was determined electrophysiologically by their effects on Ca^{2+}-activated Cl^- channels. In order for this method to be of use in the cloning of other receptor types, specific ligand binding must result in the activation of a readily detectable ion channel (either directly or indirectly via a second messenger). This is

probably an appropriate route for investigating LHRH receptors (as ligand binding results in an elevation of cytosolic Ca^{2+} levels which may well be sufficient to activate the large conductance chloride channels utilised in the $5\text{-}HT_{IC}$ and substance K receptor studies). Given the difficulties encountered by ourselves and other groups with protein purification, such an approach may therefore prove to be the strategy of choice in the cloning of the LHRH receptor, provided that a suitable mRNA library containing the message for functional LHRH receptors (in sufficient abundance) can be produced.

If such a strategy were successful, it would be interesting to compare the sequences of anterior pituitary LHRH receptors with the low affinity receptors found elsewhere, such as the placenta, and also with those responsible for the actions of LHRH in the CNS. It is possible that, similar to other receptors such as the muscarinic cholinergic receptor subtypes (Kerlavage, Fraser and Venter, 1987) or the β_1 and β_2 adrenergic receptors (Frielle, Kobilka, Lefkowitz and Caron, 1988), receptor proteins for the same endogenous ligand but with different pharmacological characteristics are in fact different gene products. Whether receptor heterogeneity was the result of differential splicing (as seen for Drosophila A-channels; Timpe, Schwarz, Tempel, Papazian, Jan and Jan, 1988), differential post-translation modification, or the existence of more than one related gene could then be determined.

If the LHRH receptors present in the pituitary and other tissues were found to be structurally different, then it may be possible to develop selective drugs. Clinically these may be of use in selectively modifying either the pituitary-gonadal axis, CNS aspects

or other elements of LHRH action. For instance, LHRH agonists have been found to be useful in the treatment of premenstrual syndrome, a phenomenon that appears unlikely to be a direct effect of either oestradiol or progesterone (Bancroft, Boyle and Fraser, 1986). One aspect of the syndrome are the psychological symptoms, including depression or irritability. Agonists selective for LHRH receptors responsible for the beneficial effects in this syndrome (possibly distinct from receptors in the anterior pituitary) may prove effective in treating the syndrome in the absence of suppression of pituitary gonadotroph release.

7.4 Conclusions

The experiments carried out in this Thesis have contributed to the understanding of the properties of the LHRH receptor and its transducing systems. Preliminary evidence (but the first of its kind) supporting the hypothesis that the receptor interacts directly with a G-protein is provided, whereas the involvement of K^+ channels in LHRH action can neither be confirmed nor refuted. A high yielding method of solubilisation of the rat anterior pituitary LHRH receptor was devised and multifunctional biotinylated covalent ligands were designed, synthesised and used in a novel strategy for receptor purification. Nevertheless, subsequent technical difficulties and the lack of availability of abundant sources of receptor protein, prevented purification of sufficient receptor protein for partial sequencing. The results suggest that other strategies for molecular characterisation of the LHRH receptor protein may prove to be more productive.

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Appendices

Appendix I Iodination of LHRH Analogues

All LHRH analogues were iodinated by the chloramine T method (Hunter and Greenwood, 1962) as described for LHRH and buserelin (Nemann and Sandow, 1973; Sandow and Konig, 1979) with modifications.

Reagents

0.01M phosphate buffered saline (PBS): 8.17g NaCl
0.1g Na merthiolate
0.25g NaH_2PO_4, $2H_2O$
1.393g Na_2HPO_4 anhydrous
per litre distilled H_2O, pH 7.6

LHRH analogues:

buserelin	5µg/10µl PBS
XBAL	5µg/10µl PBS
PBL	5µg/3µl MeOH
PBAL	5µg/10µl MeOH, made up to 20µl with 0.01M acetic acid
LHRH antagonist	5µg/10µl PBS

Chloramine T 2.5mg/ml PBS
(N-Chloro-p-toluenesulphonamide-Na)
Sodium metabisulphite 2.5mg/ml PBS
$Na^{125}I$ Amersham IMS 30: 3.7GBq/ml
0.5M Phosphate pH 7.5:

Protocol: i) 10µl (82µl for PBL) 0.5M phosphate buffer added to peptide solution (volumes as above) in eppendorf.
ii) 10µl (5µl for PBL) $Na^{125}I$ (37MBq) added.
iii) 20µl chloramine T added, mix gently.

iv) Leave to react for 20 seconds.

v) Add 50µl sodium metabisulphite, mix gently.

Separation of Iodinated Analogue

The iodinated analogue was usually separated from free iodine by affinity chromatography using a G-25 fine sephadex column. For the chemical crosslinking experiments ^{125}I-XBAL was separated from free Na^{125}I using HPLC.

1) Affinity Chromatographgy

Column: 1 x 15cm Sephadex G-25 fine.

Eluting solutions: 0.01M acetic acid.

0.01M acetic acid/3% bovine serum albumin (for buserelin and PBL).

0.01M acetic acid/6% bovine serum albumin (for PBAL, XBAL and LHRH-antagonist).

Protocol:

i) Iodination reaction mixture applied to column.

ii) Eppendorf rinsed with 0.8ml acetic acid/BSA solution, this was also applied to the column.

iii) Free ^{125}I peak eluted with 0.01M acetic acid. 2ml fractions collected and counted.

iv) Iodinated peptide eluted with acetic acid/BSA (3% or 6%) solution. 2ml fractions collected and peak of activity tube retained.

v) Aliquot peak tube - 100µl. Store at -70°C.

2) HPLC

Apparatus - Two-channel minipump (Milton Roy Co., Riviera Beach, FL)

Sample injection valve with 1ml loop (Rheodyne Berkeley, CA)

Guard column holder (Brownlee MPLC cartridge system: Brownlee Labs, Santa Clara, CA)

3cm x 0.46cm disposable column of cyanopropyl silica (Spheri 5 cyano: Brownlee Labs)

Eluting Solvents: A 0.2% trifluoroacetic acid in distilled H_2O

B 0.2% trifluoroacetic acid in MeOH.

Protocol:

i) Prime pump and wash column with solvent B.

ii) Prime pump and wash column with solvent A.

iii) Increase iodination reaction mixture volume by adding 0.9ml KI (10mg/ml in A).

iv) Inject and load iodination reaction mixture onto column.

v) Elute with stepwise gradient of B from 0% to 100% (increments of 20%). Flow rate of 1ml/minute, collect 1 minute fractions. Six fractions at each step of gradient.

vi) Count fractions. Free ^{125}I is eluted first. Retain peak of labelled peptide and store at -70°C.

Radiolabelled buserelin stored at -70°C was active for up to 12 weeks after iodination. Other iodinated analogues were used within

3 weeks of iodination.

In the case of the photo-sensitive analogues PBL and PBAL all steps were carried out in subdued lighting or in the dark with a safety light wherever practicable.

Appendix II SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

The method of SDS PAGE used was that first described by Laemmli (1970).

Stock solutions

4 x separating Gel Buffer:	45.4g Tris/HCl (pH8.8)
	1.0g sodium dodecyl sulphate
	into 200mls distilled H_2O
4 x stacking Gel Buffer:	12.1g Tris/HCl (pH6.8)
	0.8g sodium dodecyl sulphate
	into 200mls distilled H_2O
SDS Sample Buffer:	10g glycerol
	5ml β-mercaptoethanol
	2.3g sodium dodecyl sulphate
	0.75g Tris/HCl (pH6.8)
	made up to 100mls with distilled H_2O
30% Acrylamide:	29.2g acrylamide
	0.8g bis-acrylamide
	made up to 100mls with distilled H_2O

TEMED: N,N,N',N'-Tetramethylethylenediamine.

These stock solutions were kept at 4°C (the acrylamide stock in the dark).

10% Ammonium persulphate: w/v in distilled H_2O

Made fresh for each experiment.

7% Separating Gel	4 x buffer	12mls
	30% Acrylamide	12mls
	distilled H_2O	24mls
	10% persulphate	160µl
	TEMED	24µl
4.5% Stacking Gel	4 x buffer	6mls
	30% Acrylamide	3.2mls
	distilled H_2O	14.8mls
	10% persulphate	80µl
	TEMED	12µl
Tank Buffer	Tris	15.15g
	glycine	77g
	sodium dodecyl sulphate	5g
	made up to 5 litres with distilled H_2O	
Fixer	methanol	40%
	acetic acid	7%
	in distilled H_2O	

Preparation: 1) Polyacrylamide Gel.

a) Constituents of the separating gel (as above) were mixed, the ammonium persulphate and TEMED being added immediately before the gel was poured.

b) The gel solution was poured into the prepared

gel plates. (Two 20 x 20cm glass plates separated by a 0.7mm spacer. Edges sealed with tape and 1% agarose.)

Overlay with butanol.

c) Leave to polymerise for an hour.

d) Mix stacking gel - adding ammonium persulphate and TEMED immediately before pouring.

e) Decant butanol overlay and dry inside of plates.

f) Put well former (100µl wells) into place and pour stacking gel.

g) Leave to polymerise for an hour.

h) Remove tape from bottom edge of gel plates and well former from stacking gel.

2) Samples and molecular weight standards.

a) Samples were dissolved into 100µl sample buffer, add 5µl of 1% bromophenol blue.

b) Add 8µl of sample buffer and 1µl 1% bromophenol blue to 3µl ^{14}C-molecular weight markers (Amersham, 2,350-200,000MWT).

Running SDS PAGE: Apparatus: GE-214 LS vertical electrophoresis apparatus (Pharmacia Fine Chemicals Ltd)

LKB 2197 power supply

LKB 2219 Multitemp II thermostatic circulator.

Protocol: i) Samples (80µl) and standards (10µl) are applied to the wells of the stacking gel.

ii) Gel placed in apparatus, with the tank circulating, cooled to 10°C and the power supply on - constant current 40mA. Run for 10-12 hr, (until dye front was within 4cm of bottom of separating gel.)

Fixing Gel and Autoradiography

i) Fixed (40% methanol, 7% acetic acid) for at least 2 hrs.

ii) Dried under vacuum.

iii) Placed in Kodak X-Omatic cassette (Rapid x 2) with X-Omat S film, exposed for 4 weeks at -70°C.

iv) Developed using Kodak DX-80 developer, FX-40 X-ray liquid fixer (Kodak Ltd, Glasgow).

Appendix III Synthesis of ligands PBL, PBAL and XBAL

(All peptide synthesis work was done by Dr C M Bladon)

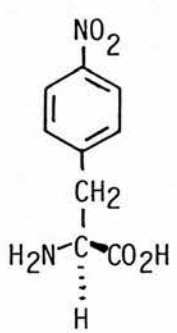
The ligands (see fig. AIII.1: 1-3) were prepared by coupling either of one of the two reagents (4,5) with one of the peptide backbones (6,7).

1. Synthesis of reagents

Both reagents, biotinyl-p-azidophenylalanine N-hydroxy-succinimide ester (4) and biotinyl-t-butoxycarbonylaminoethylglycine N-hydroxysuccinimide ester (5) were prepared from biotin and either L-p-nitrophenylalanine or L- 2,4-diaminobutyric acid as outlined in schemes (1) and (2) respectively.

Note: the compounds are written in shorthand form, eg,

H-Phe(NO_2)-OH $\equiv$



2. Synthesis of [D-Lys^6, desGly^{10}]-LHRH

This peptide was prepared by solid-phase synthesis using Fmoc-t-butyl-polyamide chemistry (Eberle, Atherton, Dryland and Sheppard, 1986). After purification by ion-exchange chromatography the peptide was approximately 95% pure (by HPLC) and showed the expected amino acid composition and molecular weight (Glu_1 1.02, His_1 0.96, Ser_1 0.89, Tyr_1 0.97, D-Lys_1 1.00, Leu_1 1.00$_1$ Arg, 1.00$_1$ Pro_1 0.95, FAB-MS: found MH^+ 1225, calc. MH^+ C_{59} H_{86} N_{17} O_{12} 1225).

(xix)

PGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ϵ}-biot-Phe(N_3)]-Leu-Arg-Pro-Gly-NH_2

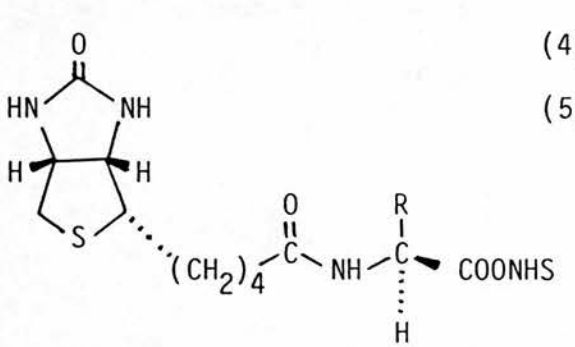
PBL (1)

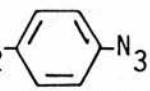
pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ϵ}-biot-Phe(N_3)]-Leu-Arg-Pro-NHEt

PBAL (2)

pGlu-His-Trp-Ser-Try-D-Lys-[N^{ϵ}-biot-Gly($CH_2CH_2NH_2$)]-Leu-Arg-Pro-NHEt

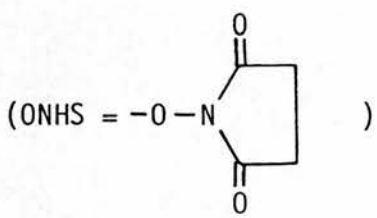
XBAL (3)



(4) R = CH_2- 

(5) R = CH_2-CH_2-NHBoc

(Boc =)



pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2

(6) D-Lys^6-LHRH

pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt

(7) [D-Lys^6, desGly^{10}]-LHRH ethylamide.

Figure A.III.1

3. Synthesis of conjugates

The conjugates PBL, PBAL and XBAL were all prepared by linking the reagent via a peptide bond to the amino group of the D-lysine residue at position 6. For example, PBL was synthesised by first dissolving D-Lys^{6}-LHRH (2mg, 1.6μmol) in freshly distilled DMF containing triethylamine (2 drops). A 5-fold excess of the reagent (4)(5mg, 9.45μmols) in DMF (1ml) was added and the resulting mixture was left standing in the dark for 1 hour. The DMF was evaporated under high vacuum and the residue was triturated with ethyl acetate (3 x 2ml) to remove excess reagent. The crude product was purified by semi-preparative HPLC to yield 1.84mg (69%) of pure (single peak on analytical HPLC) conjugate. The amino acid compositions of the three conjugates are given in the table.

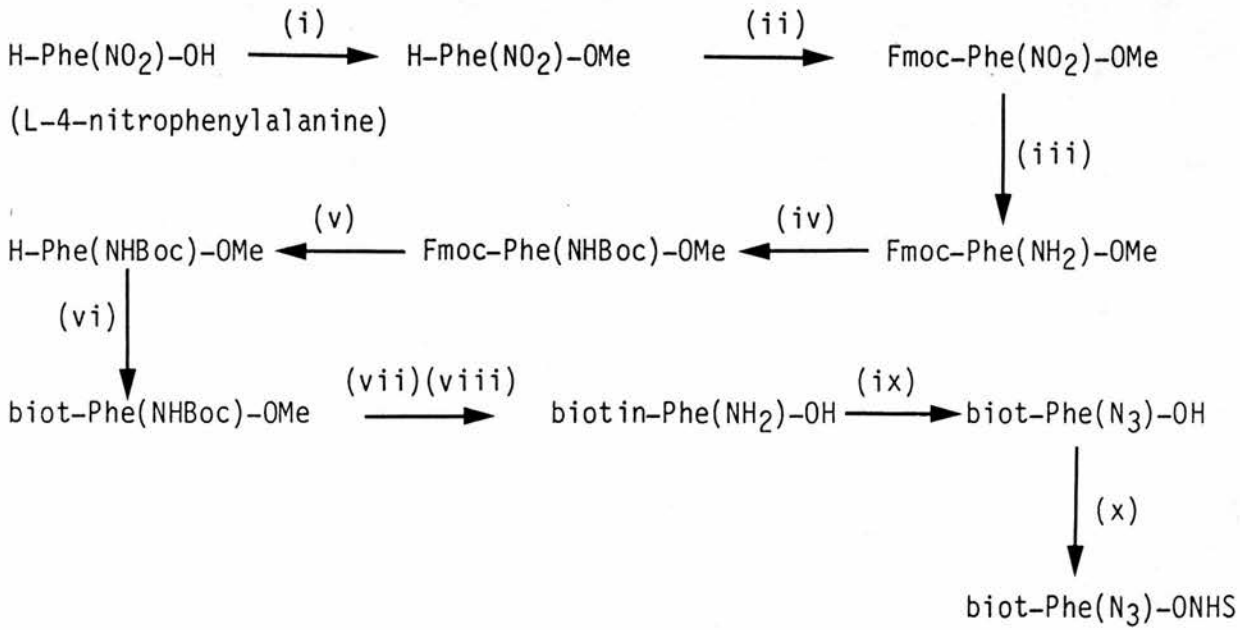
Note

1. Reactions involving the p-azido reagent(4) were performed in the dark and all handling of the p-azido conjugate was carried out in subdued lighting.

2. The amino group of the second reagent (5) was protected with the t-butoxy-carbonyl group during coupling to the peptide, and was removed, with TFA, from the conjugate.

3. HPLC condition: semi-preparative Aquapore RP300 column; eluant A, 0.1% aq TFA: B, 90% CH_3CN 10% A. The column was eluted isocratically for 2 min with 10% B and then with a linear gradient of 10-70% B over 30 min, flow rate 3ml/min. Optical density was monitored at 230nm. During purification of the azido conjugate (1,2) the detector was switched off whilst the product peak was collected.

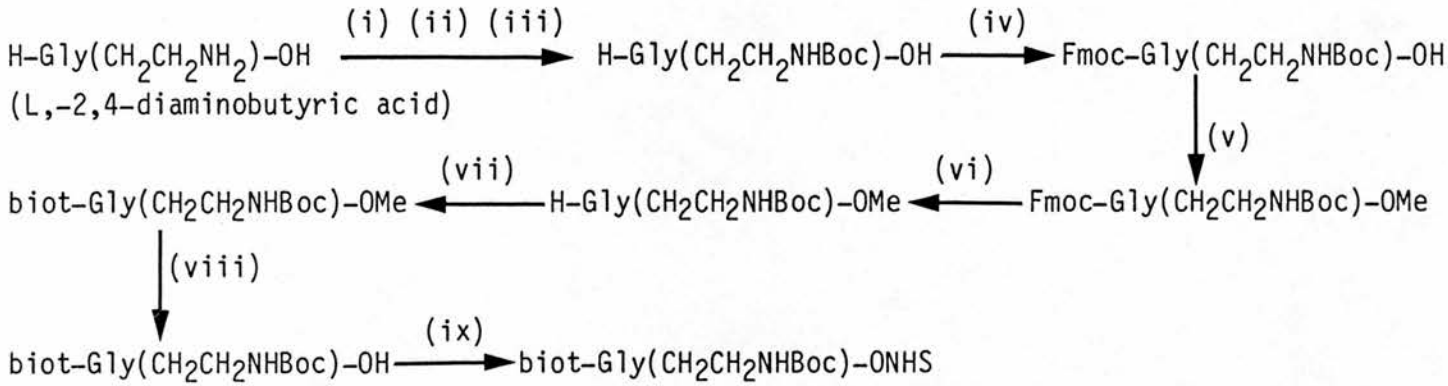
Scheme 1.



(xxi)

Conditions: (i) $SOCl_2$-MeOH; (ii) FmocCl-K_2CO_3-H_2O-dioxan; (iii) H_2-5%Pd-C-AcOH; (iv) 10% $NaHCO_3$-$(Boc)_2O$-H_2O-dioxan; (v) 20% piperidine in DMF; (vi) biotin pentafluorophenyl ester-HOBT-DMF; (vii) NaOH-H_2O-MeOH; (viii) aq TFA; (ix) $NaNO_2$-NaN_3-1M HCl; (x) N-hydroxysuccinimide-DCC-dioxan.

Scheme 2.



Conditions: (i) $CuCO_3$-$Cu(OH)_2$-H_2O; (ii) MgO-$(Boc)_2O$-MeOH; (iii) H_2S-H_2O-NH_4OH;
(iv) FmocCl-K_2CO_3-H_2O-dioxan; (v) CH_2N_2-Et_2O; (vi) 20% piperidine in DMF;
(vii) biotin pentafluorophenyl ester-HOBT-DMF; (viii) NaOH-H_2O-MeOH;
(ix) N-hydroxysuccinimide-DCC-dioxan.

Table

	pGlu	His	Ser	Tyr	D-Lys	Leu	Arg	Pro	Gly	Phe(N_3)*	Gly($CH_2CH_2NH_2$)
1.	1.07	1.00	0.81	1.07	1.01	1	1.00	0.96	1.04	0.30	-
2.	1.05	0.96	0.83	0.95	1.02	1	0.89	0.91	-	0.20	-
3.	1.09	1.01	0.91	1.01	1.07	1	0.96	0.99	-	-	0.94

* largely decomposed in hydrolysis.

Appendix IV Protein Assay

All protein assays followed the method of Geiger and Bessman, (1972).

Reagents

0.1g $CuSO_4$, $5H_2O$

0.54g NaK-D-tartarate

made up to 20ml, distilled H_2O

4g NaOH

20g $NaHCO_3$

made up to 200ml, distilled H_2O

Alkaline copper reagent was prepared by mixing the above two solutions together.

Folin-Ciocalteau reagent - 1:10 dilution in distilled H_2O.

Protocol: i) Standards (0-20µg) of bovine serum albumin and samples (5 - 100µl) were made up to 200µl with distilled H_2O in LP3 tubes.

ii) 500µl alkaline copper reagent was added and mixed well.

Tubes were left for 20 minutes.

iii) 1µl of diluted Folin-Ciocalteau reagent was added and tubes rapidly mixed.

Tubes were left for 20 minutes.

iv) Absorbance read at 725nm in a spectrophotometer and a standard curve constructed.

v) Samples were read off standard curve.

Preparation of Samples

As buffers, such as Tris and Hepes, and detergents may interfere with the colour formation in the protein assay (Thorne, 1978), samples in such solutions were precipitated and resuspended prior to protein assay. Where samples were in protein containing solutions (such as the CHAPS/NaCl solubilisation solution) standards were prepared by dissolving bovine serum albumin into this solution. Following precipitation and resuspension the standards were used as above to prepare a standard curve.

Protocol: i) TCA (25%) was added to the sample aliquot (10-250µl) to give a 5% solution.

ii) Samples left for 10 minutes.

iii) Centrifugation 15 minutes at 1,600g, 4°C to separate precipated proteins.

iv) Samples redissolved in 50µl 0.03M NaOH for use in protein assay as above.

Appendix V Avidin-Sepharose CL-4B Coupling

The coupling of avidin to Sepharose CL-4B was carried out according to the method of Beaty and Lane (1982) after activation of the Sepharose CL-4B by the cyanogen-bromide method described by March, Parikh and Cuatrecasas (1974).

Cyanogen bromide activation

Reagents

Sepharose CL-4B (Pharmacia Ltd, Bucks) preswollen

Na_2CO_3 2M in distilled H_2O

CNBr 2mg/ml in CH_3CN (dried and redistilled)

$NaHCO_3$ 0.1M, pH 9.5

$NaHCO_3$ 0.2M, pH 9.5

Protocol

i) 25ml of Sepharose CL-4B - washed (with distilled H_2O) on a coarse sintered glass funnel and resuspended into 25ml distilled H_2O.

ii) 50ml 2M Na_2CO_3 added. Stirred to mix.

iii) 5ml $CNBr/CH_3CN$ solution added (on ice in fume cupboard). Stirred vigorously for 2 minutes.

iv) Washed on coarse sintered glass funnel with 5-10 volumes of 0.1M $NaHCO_3$, distilled H_2O, 0.2M $NaHCO_3$.

v) Filtered to moist compact cake under vacuum.

Coupling of activated gel to avidin

Reagents

CNBr activated Sepharose CL-4B (see above).

Avidin (Sigma Chemical Company) - 10µg/ml in 0.01M Na/PO_4 pH 7.

0.1M $NaHCO_3$ pH 8.5.

0.1M Na/PO_4 pH 7 - 30.5ml 0.2M $NaHPO_4$

19.5ml 0.2M NaH_2PO_4

mixed and made up to 100ml in distilled H_2O

0.01M Na/PO_4 pH 7 - 1:10 dilution of 0.1M Na/PO_4 ph 7.

0.1M $NH_2C_2H_4OH$ (Aldrich Chemical Company, Dorset) in 0.01M Na/PO_4 pH 7.

Protocol

i) 10ml activated gel washed by filtration with 2 volumes each 0.1M $NaHCO_3$, pH 8.5, distilled H_2O, 0.1M Na/PO_4 pH 7.

ii) Resuspended into 7.5ml 0.1M Na/PO_4 pH 7 in plastic bottle.

iii) 2.5ml avidin solution added, agitated gently for 20-26 hours at 4°C.

iv) Washed (filtration) with 5 volumes of 0.01M Na/PO_4 pH 7.

v) Resuspended into 1 volume 0.1M $NH_2C_2H_4OH$ solution. Agitated gently for 24 hours at 4°C.

vi) Washed by filtration with 20 volumes of 0.01M Na/PO_4 pH 7. Resuspended into 10ml 0.01M Na/PO_4 pH 7 and stored at 4°C.

Appendix VI Determination of the specific activity of ^{125}I-XBAL

A commonly used method to determine the specific activity of an iodinated ligand is by radioimmunoassay. Such a method has previously been used to determine the specific activity of iodinated LHRH (Marshall and Odell, 1975) and it was this method that was initially used to determine the specific activity of ^{125}I-XBAL. The antibody chosen was F86 (kindly provided by S Lynch, Birmingham Hospital for Women) shown by Clayton, Shakespear, Duncan and Marshall(1979(a)) to bind to LHRH analogues with changes in amino-acids in positions 7-10. This antibody was found to bind both unlabelled XBAL and ^{125}I-XBAL. However, construction of standard curves for both XBAL and ^{125}I-XBAL revealed that these were not parallel, that is the antibody F86 did not bind to unlabelled and iodinated XBAL in the same manner. A different method of determining its specific activity was therefore required.

Another way of estimating specific activity is from the iodination precedure data, a method commonly used by the MRC Brain Metabolism Unit Radioimmunoassay group. Iodination data for ^{125}I-XBAL iodinated and separated by HPLC as described in Appendix I used to give an estimate (to within 20%) of its specific activity.

Data (representative)

Total XBAL used	5µg (3.2nmoles)
Total ^{125}I-Na added	770,220 cp10sec
	(1,000,000 cp10sec 37MBq)
Counts left in Eppendorf	106,400 cp10sec
Therefore total applied to HPLC	663,730 cp10sec
Background counts - 97 cp10sec	

Fractions off HPLC

	cp10secs		cp10secs		cp10secs
1	65,210	13	210	25	1,211
2	53,152	14	219	26	976
3	580	15	544	27	846
4	147	16	1,741	28	371
5	160	17	3,168	29	251
6	149	18	4,823	30	222
7	144	19	4,291	31	181
8	141	20	86,640	32	125
9	269	21	171,542	33	109
10	291	22	20,445	34	98
11	196	23	4,609	35	88
12	173	24	2,044	36	95

Estimation procedure	cp10secs
Free ^{125}I-Na elute off column (fractions 1-3)	118,942
Total ^{125}I- reacted with peptide (fractions 15-27)	302,880
plus counts left in eppendorf	106,490
[Efficiency of counting 200µl aliquots compared to reaction volumes - 75% (average of 6 determinations). Therefore correcting eluted values for efficiency of counting:	
Total ^{125}I-XBAL (302,880 x 0.75) + 106,490]	510,330

Therefore specific activity of ^{125}I-XBAL is:

510,330 cp10secs/3.2nmoles

As 1,000,000 cp10secs = 37MBq (for counter used)

Specific activity of ^{125}I-XBAL = 18.9MBq/3.2nmols

or 5,896 GBq/nmol

Mean specific activity (from 4 separate iodinations):

6,489 (± 680) GBq/mmol

(γ counter used for ligand binding assays, counting efficiency 75%, therefore specific activity of ^{125}I-XBAL as detected on γ counter = 2.65 x 10^5cpm/pmol.)

Publications

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A NOVEL STIMULATORY ACTION OF SOMATOSTATIN ON PROLACTIN SECRETION

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Several actions of somatostatin on the secretion of anterior pituitary hormones have been described (Reichlin, 1983). Further to the classical inhibition of GH secretion (Brazeau et al, 1973), inhibitory effects on basal - (or more prominently, stimulus-induced) - secretion of TSH and ACTH have been reported. Early experiments observed only weak and inconsistent effects oN prolactin secretion (Vale et al, 1974), but employed long incubation periods, likely to obscure the initial response.

We have investigated the effects of somatostatin analogues on secretion of prolactin and GH, using a rapid superfusion technique which allows the measurement of transient effects.

Anterior pituitary glands from male Wistar rats were chopped into 500μm prisms and incubated for 1 hour at 37°C in oxygenated Krebs bicarbonate medium, pH 7·4 containing 0·1% BSA, 2 g/l glucose and 30 μg/l bacitracin. Tissue was then superfused with medium at 0.5 ml/min. Fractions (2 min) were collected serially and hormone concentrations were measured by radioimmunoassay.

After 90 min, a relatively steady baseline was reached and drugs were added. Somatostatin-14 produced a marked increase in prolactin secretion in experiments where GH secretion was concomitantly inhibited. The response gradually diminished towards baseline over about 1 hour, even in the continued presence of somatostatin. The effect was concentration-dependent from 20-250 nM but declined at higher levels. Somatostatin-28 mimicked this response over a similar concentration range with some 65± 10% increase at 100nM. Neither somatostatin-28_{1-12} (Benoit et al 1982) nor d-Trp^8 somatostatin-14 produced any significant increase in prolactin secretion at concentrations up to 300nM.

The response to 100nM somatostatin-28 was significantly attenuated to 16 ± 4% increase by the putative somatostatin antagonist
cyclo [7-aminohepantanoyl- Phe-DTrp-Lys-Thr(Bzl)] (Fries et al, 1982) at 60nM.

These results demonstrate a novel stimulatory effect of somatostatin on prolactin secretion, which contrasts with previously described effects on other anterior ptiuitary hormones in that they are all inhibitory. Both somatostatin-14 and somatostatin-28 but not the fragment somatostatin-28_{1-12}, are active at this site and it is susceptible to the putative somatostatin antagonist. The novel stimulatory influence exerted on lactotrophes suggests that this somatostatin recognition site may have different properties from those on other anterior pituitary cells.

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Investigation of the mechanism of somatostatin action on growth hormone secretion in rats

By R. Mitchell and S.-A. Ogier. *M.R.C. Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh, EH8 9JZ*

Studies on the mechanism of somatostatin (SS) action have yielded conflicting results (see Hayasaki-Kimura & Takahashi, 1979). We have investigated this problem using a rapid superfusion technique. Anterior pituitary glands from male Wistar rats were sliced into 500 μm prisms and after preincubation were continuously perfused with modified Krebs bicarbonate medium (Mitchell & Ogier, 1985). GH was measured by radioimmunoassay.

Both SS-14 and SS-28 caused concentration-dependent inhibition of GH secretion, in the range 0·2–300 nM, with SS-28 apparently being of much greater potency. The effect was mimicked by the analogues D-Trp^8 SS-14 and D-Trp^8, D-Cys^{14} SS-14, unlike the action of SS on prolactin secretion (Mitchell & Ogier, 1985) where these are inactive. The K^+ channel blockers TEA (10 mM) and quinine (200 μM) caused large increases in GH secretion of around 5-fold and 2-fold respectively. However, they caused no attenuation of the inhibitory effect of 10 nM SS-28 (control 62 ± 6 % of prior baseline; TEA 52 ± 3 %, quinine 41 ± 2 %, $n = 4$–6). Dibutyryl cyclic AMP (dbcAMP) (1 mM) increased GH release by some 29 ± 7 %, but after exposure to 200 nM SS-14 (which had itself slightly reduced secretion) this was completely blocked (-9 ± 6 %, $n = 6$).

It appears that K^+ channels sensitive to TEA or quinine are not of major importance in the action of SS on somatotrophes, in contrast to observations on mouse pancreatic islet β cells (Pace & Tarvin, 1981). The effect of SS on GH secretion is reported to be attenuated by pertussis toxin (Cronin, Rogol, Myers & Hewlett, 1983) suggesting an inhibitory coupling with adenylate cyclase. However, responses to dbcAMP (which should by-pass any sites of adenylate cyclase regulation) are readily inhibited by SS (see also Hayasaki-Kimura & Takahashi, 1979). Apparently, neither activation of these K^+ channels nor inhibition of adenylate cyclase can account for the effects of SS on GH secretion.

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EVIDENCE FOR SEX DIFFERENCES IN GnRH RECEPTORS AND MECHANISM OF ACTION

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INTRODUCTION

Over recent years, a number of hypotheses have been proposed for the biochemical mechanism of action of Gonadotropin-Releasing Hormone (GnRH). Although adenosine 3',5'-monophosphate (cAMP) has been implicated as a second messenger in GnRH action (Borgeat et al, 1972), this has been refuted (Conn et al., 1979) and Cronin et al (1983) have shown that changes in Luteinizing Hormone (LH) secretion are not temporally coupled to changes in cAMP levels. An apparently direct effect of cAMP derivatives on GnRH receptors (Smith et al, 1982) may have contributed to some of these results.

In contrast, there is evidence that Ca^{2+} is necessary for the post-receptor process coupling LH secretion to occupancy of GnRH receptors. While most groups have described a strict dependence of GnRH-stimulated LH release on extracellular Ca^{2+} concentration (Marian and Conn, 1979; Pickering and Fink, 1979; Naor et al, 1980; Bourne and Baldwin, 1980; Borges et al, 1983), it has been reported that this is less marked than the Ca^{2+}-dependence of depolarisation-induced release (Hopkins and Walker, 1978). Radioactive ion flux experiments have indicated that GnRH can mobilise Ca^{2+} from extracellular-, but probably also intracellular sources (Williams, 1976; Hopkins and Walker, 1978; Conn et al, 1981). A number of studies have reported marked inhibition of GnRH-stimulated LH secretion by verapinoid calcium antagonists, although in many instances this appears to be incomplete, even at very high antagonist concentrations (Hopkins and Walker, 1978; Marian and Conn, 1979; Pickering and Fink, 1979; Naor et al, 1980), again implying a component of intracellular Ca^{2+} mobilisation. An electrophysiological study on (trypsin-dispersed) gonadotrophes of the ovine pars tuberalis, (Mason and Waring, 1985) has reported membrane voltage fluctuations in response to GnRH which were suggested to reflect the activation of a Ca^{2+} channel or a Ca^{2+}-dependent channel.

Recent evidence suggests that inositol 1,4,5-trisphosphate (formed in the metabolic turnover of inositol phospholipids) can act as a second messenger signalling for Ca^{2+} mobilisation (Streb et al, 1984; Berridge and Irvine, 1984). GnRH has been reported to stimulate phospholipid metabolism in gonadotrophes, with increased turnover of phosphatidyl inositol (Snyder and Bleasdale, 1982; Naor et al, 1984). This response is likely to be multifunctional, in that diacylglycerol (an activator of protein kinase C) is also formed (Nishizuka, 1984). Both diacylglycerols and phorbol esters (which also activate protein kinase C) can stimulate LH secretion (Smith and Vale, 1980; Catt et al, 1984; Conn et al, 1985). Arachidonic acid is also liberated in the GnRH-induced turnover of inositol phospholipids and whilst the possible involvement of prostaglandins in GnRH-induced LH secretion appears to have been eliminated, a number of novel epoxygenated metabolites have been recently implicated (Snyder et al, 1983). A synergistic interaction between protein kinase C and elevated intracellular Ca^{2+} concentration as LH secretagogues has recently been suggested (Harris et al, 1985).

Despite all this evidence implicating Ca^{2+} as a second messenger in GnRH action, it is important to consider that mobilisation of Ca^{2+} is likely to be a ubiquitous requirement in secretory response processes (Moriarty, 1978). Great caution is required therefore, in attributing to this the role of the primary mechanism transducing the signal of GnRH receptor occupancy by an agonist.

Extensive electrophysiological analysis of GnRH action at a neuronal site has been carried out using the bullfrog sympathetic ganglion. Although the GnRH-like peptide found there endogenously may be teleost GnRH rather than the mammalian sequence (Eiden et al, 1982), it is clear that exogenous GnRH can mimic the synaptically-mediated response of a late slow depolarisation of the principal cells of the ganglion (Jan et al, 1980). Adams and Brown (1980) have reported that the observed depolarisation is associated with inhibition of the potassium M-current, which appears to show quite distinct electrical. kinetic and pharmacological properties (Adams et al, 1982a,b). A minority of GnRH-sensitive cells, either additionally or separately, show conductance changes that cannot be accounted for by M-current blockade (Katayama and Nishi, 1982).

Clearly therefore, there may be a number of membrane ion channels involved in GnRH action, especially potassium M-currents, which have not so far been investigated in gonadotrophes. It is not clear whether any one ionic or chemical signal will prove to be the primary co-ordinating event ensuing from GnRH receptor activation, but it now seems likely that a number of components contribute to the overall response observed.

Our approach was to attempt an assessment, by pharmacological means, of the role of membrane K^+- and Ca^{2+}-channels in the secretory response of gonadotrophes to GnRH. It is feasible that the receptors could interact relatively directly with particular ion channels within some membrane molecular complex, so we also sought any allosteric effects of appropriate agents on ligand binding to receptor sites. In relation to our other studies on the GnRH priming response, we were interested to compare the mechanism of the basic LH secretory response in male and female gonadotrophes with the premise that subtle but distinct differences may be concerned with conferring the property of "primability".

TECHNIQUES

Rapid superfusion of anterior pituitary tissue in vitro

Adult Wistar rats were used for all studies; either males, or females pooled in equal proportions from metoestrus and dioestrus days of the 4 day cycles.

Animals were stunned, decapitated, and the pituitaries were removed. The neurointermediate lobe was discarded and the anterior lobe was mechanically chopped at 500μm intervals in two directions at 90°. Tissue was then incubated in Krebs Bicarbonate medium (composition in mM: NaCl, 127; KCl, 3.83; $CaCl_2$, 1.8; KH_2PO_4, 1.18; $MgCl_2$, 1.18; $NaHCO_3$, 20; with $2gl^{-1}$ glucose, 30mg l^{-1} bacitracin and 0.1% BSA, pH 7.4) under 95%O_2/5% CO_2 at 37°C. After one hour of static incubation, tissue equivalent to one pituitary gland transferred into a superfusion chamber, where it was supported on a Millipore AP20 prefilter. Tissue was then superfused with oxygenated medium at 0.5ml/min, at 37°C, and 2 minute fractions were collected. After 60 min of superfusion, inlet lines were changed to medium containing drugs or ions as appropriate. Subsequent challenges with GnRH were carried out after some 45 min in the presence of the relevant substances. Luteinizing Hormone was estimated by double antibody radioimmunoassay.

Binding of [^{125}I] buserelin to GnRH receptors

[D-Ser(tBu)^6] des Gly^{10}-GnRH ethylamide (buserelin) was iodinated using the Chloramine-T procedure and the product separated from reactants according to Sandow and Konig (1979) with modifications.

Anterior pituitary glands were homogenised in 100 volumes of cold Tris HCl buffer (25mM), pH 7.6. Membrane fragments were washed once by centrifugation (48,000g, 10 min) and resuspension in assay buffer, (which additionally contained 0.1% BSA). Assays were carried out in triplicate or duplicate in a total volume of 500μl, comprising: ~0.5-1mg tissue equivalent, [^{125}I] buserelin to a final concentration of ~25pM (~30,000-50,000 cpm), and drugs or ions as appropriate, with 300nM GnRH for determination of non-specific binding. After 90min at 4°C, bovine γ-globulin (to 0.025%) and polyethylene glycol 8000 (to 15%) were added, tubes vortexed and left for 15 min at 4°C before centrifugation and aspiration of the supernatant.

RESULTS AND DISCUSSION

GnRH-stimulated LH secretion

The superfusion protocol allowed a consistently steady baseline of LH secretion to be reached, with basal levels of 2-4ng/ml from male and female tissue. GnRH (1-1000μM) caused a prompt stimulation of LH secretion, in a concentration-dependent manner, from both male and female tissue. Near-maximal responses of around 80-100% increase were obtained at a concentration of 1000nM and a standard stimulus of 300nM was used in all experiments here (Table 1).

Membrane Ca^{2+} channels and GnRH action

Verapinoid Ca^{2+} channel antagonists, but not 1,4 dihydropyridines or diltiazem, have been previously reported to inhibit GnRH-evoked LH secretion in female weanling rats (Conn et al., 1983). Ro5-4864 (4'-chlorodiazepam) belongs to a new class of putative Ca^{2+} channel antagonist (Mestre et al., 1985). Surprisingly, both (±) verapamil and

Table 1. GnRH-stimulated LH secretion.

Time after addition of 300nM GnRH (min)	LH secretion [a] % of mean control baseline)	
	male	female
-4	99 ± 2	100 ± 1
-2	101 ± 2	98 ± 1
0	100 ± 2	99 ± 1
2	162 ± 8	170 ± 8
4	161 ± 9	174 ± 8
6	162 ± 8	178 ± 9
8	156 ± 8	177 ± 15
10	158 ± 8	180 ± 12
12	148 ± 6	188 ± 15
14	151 ± 6	187 ± 16

[a] Mean ± S.E.M., n = 5 - 10 in all experiments.

Table 2. Effects of Ca^{2+} channel antagonists on GnRH-stimulated secretion

drug (concentration)	% of control [a] response to 300nM GnRH	
	male	female
GnRH + (±) verapamil (100µM)	14 ± 7* **	51 ± 7*
GnRH + Ro5-4864 (5µM)	41 ± 10* **	112 ± 15

[a] values for 10 min after GnRH addition
* Significant inhibition of response
** significant difference between extent of inhibition in male/female.
($P < 0.05$, Mann-Whitney U test).

Ro5-4864 were significantly more effective at blocking the GnRH response in male rather than female gonadotrophes (Table 2), suggesting that there may be a greater involvement of membrane Ca^{2+} channels in the male response. Barium ions are reported to enhance the unitary conductance through membrane Ca^{2+} channels* (Hagiwara and Byerly, 1981) and so might be expected to facilitate any response involving them. A profound secretagogue action of Ba^{2+} is widely reported (Douglas et al., 1983) which presumably also involves such a mechanism. Male gonadotrophes are anomalous in that they fail to show any secretagogue response to Ba^{2+} (Mitchell and Anderson, 1985), although such a response is seen with female weanling gonadotrophes (Conn et al., 1980). In the present experiments, Ba^{2+} released LH from adult female but not male tissue (Table 3); a result apparently at variance with the Ca^{2+} channel antagonist data.

Membrane K^+ channels and GnRH action

One explanation of these apparently disparate results might be that an action of Ba^{2+}, not directly related to Ca^{2+} channels, is involved. One such action is its powerful and selective blockade of potassium M-currents (Adams et al., 1982b), equivalent to the proposed mechanism of GnRH action in bullfrog sympathetic ganglion. Another selective blocker of M-currents, uridine 5'-triphosphate (Adams et al., 1982b) was also able to produce a small increase in LH secretion from female but not male tissue. It may be therefore, that closure of potassium M-currents (as mimicked by Ba^{2+}) could account for a part of the secretory response to GnRH in female but not male gonadotrophes. This would be in accordance with a significant proportion of the female gonadotrophe response being resistant to Ca^{2+} channel antagonists.

Table 3. Effects of some K^+ channel antagonists on basal LH secretion

drug concentration	LH secretion (peak % of mean control baseline)	
	male	female
Ba^{2+} (3mM)	106 ± 4	164 ± 7* **
4-AP (1mM)	441 ± 25*	170 ± 7* **

* significant increase
** significant difference between extent of increase in male/female.
($P < 0.05$, Mann-Whitney U test)

* A recent report (Nilius et al., 1985) describes a novel class of Ba^{2+}-insensitive Ca^{2+} channel.

When GnRH stimuli were applied to gonadotrophes that had been previously exposed to Ba^{2+} or UTP, again a marked difference was seen between responses of male and female tissue. The amplitude of stimulus induced release was profoundly enhanced in female tissue but patently unaffected in male (Table 4). If Ba^{2+}, UTP and GnRH are all acting on female but not male gonadotrophes to inhibit potassium M-currents, then it implies that the huge potentiation seen here represents a synergistic interaction of the modes by which GnRH and the other agents influence M-currents. Alternatively, it is possible that M-currents serve only as an inhibitory modulator rather than mediator of GnRH action in female gonadotrophes. Detailed analysis of the interaction will be required to resolve this.

Other K^+ currents, such as the transient outward current (I_A), the delayed rectified (I_K) and Ca^{2+}-activated currents (including the classical I_C), (Thompson, 1977) could potentially be involved. Preliminary experiments however, indicated no major effects of tetraethylammonium or quinine on either basal or GnRH-stimulated LH secretion, suggesting that currents of the latter two categories may not be significantly involved. Nevertheless, the selective inhibitor of A-currents, 4-aminopyridine (4-AP) (Thompson, 1977), caused both prominent changes in baseline LH secretion and a modification of the GnRH response. At a concentration of 1mM, 4-AP caused a vast, but transient increase in LH secretion from male gonadotrophes, with a much more minor effect on female tissue (Table 3). This perhaps suggests that more K^+ A-current channels are normally present in an activated state in the male gonadotrophe. After the baseline had recovered (within 30 min), the response to GnRH of male but not female gonadotrophes was significantly attenuated in the initial rapidly

Table 4. Effects of some K^+ channel antagonists on GnRH-stimulated LH secretion

drug (concentration)	% of control response to 300nM GnRH	
	male	female
GnRH + Ba^{2+}(3mM)	[a] 89 ± 9	345 ± 51*
GnRH + UTP(100μM)	[a] 82 ± 9	226 ± 25*
GnRH + 4-AP (1mM)	[b] 44 ± 4*	126 ± 15

[a] Values for 10 min. after GnRH addition

[b] Values for 2 min after GnRH addition

* significantly different from control response ($P < 0.05$, Mann-Whitney U test.

peaking phase of the response (Table 4). This is consistent with closure of an ongoing A-current representing a component of the initial response to GnRH of male but not female gonadotrophes. Although experiments on bullfrog sympathetic ganglion failed to implicate A-currents in GnRH action (Adams et al., 1982a), the A-current there is unusual in being insensitive to 4-AP and other (as yet unidentified) currents have been considered to contribute to the response (Jan et al., 1980).

Effects of ions on GnRH receptors

Degradation-resistant analogues of GnRH bind almost entirely to a single high affinity site in anterior pituitary membranes (Clayton et al., 1979). A number of cations can substantially influence the binding of [^{125}I]-labelled ligands (Marian and Conn, 1980; Hazum, 1981). In none of these experiments however, was a comparison made of membranes from male and female anterior pituitaries. We addressed this problem and found that while some cations (Na^+, Ca^{2+}) had identical effects on [^{125}I]buserelin binding, there appeared to be a sex-related difference in the effect of K^+ ions (Table 5). Under these conditions, any allosteric influences from other membrane components closely associated with GnRH receptors may be detectable. The selective inhibitory effect of K^+ on binding to the male GnRH receptor parallels the selective inhibition by a potassium A-current blocker of part of the male secretory response to GnRH. It may be therefore, that in the male, the effect on [^{125}I] buserelin binding represents an allosteric influence of an element of the ion channel mediating A-currents on GnRH receptors, thereby reflecting the close functional interaction of these sites.

Table 5. Effects of some cations on [^{125}I] buserelin binding to pituitary GnRH receptors.

ion (concentration)	% of control specific binding	
	male	female
Ca^{2+} (3mM)	[a] 72 ± 6*	74 ± 5*
Na^+ (50mM)	94 ± 3	94 ± 4
K^+ (50mM)	56 ± 6*	85 ± 5

* significant inhibition of specific binding ($P < 0.05$, Mann Whitney U-test)

(3mM Ca^{2+} also inhibited non-specific binding to a similar extent)

Summary

Whilst it is clear that Ca^{2+} is involved in GnRH action, it is now apparent that membrane K^+ channels are also of major importance. There is evidence for a role of potassium A- and M- currents both in contributing to the basal membrane state of gonadotrophes and in either mediating (or modulating) GnRH responses. At each of these levels, there appears to be a quantitative or qualitative difference between male and female gonadotrophes. While Ca^{2+} channel opening and potassium A-current blockade appear to account for a large part of the male response to GnRH, potassium M-current blockade and to a lesser extent Ca^{2+} channel opening appear important in the female. Experiments on properties of the receptors as labelled by [^{125}I] buserelin suggested an allosteric interaction between potassium A-channels and GnRH receptors in the male but not female, and no evidence of such close molecular interactions with Ca^{2+} channels. We believe this sex difference in the ionic/biochemical mechanism of action of what is empirically a single receptor, to be unprecedented.

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LHRH-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro* can be reduced by activation of protein kinase C

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The action of luteinizing hormone releasing hormone (LHRH) on LH secretion is partly dependent on the presence of extracellular Ca^{2+} and its influx into gonadotrophs through verapamil-sensitive channels (Pickering & Fink, 1979; Hopkins & Walker, 1978). LHRH effects are likely to be mediated by stimulation of phosphoinositide hydrolysis (Schrey, 1985) which may lead to activation of protein kinase C (PKC). We have investigated the role of PKC in the rapid stimulation of $^{45}Ca^{2+}$ influx into anterior pituitary gland.

The animals used were adult Wistar rats of approximately 200 g body wt., maintained under controlled lighting (lights on 05.00–19.00 h) and temperature (22 °C) and given free access to Diet 41 B and tap water. Uptake of $^{45}Ca^{2+}$ into 500 μm prisms of anterior pituitary glands removed at 13.00 h was measured over 30 s at 37 °C in an oxygenated buffer containing NaCl 154 mM, KCl 5·4 mM, $CaCl_2$ 1·5 mM, glucose 11·0 mM, Hepes 6·0 mM, pH 7·4. Incubations were quenched with ice-cold buffer which contaned 2 mM EGTA instead of $CaCl_2$, filtered through cellulose membrane and extensively washed with cold buffer.

In prisms from male rats and female rats in each day of the oestrous cycle, LHRH (< 1–100 nM) elicited a concentration-dependent increase in $^{45}Ca^{2+}$ uptake. The LHRH-induced $^{45}Ca^{2+}$ influx was greatly reduced at higher concentrations of LHRH (> 100 nM) in dioestrous, pro-oestrous and male but not oestrous or metoestrous rats. The inhibitory effects on $^{45}Ca^{2+}$ influx of high LHRH concentrations were mimicked by phorbol 12-myristate 13-acetate (100 nM) and 1-oleoyl 2-acetyl-rac-glycerol (125 μM) and were reversed by polymyxin B (10 μM), indicating the involvement of PKC. The PKC-mediated restriction of LHRH effects on membrane Ca^{2+} channels, which occurred at high agonist concentrations, is analogous to observations on the control of cytosolic free calcium by the TRH receptor which is also linked to phospho-inositide hydrolysis (Drummond, 1985).

These results show that LHRH can produce a biphasic influence on Ca^{2+} influx and that this influence is likely to be hormone-dependent.

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Protein kinase C has inverse effects on LHRH- and depolarization-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*

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Luteinizing hormone releasing hormone (LHRH) may act by hydrolysis of phospho-inositides. Subsequent Ca^{2+} mobilization from both intracellular and extracellular pools appears to be required for luteinizing hormone (LH) secretion. LHRH rapidly elicits an influx of Ca^{2+} into anterior pituitary tissue (Fink, Johnson, Minaur, Mitchell & Ogier, 1986), but the relationship between this and depolarization-induced influx is unclear.

The measurement of $^{45}Ca^{2+}$ influx has been described previously (Fink *et al.* 1986). Briefly, 500 μm prisms of male tissue were incubated at 37 °C in oxygenated physiological buffer containing $^{45}Ca^{2+}$ (2 μM), usually for 30 s. After quenching with ice-cold EGTA-buffer the tissue was filtered and extensively washed. The influx of $^{45}Ca^{2+}$ induced by LHRH (100 nM) was inhibited by phorbol 12-myristate 13-acetate (PMA) (1–1000 nM), or by 1-oleoyl 2-acetyl-rac-glycerol (63μM) but not 4β-phorbol (10 μM), indicating that protein kinase C (PKC) may inhibit the LHRH-induced influx of $^{45}Ca^{2+}$. In contrast, the influx of $^{45}Ca^{2+}$ by K^+ depolarization (60 mM-K^+) was greatly facilitated by these activators of PKC.

Inhibition of receptor responses by PKC has also been reported for other phospho-inositide linked receptors such as α_1 adrenoreceptors (Lynch, Charest, Bocckino, Exton & Blackmore, 1985). Reduction in receptor numbers accounts for many of these observations, but that is not the case here since we have shown using [^{125}I]buserelin binding that LHRH receptor numbers are not affected by exposure to PMA. The facilitated voltage-activation of Ca^{2+} channels matches observations in smooth muscle (Menkes, Baraban & Snyder, 1986), adrenal medulla (Wakade, Malhotra & Wakade, 1986) and *Aplysia* neurones (De Riemer, Strong, Albert, Greengard & Kaczmarek (1985), but contrasts with one report using GH_3 cells (Drummond, 1985). Our results indicate two sites of action of PKC with respect to membrane Ca^{2+} channels: one related to voltage-sensitive Ca^{2+} channels and another within the LHRH-activated transduction mechanism.

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High yield solubilization of undenatured luteinizing hormone-releasing hormone receptors from rat anterior pituitary

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The solubilization of receptors in high yield is an important initial step in their purification. In the case of the luteinizing hormone-releasing hormone (LHRH) receptors, solubilization has previously given low yields, such as 8% using 5 mM-3-[3(cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (Chaps) (Perrin *et al.*, 1983). Higher yields have been reported (Winiger *et al.*, 1983) but in that case the criteria for authentic solubilization are questionable.

For the rat brain serotonin S_2 receptor, significant increases in solubilization yield were achieved by the addition of high concentrations of NaCl to the detergent (Chaps) solution (Wouters *et al.*, 1985). Increasing Chaps concentration above 5 mM, does not increase yields, and in some cases seems to diminish them (Perrin *et al.*, 1983: Winiger *et al.*, 1983; Wouters *et al.*, 1985).

Studies on photoaffinity-labelled LHRH receptors using SDS/polyacrylamide-gel electrophoresis have indicated an apparent molecular weight of about 60 000 Da (Hazum, 1981). However, under these conditions receptors are denatured. Radiation inactivation of the LHRH receptor *in situ* indicates an apparent molecular mass of 136 000 Da (Conn & Venter, 1985), suggesting the possibility of a complex of subunits. Efficient solubilization of the LHRH receptor using non-denaturing detergent conditions would allow determination by gel filtration of the apparent molecular mass of the isolated, undenatured receptor complex.

Anterior pituitaries from male Wistar rats were sonicated in 100 vol. of 25 mM-Tris/HCl pH 7.4, centrifuged for 15 min at 60 000 ***g*** at 4°C and resuspended in either 20 vol. of 25 mM Tris/HCl pH 7.4/0.1% bovine serum albumin for binding assay or 20 vol. of the solubilization solution (25 mM-Tris/HCl pH 7.4, 5 mM-Chaps, 1.5 M-NaCl, 50 μg of soyabean trypsin inhibitor/ml, 400 k.i.u. of trasylol/ml). For solubilization, samples were agitated for 90 min at 4°C then centrifuged for 2 h at 60 000 ***g***, 4°C to separate solubilized from membrane-retained material. The supernatant was used in binding assay or applied to a calibrated Sepharose 6B gel column. Before ligand-binding assay, solubilized proteins were precipitated from the detergent solution using polyethylene glycol 8000 (PEG). PEG and bovine γ-globulin were added to 15% and 0.035% respectively followed by incubation on ice for 20 min and centrifugation to collect the precipitate.

Binding was carried out in 500 μl containing 10–60 μg of protein and approx. 25 pM-[^{125}I]buserelin, iodinated by the chloramine-T method (Mitchell *et al.*, 1985). Non-specific binding was determined in the presence of 100 nM-LHRH. Incubation, 90 min at 4°C, was followed by PEG precipitation as above to separate unbound ligand and the remaining pellet was counted.

The absence of Chaps and NaCl from the solubilization solution resulted in the retention of all specific [^{125}I]buserelin binding in the pellet. Addition of 5 mM-Chaps caused the solubilization of 14 ± 1% of the specific binding, rising to 16 ± 4% and 23 ± 3% of 0.2 M- and 0.6 M-NaCl respectively. 1.5 M-NaCl/5 mM-Chaps gave 73 ± 10% of the specific [^{125}I]buserelin binding in the supernatant. This was not precipitated even on centrifugation for 60 min at 263 000 ***g*** at 4°C, and was therefore considered to be truly solubilized.

In our hands, the solubilization procedure of Winiger *et al.* (1983) gave very low yields (22 ± 3%) of authentically solubilized receptor. Low-speed centrifugation of membrane fragments through 0.32 M-sucrose/30% glucerol, as employed by Winiger *et al.* (1983), produced a large artefact of 20 ± 2% of binding apparently being retained in the supernatant, presumably due to microdispersed fragments of tissue.

The affinities of several LHRH analogues were determined both on anterior pituitary membranes and the solubilized receptor preparation. Affinity profiles were very similar in both preparations although small relative changes were observed.

The solubilized receptor showed decreased affinity for the agonists, buserelin and LHRH, and increased affinity for the antagonist, [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]LHRH, with little change for the partial agonist (des-pGlu^1]LHRH. Removal of the receptor from its associated effector mechanism in membranes may account for these changes.

Specific [^{125}I]buserelin binding indicated the presence of the solubilized receptors in non-void fractions eluted from the Sepharose 6B column using the same buffer as for solubilization. The peak binding fraction corresponded to an apparent molecular mass of 155–170 kDa. This is similar to that found by radiation inactivation of receptors *in situ* but somewhat more than twice that found by SDS/polyacrylamide-gel electrophoresis. These observations, together with evidence for two contiguous binding sites being involved in LHRH receptor activation (Conn *et al.*, 1982), suggest that the LHRH receptor may exist *in situ* as a dimer.

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Abbreviations used: LHRH, luteinizing hormone-releasing hormone; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid; PEG, polyethylene glycol 8000.

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Dual effects of protein kinase C on gonadotropin secretion from rat anterior pituitary gland

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Luteinizing hormone-releasing hormone (LHRH) acts on pituitary gonadotrophs via its receptor to cause the release of luteinizing hormone (LH) and follicle stimulatory hormone (FSH). There is evidence that LHRH receptor activation causes phosphoinositide hydrolysis giving rise to inositol phosphates (Schrey, 1985) and presumably also diacylglycerol (DAG). These breakdown products can act as 'second messengers' in the cell, the inositol phosphates involved in mobilizing intracellular Ca^{2+} stores (Berridge & Irvine, 1984) while DAG activates the Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (Nishizuka, 1984) leading to protein phosphorylation.

Phorbol 12-myristate 13-acetate (PMA) also activates PKC, and, while not altering cytosolic free Ca^{2+} levels in pituitary cells (Drummond, 1985), does give rise to secretion of LH (Smith & Vale, 1980). We have investigated the potential regulatory control of PKC in gonadotropin secretion using activators and inhibitors of this enzyme.

Pituitary glands from pro-oestrous COB/Wistar rats removed and the anterior lobes were separated and bisected. The hemipituitary glands (two/flask) were incubated in 2 ml of Hepes-buffered minimal essential medium with Earles salts, which had 1 mM-L-glutamine added, at 37°C in a shaking water bath under 95% O_2/5% CO_2. After a pre-incubation for 20 min the medium was changed. The replacement medium had no additives or contained polymyxin B (PMB), a selective inhibitor of PKC (Wooten & Wrenn, 1984), or 8-(*N*,*N*-diethyl amino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) which has also been shown to inhibit phorbol ester-evoked secretion (Simpson *et al.*, 1984). After 5 min a sample of 200 μl of medium was taken and drugs were added in the 200 μl replacement medium to give the appropriate final dilution. The flasks were then incubated for an hour before repeating the procedure. Usually an hour of baseline release was followed by successive hours with drugs. All samples were frozen (−40° C) until assayed for LH or FSH by radioimmunoassay.

The PKC activators PMA and phospholipase C (PLC) both effected a dose-dependent release of LH and FSH. The response to PMA was delayed though, and secretion was not significant until the pituitary glands had been in the presence of PMA for at least 2 h (PMA 100 nM, 1 μM, LH $P < 0.01$, FSH $P < 0.01$). This was not the case for PLC which, in addition, produced much greater secretion of both LH and FSH (Table 1). The larger response to PLC may reflect the presence of not only DAG, but also the Ca^{2+}-mobilizing inositol phosphates. The PKC inhibitor PMB whilst not affecting baseline release of gonadotropins, augmented the LHRH-induced release of LH and FSH (Table 1). This was also a concentration-dependent effect which was maximal at 10–100 μM. TMB-8 caused a similar increase in LHRH-induced gonadotropin release (Table 1) (maximal 20 μM) but at higher concentrations (> 20 μM) it increased basal release of LH but not FSH. At 100 μM both the release of LH and FSH decreased towards control levels although LH levels were still significantly raised ($P < 0.05$). At concentrations of 50 μM, TMB-8 has been shown to inhibit respiration in rat thymocytes (Brand & Felber, 1984) and at higher concentrations still (> 500 μM), it may block intracellular Ca^{2+} mobilization (Simpson *et al.*, 1984). These observations may explain the reversal of the augmented response at 100 μM-TMB-8.

Table 1. *Concentrations of LH and FSH released from the pituitary gland in vitro with different inhibitors and activators of protein kinase C*

Results are means ± S.E.M. of $n > 5$. All values (except PMA first hour), are significantly ($P < 0.1$) elevated from basal and/or LHRH (0.85 nM) levels (Students t test).

Drug	LH (ng/ml)	FSH (ng/ml)
Basal	22.7 ± 3.8	4.48 ± 0.15
LHRH	82.0 ± 10.8	11.03 ± 0.67
LHRH + PMB (10 μM)	220.4 ± 13.6	18.95 ± 1.34
Basal	11.1 ± 0.96	6.61 ± 0.18
LHRH	142.3 ± 16.2	20.65 ± 0.61
LHRH + TMB-8 (10 μM)	247.1 ± 19.8	27.33 ± 1.74
Basal	10.1 ± 1.4	5.84 ± 0.72
PLC (0.25 unit/ml)	262.4 ± 39.4	26.12 ± 1.89
PMA (1 μM) First hour	10.1 ± 0.5	7.66 ± 0.83
Second hour	30.7 ± 5.0	15.25 ± 1.25

These results indicate that PKC activators can cause gonadotropin release, although PLC, which can increase levels of inositol phosphates and hence raise intracellular Ca^{2+} levels (Drummond, 1984), is more effective than PMA. This is in accordance with the synergistic affects of PMA and Ca^{2+} ionophores on LH release from gonadotrophs (Naor & Eli, 1985). However, the antagonists of PKC action, PMB and TMB-8, can greatly increase LHRH-induced release of LH and FSH. This suggests that PKC has a dual role in the control of gonadotropin secretion: PKC may stimulate gonadotropin secretion by an unknown-mechanism and yet also down-regulate secretion evoked by LHRH receptor activation.

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Abbreviations used: LHRH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; FSH, follicle stimulatory hormone; DAG diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMB, polymyxin B; PLC, phospholipase C. TMB-8, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate.

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Luteinizing hormone-releasing hormone rapidly elicits an opening of membrane Ca^{2+} channels which is suppressed by protein kinase C

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Several groups have demonstrated that the stimulation by luteinizing hormone-releasing hormone (LHRH) of luteinizing hormone (LH) secretion from the anterior pituitary is dependent on extracellular Ca^{2+} (Marian & Conn, 1979; Pickering & Fink, 1979; Naor *et al.*, 1980). Hopkins & Walker (1978); Marian & Conn (1979) and Naor *et al.* (1980) showed that La^{3+} inhibited LHRH-induced (but not basal) LH secretion and organic Ca^{2+}-channel blockers of the verapinoid series are also effective (Marian & Conn, 1979; Naor *et al.*, 1980). Therefore Ca^{2+} has been extensively proposed as the second messenger mediating LHRH action (Marian & Conn, 1979).

Nevertheless, LHRH-induced LH release appears rather less sensitive to removal of extracellular Ca^{2+} than depolarization-induced release (Hopkins & Walker, 1978) and the verapinoid inhibitors of release described were generally only partial (Marian & Conn, 1979; Naor *et al.*, 1980). These findings suggest that LHRH can mobilize intracellular as well as extracellular Ca^{2+}. This is consistent with evidence that LHRH can evoke phosphoinsitide hydrolysis (Schrey, 1985), thus generating both inositol phosphates, that may mobilize intracellular Ca^{2+}, and diacylglycerol, that may activate protein kinase C (PKC).

It has been suggested that the mobilization by phosphoinositide linked receptors of intra- and extra-cellular Ca^{2+} is intimately linked (Putney, 1986). We therefore aimed to assess whether elements of the phosphoinositide response, particularly activation of PKC, participate in causing the LHRH-induced influx of $^{45}Ca^{2+}$, described briefly by Hopkins & Walker (1978).

COB Wistar rats were maintained under controlled lighting (light 05:00–19:00h) and temperature (22°C) and given free access to Diet 41B and tap water. Oestrous cycles were assessed by daily vaginal smears. Anterior pituitaries were chopped into 500 μm prisms and suspended in a pre-warmed and pre-oxygenated Ca^{2+}-uptake buffer with 0.1% bovine serum albumin and 35% Percoll. The buffer contained (concentrations in mM): NaCl 154, KCl 5.4, $CaCl_2$ 1.5, glucose 11.0 and Hepes 6.0 [buffered to pH 7.4 with solid Tris (hydroxymethyl)-aminomethane base]. Aliquots of tissue suspension were added to equal volumes of buffer, then preincubated at 37°C and gassed with 100% O_2 for approx. 20 min before initiation of the experiment. Pre-warmed buffer containing $^{45}Ca^{2+}$ to give a final concentration of 2 μM was added and then after precise time intervals, $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of an ice-cold wash. This was uptake buffer containing 2 mM-EGTA instead of Ca^{2+}, similar to those used by other workers to halt $^{45}Ca^{2+}$ uptake in smooth muscle. After quenching, the contents of each tube were filtered and the tissue received another 3 ml of the relevant wash immediately, as an acute wash. The filtering was carried out on a Millipore filter block under vacuum, using Millipore cellulose acetate filters supported on GF/B. Three further 2 min washes followed. The $^{45}Ca^{2+}$ uptake was measured by liquid scintillation counting.

The amount of $^{45}Ca^{2+}$ accumulated by unstimulated tissue increased linearly in a time-dependent fashion, but the excess uptake due to 100 nM-LHRH or 60 mM-K^+ was maximal within 30 s (the incubation time used for further studies. LHRH increased $^{45}Ca^{2+}$ influx in a concentration-dependent fashion from 0.1 to 100 nM. Maximal responses to 100 nM-LHRH (as percentage increase over controls) varied with tissue from different stages of the oestrous cycle: pro-oestrus, 99 ± 14; oestrus, 124 ± 15; met-oestrus, 74 ± 10; di-oestrus, 35 ± 7; male, 40 ± 9 (n = 6–8, means ± S.E.M.). Control unstimulated uptake was no different in tissue from these various sources.

The activators of PKC, phorbol 12-myristate 13-acetate (100 nM) and 1-oleoyl 2-acetyl rac-glycerol (63 μM) (incubated with tissue for 10 min before uptake) drastically inhibited the LHRH response with no effect on basal $^{45}Ca^{2+}$ uptake. In oestrus tissue the response to 100 nM-LHRH in the presence of these activators was reduced to 30 ± 10% and −4 ± 7% respectively (n = 5, means ± S.E.M.). Similar effects were observed in male tissue where concentration-dependent inhibition of the response to 100 nM-LHRH by phorbol 12-myristate 13-acetate was observed. This was maximal by 100 nM and was not mimicked by 10 μM-4β-phorbol.

LHRH receptor properties in tissue subjected to these PKC activators were investigated using equilibrium binding of [^{125}I]buserelin (Mitchell *et al.*, 1985). Neither affinity nor number of sites were altered (male tissue), suggesting that the locus of PKC action must be at some post-receptor site. PKC activation clearly therefore does not act to mediate the opening of membrane Ca^{2+} channels by LHRH but rather acts as a regulatory inhibitor of this effect. Whether other consequences of the phosphoinositide response, such as the production of inositol phosphates, are responsible for Ca^{2+} channel opening, is not clear.

Abbreviations used: LHRH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; PKC, protein kinase C.

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Sandwich hybridization using immobilized DNA

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The most common method currently used for the detection of specific DNA sequences is the Southern blot (Southern, 1975). This technique is reliable and sensitive, but has the disadvantages that it is not rapid and cannot be readily automated. Clearly, an alternative eliminating these problems should bring the diagnosis of genetic defects into the realm of routine clinical medicine. We have developed such an alternative, using DNA fragments chemically coupled to resins and a sandwich hybridization assay. The major advantage of this type of assay is that it does not require the time-consuming immobilization of sample DNA. Our technique uses two non-overlapping restriction fragments: fragment A is attached covalently to a resin by a diazotization reaction (Seed, 1982), and fragment B is radiolabelled to 10^8–10^9 c.p.m./μg of DNA by random priming with hexadeoxynucleotides (Feinberg & Vogelstein, 1983, 1984). A and B will not cross hybridize, but since both hybridize with the nucleic acid sequence we are searching for (the 'target'), the resin becomes labelled. Digestion of target with the enzyme *Dde*I, followed by hybdridization with A and B and washing, allows us to discriminate between the 'normal' and 'sickle' genotypes (Langdale & Malcolm, 1985).

The sandwich hybridization assay has been used previously to determine the presence or absence of specific DNA sequences in non-homologous mixtures (Ranki *et al.*, 1983). The technique is suitable for probing variations within a DNA sequence. The restriction fragment length polymorphism associated with the single base substitution causing sickle-cell anaemia was chosen as a model system. Restriction fragments of the human β-globin gene taken from either side of, but not overlapping with, the mutation site were used as A and B (Langdale & Malcolm, 1985).

In order to refine our method, we have to (i) identify the most suitable fragments to use as A and B, (ii) find the most suitable resin, and (iii) determine the sensitivity of the assay with plasmid DNA and with human samples.

Suitable available fragments were of lengths 879, 341 and 201 bp. It was found that fragment size is not an important factor in diazotization reactions, and it does not affect hybridization efficiency. The 341 bp fragment was chosen as A and the 201 bp fragment as B (Langdale & Malcolm, 1985).

The diazotization reaction relies on the presence of free hydroxyl groups on the surface of the supporting resin. The hydroxyl groups first react with a di-epoxide linker (2,4-butanediol diglycidyl ether), which subsequently reacts itself with an aromatic amine (2-aminothiophenol). The amine is diazotized with nitrous acid and the DNA coupled via the bases. Of several suitable resins tested, including Sephacryls S-500 and S-1000, Sepharose CL4B, Sephadex G-50 and magnetic Dynospheres M450, Sephacryl S-1000 was found to be the most suitable, binding the most DNA covalently. Approx. 75% of the coupled DNA was found to be available for hybridization.

Our results clearly indicate that it is possible to reproducibly detect as little as 20 amol of β-globin DNA in a complex mixture of non-homologous sequences (approximately equivalent to the amount of single copy gene present in 40 μg of human DNA). Normal, heterozygous and sickle homozygous individuals can be readily distinguished, and the technique is almost as sensitive as Southern blotting. However, there is a significant improvement in the ease of sample manipulation and time taken to achieve the result (under 24 h).

The advantage of this technique should ensure that the clinical processing of large numbers of samples will not prove to be a problem. It is commonly accepted that DNA probe technology will be based on the use of non-radioactive detection methods. Our technique is adaptable for use with such probes.

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Abbreviation used: bp, base pairs.

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Pyrrolidine-2-phosphonic acid: a new inhibitor of kidney prolidase

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Prolidase (iminodipeptidase, EC 3.4.13.9) hydrolyses dipeptides with a *C*-terminal proline or hydroxyproline residue, characterized by a –CO–N< bond. Glycyl-L-proline and L-alanyl-L-proline were shown to be the two main substrates of the enzyme (Sjöström, 1974; Hui & Lajtha, 1978). Prolidase is a dimeric Mn^{2+}-dependent enzyme with a 53 kDa subunit. This widespread cytoplasmic enzyme plays a role in collagen degradation (Bergmann & Fruton, 1937); biological considerations and the properties of prolidase were recently reviewed by Myara *et al.* (1984). The enzyme is inhibited by proline, by divalent metal ions which compete with Mn^{2+} and by sulphydryl reagents (Davis & Smith, 1957).

A porcine kidney prolidase with a specific activity of 180 units/mg of protein (Sigma Chemical Co, St. Louis, U.S.A.) was used in all experiments. Enzymic assays were performed in 100 mM-Tris/HCl buffer (pH 8) containing 5 mM-$MnCl_2$, 2–50 mM-Gly-Pro, 0–20 mM inhibitor and the enzyme dilution in a final volume of 0.25 ml. To obtain its full activity, the enzyme was preincubated in the buffer in the presence of Mn^{2+} for 10 min at 37°C, before the addition of the potential inhibitor; the substrate was added finally to initiate the reaction. After an incubation of 8 min at 37°C, 2 M-HCl (12.5 μl) was added to stop the reaction. The

Abbreviation used: Pro-P, pyrrolidine-2-phosphonic acid.

Solubilization of a large molecular weight form of the rat LHRH receptor

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ABSTRACT

The LHRH receptor has been solubilized from male rat anterior pituitary glands, using the zwitterionic detergent 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate in the presence of a high concentration of sodium chloride. This method gave high yields (up to >70%) of the LHRH-binding site from the membrane preparation. Ligand binding studies using LHRH analogues were carried out to determine dissociation constants for LHRH receptors both *in situ* in the membrane preparation and for solubilized LHRH receptors. For all the analogues the binding characteristics were similar in both preparations, suggesting that the solubilization procedure left the LHRH receptor undenatured. Gel filtration revealed an apparent molecular weight for the LHRH receptor of 100 000–160 000, with the mean value being approximately twice that found by others using sodium dodecyl sulphate–polyacrylamide gel electrophoretic techniques. The results indicate that the LHRH receptor probably exists in gonadotroph membranes as a large complex of more than one subunit.

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INTRODUCTION

The release of gonadotrophins from the pituitary is mediated by the hypothalamic peptide luteinizing hormone-releasing hormone (LHRH). This acts through a cell-surface receptor on gonadotrophs to cause the release of luteinizing hormone and follicle-stimulating hormone, presumably by means of the increased phosphoinositide turnover which occurs in response to LHRH (Conn, Staley, Harris *et al.* 1986). Purification and molecular characterization of the LHRH receptor molecule would greatly facilitate the understanding of its structure and those structural elements involved in its mode of action.

An important initial step in the purification of any receptor type is the effective solubilization of the receptor from its phospholipid environment in cell membranes. In the case of the LHRH receptor, several previous attempts at solubilization have been documented, although generally these have achieved only low yields. For example, 8% of available sites from bovine pituitaries (Perrin, Haas, Rivier & Vale, 1983) and 20% from rat ovaries (Capponi, Aubert & Clayton, 1984) were solubilized with 3-((3-cholamidopropyl) -dimethylammonio)-1-propanesulphonate (CHAPS; 5 mmol/l). Where higher yields have been reported (Winiger, Birabeau, Lang *et al.* 1983) the solubilization criteria may not have been sufficiently stringent (Ogier, Mitchell & Fink, 1987).

In the case of the rat brain serotonin receptor a 75% increase in yield on solubilization was achieved by the addition of NaCl (1·4 mol/l) to the detergent solution (Wouters, van Dun, Leysen & Laduron, 1985). Similar observations have recently been made with the D_2-dopamine receptor (Hooper, 1986). Salt alone or higher concentrations of detergent alone were less effective. In previous reports on the LHRH receptor, increasing concentrations of CHAPS above 5 mmol/l did not increase yields, but in some cases appeared to diminish them (Perrin *et al.* 1983; Winiger *et al.* 1983).

The molecular weight of the LHRH-binding site has been investigated by several groups using photoaffinity labelling before gel electrophoresis under denaturing conditions. The receptor from rat pituitary gland was demonstrated to display an apparent molecular weight of 60 000 (Hazum, 1981), although more recent studies (Iwashita & Catt, 1985) describe a doublet at 63 000 and 52 000, the latter of which was suggested to show greater specificity. A ligand immunoblotting technique under similar denaturing conditions also indicated a molecular weight of around

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60 000 (Eidne, Hendricks & Millar, 1985). The denaturing/dissociating conditions of all of these studies, however, would not reveal the existence of oligomeric complexes, such as dimers, of a binding subunit or association of the binding subunit with non-binding accessory subunits. In order to assess the molecular size of the native LHRH receptor complex we have carried out gel filtration studies of the solubilized receptor under non-denaturing conditions.

MATERIALS AND METHODS

Membrane preparation

Male Wistar rats (200–250 g) were stunned, decapitated and their anterior pituitary glands rapidly removed. These were placed in 100 volumes ice-cold Tris–HCl (25 mmol/l; pH 7·4) and sonicated, followed by centrifugation at 60 000 ***g*** for 15 min at 4 °C. The washed membranes were resuspended either as for solubilization (see below) or in 20 volumes Tris–HCl (25 mmol/l; pH 7·4) with 0·1% (w/v) bovine serum albumin (BSA) (Tris/BSA; Sigma Chemical Company Ltd, Poole, Dorset) for ligand binding assay.

Receptor solubilization

Washed membranes were resuspended in 20 volumes ice-cold solubilization solution (Tris–HCl; 25 mmol/l; pH 7·4) containing CHAPS (5 mmol/l; Sigma), NaCl (1·5 mol/l), soybean trypsin inhibitor (50 μg/ml; Sigma), aprotinin (400 KIU/ml; Sigma) or in this solution with variations in the detergent and NaCl concentrations as indicated. Samples were then agitated for 90 min at 4 °C, followed by centrifugation for 2 h at 60 000 ***g*** at 4 °C to separate solubilized from non-solubilized membrane constituents. Other detergents tested for receptor solubilization were N,N-di-(propylamido-(2,3,4,5,6-pentahydroxyhexanoyl)) cholamide (deoxy-BIGCHAP; Pierce Chemical Co., Chester, Cheshire), nonanoyl-N-methyl glucamide (MEGA-9; Cambridge Research Biochemicals, Cambridge), (N-dodecyl)-sulphobetaine (Zwittergent 3-12; Calbiochem Brand Biochemicals, Cambridge), digitonin (Sigma), sodium deoxycholate (Sigma), lauryl maltoside (Boehringer Mannheim, Lewes, East Sussex), polyoxyethylene sorbitan mono-oleate (Tween 80; Sigma) and (iso-octyl phenoxy)deca-ethoxyethanol (Triton X-100; Sigma). All detergents were used at concentrations including those approximately equal to their published critical micellar concentrations. The supernatant (the solubilized preparation) was used either for ligand binding assay to characterize the solubilized LHRH-binding site or applied to the gel filtration column for determination of molecular weights. In some cases the pellet was resuspended in 20 volumes Tris/BSA for ligand binding.

Determination of apparent molecular weight of the LHRH receptor

The apparent molecular weight of the LHRH receptor was determined on a Sepharose 6B (Pharmacia Fine Chemicals, Milton Keynes, Bucks) column (26 mm × 70 cm) run at 19·5 ml/h at 4 °C. The column was calibrated using Pharmacia Calibration Kits for proteins of high and low molecular weights. The eluting buffer used was the same as the solubilization buffer. Sucrose (50 mg/ml) was added to the solubilized preparation before its application to the column to facilitate its entry into the gel bed as a well-defined band. Fractions of the eluate were collected every 15 min, from which 1·3 ml aliquots were taken and the proteins precipitated from them before ligand binding. A polyethylene glycol (PEG) precipitation was used as in the equilibrium binding assay (described below): 17·9 ml 30% (w/v) PEG 8000 (Sigma) in Tris–HCl (25 mmol/l; pH 7·4) (15% in final solution), 5·1 ml 0·25% (w/v) bovine γ-globulin (Sigma) in Tris–HCl (25 mmol/l; pH 7·4) (0·035% in final solution) and 11·5 ml Tris/BSA were added to the aliquots, after which the tubes were vortexed and left for 20 min on ice. The precipitated proteins were then separated from the detergent solution by centrifugation (30 min at 15 000 ***g***) followed by resuspension in 1·8 ml Tris–HCl (25 mmol/l; pH 7·4). Aliquots of 300 μl were used in the subsequent ligand binding assays. The PEG precipitation method of CHAPS-solubilized LHRH receptors has been shown previously (Winiger *et al.* 1983; Ogier *et al.* 1987) to permit recovery of fully functional LHRH-binding sites from the solubilization solution.

Equilibrium binding assay

Standard equilibrium binding assays were carried out with the protected LHRH analogue buserelin (D-Ser(Bu^t)^6 des Gly^{10} LHRH-ethylamide; a generous gift from Hoechst A.G., Frankfurt, F.R.G.), which was iodinated with ^{125}I to a specific activity of about 1100 Ci/mmol by the chloramine T method (Mitchell, Ogier, Johnson *et al.* 1985). Luteinizing hormone-releasing hormone and its analogues [des pGlu^1]-LHRH and [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-LHRH were obtained from Sigma. Aliquots (50 μl) of the solubilized preparation for use in ligand binding assays were first subjected to PEG precipitation of the proteins as described above for the column fractions (but in a final volume of 1·4 ml). This was necessary as the presence of the detergent–NaCl solution in the assay caused marked suppression of ^{125}I-labelled buserelin binding.

Assays were carried out in a total volume of 500 µl containing 10–60 µg protein, ~40 000 c.p.m. ^{125}I-labelled buserelin (~25 pmol/l) and LHRH analogues, as appropriate, in Tris/BSA. Non-specific binding was determined in the presence of 1 µmol LHRH/l. Duplicate or, in some cases, triplicate determinations were carried out. Incubation for 90 min at 4 °C was followed by PEG precipitation as above (final volume 1·4 ml). The resulting pellet, containing bound ligand, was counted by γ-spectrometry. Displacement of specific ^{125}I-labelled buserelin binding by LHRH and buserelin (agonists), [des pGlu^1]-LHRH (a partial agonist) and [D-pGlu^1,D-Phe^2, D-Trp^{3,6}]-LHRH (antagonist) was determined. The affinities of these analogues were compared in solubilized and native (membrane) LHRH receptors.

Protein assay

Protein assays were carried out according to Geiger & Bessmann (1972).

RESULTS

Solubilization conditions

The absence of NaCl and CHAPS from the solubilization solution resulted in the retention of all specific ^{125}I-labelled buserelin binding in the pellet and none in the supernatant of the solubilization preparation (Fig 1). Addition of 5 mmol CHAPS/l led to the appearance of a small amount (14%) of the specific binding in the supernatant. This was marginally increased on addition of 0·2 and 0·6 mol NaCl/l to the 5 mmol CHAPS/l. The combination of 5 mmol CHAPS/l and 1·5 mol NaCl/l, however, extracted 73 ± 5% (S.E.M.) of the specific binding sites for ^{125}I-labelled buserelin into the supernatant. The sites were retained in solution after 2 h of centrifugation at 60 000 ***g*** and were regarded as truly solubilized. In some experiments, centrifugation for 1 h at over 230 000 ***g*** caused no further precipitation of the binding sites in the supernatant, confirming their authentic solubilization.

Although most previous studies on LHRH receptor solubilization have described very low yields, Winiger *et al.* (1983) reported efficient solubilization of LHRH receptors from bovine pituitary glands using 10 mmol CHAPS/l in a sucrose (0·32 mol/l) and 30% glycerol solution. Separation of solubilized from non-solubilized specific binding sites was determined by centrifugation at 45 000 ***g*** for 1 h. In our hands, using rat tissue, this procedure gave an artifactual result of 20 ± 2% of specific binding in the supernatant in the absence of any detergent (Ogier *et al.* 1987). This was presumably due to the retention of microdispersed fragments of tissue in the dense solution. Yields of authentically solubilized receptor were found to be low (22 ± 3%), similar to those reported by Capponi *et al.* (1984) who used similar conditions but more intensive centrifugation.

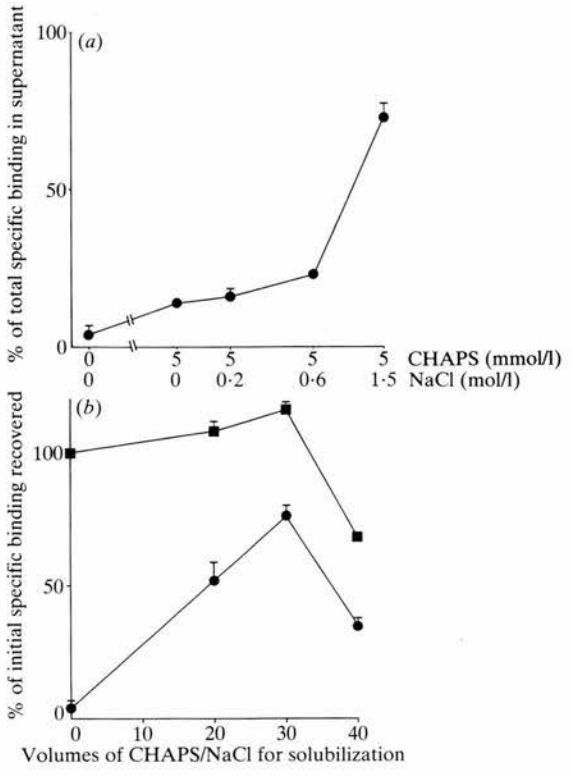

FIGURE 1. (*a*) Effects of increasing concentrations of NaCl on solubilization of rat LHRH receptors from anterior pituitary membranes. Anterior pituitary membranes were shaken with 30 volumes solubilization solution for 90 min at 4 °C. Detergent and salt concentrations were varied as indicated. Solubilized receptors were separated from residual membrane sites by high-speed centrifugation. Using the equivalent of approximately 0·25 anterior pituitary gland per tube, specific binding to the 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate (CHAPS; 5 mmol/l)/NaCl (1·5 mol/l)-solubilized supernatant was around 6000 c.p.m., with some 50% of total binding being specific. Values are means ± S.E.M. for three separate determinations. (*b*) Effect of detergent/protein ratio on receptor recovery and efficiency of solubilization. Receptor solubilization was carried out as in *a* using CHAPS (5 mmol/l)/NaCl (1·5 mol/l), but at a varying ratio of volumes of solubilizing solution to initial tissue volume (as indicated). Solubilized and residual sites were again separated by centrifugation before polyethylene glycol precipitation and ^{125}I-labelled buserelin binding. Squares indicate total recovery of binding (values for supernatant plus pellet as a percentage of initial membrane binding); circles indicate solubilized binding (values for supernatant as a percentage of initial membrane binding). Values are means ± S.E.M. for three separate determinations.

Both the percentage recovery of viable binding sites and the percentage of binding sites successfully solubilized were dependent upon the detergent:protein ratio during solubilization (Fig. 1*b*). Optimal conditions for solubilization with 5 mmol CHAPS/l plus 1·5 mol NaCl/l were found at a ratio of 30 volumes solubilizing solution to tissue. Under these conditions there was full recovery of binding sites and maximal solubilization of around 70% of the sites. This corresponds to a detergent:protein ratio of approximately 1·6 (w/w), similar to the value of 2 recently reported by Hazum, Schvartz, Waksman & Keinan (1986) to be optimal for solubilization with CHAPS alone.

Various other detergents, in the presence and absence of 1·5 mol NaCl/l, were screened for their ability to solubilize the LHRH receptor whilst retaining its capacity to bind ^{125}I-labelled buserelin after PEG precipitation. The detergents tested included examples of non-ionic detergents, bile salts and zwitterionic sulphobetaines with alkyl and cholate side groups (Table 1). Zwittergent 3-12 and sodium deoxycholate were as efficient as CHAPS in protein solubilization from the pituitary membrane preparation. However, no specific binding of ^{125}I-labelled buserelin was detectable in the supernatant, whilst that in the pellet was much reduced (less than 10% of that found in pellets not treated with detergent). This suggests that the receptor is being denatured or the binding site otherwise damaged. Similar effects on specific binding of ^{125}I-labelled buserelin were found using digitonin, lauryl maltoside (1·8 mmol/l), Triton X-100 (1%) or MEGA-9 (5%), with minimal recovery of functional binding sites and only small fractions of these being found in the supernatant. In other cases (lauryl maltoside, 0·18 mmol/l; Triton X-100, 0·16%; MEGA-9, 0·5%; Tween-80, 0·13 and 1·3%) there were no deleterious effects on binding site recovery, but also little solubilization of the binding sites. Only CHAPS produced a marked solubilization together with only modest losses in recovery. This efficiency of solubilization was also seen with deoxy-BIGCHAP, but recovery was further reduced. The addition of 1·5 mol NaCl/l to the detergents generally caused a small increase in the amount of protein solubilized. Percentage recovery of binding sites was generally less than corresponding values without NaCl present. Prominent reductions were seen with lauryl maltoside (0·18 mmol/l), Triton X-100 (0·16%), digitonin, sodium deoxycholate and MEGA-9 (0·5%). The efficiency of binding site solubilization was generally similar in the presence or absence of NaCl. In the case of CHAPS, however, the presence of NaCl effected a two- to fourfold increase in solubilization of the receptor.

Providing that solubilization had been carried out in 20–30 volumes detergent solution, PEG precipitation gave full recovery of the CHAPS/NaCl-solubilized receptor. Other detergents investigated (Table 1; Winiger *et al.* 1983) were not compatible with significant recovery of solubilized receptor by PEG precipitation. There was marked suppression of binding of ^{125}I-labelled buserelin if CHAPS or CHAPS/NaCl was present during the binding assay (at concentrations used for solubilization) (Fig. 2*a*). When the concentrations of CHAPS and CHAPS/NaCl were reduced in the binding assay, the amount of ^{125}I-labelled buserelin bound was increased. Values reached only 50% of detergent/NaCl-free determinations, however, even when the CHAPS/NaCl levels were reduced tenfold. Binding in the presence of reduced concentrations of CHAPS was less impaired, but CHAPS alone was so much less efficient as a solubilizer that the overall yield of binding in the presence of 0·5 mmol CHAPS/l, for example, was only 12 ± 1% of initial membrane binding. When receptor preparation solubilized in CHAPS (5 mmol/l)/NaCl (1·5 mol/l) was diluted to lower levels of detergent/NaCl and then subjected to high-speed centrifugation (Fig. 2*b*), it was clear that, at any concentration compatible with efficient binding, little of the receptor was retained in solution. Binding to the solubilized receptor was therefore not routinely measured in the presence of even diluted solubilization solution, but, instead, after the removal of detergent/NaCl by PEG precipitation.

Characterization of the binding site solubilized by CHAPS/NaCl

Comparison of the binding characteristics of the solubilized receptor with those of the receptor *in situ* in the membrane preparation shows that the receptor was essentially unaltered and clearly not denatured by the procedure. Under the conditions described, binding of ^{125}I-labelled buserelin to both membrane-bound and solubilized receptors was maximal within 90 min. Displacement curves with agonist, partial agonist and antagonist analogues were very similar in solubilized and membrane preparations and dissociation constant (K_d) values derived by computer analysis were essentially the same (Fig. 3). Since displacement of ^{125}I-labelled buserelin by unlabelled buserelin was essentially identical after solubilization, determinations at a single concentration of ligand could be appropriately used to estimate the proportion of sites solubilized.

Apparent molecular weight determination

Application of the solubilized LHRH receptor preparation to a Sepharose 6B column revealed that the major peak of specific binding of ^{125}I-labelled buserelin in the eluate corresponded to a molecular weight of

TABLE 1. Effects of different detergents on solubilization of the rat LHRH receptor. Values are means ± S.E.M. for three separate determinations

	Solubilizing conditions		Recovery of binding sites (% of membrane binding)	Solubilized binding sites (% of total recovered)	Solubilized protein (% of total)
	Concentration	With(+) or without(−) NaCl (1·5 mol/l)			
Detergent					
CHAPS	5 mmol/l	−	46±1	19±3	53±6
	5 mmol/l	+	58±3	56±1	52±2
DeoxyBIGCHAP	1·4 mmol/l	−	36±1	24±1	20±2
	1·4 mmol/l	+	34±4	24±1	35±4
MEGA-9	0·5%	−	103±1	5±1	31±4
	0·5%	+	1±1	1±1	40±4
	5%	−	3±1	1±1	74±9
Zwittergent 3-12	4 mmol/l	−	1±1	ND	62±5
	4 mmol/l	+	3±2	ND	67±6
Digitonin	0·5%	−	1±0·5	ND	28±2
	0·5%	+	1±1	ND	31±18
Sodium deoxycholate	4 mmol/l	−	11±1	ND	46±6
	4 mmol/l	+	2±1	ND	68±10
Triton X-100	0·16%	−	92±1	7±1	24±7
	0·16%	+	18±1	4±1	33±8
	1%	−	10±1	4±1	64±6
Tween 80	0·13%	−	88±1	7±13	24±6
	0·13%	+	81±2	10±1	43±12
	1·3%	−	108±3	4±1	25±8
Lauryl maltoside	0·18 mmol/l	−	100±1	13±1	24±5
	0·18 mmol/l	+	67±2	14±1	21±2
	1·8 mmol/l	−	12±1	3±1	22±6

Male rat anterior pituitary membranes were shaken for 90 min at 4 °C with 40 volumes solubilization solution, containing detergent and NaCl as indicated. Following high-speed centrifugation, supernatant samples were precipitated with polyethylene glycol before determination of ^{125}I-labelled buserelin specific binding. Values for the recovery of binding sites are the binding recovered in supernatant plus pellet as a percentage of initial membrane binding. The solubilized binding sites are those recovered in the supernatant as a percentage of the total recovered. The values for protein are calculated similarly.

ND, no detectable specific binding; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate; deoxyBIGCHAP, N,N-di-(propylamido-(2,3,4,5,6-pentahydroxyhexanoyl)) cholamide; MEGA 9, nonanoyl-N-methyl glucamide; Zwittergent 3-12, (N-dodecyl)-sulphobetaine; Tween 80, polyoxyethylene sorbitan mono-oleate; Triton X-100, (iso-octyl phenoxy)-deca-ethoxyethanol.

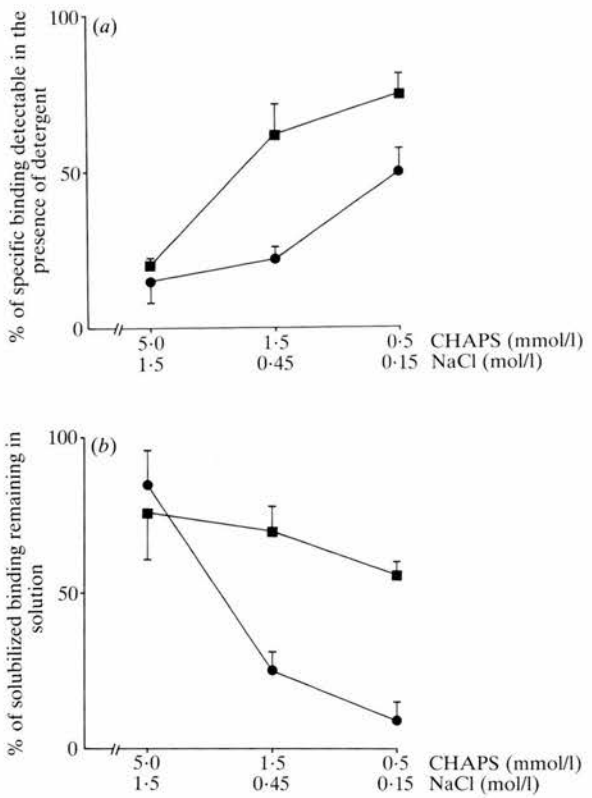

FIGURE 2. (*a*) Effects of detergent/NaCl on binding of ^{125}I-labelled buserelin to solubilized rat LHRH receptor. After solubilization in 20 volumes 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate (CHAPS; 5 mmol/l) (■) or CHAPS (5 mmol/l) plus NaCl (1·5 mol/l) (●), and centrifugation, supernatant samples were assayed for binding of ^{125}I-labelled buserelin. Assays were adjusted to the detergent and salt concentrations indicated and parallel determinations, after polyethylene glycol (PEG) precipitation, were always carried out. The binding detectable in the presence of detergent/NaCl is expressed as percentage of that detected after removal of detergent/NaCl by PEG precipitation. Values are means ± S.E.M. for three separate determinations. This experiment was carried out on solubilized receptors rather than on a membrane receptor preparation, in case their ability to bind ligand was affected differently by the presence of detergent/NaCl. Very similar results were, in fact, obtained with membrane receptor preparations. (*b*) Ability of reduced detergent/NaCl concentrations to retain solubilized receptors in solution. Receptors were solubilized in CHAPS (5 mmol/l (■) or CHAPS (5 mmol/l) plus NaCl (1·5 mol/l) (●) as above. The supernatant fraction was adjusted to the detergent/NaCl concentration indicated, vortexed and then subjected to further high-speed centrifugation to pellet any proteins which had precipitated under the new conditions. The binding in PEG precipitates of the supernatant from such a second centrifugation is expressed as a percentage of that in PEG precipitates of the first supernatant. This value indicates what proportion of the sites remain in solution after dilution of the detergent/NaCl. Values are means ± S.E.M. for three separate determinations.

100 000–160 000 (Fig. 4). There was notably minimal specific binding in the molecular weight region of 60 000. The specific activity of the peak fraction for ^{125}I-labelled buserelin binding (fmol specific binding/μg protein) had increased approximately sevenfold over that found in the crude membrane preparation (7·76 fmol/mg protein in the membrane and 53·3 fmol/mg protein in the gel column eluate; Fig. 4). Some losses of specific binding were incurred in the large-scale PEG precipitation of the column eluate (possibly due to incomplete sedimentation of the precipitated proteins under the conditions employed). The calculated factor of purification may therefore be somewhat underestimated.

DISCUSSION

Using the zwitterionic detergent CHAPS in the presence of high NaCl concentrations, conditions have been achieved which resulted in the solubilization of LHRH receptors from rat anterior pituitary membranes at a yield (>70%) far greater than that achieved previously. The best yields previously described were obtained with CHAPS (5 mmol/l), but values as different as 8% (Perrin *et al.* 1983) and 40% (Hazum *et al.* 1986) have been reported. It is a consistent finding (Perrin *et al.* 1983; Winiger *et al.* 1983; Hazum *et al.* 1986) that increasing the CHAPS concentration causes no improvement in the yield. The combination of CHAPS (5 mmol/l) and NaCl (1·5 mol/l) increased yields by two- to fourfold in the present study. The addition of high NaCl concentrations to the detergent solutions generally resulted in a more efficient solubilization of membrane proteins, presumably by its influence on the effective critical micellar concentrations of the detergents. In several cases, however, this increased efficiency for protein solubilization was associated with an increased denaturing effect, seen as a reduction in the total detectable specific binding of ^{125}I-labelled buserelin. For example, 91% of total control binding was found in the pellet and supernatant treated with Triton X-100 (0·16%) but only 18% in the presence of 1·5 mol NaCl/l. Similar results were found with MEGA 9. Some of the detergents, such as digitonin, sodium deoxycholate and Zwittergent 3-12, caused profound loss of receptor viability both in the presence and absence of NaCl. Other detergents, such as Tween 80, had no deleterious effects on the LHRH receptor, but (either with or without salt) failed to solubilize the binding sites. Only CHAPS, in the presence of salt, resulted in a high degree of receptor solubilization in combination with a low level of receptor damage. The increased receptor solubilization due to salt (in the order of two- to fourfold) seemed to be

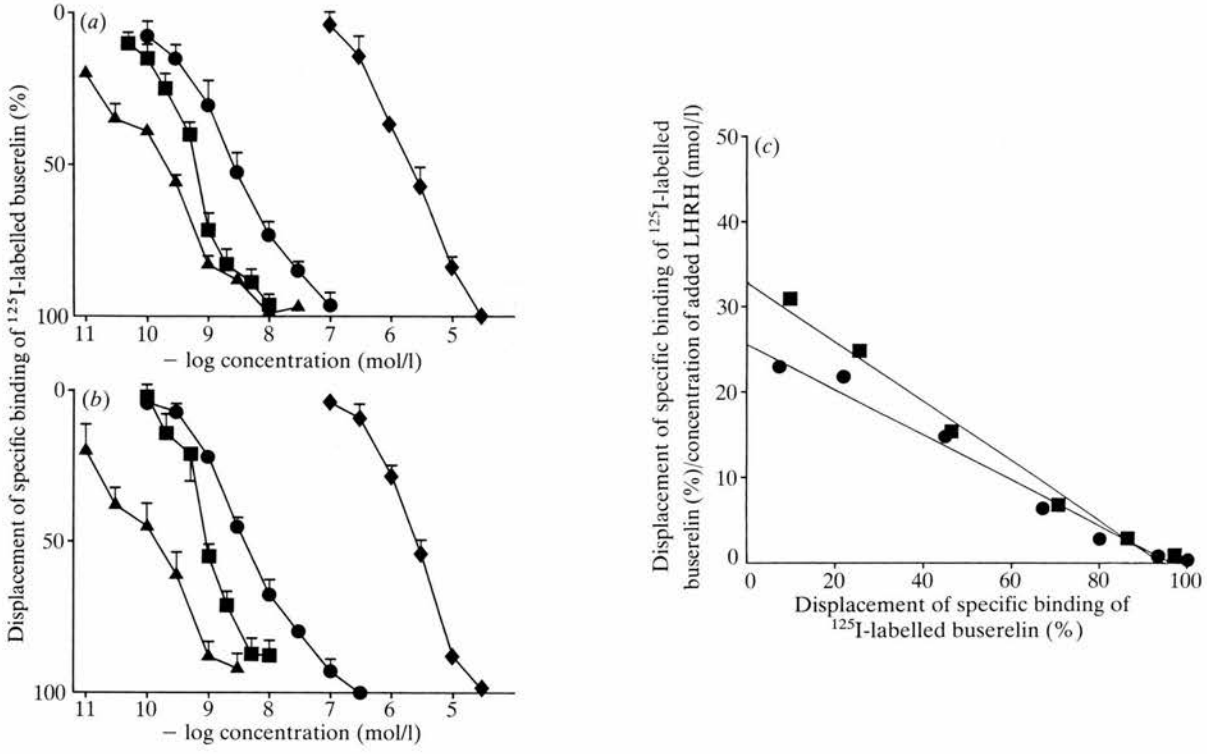

FIGURE 3. Comparison of pharmacological profiles of solubilized and membrane LHRH receptors of the rat. (*a*) Displacement of ^{125}I-labelled buserelin binding to membrane receptors by a series of LHRH analogues: [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-LHRH (▲), buserelin (■), LHRH (●) and [des pGlu^1]-LHRH (◆). Values are means ± S.E.M. for four to ten separate determinations. (*b*) Displacement of ^{125}I-labelled buserelin binding to solubilized receptors. Receptors in the supernatant fraction after solubilization in 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate (CHAPS; 5 mmol/l) plus NaCl (1·5 mol/l) were precipitated by polyethylene glycol before the ^{125}I-labelled buserelin binding assay. Analogues are as in *a*. Values are means ± S.E.M. for three to eight separate determinations. (*c*) Hofstee plot of examples of LHRH displacement of ^{125}I-labelled buserelin binding in membrane (■) and solubilized (●) preparations. Results are typical of six sets of observations. LHRH (0·1–100 nmol/l) displaced ^{125}I-labelled buserelin from both preparations as a single component from which the equilibrium dissociation constant (K_d) was determined by an error-weighted computer programme (Zivin & Waud, 1982). Values for membrane and solubilized preparations were not significantly different (2·20 ± 0·72 and 2·91 ± 0·4 nmol/l respectively). Values of K_d for buserelin (0·47 ± 0·14 nmol/l), [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-LHRH (0·15 ± 0·01 nmol/l) and [D-pGlu^1]-LHRH (1·52 ± 0·26 μmol/l) on the membrane preparation were similar to those found on the solubilized preparation (0·66 ± 0·3 nmol/l, 0·11 ± 0·02 nmol/l and 2·53 ± 0·48 μmol/l respectively). All values are means ± S.E.M. for three to ten separate determinations.

rather selective for this protein, since it was accompanied by only a minor increase in general protein solubilization.

This high-yield solubilization technique (in which the receptor is clearly not denatured) will greatly facilitate subsequent purification of the LHRH receptor protein, an important step in the elucidation of its chemical and structural characteristics. The suitability of the CHAPS/NaCl solubilization for preserving LHRH receptor properties was confirmed by ligand binding studies using the LHRH analogues which give similar K_d values for both the crude membrane preparations and the solubilized LHRH receptors (Fig. 3). Any minor differences in absolute values may have been due to the removal of the receptor from its membrane environment.

The apparent molecular weight of the LHRH receptor, as revealed by the specific binding peak in the gel column eluate, was 100 000–160 000. This is the first time that a value in this range has been described for the receptor in a solubilized form. Our estimate agrees well with the molecular weight of 135 000 obtained by Conn & Venter (1985) using target-size analysis of radiation-inactivated LHRH

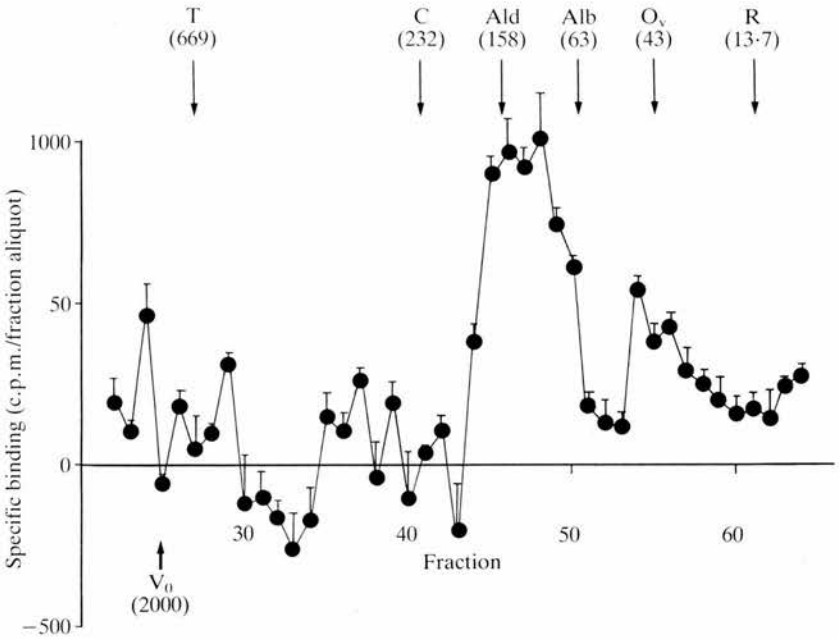

FIGURE 4. Estimation of apparent molecular weight of solubilized rat LHRH receptors using gel chromatography. After solubilization in 3-((3-cholamidopropyl)-dimethyammonio)-1-propanesulphonate (CHAPS; 5 mmol/l)/NaCl (1·5 mol/l), the supernatant fraction was loaded onto a Sepharose-6B column using a sample applicator (SA-5; Pharmacia Ltd). The column was equilibrated, calibrated and eluted using the CHAPS (5 mmol/l)/NaCl (1·5 mol/l) solution. The molecular weight standards and their weights ($\times 10^{-3}$; in parentheses) are as indicated: V_o, blue dextran; T, thyroglobulin; C , catalase; Ald, adolase; Alb, albumin; Ov, ovalbumin; R, ribonuclease. Fractions of the eluate were precipitated with polyethylene glycol and aliquots used in the ^{125}I-labelled buserelin binding assay for duplicate determinations of specific and non-specific binding. The specific binding in the peak fractions (45–48) represented approximately 35% of total. Solubilization of 30 pituitaries gave 24 000 c.p.m. of specific binding in the peak fraction of eluate, which when corrected for protein values indicated at least a sevenfold purification of the receptor over membrane values. The elution profile shows means ± S.E.M. of specific binding in aliquots of fractions from four separate gel elutions.

receptors *in situ*. These two results contrast with those obtained using sodium dodecyl sulphate–polyacrylamide gel electrophoretic molecular weights techniques, where values in the range of 60 000 are obtained consistently (Hazum, 1981; Jansem de Almeida Catanho, Berault, Theoleyre & Jutisz, 1983; Eidne *et al.* 1985; Iwashita & Catt, 1985). This suggests that our procedure succeeded in solubilizing the LHRH receptor complex as it exists *in situ*, in an undenatured form. A second much smaller peak of specific binding was also found, corresponding to a molecular weight of 46 000–53 000, similar to the lower molecular weight unit of Iwashita & Catt (1985). The native complex may contain a binding subunit with a molecular weight of approximately 60 000 and an accessory subunit(s), or may feasibly exist as a dimer of binding subunits. The latter suggestion could be construed as consistent with evidence for a role of micro-dimerization of binding sites in LHRH agonist action. Conn, Rogers, Stewart *et al.* (1982) used a constructed ligand consisting of two antagonist molecules joined through part of the structure not required for binding. A monovalent antibody could interact with one of the antagonist molecules in the bridged analogue, leaving the other available for receptor interaction, where it still displayed an antagonist profile. However, a divalent antibody, which was considered to present two antagonist molecules (separated by ~15 nm) for receptor interaction, evoked an agonist-like response. Although it can be inferred that LHRH agonist action requires receptor dimerization, the large receptor complex in our study had not been exposed to LHRH analogues before the gonadotroph cell membranes were disrupted. It could

be that a proportion of the complexes already exists in a dimerized form, perhaps on account of in-vivo exposure to LHRH. It is possible then that the LHRH receptor exists *in situ* at least in part as a dimer of two 60 000 molecular weight binding units presenting two contiguous binding sites for occupancy by ligands. Alternatively, it could be that the large native LHRH receptor complex identified in the present study and by Conn & Venter (1985) represents the monomeric form of a receptor which consists of several subunits. In-vitro exposure to agonists/antagonists before solubilization may help to resolve this question.

ACKNOWLEDGEMENTS

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NEUROSCIENCE LETTERS

SUPPLEMENT 29 (1987)

PREPARATION AND USE OF A MULTIFUNCTIONAL COVALENT LIGAND FOR LHRH RECEPTOR PURIFICATION.

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We have devised a strategy for purification of the LHRH receptor. A novel photoaffinity biotinylated ligand ([biotinyl-p-azido-Phe- DLys^6]- LHRH)(PBL) was synthesised and utilised through avidin-affinity chromatography for receptor purification. This ligand binds competitively to the LHRH receptor site in the rat anterior pituitary, displacing [^{125}I] Buserelin with a K_D of 1.55nM (not significantly different to that of DLys^6 LHRH, 3nM). Exposure of the preparation to UV light 366nm for 2 min, resulted in the binding of PBL to the receptor becoming irreversible, (no reduction in the number of occupied sites over 24 hrs). PBL was iodinated using the Chloramine T method. Sepharose CL-4B, activated by cyanogen bromide, was coupled to avidin. Biotin displacement of [^{3}H] biotin bound to the avidin-sepharose in the presence of 5mM CHAPS/1.5M NaCl showed that the matrix had umolar affinity for biotin under these conditions. Anterior pituitary membranes prepared from male rats were labelled with the [^{125}I] PBL. After washing to remove unbound ligand and UV activation (2 mins at 366nm) the membranes were shaken in 5mM CHAPS / 1.5M NaCl at 4°C for 90 mins to solubilise membrane proteins. The supernatant from centrifugation for 2hr at 60,000g was added to 1ml of the avidin-sepharose matrix and left shaking gently for 12-18hrs at 4°C. The gel, packed in a 1ml column, was washed using 20 volumes of CHAPS/NaCl solution. Excess biotin (2mM) was used to elute PBL-labelled protein from the column. PBL- labelled sites were purified by ≈700 fold compared with those in native membranes.

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192 SYNTHESIS OF BIOTINYLATED REAGENTS AND THEIR USE IN LHRH RECEPTOR PURIFICATION

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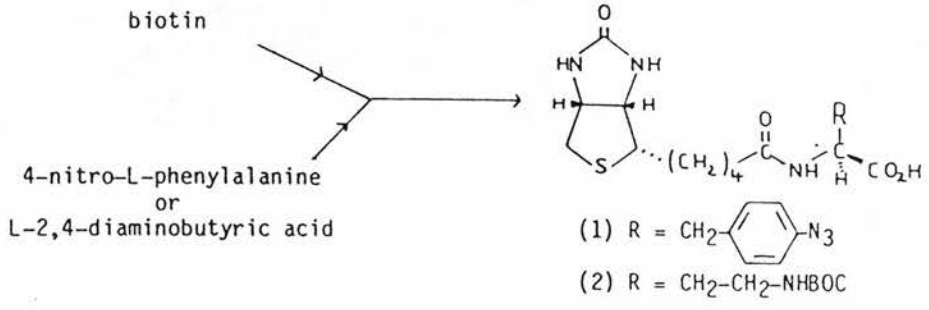
In order to further our studies on the LHRH receptor we have prepared, and coupled to LHRH analogues, two novel biotinylated reagents (1,2). We coupled our initial photolabile compound (1) to DLys^6-LHRH but the conjugate [biotinyl-p-azido-Phe-DLys^6]-LHRH lacked appreciable specific binding in rat anterior pituitary membranes. To overcome this difficulty the peptide backbone was switched to DLys^6,desGly^{10}-LHRH ethylamide (synthesised by the FMOC/polyamide solid phase method) and the reactive grouping to an amino moiety (reagent 2). This second conjugate [biotinyl-aminobutyl-DLys^6, desGly^{10}]-LHRH ethylamide showed approximately 50% specific binding to rat anterior pituitary membranes and could be covalently crosslinked with EGS (ethylene glycolbissuccinimidylsuccinate).



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231 REGULATION OF Ca^{2+} MOBILISATION IN GONADOTROPHES

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The secretion of gonadotrophins induced by Luteinizing Hormone-Releasing Hormone (LHRH) requires both extracellular and intracellular Ca^{2+}. We have been investigating the mechanisms through which activation of phospho-inositide metabolism in response to LHRH, may lead to the different components of Ca^{2+} mobilisation. Experiments were designed to investigate both influx of $^{45}Ca^{2+}$ (through nimodipine-sensitive Ca^{2+} channels) and the efflux of $^{45}Ca^{2+}$ (from endoplasmic reticulum stores) that occurred in rat anterior pituitary slices in response to LHRH.

Activators of protein kinase C (PKC) such as phorbol 12-myristate, 13-acetate (10-300nM) profoundly inhibited LHRH-induced $^{45}Ca^{2+}$ influx but not efflux, in a manner reversible by the PKC inhibitor H7(10μM). Biphasic concentration-response curves for LHRH-induced $^{45}Ca^{2+}$ influx suggested that PKC may exert feedback inhibition of LHRH-induced mobilisation of extracellular Ca^{2+}(1,2). In contrast, the priming effect of LHRH involves a facilitated mobilisation of intracellular but not extracellular Ca^{2+}, with a parallel increase in inositol phosphate production. These data reveal two quite different modes of regulation of LHRH-induced Ca^{2+} mobilisation.

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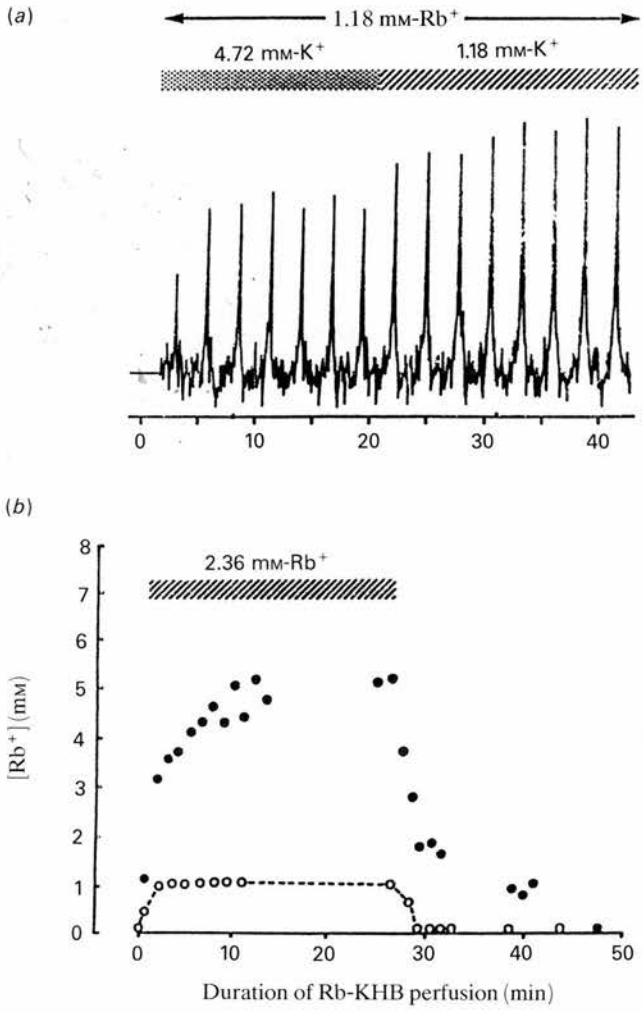

Fig. 1. *$^{87}Rb^+$ in perfused rat kidney*

Single-pass perfusion of right kidneys from 350 g Wistar rats was initiated with KHB. At time 0 min the perfusate was switched to Rb-KHB. (*a*) Paired spectra (20 480 scans each, duration 174 s, line broadening 150 Hz) during perfusion with 1.18 mM-Rb^+ and 4.72 mM-K^+; at 20 min the perfusate K^+ was reduced to 1.18 mM while maintaining the Rb^+ concentration constant. (*b*) Total renal Rb^+ concentration during perfusion with 2.36 mM-Rb^+ and 2.36 mM-K^+ (●); at 27 min the perfusate was switched back to Rb-free KHB. Also shown in (*b*) is the total renal Rb^+ concentration during perfusion of a glutaraldehyde-fixed kidney perfused at the same flow rate and with the same concentration of Rb^+ (○).

can be used to estimate cellular Rb^+ fluxes with a time resolution of approximately 90 s.

This work was supported by the Medical Research Council.

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Regulation of receptor-operated Ca^{2+} influx by protein kinase C

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Activation of receptors for luteinizing hormone-releasing hormone (LHRH) is accompanied by increased phosphoinositide turnover and results in mobilization of Ca^{2+} from both intracellular and extracellular stores (Naor *et al.*, 1986). We have been particularly interested in the mechanism responsible for the receptor-operated Ca^{2+} influx component of this response. Although the involvement of a novel inositol phosphate (Irvine & Moor, 1986) or of a kinetic coupling between different calcium pools (Putney, 1986) have been suggested, there is no firm evidence that they are in fact responsible. We have investigated instead whether protein kinase C (PKC) (which is also activated in response to LHRH) might participate in the relevant signalling mechanism (Mitchell *et al.*, 1986; Fink *et al.*, 1987). The present experiments extend our analysis of the role of PKC, employing the $^{45}Ca^{2+}$ influx protocol described in detail previously (Mitchell *et al.*, 1986).

Abbreviations used: LHRH, luteinizing hormone releasing hormone; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine hydrochloride.

Male COB Wistar rats were maintained under controlled lighting (light 05.00–19.00 h) and temperature (22°C) and given free access to Diet 41B and tap water. Anterior pituitaries were chopped into 0.5–1.0 mm prisms and suspended in buffer with 0.05% (w/v) bovine serum albumin. The buffer contained (mM): NaCl, 154; KCl, 5.4; $CaCl_2$, 1.5; glucose, 11.0 and Hepes 6.0 (buffered to pH 7.4). Tissue was preincubated at 37°C and gassed with O_2 for approx. 20 min before initiation of the experiment. Prewarmed buffer containing $^{45}Ca^{2+}$ to give a final concentration of 2 μM was added and then after 30 s, $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of an ice-cold wash. This was uptake buffer containing 2 mM-EGTA instead of Ca^{2+}. After quenching, the contents of each tube were filtered and the tissue received another 3ml of wash immediately. The filtering was carried out on a Millipore filter block under vacuum, using Millipore cellulose acetate filters supported on Whatman GF/B glass fibre filters. Three further 2 min washes followed. The $^{45}Ca^{2+}$ uptake was measured by liquid scintillation counting.

LHRH (100 nM) caused 69 ± 8% increase over basal $^{45}Ca^{2+}$ accumulation ($n = 7$, mean ± S.E.M.), but the selective activator of PKC, phorbol 12-myristate 13-acetate (PMA)

was unable to cause any significant increase in $^{45}Ca^{2+}$ influx until concentrations in excess of 3 μM. At 30 μM, a 52 ± 10% increase in $^{45}Ca^{2+}$ influx was observed ($n = 5$, mean ± S.E.M.), but phorbol ester actions at such concentrations are grossly non-specific. In contrast, low concentrations of PMA (3–300 nM) inhibited LHRH-induced $^{45}Ca^{2+}$ influx in a manner reversible by the PKC inhibitors H7 [1-(5-isoquinolinesulphonyl)-2-methyl piperazine hydrochloride] and polymyxin B. LHRH-induced $^{45}Ca^{2+}$ influx was reduced to 9 ± 10% increase over basal by 100 nM-PMA and in the presence of H7 (10 μM) this was restored to 61 ± 8% ($n = 5$, means ± S.E.M.). Additional experiments with diacylglycerols and an inhibitor of diacylglycerol kinase further support the idea that PKC is not the intracellular signal mediating receptor-induced Ca^{2+} influx, but instead can act as a negative regulator of this response.

To assess the route of $^{45}Ca^{2+}$ influx here, experiments were carried out with the dihydropyridine antagonist of L-type Ca^{2+} channels, nimodipine (Nowycky *et al.*, 1985) and the inhibitor of the Na^+/Ca^{2+} exchanger, amiloride (Kaczorowski *et al.*, 1984). LHRH-induced $^{45}Ca^{2+}$ influx in these experiments was inhibited by nimodipine with an IC_{50} (concentration required to inhibit by 50%) of 6 ± 1 nM ($n = 4$, mean ± S.E.M.). Amiloride, however, in the range 0.25–4 mM (sufficient for blockade of the Na^+/Ca^{2+} exchanger as well as the Na^+/H^+ exchanger, Kaczorowski *et al.*, 1984), did not block, but enhanced LHRH-induced $^{45}Ca^{2+}$ influx. In contrast, it has been suggested that thrombin-induced Ca^{2+} influx in platelets is inhibited by an amiloride analogue (Siffert & Akkerman, 1987) and interestingly platelets are reportedly devoid of dihydropyridine binding sites (Erne *et al.*, 1984). The mechanisms involved in receptor-operated Ca^{2+} influx (and in its regulation) may therefore be quite different in different cell types.

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Effects of neomycin on K^+ transport into inside-out erythrocyte membrane vesicles

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Aminoglycoside antibiotics, such as neomycin, are ototoxic and have been shown to reversibly inhibit receptor potentials in vertebrate hair cells (Kroese & van den Bercken, 1980; Hudspeth, 1982). Since Ca^{2+}-activated K^+ conductance is an important component of the transduction process in cochlear hair cells (Ashmore & Meech, 1986) and since neomycin and Ca^{2+} compete for binding sites in a wide variety of tissues, we wished to examine what effect neomycin might have on Ca^{2+}-activated K^+ transport.

The mammalian erythrocyte membrane is a readily accessible system for studying Ca^{2+}-activated K^+ transport. We prepared inside-out vesicles (IOVs) from human erythrocyte membranes using the method of Lew *et al.* (1982) as modified by Alvarez *et al.* (1984). K^+ transport was assessed from measurements of uptake of $^{86}Rb^+$ (Alvarez *et al.*, 1984). Incubations were performed at room temperature in a medium containing 18 mM-KCl, 16.5 mM-Hepes and 0.04 mM-EGTA at pH 7.5. Ca^{2+}, neomycin, quinine or valinomycin were added to the incubation medium as required.

The uptake of $^{86}Rb^+$ in the presence of Ca^{2+} was rapid, achieving equilibrium after 10 min. All subsequent measurements of uptake were done after 10 min incubation. $^{86}Rb^+$ uptake was stimulated by Ca^{2+} with an EC_{50} of 4×10^{-7} M and a maximum response at 10^{-5} M. Initially it was difficult to establish what effect, if any, neomycin had on this Ca^{2+}-activated $^{86}Rb^+$ uptake because neomycin alone stimulated $^{86}Rb^+$ uptake and whereas the effect of Ca^{2+} was similar when IOVs were prepared from erythrocytes from different donors ($n = 5$), the effect of neomycin was very variable depending on the donor of the blood. Consequently, we investigated this effect of neomycin on $^{86}Rb^+$ uptake. In IOVs prepared from one donor a dose–response to neomycin was established with an EC_{50} of 9×10^{-6} M and a maximum response at 10^{-4} M. In IOVs prepared from four of five donors, the maximum response produced by 10^{-4} M-neomycin averaged 41% (range 26–60%) of that produced by 10^{-5} M-Ca^{2+}. However, in IOVs from the fifth donor 10^{-4} M-neomycin consistently stimulated an uptake of $^{86}Rb^+$ equivalent to that produced by 10^{-5} M-Ca^{2+}. Quinine (10^{-3} M), had no effect on the neomycin-stimulated $^{86}Rb^+$ uptake although it inhibited by 90% the uptake stimulated by 10^{-6} M-Ca^{2+}.

Neomycin(10^{-4} M), antagonized the effect of Ca^{2+} on $^{86}Rb^+$ uptake, but this effect was partially masked by coexisting stimulation of $^{86}Rb^+$ uptake by neomycin and was overcome at high concentrations of Ca^{2+} ($> 5 \times 10^{-4}$ M). There appeared to be little or no effect of neomycin on the rate of Ca^{2+}-activated $^{86}Rb^+$ uptake (half-time to equilibrium was unchanged), but the amount of $^{86}Rb^+$ associated with IOVs at equilibrium (10 min) was reduced. The antagonism produced by neomycin was more evident at high concentrations: 10^{-3} M-neomycin inhibited by 82% $^{86}Rb^+$ uptake stimulated by 10^{-5} M-Ca^{2+} ($P < 0.01$, paired t-test). However, in these experiments neomycin alone produced little stimulation of $^{86}Rb^+$ uptake, allowing the inhibitory effect on Ca^{2+}-activated uptake to be clearly seen. In parallel experiments we have examined the effect of furosemide on $^{86}Rb^+$ uptake stimulated by another aminoglycoside antibiotic, gentamicin. When furosemide (10^{-3} M), was incorporated into the IOVs during vesiculation, a significant inhibition of gentamicin-stimulated $^{86}Rb^+$ uptake was achieved, whereas there was little, or no effect of furosemide on Ca^{2+}-activated $^{86}Rb^+$ uptake. As a consequence of this finding, the effect of 10^{-4} M-neomycin on Ca^{2+}-activated $^{86}Rb^+$ uptake was

Abbreviations used: IOVs, inside-out erythrocyte membrane vesicles; EC_{50}, concentration producing half-maximal activation.

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191 USE OF DIVALENT CROSSLINKERS TO AFFINITY LABEL LHRH RECEPTORS.

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We have recently described a high yielding method of solubilisation for the rat anterior pituitary LHRH receptor (Ogier, Mitchell and Fink, 1987), the initial step in purification and molecular characterisation of this receptor. The method of choice for receptor purification is usually affinity chromatography. However for this approach to be viable, detergent conditions must be used that allow the retention of the receptor in a solubilised state and at the same time do not disrupt affinity for the ligand utilised. In the case of the LHRH receptor, extensive experiments have failed to reveal mutually compatible conditions. In comparison the biotin-avidin interaction is very resilient to the presence of detergents. Hoffmann & Finn (1985) and Kohanski & Lane (1985) describe the use of biotinylated insulin derivatives to purify insulin receptors via avidin columns. As used, the method is still dependent on the retention of substantial affinity for the analogue in the presence of detergent. In an attempt to circumvent the problem and adapt the method for LHRH receptor purification, we have covalently attached the biotinylated ligand to the receptor. Several divalent crosslinkers of varying length were used in order to obtain the highest efficiency of covalently labelling the rat anterior pituitary LHRH receptor in situ with biotinylated LHRH analogues.

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Facilitated calcium mobilization and inositol phosphate production in the priming effect of LH-releasing hormone in the rat

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ABSTRACT

The ability of LHRH to induce Ca^{2+} mobilization and production of inositol phosphates in rat anterior pituitary tissue *in vitro* was investigated in relation to the self-priming effect of LHRH. Prior exposure to LHRH (which caused a characteristic potentiation of subsequent secretory responses) specifically enhanced LHRH-induced inositol phosphate production and mobilization of intracellular Ca^{2+} stores. LHRH-induced influx of Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels was unaltered, as was ligand binding to LHRH receptors. These data suggest that a novel facilitation of signalling may occur in the phospho-inositide-Ca^{2+} mobilization response mechanism during LHRH priming, and that this may represent an important means of regulating cellular responsiveness in gonadotrophs.
J. Endocr. (1988) **119,** 293–301

INTRODUCTION

Luteinizing hormone-releasing hormone (LHRH) coordinates gonadotrophin secretion partly through a unique self-priming effect on the anterior pituitary gland whereby LHRH significantly increases the responsiveness of gonadotrophs to itself (Aiyer, Chiappa & Fink, 1974). The priming effect of LHRH plays an important role in the mechanism of the spontaneous ovulatory surge of luteinizing hormone (LH) (Fink, 1979) and explains why in man and animals the LH response to exogenously administered LHRH is correlated with the preinjection plasma LH concentrations (Aiyer *et al.* 1974; Fink, 1979). The cellular mechanism responsible is unknown, but is clearly quite distinct from that of normal LHRH-induced gonadotrophin secretion (Pickering & Fink, 1976*a*, 1979; Waring & Turgeon, 1983). The magnitude of priming varies throughout the oestrous cycle (Aiyer *et al.* 1974; Fink, 1979; Waring & Turgeon, 1980) and the effect is dependent both on protein synthesis and on the integrity of microfilaments, but is independent of extracellular Ca^{2+} (Pickering & Fink, 1976*a*, 1979). A significant synthesis of LH does not seem to occur in the priming effect, but instead there is synthesis of a novel protein and probably post-translational modifications of others (Pickering & Fink, 1979; Curtis, Lyons & Fink, 1985). The precise nature of the altered intracellular signalling involved in the priming effect is not established, but the changes can only be evoked by LHRH, not by any other secretagogue investigated (Pickering & Fink, 1979).

Nevertheless, it is clear that calcium is required from both extracellular and intracellular sources for LHRH-induced gonadotrophin secretion (Pickering & Fink, 1979; Bourne & Baldwin, 1980; Bates & Conn, 1984; Limor, Ayalon, Capponi *et al.* 1987). The cellular response to LHRH involves phospho-inositide hydrolysis, and release of intracellular Ca^{2+} together with activation of protein kinase C (PKC) (Hirota, Hirota, Aguilera & Catt, 1985; Schrey, 1985; Limor *et al.* 1987). Since in most cases the critical (though not exclusive) signal for exocytotic secretion appears to be an increase in cytosolic free Ca^{2+} levels (Baker & Knight, 1986) the present series of experiments was carried out to examine whether any modification of LHRH-induced Ca^{2+} mobilization occurs in priming. Our approach to this was to measure LHRH-induced movements of $^{45}Ca^{2+}$ rather than to use fluorescent indicators of cytosolic free Ca^{2+} levels (Limor *et al.* 1987) since the priming effect is impaired in isolated cells required for such studies (Speight & Fink, 1981).

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MATERIALS AND METHODS

Standard laboratory chemicals were of Analar grade obtained from BDH Chemicals Ltd, Dagenham, Essex, U.K. All other compounds and drugs were from Sigma Chemical Company Ltd, Poole, Dorset, U.K., unless otherwise indicated. Ryanodine was purchased from Progressive Agri-Systems Inc., Wind Gap, PA, U.S.A. Buserelin, nimodipine and dantrolene were gifts from Hoechst UK, Hounslow, Middx, U.K.; Bayer AG, Wuppertal, F.R.G. and Norwich-Eaton Pharmaceuticals Inc., Norwich, NY, U.S.A.

LHRH priming

Female Wistar–COB rats (approximately 200 g) purchased from Charles River UK Ltd, Margate, Kent, U.K. were maintained under controlled lighting (lights on from 05.00 to 19.00 h) and temperature (22 °C) and allowed free access to diet 41B (Oxoid Ltd, Basingstoke, Hants, U.K.) and tap water. The rats had shown two consecutive 4-day oestrous cycles immediately before use. Animals were anaesthetized with sodium pentobarbitone (Sagatal; 30 mg/kg; May & Baker Ltd, Dagenham, Essex, U.K.) at 13.30 h, usually on the day of pro-oestrus (when the priming effect is optimal). Hemisected anterior pituitary glands were incubated in Hepes-buffered Minimal Essential Medium with Earle's salts (and L-glutamine, 1 mmol/l) as described previously (Pickering & Fink, 1979). After 20 min preincubation and 1 h with fresh medium, tissue was exposed to LHRH (0·85–8·5 nmol/l) (Peninsula Labs Europe Ltd, St Helens, Merseyside, U.K.) or control medium for one or two more consecutive periods of an hour, designated I_1 and I_2 respectively. Parallel incubations were used for determination of LH secretion using radioimmunoassay procedures decribed in detail previously (Pickering & Fink, 1979) or for $^{45}Ca^{2+}$ flux and [^{3}H]inositol-labelling experiments. In order to enable manipulation of the K^+ concentration, some incubations were carried out in a simplified Hepes-buffered medium, normally of the following composition (concentrations in mmol/l): NaCl, 135; KCl, 4·5; $CaCl_2$, 1·5; $MgCl_2$, 0·5; glucose, 5·6; Hepes, 10; pH 7·4.

Influx of $^{45}Ca^{2+}$

Procedures were based on those described previously (Hopkins & Walker, 1978; Fink, Johnson, Minaur *et al.* 1986). Following incubation in I_1 with or without LHRH (8·5 nmol/l) hemipituitaries were washed three times with 2 ml medium, quartered, and then rapidly transferred to tubes containing 1 ml 'calcium-uptake medium' of the following composition (Fink *et al.* 1986) (concentrations in mmol/l): NaCl, 154; KCl, 5·4; $CaCl_2$, 1·5; glucose, 11; Hepes, 6; pH adjusted to 7·4 with Tris base and with the addition of 0·1% (w/v) bovine serum albumin (BSA). After 20 min preincubation at 37 °C under O_2, 1 ml medium containing $^{45}Ca^{2+}$ (4 μmol/l; ~3 μCi per tube; Amersham International plc, Amersham, Bucks, U.K.; specific activity 17 mCi/mg) with or without LHRH was rapidly added. After 30 s incubation, influx was quenched with 3 ml ice-cold medium (2 mmol EGTA/l replacing Ca^{2+}) (Meisheri, Palmer & Van Breemen, 1980) and tissue was separated by filtration through Millipore SCWP cellulose acetate/nitrate filters (8 μm pore size) underlain by GF/B filters on Millipore 1225 Sampling Manifolds (Millipore UK Ltd, Harrow, Middx, U.K.). Samples were washed once immediately under vacuum with 3 ml ice-cold EGTA medium and then a further three times for 2 min each. Preliminary experiments revealed that these conditions gave the optimal signal to noise ratio with LHRH or 60 mmol K^+/l medium. Stimulus-induced influx in excess of basal controls was maximal within 30 s, suggesting that it represented specific response-triggered flux rather than adsorption or steady accumulation by storage pools.

Unidirectional efflux of $^{45}Ca^{2+}$

The protocol used for these experiments was based on previous studies of pseudo-unidirectional $^{45}Ca^{2+}$ efflux (Williams, 1976; Masters, Quinn & Brown, 1985), but here included EGTA (Meisheri *et al.* 1980) to minimize any exchange-induced efflux. Tissue was not permeabilized in case this disrupted the intracellular changes responsible for priming.

After incubation in I_1 with or without LHRH (8·5 nmol/l) and extensive washing, pairs of hemipituitaries were preincubated for 20 min in 0·5 ml 'calcium uptake medium' with 0·05% BSA before addition of 1 ml medium containing 6 μmol $^{45}Ca^{2+}$/l (~4·5 μCi/tube). Samples were incubated for 1 h at 37 °C under O_2 before the labelling solution was aspirated. Warm oxygenated 'EGTA medium' (3 ml) was added and the tissue captured by filtration through GF/B filters on a Millipore 1225 Sampling Manifold. Following five rapid 3 ml washes, the manifold was reassembled with tubes to collect the filtrate, and 3 ml fresh oxygenated 'EGTA' medium (37 °C) added for 2 min before filtration. This process was repeated a further nine times, with LHRH present when required from the sixth fraction. Results were calculated in terms of the fractional rate coefficient (FRC) of $^{45}Ca^{2+}$ efflux per fraction (i.e. d.p.m. per fraction divided by total d.p.m. recovered).

Radioligand binding to LHRH receptors

Experiments were carried out to assess whether any changes in the affinity or number of LHRH binding sites occurred due to priming *in vitro* or due to

ovariectomy. After incubation with or without LHRH (8·5 nmol/l) in period I_1, anterior pituitary glands were sonicated in ice-cold Tris–HCl buffer (25 mmol/l, pH 7·6) containing 0·05 g soybean trypsin inhibitor/l, 0·016 g trasylol/l and 8·5 nmol LHRH/l, before incubation for 90 min on ice to ensure equal occupancy of LHRH receptors in control and primed tissue. This was carried out to avoid any artefacts due to residual occupancy of receptors by LHRH in primed but not control tissue. Membranes were then washed once by centrifugation at 48 000 ***g*** for 10 min and resuspended in 1000 volumes fresh buffer before allowing 12 h at 4 °C for dissociation of the majority of bound LHRH. Then, after centrifugation and resuspension in fresh buffer (with 0·1 g BSA/l), equilibrium binding of the LHRH analogue [^{125}I]buserelin ([D-Ser(Bu^t)^{6}desGly^{10}]LHRH ethylamide) was determined as described previously (Mitchell, Ogier, Johnson *et al.* 1986). Approximately one-tenth of a pituitary equivalent was used per tube with a label concentration of ~30 pmol/l (specific activity ~1100 Ci/mmol). Data was obtained from experiments with duplicate determinations at ten unlabelled buserelin concentrations in the range 0·05–5·nmol/l, and then analysed by an error-weighted curve-fitting programme (Zivin & Waud, 1982) to yield values for number of binding sites and affinity in each case.

Inositol phosphate production

Following incubation in period I_1, with or without LHRH (8·5 nmol/l), hemipituitaries were washed five times with 2 ml of the Hepes-buffered medium (as described for K^+ depolarisation studies above) but with 10 mmol glucose/l and 0·1 g BSA/l added and then cut into quarters. Tissue was added to 100 µl of the same medium in capped 1·5 ml polypropylene tubes at 37 °C under O_2. Then 10 µCi myo-[^{3}H]inositol (Amersham International plc; 80–120 Ci/mmol) was added to each tube and incubation continued for 1 h. Incorporation of label into inositol phosphates was measured according to well-characterized procedures (Berridge, Dawson, Downes *et al.* 1983; Batty, Nahorski & Irvine, 1985; Schrey, 1985). Tissue was washed twice with 1 ml fresh medium and resuspended in 100 µl. An equal volume of medium containing 200 nmol LHRH/l and 20 mmol LiCl/l (or LiCl alone) was added for 10 min (Schrey, 1985) before stopping the incubation with 100 µl ice-cold trichloracetic acid (22·5 g/l). After homogenization and 15 min on ice, tubes were centrifuged (16 000 ***g***; 5 min) and an aliquot of the supernatant was removed. This was washed five times with two volumes of water-saturated diethyl ether and neutralized to pH 7–8 with solid $NaHCO_3$ (Batty *et al.* 1985). Samples were loaded onto 0·5 ml columns of Dowex AG 1-X8 (200–400 mesh, formate form; Bio-Rad Laboratories Ltd, Watford, Herts, U.K.) ion exchange columns and eluted sequentially with buffers established by other workers (Berridge *et al.* 1983; Batty *et al.* 1985) to separate different inositol phosphates. Fractions of eluate were collected (Batty *et al.* 1985) corresponding to glycerol inositol phosphate, inositol monophosphate, inositol bisphosphate and inositol trisphosphate.

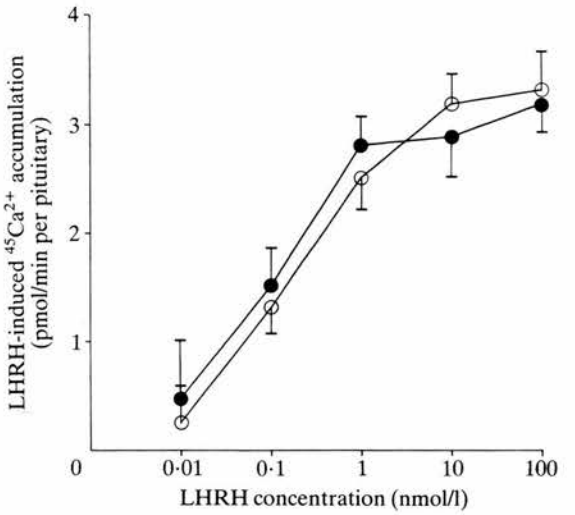

FIGURE 1. Effect of LHRH priming on LHRH-induced $^{45}Ca^{2+}$ influx into anterior pituitary tissue of the rat *in vitro*. Values are means ± S.E.M. from four to eight separate experiments. Accumulation of $^{45}Ca^{2+}$ in the presence of 100 nmol LHRH/l was typically around 6000 d.p.m., of which adsorption to the filter was responsible for around 2000 d.p.m. Basal $^{45}Ca^{2+}$ accumulation by tissue in the absence of LHRH was 1·45 ± 0·21 (n = 12) and 1·43 ± 0·27 (n = 8) pmol/min per pituitary for control and primed tissue respectively. The figure shows concentration–response data for LHRH-induced $^{45}Ca^{2+}$ accumulation following incubation with medium (○) or 8·5 nmol LHRH/l (●) for a 60-min period (incubation I_1) and extensive washing.

RESULTS

$^{45}Ca^{2+}$ influx and LHRH primimg

A rapid influx of $^{45}Ca^{2+}$ into anterior pituitary tissue *in vitro* could be induced by LHRH (Fig. 1) or by depolarising concentrations of extracellular K^+ (Hopkins & Walker, 1978; Fink, Johnson, Minaur *et al.* 1986, 1987). The $^{45}Ca^{2+}$ influx induced by LHRH (1 nmol/l) or K^+ (30 mmol/l) was potently inhibited by the L-type Ca^{2+}-channel blocker, nimodipine (Nowycky, Fox & Tsien, 1985), which caused 50% inhibition at nimodipine concentrations of 6 ± 2 nmol/l and 7 ± 1 nmol/l respectively (means ± S.E.M.; n = 4). However, exposure of tissue to LHRH in incubation

TABLE 1. The priming effect of LHRH on LH secretion under different conditions in the rat *in vitro*. Values are means ± S.E.M. and numbers of experiments are shown in parentheses

	Incubation conditions		**Increments in LH during period** (μg/l)	
	I_1	I_2	I_1	I_2
Tissue				
Pro-oestrous	Medium	LHRH(8)	6±3	127±13
	LHRH	LHRH(19)	144±15	335±57
Oestrous	LHRH	LHRH(8)	111±19	210±43*
Ovariectomized	LHRH	LHRH(10)	164±10	157±24*
Pro-oestrous (simplified medium)	LHRH	LHRH(4)	96±24	313±15
Pro-oestrous	LHRH	LHRH(8)	125±15	449±58
	LHRH+ nimodipine	— (8)	65±17†	—
	LHRH	LHRH+ nimodipine(8)	136±14	396±53

Tissue was exposed to medium, LHRH (0·85 nmol/l) or nimodipine (300 nmol/l) as indicated in two successive 60-min incubation periods, I_1 and I_2.
*$P<0·01$ compared with corresponding value for pro-oestrous tissue; †$P<0·05$ compared with corresponding value for LHRH alone (Student's *t*-test).

I_1 (which caused priming of LH secretion in I_2 (Table 1)) produced no detectable change in the concentration–response curve for LHRH-induced $^{45}Ca^{2+}$ influx (Fig. 1). Since there is no increase in nimidopine-sensitive $^{45}Ca^{2+}$ influx under conditions of priming, the excess secretory responsiveness of primed tissue may be expected to be insensitive to nimodipine (Mitchell, Johnson & Fink, 1987). Accordingly, whilst a maximally effective concentration of nimodipine (300 nmol/l) blocked approximately 50% of LHRH-induced LH secretion in unprimed tissue, the increased responses in primed tissue were inhibited only marginally by nimodipine (Table 1).

$^{45}Ca^{2+}$ efflux and LHRH priming

Following an initial phase of rapid washout, the basal efflux of $^{45}Ca^{2+}$ declined slowly to an almost steady rate, after which addition of 100 nmol LHRH/l elicited a clear but transient increase in $^{45}Ca^{2+}$ efflux (Fig. 2). Efflux in the presence of LHRH was significantly greater than basal for the first two fractions after LHRH addition (Fig. 2*a*). In tissue that had been previously exposed to LHRH, the subsequent LHRH-induced $^{45}Ca^{2+}$ efflux was clearly facilitated, although basal efflux was unaltered. The LHRH-induced increment in FRC from such primed tissue was significantly greater than that from unprimed tissue in the first three fractions after the addition of LHRH (Fig. 2*b*). Concentration–response data (Fig. 2*c*) showed that enhanced responsiveness was significant at 10–100 nmol LHRH/l with a similar trend at lower concentrations.

The $^{45}Ca^{2+}$ efflux response to LHRH (either with or without previous exposure to LHRH) was inhibited by 10 min prior exposure to dantrolene (10 μmol/l), caffeine (10 mmol/l), ryanodine (100 μmol/l) or by $^{45}Ca^{2+}$-loading in the presence of 10 μmol but not 1 μmol Ruthenium Red/l, whereas the $^{45}Ca^{2+}$ efflux induced by the mitochondrial poison carbonyl cyanide *m*-chlorophenylhydrazone (5 μmol/l) was abolished by just 1 μmol Ruthenium Red/l (R. Mitchell & M. Johnson, unpublished observations). These observations suggest (Kojima, Kojima, Kreutter & Rasmussen, 1984; Biden, Wollheim & Schlegel, 1986; Meissner, 1986; McBurney & Neering, 1987) that the LHRH-induced $^{45}Ca^{2+}$ efflux response here originates from a non-mitochondrial pool which may be endoplasmic reticulum.

The effect of priming on LHRH-induced $^{45}Ca^{2+}$ efflux was further investigated under a variety of conditions reported to influence the magnitude of the secretory change in primed tissue (Table 1). In agreement with earlier observations (Fink, 1979; Pickering & Fink, 1979; Waring & Turgeon, 1980) priming of LH secretion was significantly less in oestrous than in pro-oestrous rats (Table 1). Using tissue from oestrous rats, incubation with LHRH during period I_1 correspondingly caused no significant enhancement of subsequent LHRH-induced $^{45}Ca^{2+}$ efflux (Table 2). Tissue from rats which had been ovariectomized 3 weeks previously showed high basal LH output and no apparent priming (Table 1), as has also been described previously (Aiyer, Sood & Brown-Grant, 1976). Basal $^{45}Ca^{2+}$ efflux was unaltered in tissue from ovariectomized rats and $^{45}Ca^{2+}$ efflux responses to LHRH were very much greater than in pro-oestrous

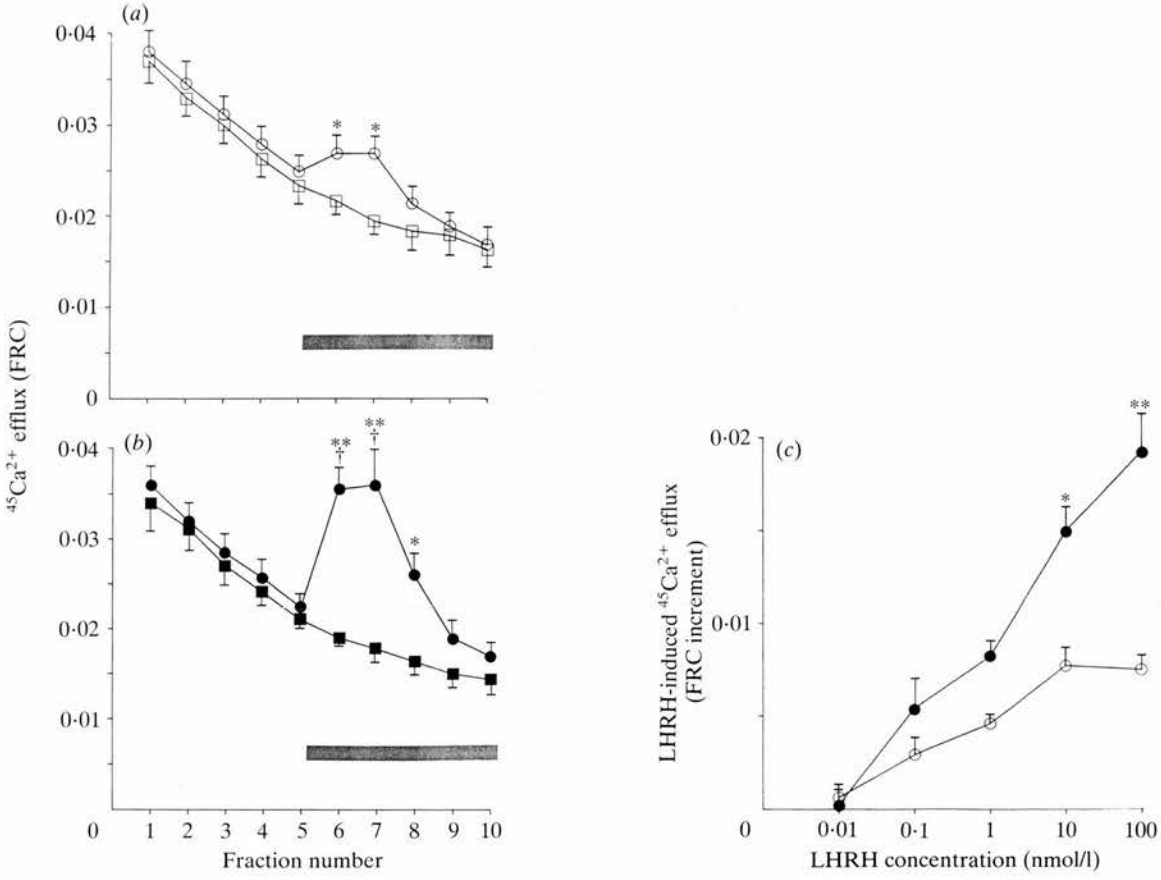

FIGURE 2. Effect of LHRH priming on LHRH-induced unidirectional efflux of $^{45}Ca^{2+}$ from anterior pituitary tissue of the rat *in vitro*. Efflux of $^{45}Ca^{2+}$ expressed as the fractional rate coefficient (FRC) i.e. radioactivity per fraction divided by total recovered radioactivity. Control basal efflux represented approximately 1500 d.p.m. at fraction 7. LHRH was present during the period shown by the horizontal bars. (*a*) Basal efflux (□) and the response to 100 nmol LHRH/l (○) in tissue previously exposed to medium alone for a 60-min period (incubation I_1); (*b*) basal efflux (■) and the response to 100 nmol LHRH/l (●) in tissue previously exposed to 8·5 nmol LHRH/l in incubation I_1. Values are means ± S.E.M., $n=7$. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ compared with FRC for corresponding basal efflux (Student's *t*-test); $\dagger P<0{\cdot}01$ compared with increment in FRC for tissue not pre-exposed to LHRH (Student's *t*-test); (*c*) concentration–response data for the LHRH-induced increment in FRC, calculated at fraction 7 of experiments like those shown in (*a*) and (*b*). Tissue had been previously exposed to medium (○) or to 8·5 nmol LHRH/l (●) in incubation I_1. Values are means ± S.E.M., ($n=4$–13). $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ compared with increment in FRC for tissue not previously exposed to LHRH (Student's *t*-test).

rats (Table 2). However, incubation in I_1 with LHRH rather than medium alone caused no significant increase in the subsequent LHRH-induced $^{45}Ca^{2+}$ efflux using tissue from ovariectomized rats (Table 2). Blockade of protein synthesis with cycloheximide, which prevents the priming effect of LHRH (Pickering & Fink, 1979; Curtis *et al.* 1985; Turgeon & Waring, 1986), eliminated the increase in subsequent LHRH-induced $^{45}Ca^{2+}$ efflux from pro-oestrous tissue exposed to LHRH rather than medium in I_1 (Table 2). Depolarization with K^+ in period I_1 fails to mimic the priming effect caused by exposure to LHRH (Pickering & Fink, 1976*b*), and also failed here to modify subsequent LHRH-induced $^{45}Ca^{2+}$ efflux (Table 2).

LHRH receptors and priming

Specific equilibrium binding of [^{125}I]buserelin was determined in tissue from pro-oestrous rats that had been exposed to either LHRH or medium in I_1 or from ovariectomized rats. In each case, saturation data showed excellent fit to a single component of binding. Neither the affinity nor the number of sites in pro-oestrous tissue were altered by exposure to LHRH in period I_1, equivalent to that which would

TABLE 2. LHRH-induced unidirectional efflux of $^{45}Ca^{2+}$ and binding of [^{125}I]buserelin under different conditions in the rat *in vitro*. Values are means ± S.E.M. and numbers of experiments are shown in parentheses

	I_1 incubation conditions	LHRH-induced $^{45}Ca^{2+}$ efflux (peak increment in FRC × 10^2)	[^{125}I]buserelin binding	
			Affinity constant (nmol/l)	Total no. of sites (fmol/pituitary)
Tissue				
Pro-oestrous	Medium	0·75 ± 0·08(7)	0·25 ± 0·03	161 ± 10(4)
	LHRH	1·92 ± 0·21(7)*	0·24 ± 0·02	147 ± 12(4)
Oestrous	Medium	0·66 ± 0·10(4)	—	—
	LHRH	0·85 ± 0·12(4)†	—	—
Ovariectomized	Medium	2·40 ± 0·42(6)‡	0·22 ± 0·02	248 ± 16‡(4)
	LHRH	2·35 ± 0·35(6)	—	—
Pro-oestrous	Cycloheximide	0·68 ± 0·13(7)	—	—
	LHRH + cycloheximide	0·61 ± 0·10(7)†	—	—
Pro-oestrous	Simplified medium	0·81 ± 0·09(6)	—	—
	Simplified K^+ medium	0·89 ± 0·11(6)†	—	—

Tissue was exposed in a 60-min incubation period (I_1) to medium, LHRH (8·5 nmol/l), high-K^+ medium (30 mmol/l) or cycloheximide (50 μmol/l) before $^{45}Ca^{2+}$ efflux or [^{125}I]buserelin binding experiments. For pro-oestrous tissue, incubation with LHRH in I_1 consistently caused priming of LHRH-induced LH secretion in a subsequent 60-min incubation period (I_2) (see Table 1). The values for $^{45}Ca^{2+}$ efflux show the peak increment in fractional rate coefficient (FRC) (induced by 100 nmol LHRH/l) over corresponding basal efflux curves (taken at fraction seven).

*$P < 0·01$ compared with the increment in corresponding controls unexposed to LHRH in I_1; †$P < 0·01$ compared with the corresponding increment for pro-oestrous tissue exposed to LHRH in I_1; ‡$P < 0·01$ compared with the corresponding value for pro-oestrous tissue (Student's *t*-test).

cause priming of LH secretion (Table 2). Ovariectomy, however, significantly increased the number of available sites above pro-oestrous values (which are normally the highest of the oestrous cycle (Marian, Cooper & Conn, 1981) (Table 2).

Inositol phosphate production and LHRH priming

The incorporation of [^{3}H]inositol into inositol phosphates was measured in response to 100 nmol LHRH/l using pro-oestrous tissue that had been exposed to medium or LHRH in period I_1 (Fig. 3). Basal levels of incorporation were unaltered by the previous exposure to LHRH. However, LHRH-induced labelling of the inositol trisphosphate fraction was significantly increased by previous exposure to LHRH, and similar trends were seen in the bisphosphate and monophosphate fractions. Values for glycerol inositol monophosphate and unincorporated inositol were unchanged. Under the present experimental conditions, no significant levels of label could be detected that eluted under the conditions described (Batty *et al.* 1985) for inositol tetrakisphosphate, although other experimental protocols and time-courses were not investigated.

DISCUSSION

Under the present experimental conditions, either LHRH-induced or depolarization-induced influx of $^{45}Ca^{2+}$ was sensitive to concentrations of nimodipine in the nmol/l range. The functional importance of such dihydropyridine-sensitive channels in LHRH action is suggested by partial blockade of LHRH-induced LH secretion by nitrendipine (Chang, McCoy, Graeter *et al.* 1986) or nimodipine (Table 1). However, it is clear that LHRH priming causes no change in the LHRH-induced influx of $^{45}Ca^{2+}$ through these channels (Fig. 1), and accordingly that the excess LHRH-induced LH secretion from primed gonadotrophs is insensitive to nimodipine (Table 1). We therefore found no evidence here to suggest that LHRH-induced influx of extracellular Ca^{2+} is promoted by priming. Nevertheless, other classes of membrane Ca^{2+} channel (Nowycky *et al.* 1985), cation exchanger (Kaczorowski, Costello, Dethmers *et al.* 1984) or Ca^{2+} extrusion pump (Lotersztajn, Hanoune & Pecker, 1981) could potentially by influenced by priming, and their role may not be detected by our particular experimental protocol.

In contrast, the LHRH-induced mobilization of previously accumulated $^{45}Ca^{2+}$ was markedly facili-

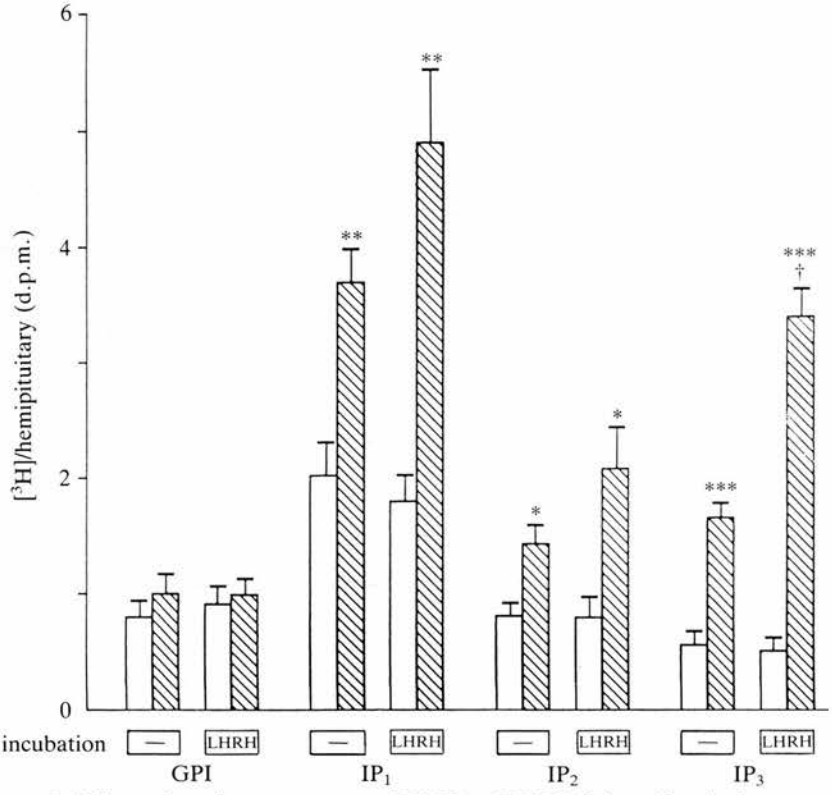

FIGURE 3. Effects of previous exposure to LHRH on LHRH-induced inositol phosphate production in rat anterior pituitary tissue *in vitro*. Inositol phosphates were separated, as described, into fractions corresponding to glycerol inositol phosphate (GPI), inositol monophosphate (IP_1), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3). The figure shows the radioactivity recovered in each of these fractions as mean ± S.E.M. from seven experiments. Values obtained in the presence of 10 mmol LiCl/l alone or 10 mmol LiCl/l with 100 nmol LHRH/l are shown by the open or hatched columns respectively. The previous conditions in a 60-min incubation period (I_1) are indicated beneath the columns. In I_1, LHRH was present at a concentration of 8·5 nmol/l. $*P<0{\cdot}05$, $**P<0{\cdot}01$, $***P<0{\cdot}001$ compared with LiCl alone; $\dagger P<0{\cdot}01$ compared with response to LHRH in tissue not previously exposed to LHRH during I_1 (Student's *t*-test).

tated by previous exposure to LHRH sufficient to cause priming of LH secretion (Fig. 2). The $^{45}Ca^{2+}$ efflux occurring in response to LHRH is transient, suggesting that it originates from a finite cellular pool, and the pharmacological properties of this pool suggest that it relates to endoplasmic reticulum rather than to mitochondria. It is possible that the increased $^{45}Ca^{2+}$ efflux could result from changes in Ca^{2+} sequestration, extrusion or pool sizes (Lotersztajn *et al.* 1981; Jy & Haynes, 1984; Adunyah & Dean, 1986) although little is known about whether these can be receptor-regulated under physiological conditions.

An alternative explanation is that the priming effect of LHRH involves facilitating the efficacy with which subsequent LHRH challenges can mobilize intracellular Ca^{2+} stores. Either receptor-induced production of the second messenger inositol 1,4,5-trisphosphate (IP_3) or its action on endoplasmic reticulum Ca^{2+} stores might be enhanced. Two lines of evidence strongly suggest that a facilitation of LHRH-induced second messenger production is responsible for the present observations. First, our data showing that LHRH-induced labelling of inositol phosphates is increased in tissue previously exposed to LHRH (Fig. 3) and secondly, the specific demonstration that dantrolene (which blocks LHRH-induced $^{45}Ca^{2+}$ efflux here) prevents IP_3-induced Ca^{2+} mobilization in permeabilized cells (Kojima *et al.* 1984).

The parallel increases in LHRH-induced inositol phosphate production and intracellular Ca^{2+} mobilization could in principle be due to changes in affinity or numbers of LHRH receptors. An up-regulation of

LHRH receptors may explain the increased LHRH-induced $^{45}Ca^{2+}$ efflux in tissue from ovariectomized rats (Table 2). A quite different explanation is needed, however, for the increased LHRH-induced $^{45}Ca^{2+}$ efflux from pro-oestrous tissue previously exposed to LHRH, as there is no corresponding change in receptor properties (Table 2). Our hypothesis is that a novel phenomenon—a facilitated coupling of the LHRH receptor to its effector (phospho-inositidase C)—may be responsible for the changes observed. Changes in the activity of inositol lipid kinases or of the enzymes involved in inositol phosphate metabolism could be alternative explanations, but the specific changes in LHRH-induced, rather than basal, inositol phosphate production suggest that they are less likely. However the altered signalling is brought about, it is clear that its importance extends to a functional alteration of receptor-induced Ca^{2+} mobilization in gonadotrophs.

The degree to which changes in LHRH-induced intracellular Ca^{2+} mobilization may directly account for the enhanced stimulus–secretion coupling in the priming effect is difficult to establish. Nevertheless, a close correlation exists between factors influencing the priming of LH secretion and those affecting the enhancement of LHRH-induced $^{45}Ca^{2+}$ efflux in tissue previously exposed to LHRH (Tables 1 and 2). Furthermore, it is clear that significant priming of gonadotrophs to LHRH still occurs in conditions where only intracellular and not extracellular Ca^{2+} mobilization can be enlisted (Pickering & Fink, 1979). Whilst facilitated mobilization of intracellular Ca^{2+} may be crucial for primed responses to the natural secretagogue LHRH, other changes may occur in the secretory apparatus of primed gonadotrophs (Lewis, Morris & Fink, 1985). These may be responsible for the enhancement of responses to non-specific secretagogues such as K^+ or Ca^{2+} ionophores seen after LHRH priming (Pickering & Fink, 1979; Turgeon & Waring, 1986). Recent experiments have revealed that LHRH priming causes a significantly greater degree of enhancement of subsequent responses to LHRH rather than to K^+ or ionomycin (R. Mitchell & M. Johnson, unpublished data). Kinetic analysis of these data indicates that the greater degree of enhancement of LHRH responses involves two distinct components, one of which is also apparent with non-specific secretagogues and the other which is specifically receptor-related (like the changes described here).

If enhanced activation of phospho-inositidase C were occurring in primed tissue it may well lead to increased activity of PKC as well as to changes in Ca^{2+} mobilization. Secretion of LH is slowly increased by phorbol esters (which activate PKC) (Smith & Vale, 1981; Turgeon & Waring, 1986; Johnson, Mitchell & Fink, 1988) in a manner which is synergistic with raised cytosolic Ca^{2+} levels (Harris, Staley & Conn, 1985). Responses to LHRH can also be facilitated by phorbol esters (Turgeon & Waring, 1986), although our studies with inhibitors as well as activators of PKC (Johnson *et al.* 1988) reveal that PKC plays no apparent role in the priming effect. Our evidence therefore suggests that one element of the priming effect of LHRH is a quite novel facilitation of receptor-induced inositol phosphate production and intracellular Ca^{2+} mobilization, whilst any concomitant increase that may occur in PKC activity does not play an important role in the enhanced responsiveness of gonadotrophs.

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PREPARATION AND USE OF BIOTINYLATED LIGANDS FOR LHRH RECEPTOR PURIFICATION.

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Summary: The synthesis of biotinylated analogues of LHRH is described in which the peptides simultaneously combine biotin and either a photolabile or an amino substituent. In rat anterior pituitary membranes the conjugate [biotinyl-aminoethylglycyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide showed approximately 50% specific binding and could be covalently crosslinked to the LHRH receptor site with ethylene glycolbis(succinimidylsuccinate).

Photoaffinity labelling has developed into a regular technique for the investigation of neuropeptide-receptor interactions. In this procedure a ligand containing a photolabile species, normally an azido group, is activated by photolysis and the resulting nitrene covalently binds to the receptor[1,2]. This technique combined with affinity chromatography based on the biotin-avidin/streptavidin interaction[3-5] provides a method for the identification, localisation and purification of hormone-receptor complexes[6]. We have adopted and extended this approach for the purification of the luteinizing hormone releasing hormone (LHRH) receptor. In addition to investigating the photoaffinity approach to receptor labelling we have developed an alternative method in which an amino group can be covalently attached to the receptor with bifunctional crosslinkers[7]. LHRH(1), a hypothalamic decapeptide, plays a key role in the mammalian reproductive cycle and knowledge of the structure of the receptor binding site would further our understanding of the mechanism of cellular response. In this report we describe the synthesis of two novel biotinylated reagents, their conjugation to LHRH analogues and preliminary biological studies of the peptide derivatives in rat anterior pituitary membranes.

Two active LHRH analogues, D-Lys^6-LHRH(2) and [D-Lys^6, des Gly^{10}]-LHRH ethylamide(3) were selected for this study. The ε-amino moiety of the D-Lys^6 residue provides a convenient group on which to couple reagents. The latter peptide was chosen as previous reports[8] indicate that better specific:non-specific binding ratios are observed with the des Gly^{10}-ethylamide C-terminus. D-Lys^6-LHRH was obtained commercially (Peninsula) and [D-Lys^6, des Gly^{10}]-LHRH ethylamide was synthesised by the solid-phase method using Sheppard's Fmoc-t-butyl-polyamide chemistry[9]. After purification by ion-exchange chromatography this peptide showed the expected amino acid composition and molecular weight (Glu_1 1.02, His_1 0.96, Ser_1 0.89, Tyr_1 0.97, D-Lys_1 1.00, Leu_1 1.00, Arg_1 1.00, Pro_1 0.95; FAB MS: found MH^+ 1225, calc. MH^+ $C_{59}H_{86}N_{17}O_{12}$ 1225).

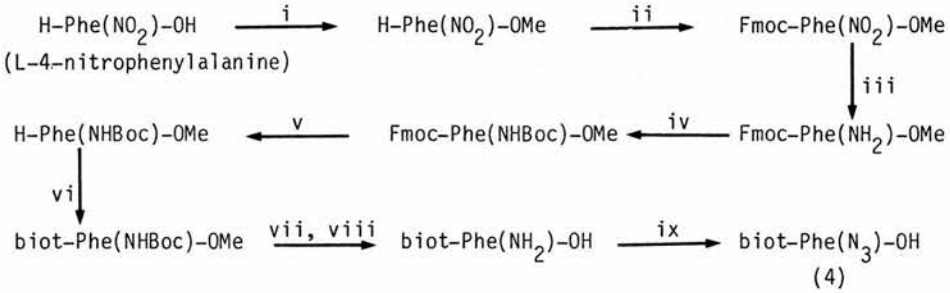
(1) pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

(2) pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2

(3) pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt

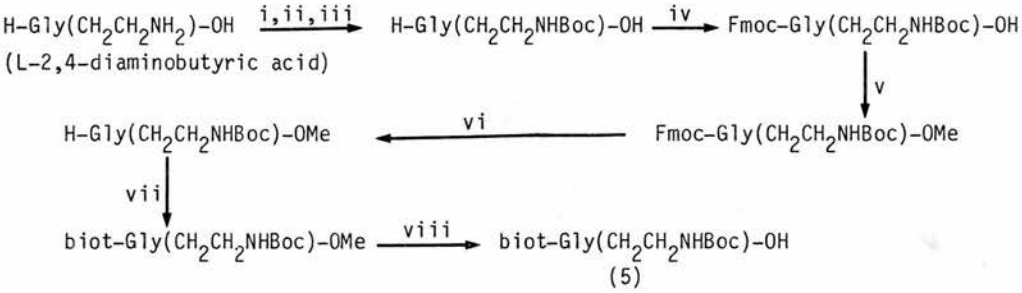
The reagents biotinyl-p-azidophenylalanine (4) and biotinyl-t-butoxycarbonylamino-ethylglycine(5)[10] were prepared from L-p-nitrophenylalanine and L-2,4-diaminobutyric acid respectively and the syntheses are outlined in schemes 1 and 2. Both reagents were converted to their N-hydroxysuccinimide esters for coupling to the peptide backbone[11].

Scheme 1, Synthesis of biotinyl-p-azidophenylalanine (4)



Conditions: i, $SOCl_2$-MeOH; ii, FmocCl-K_2CO_3-H_2O-dioxan; iii, H_2-5%Pd-C-AcOH; iv, 10%$NaHCO_3$-$(Boc)_2O$-H_2O-dioxan; v, 20% piperidine in DMF; vi, biotin-pentafluoro-phenyl ester-HOBT-DMF; vii, NaOH-H_2O-MeOH; viii, aq TFA; ix, $NaNO_2$-NaN_3-1MHCl.

Scheme 2, Synthesis of biotinyl-t-butoxycarbonylaminoethylglycine (5)



Conditions: i, $CuCO_3.Cu(OH)_2$-H_2O; ii, MgO-$(Boc)_2O$-MeOH; iii, H_2S-H_2O-NH_4OH; iv, FmocCl-K_2CO_3-H_2O-dioxan, v, CH_2N_2-Et_2O; vi, 20% piperidine in DMF; vii, biotin-pentafluorophenyl ester-HOBT-DMF; viii, NaOH-H_2O-MeOH

Three conjugates were prepared, [biotinyl-p-azidophenylalanyl-D-Lys^6]-LHRH (6) [biotinyl-p-azidophenylalanyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide (7) and [biotinyl-aminoethylglycyl-D-Lys^6, des Gly^{10}]-LHRH ethylamide (8), and the coupling procedure was similar in each case. For example, D-Lys^6-LHRH (2mg, 1.6μmol) was dissolved in freshly distilled DMF (2ml) and triethylamine (2 drops) was added. Biotinyl-p-azido-phenylalanine-N-hydroxysuccinimide ester (5mg, 9.45μmols) in DMF (1ml) was added and the resulting mixture was left standing in the dark[12] for 1 h. The DMF was evaporated under high vacuum and the residue was triturated with ethyl acetate (3 x 2ml) to remove excess reagent. The crude product was purified by semi-preparative reverse-phase hplc[13] to yield 1.84mg (69%)[14] of pure (single peak on analytical hplc) conjugate. The amino acid compositions of the three conjugates are given in Table 1.

pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ϵ}-biot-Phe(N_3)]-Leu-Arg-Pro-Gly-NH_2

(6)

pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ϵ}-biot-Phe(N_3)]-Leu-Arg-Pro-NHEt

(7)

pGlu-His-Trp-Ser-Tyr-D-Lys-[N^{ϵ}-biot-Gly($CH_2CH_2NH_2$)]-Leu-Arg-Pro-NHEt

(8)

Table 1, Amino acid analysis of LHRH conjugates[15]

No.	PGlu	His	Ser	Tyr	D-Lys	Leu	Arg	Pro	Gly	Phe(N_3)*	Gly($CH_2CH_2NH_2$)
6	1.07	1.00	0.81	1.07	1.01	1	1.00	0.96	1.04	0.30	-
7	1.05	0.96	0.83	0.95	1.02	1	0.89	0.91	-	0.20	-
8	1.09	1.01	0.91	1.01	1.07	1	0.96	0.99	-	-	0.94

* largely decomposed on hydrolysis

Of the three ligands prepared, two contain a photolabile moiety (6,7) whilst the third (8) contains an amino substituent suitable for reaction with bifunctional chemical crosslinkers. The ligands were iodinated and the specificity of their binding to anterior pituitary membranes of male rats was examined by displacement with 1μM LHRH (see Table 2). Their affinity and maximal number of specific binding sites were determined (where appropriate) by saturation analysis and affinity values for the uniodinated forms were confirmed to be similar by displacement of iodinated buserelin ([D-Ser(Bu^t)^6, des Gly^{10}]-LHRH ethylamide). A proportion of the specific binding was rendered resistant to dissociation (4 hrs, 23°C in the presence of excess LHRH) either by illumination for 10 min on ice using a 4 Watt Hg lamp (ligands 6, 7) or by incubation for 30 min on ice with 5mM ethylene glycol<u>bis</u>(succinimidylsuccinate) in the case of ligand (8). Preliminary experiments have shown that membrane protein covalently labelled with (8) will subsequently bind to streptavidin-agarose columns and can be efficiently eluted with 2mM biotin.

Table 2, Biological experiments with iodinated ligands[16]

ligand No	specific binding: as % of total binding	specific binding: affinity (K_D) pM	specific binding: number of sites (Bmax) fmol/pituitary	covalent crosslinking of specific binding: as % of initial specific binding	covalent crosslinking of specific binding: overall yield as % of total ligand binding
buserelin	78 ± 4(8)	280 ± 20	92 ± 3	-	-
6	12 ± 4(4)	-	-	< 4(4)	< 0.5
7	10 ± 3(3)	-	-	28 ± 5(3)	3 ± 1
8	44 ± 6(3)	131 ± 16	63 ± 5	18 ± 3(5)	8 ± 1

values are means ± SEM; numbers of experiments in parentheses

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10. The amino group of this reagent was protected with the t-butoxycarbonyl group during coupling to the peptide and was removed, with TFA, from the conjugate.
11. All new compounds gave satisfactory spectral analyses.
12. Reactors involving the p-azido reagent were performed in the dark and all handling of the p-azido-conjugates was carried out in subdued lighting.
13. Conditions: semi-preparative Aquapore RP300 column; eluant A, 0.1% aqueous TFA; B, 90% CH_3CN 10% A. The column was eluted isocratically for 2 min with 10% B and then with a linear gradient of 10-70% B over 30 min, flow rate 3ml/min. Optical density was monitored at 230nm. During purification of the azido-conjugates (6,7) the detector was switched off whilst the product peak was collected.
14. Yield calculated from amino acid analysis.
15. Hydrolysis conditions: constant boiling HCl containing phenol in a sealed tube for 18 h at 110°C.
16. Full details of the biological experiments will be reported elsewhere, Biochem. J. (in press).

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Novel ligands for the affinity labelling of luteinizing hormone releasing hormone receptors

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A number of novel luteinizing hormone releasing hormone (LHRH) analogues incorporating biotin together with potential covalent attachment sites have been synthesized. Those based on the des-Gly^{10}-[D-Lys^{6}]-LHRH ethylamide peptide backbone resulted in the most useful characteristics of binding to the LHRH receptor in rat anterior pituitary gland membranes. Of these, des-Gly^{10}-[biotinyl-aminoethylglycyl-D-Lys^{6}]-LHRH ethylamide (XBAL) gave the best specific:non-specific binding ratio, with $44 \pm 6\%$ ($\pm$S.E.M.) of total binding being specific with a K_d of 131 ± 16 pM ($\pm$S.E.M., $n = 4$) as determined by Scatchard analysis. Two methods have been used to covalently crosslink these analogues with the LHRH receptor; photoaffinity labelling and the use of homobifunctional *N*-hydroxysuccinimide ester crosslinkers. The photoaffinity analogues gave poor specific:non-specific binding ratios. Of the chemical crosslinkers tested, ethylene glycolbis(succinimidylsuccinate) (EGS) was found to be the most efficient at covalently linking the ^{125}I-XBAL bound to the LHRH receptor site. At an EGS concentration of 5 mM, $23 \pm 3\%$ ($\pm$S.E.M.) of the specific binding of ^{125}I-XBAL was covalently crosslinked.

INTRODUCTION

Luteinizing hormone releasing hormone (LHRH) causes release of gonadotrophins from the anterior pituitary gland by a mechanism involving interaction with a membrane-bound receptor (Clayton & Catt, 1981) and a subsequent increase in the turnover of membrane phosphoinositides (Conn *et al.*, 1986). Information on the structure of the binding site and signal transduction domains of the LHRH receptor may facilitate our understanding of the signalling events occurring. We have recently described a high-yielding method of solubilization for the rat anterior pituitary LHRH receptor (Ogier *et al.*, 1987), the initial step in purification and molecular characterization of this receptor. Several receptor types have been successfully purified using the technique of ligand affinity chromatography (Strosberg, 1984), including the $GABA_A$ (Sigel & Barnard, 1984), muscarinic cholinergic (Haga & Haga, 1985), glycine (Pfeiffer *et al.*, 1982), β_2-adrenergic (Homcy *et al.*, 1983) and insulin receptors (Cuatrecasas, 1972). This approach requires conditions to be found which retain the receptor in a solubilized state without denaturing the protein or greatly reducing its affinity for the ligand utilized. In the case of the LHRH receptor, an extensive survey of different detergents failed to find conditions compatible with these criteria (Ogier *et al.*, 1987). Although detergent/salt conditions were found to successfully solubilize the receptor, ligand binding could only be observed subsequently by removal of the solubilizing agents (and consequent precipitation of the receptor protein). A novel method for purification of the solubilized LHRH receptor was therefore required.

Hofmann & Finn (1985) and Kohanski & Lane (1985) described a method of receptor affinity chromatography based on the interaction of immobilized avidin/streptavidin with biotin incorporated into specific receptor ligands. A similar biotinylated (but still reversible) ligand for LHRH receptors has also been described (Hazum *et al.*, 1986). As described, this approach is strictly dependent on the ligand–receptor interaction being viable in the presence of the detergent (the biotin/avidin interaction appears to be much more resistant to disruption by detergents; Hofmann & Finn, 1985; Kohanski & Lane, 1985). To further develop this approach for LHRH receptor purification we adopted the strategy of covalently linking biotinylated ligands to the LHRH receptor, thereby overcoming the problem of detergent suppression of reversible ligand binding.

The present study describes the design of several LHRH analogues incorporating a biotin molecule. These have been used in conjunction with the two methods of covalent labelling (photoaffinity labelling or affinity crosslinking; Pilch & Czech, 1984) to assess the optimal conditions required for the covalent affinity labelling of LHRH receptors by biotinylated LHRH analogues.

EXPERIMENTAL

Materials

The divalent crosslinkers bis(sulphosuccinimidyl)-suberate (BSSS), disuccinimidyl suberate (DSS), disuccinimidyl tartarate (DST), ethylene glycolbis(succinimidylsuccinate) (EGS) and sulphosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (sulpho-SANPAH)

Abbreviations used: LHRH, luteinizing hormone releasing hormone; buserelin, des-Gly^{10}-[D-Ser(Bu^{t})^{6}]LHRH ethylamide; PBL, [biotinyl-*p*-azidophenylalanyl-D-Lys^{6}]LHRH, PBAL, des-Gly^{10}-[biotinyl-*p*-azidophenylalanyl-D-Lys^{6}]LHRH ethylamide; XBAL, des-Gly^{10}-[biotinyl-aminoethylglycyl-D-Lys^{6}]LHRH ethylamide; sulpho-SANPAH, sulphosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate; EGS, ethylene glycolbis(succinimidylsuccinate); DSS, disuccinimidyl suberate; DST, disuccinimidyl tartarate; BSSS, bis(sulphosuccinimidyl)suberate; Boc, *t*-butoxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; chloramine-T: *N*-chloro-*p*-toluene sulphonamide, sodium salt; PEG, poly(ethylene glycol)8000.

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were purchased from Pierce and Warriner (U.K.) Ltd., Chester, U.K. The LHRH analogue buserelin (des-Gly^{10}-[D-Ser(Bu^t)^6]LHRH ethylamide) was a generous gift from Hoechst A. G., Frankfurt, F.R.G. [D-Lys^6]-LHRH was purchased from Peninsula Laboratories Europe Ltd., St. Helens, U.K. Other LHRH analogues were synthesized as described below. All other chemicals and peptides, unless otherwise stated, were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Synthesis and derivatization of peptide analogues

[des-Gly^{10}, D-Lys^6]LHRH ethylamide was synthesized by the solid-phase method using Sheppard's Fmoc-*t*-butyl-polyamide chemistry (Eberle *et al.*., 1986). After purification, the peptide had the expected amino acid composition (Glu_1, 1.02; His_1, 0.96; Ser_1, 0.89; Tyr_1, 0.97; Lys_1, 1.00; Leu_1, 1.00; Arg_1, 1.05; Pro_1, 0.95) and fast atom bombardment mass spectrum (MH^+, 1225; calc. for $C_{59}H_{85}N_{17}O_{12}$, 1224).

The biotinylated reagents (Fig. 1, **1** and **2**) were prepared in several steps from biotin and either *p*-nitrophenylalanine or 2,4-diaminobutyric acid, and were converted to their *N*-hydroxysuccinimide esters prior to coupling to the ϵ-amino group of the D-Lys^6 of the peptide backbone (Bladon *et al.*, 1988).

The synthesis of reagent **2**, (Fig. 1) (biotinyl-*t*-butoxycarbonylaminoethylglycine), required the selective protection/deprotection of the two amino groups of L-2,4-diaminobutyric acid. The γ-amino function was protected with the acid-labile *t*-butoxycarbonyl group (Boc) in a two-step process (Schwyzer & Rittel, 1961) which first involved the formation of a copper complex with the α-amino and carboxyl groups. Only the γ-amino function was then left available to react wih di-*t*-butyl dicarbonate to yield the protected derivative in 40 % yield. The copper complex was then destroyed by treatment with H_2S. The α-amino function was protected with the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group using the standard procedure (Carpino & Han, 1972), and the carboxylic acid was esterified with diazomethane. The Fmoc group was cleaved by piperidine and then the free amino derivative was treated with biotin pentafluorophenyl ester to yield biotinyl-*t*-butoxycarbonylaminothylglycine methyl ester in 66 % yield for the two-step process. The methyl ester was hydrolysed to yield reagent **2** (Fig. 1). To facilitate coupling to the peptide, the carboxylic acid was converted to its *N*-hydroxysuccinimide ester. Also, the Boc-protecting group on the γ-amino function was left in position until after the reagent was conjugated to the peptide backbone and the production purified. The same type of procedure was used for synthesis of reagent (**1**) (biotinyl-*p*-azidophenylalanine).

The conjugates [biotinyl-*p*-azidophenylalanyl-D-Lys^6]-LHRH (PBL), des-Gly^{10}-[biotinyl-*p*-azidophenylalanyl-D-Lys^6]LHRH ethylamide (PBAL) and des-Gly^{10}-[biotinyl-aminoethylglycyl-D-Lys^6]LHRH ethylamide (XBAL) were all synthesized in a similar manner. For example, PBL was synthesized by first dissolving D-Lys^6-LHRH (2 mg, 1.6 μmol) in freshly distilled dimethylformamide (2 ml) containing triethylamine (2 drops), and then an excess of biotinyl-*p*-azido-phenylalanine-*N*-hydroxysuccinimide ester (reagent **1**, Fig. 1) (5 mg, 9.45 μmol) was added. The resulting mixture was left in the dark for 1 h and then the dimethylformamide was evaporated. The residue was triturated with ethyl acetate (3 × 2 ml) to remove excess reagent and then the crude product was purified by semi-preparative reverse-phase h.p.l.c. (Column: Aquapore RP300; eluents: A, 0.1 % aqueous trifluoroacetic acid; B, 90 % acetonitrile: 10 % A; the product was eluted with a linear gradient of 10–70 % B over 30 min). Reactions utilizing the azido ligand were carried out in subdued lighting, and during purification the u.v. detector was switched off whilst the peak was collected. Amino acid compositions of the derivatives are shown in Table 1.

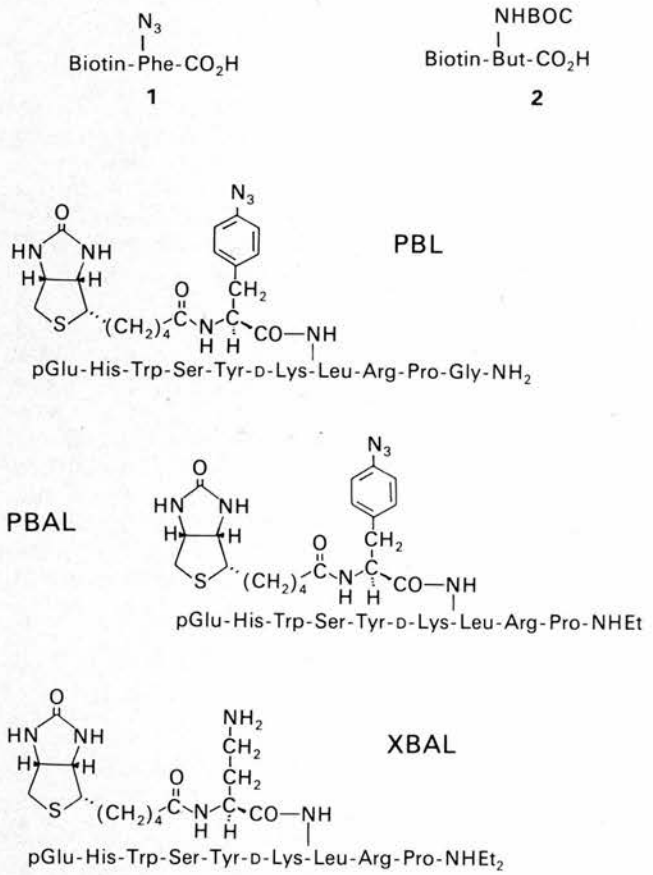


Fig. 1. Biotinyl-LHRH analogue structures

Structures of the novel biotin ligands (**1**) and (**2**) and the three LHRH analogues incorporating them: PBL, [biotinyl-*p*-azidophenylalanyl-D-Lys^6]LHRH; PBAL, des-Gly^{10}-[biotinyl-*p*-azidophenylalanyl-D-Lys^6]LHRH ethylamide; XBAL, des-Gly^{10}-[biotinyl-aminoethylglycyl-D-Lys^6]LHRH ethylamide.

Membrane preparation

Male Wistar rats (approx. 250 g) were stunned, decapitated and their anterior pituitary glands rapidly removed. These were placed in 100 vol. of ice-cold Hepes/KOH (25 mM, pH 7.4) and sonicated, followed by centrifugation at 60000*g* for 15 min at 4 °C. The resulting washed membranes were resuspended in the Hepes/KOH buffer.

Equilibrium binding studies

The binding characteristics of the LHRH analogues were determined by equilibrium displacement assays on anterior pituitary membranes. Iodination of all analogues

Table 1. Amino acid analysis of affinity analogues

Results of amino-acid analysis of the three biotinyl-LHRH analogues PBL, PBAL and XBAL.

	Amino acid										
Analogue	pGlu	His	Ser	Tyr	D-Lys	$PheN_3$*	But	Leu	Arg	Pro	Gly
PBL	1.07	1.00	0.81	1.07	1.01	0.30	–	1.00	1.00	0.96	1.04
PBAL	1.05	0.96	0.83	0.95	1.02	0.20	–	1.00	0.89	0.91	–
XBAL	1.09	1.01	0.91	1.01	1.07	–	0.94	1.00	0.96	0.99	–

* Largely decomposed on hydrolysis.

was by the chloramine-T method (Mitchell *et al.*, 1985) to a specific activity of approx. 1100 Ci/mmol. Assays were usually carried out in triplicate, in a volume of 500 μl containing 10–60 μg of protein, unlabelled analogue as appropriate and 50000 c.p.m. of iodinated analogue in 25 mM-Tris/HCl, pH 7.4, containing 0.1 % bovine serum albumin. Non-specific binding was determined in the presence of 1 μM-LHRH. After incubation for 90 min at 4 °C, equilibrium had been reached and the membrane preparation was subjected to a poly(ethylene glycol) 8000 (PEG) precipitation as previously described (Ogier *et al.*, 1987). Briefly, PEG was added to a final concentration of 15 % and bovine γ-globulin to 0.035 % in a volume of 1.5 ml. After 20 min on ice, samples were centrifuged at 1600 *g*, 6 °C for 15 min. Precipitated (bound) label was then counted by γ spectrometry. Under these conditions, total binding of the iodinated analogue ^{125}I-labelled buserelin was typically 21 ± 1 % ($\pm$S.E.M., $n = 8$) of the total radioligand added, with non-specific binding representing 28 ± 3 % ($\pm$S.E.M., $n = 8$) of this value. These values are similar to those previously reported for the binding of iodinated LHRH analogues (Marshall & Odell, 1975). For photoreactive analogues, all procedures were carried out in subdued light, using a safety light as necessary. Equilibrium binding for chemical crosslinking studies was carried out as above but in Hepes/KOH instead of Tris/HCl/bovine serum albumin. Saturation and displacement data were analysed by an error-weighted programme (Zivin & Waud, 1982).

Covalent labelling of the LHRH receptor

In all cases, covalent labelling was initiated on membrane preparations which were at binding equilibrium with an iodinated LHRH analogue. For photoaffinity labelling studies, the photoreactive conjugates were prepared prior to iodination, with the exception of ^{125}I-labelled-XBAL-sulpho-SANPAH. After an excess of the heterobifunctional reagent sulpho-SANPAH (1.5 mg) had been allowed to react for 40 min with ^{125}I-XBAL (approx. 0.03 μg in Hepes/KOH), at room temperature in the dark (total volume 200 μl), 200 μl of 0.2 M-glycine was added for 10 min to quench the reaction. The ^{125}I-XBAL-sulpho-SANPAH was then diluted in Hepes/KOH for use in membrane equilibrium binding. When binding equilibrium had been attained, the photoreactive analogue ^{125}I-XBAL-sulpho-SANPAH (or in other experiments ^{125}I-PBL or ^{125}I-PBAL) was activated by exposure to u.v. light (366 nm) at 20 cm from a 4 W mercury lamp for 0–10 min (on ice).

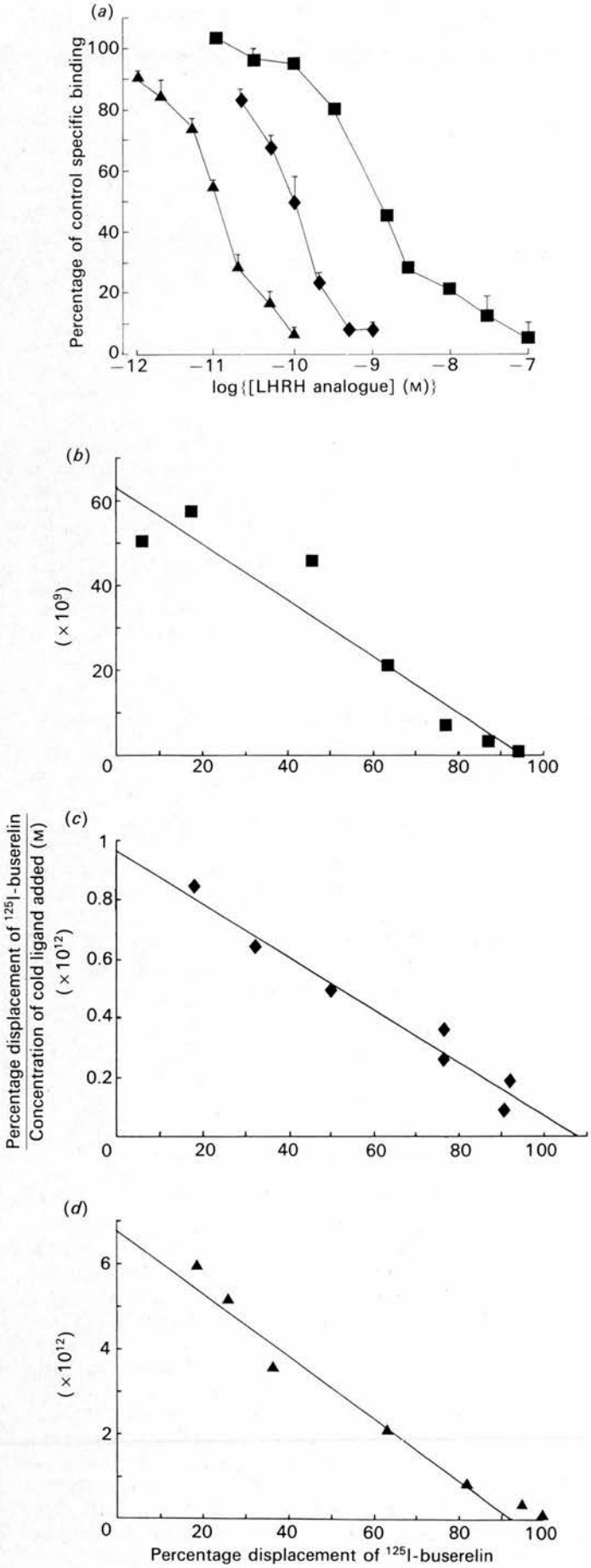
In the divalent chemical crosslinker studies, DSS, DST and EGS were dissolved into dimethyl sulphoxide immediately before use. In the case of BSSS, the crosslinker was dissolved directly into Hepes/KOH. Crosslinkers were routinely added at 1:100 dilutions and left to react for 30 min either on ice or at room temperature, before quenching with 0.25 ml of 0.2 M-glycine.

After photoaffinity or chemical crosslinking, the membrane preparation was centrifuged (45 min at 1600 *g*, 6 °C) to remove excess unbound label and crosslinker, and the membrane-bound radioactivity was determined by γ spectrometry. Photoreactive preparations were kept in subdued lighting.

Assay of crosslinking efficiency

Efficiency of the covalent crosslinking reaction was monitored by comparing the amount of bound ligand in the same samples both at the time of crosslinking and then subsequently after a period of 4 h at room temperature in the presence of 1 μM-LHRH (sufficient to cause dissociation of at least 95 % of the ligand reversibly bound to the receptor). The crosslinked membrane preparation (or crosslinker-free controls) were resuspended into 0.75 ml of Tris/HCl (25 mM, pH 7.4) containing 1 μM-LHRH, 0.1 % bovine serum albumin, soyabean trypsin inhibitor (50 μg/ml) and aprotinin (400 KIU/ml). The radioactivity present was counted. After 4 h at room temperature the membranes were again centrifuged (45 min at 1600 *g*, 6 °C) and recounted. As before, photoreactive preparations were used in subdued light.

Samples for total and non-specific binding were carried through all procedures in parallel. Since the excess amount of membrane binding in the total samples (above that in non-specific samples) initially represented occupancy of the LHRH receptor (being displaceable by LHRH), the effect of crosslinking these samples will most likely include attachment of some of this specifically-bound ligand to part of the receptor protein. Crosslinking the non-specific samples should control for the same level of spurious labelling of non-receptor proteins as occurs additionally to the specific binding in the total samples. After crosslinking, the excess specific covalent labelling in the total samples should be reflected by the value for non-dissociable binding in total samples less that in non-specific samples. The efficiency of receptor-specific crosslinking can then be expressed in terms of the fraction by which reversible specific binding is rendered irreversible after incubation with crosslinker rather than its vehicle control (that is, specific irreversible binding).



RESULTS

Characterization of LHRH analogue binding properties

The affinities of non-radioactive analogues for the LHRH receptor were assessed by the displacement of ^{125}I-buserelin as described previously (Mitchell *et al.*, 1985; Ogier *et al.*, 1987). Displacement of ^{125}I-buserelin from rat anterior pituitary membranes revealed a K_i of 0.92 nM ± 0.16 (± S.E.M., $n = 4$) for PBL (Fig. 2). This is similar to that reported for the parent peptide [D-Lys^6]-LHRH (Clayton & Catt, 1980) suggesting that the addition of the photo-biotin group in the D-Lys^6 position does not impede binding to the LHRH receptor. Binding with the iodinated derivative ^{125}I-PBL could be partially displaced by excess LHRH but only a low ratio of specific to non-specific binding could be observed (Mitchell *et al.*, 1987). Previous reports of binding using iodinated LHRH analogues have suggested that better specific:non-specific ratios are seen using analogues altered at their *C*-terminal to des-Gly^{10}-ethylamide (Perrin *et al.*, 1983). The analogue des-Gly^{10}-[D-Lys^6]-LHRH ethylamide was constructed. Again, displacement of ^{125}I-buserelin (Fig. 2) showed it to bind specifically to the LHRH receptor site, with a K_i of 98 ± 10 pM (± S.E.M., $n = 3$). Addition of the biotin-containing group at D-Lys^6 (to form the analogue XBAL) apparently enhanced binding affinity [K_i of 14 ± 1 pM (± S.E.M., $n = 6$)] (Fig. 2). Hofstee analysis of self-displacement (Hofstee, 1952) gave a K_i value of 57 ± 14 pM (± S.E.M., $n = 3$) and Scatchard analysis of saturation (Scatchard, 1949) gave a K_d of 131 ± 16 pM (± S.E.M., $n = 3$) (Fig. 3). The apparent receptor number ($B_{max.}$) value for ^{125}I-XBAL binding on male rat anterior pituitary tissue was 63 fmol/anterior pituitary, comparable to that for buserelin of 92 fmol/anterior pituitary. Under the conditions used, specific binding of ^{125}I-XBAL was 11 ± 1% (± S.E.M., $n = 4$) of total radioligand added and represented 44 ± 6% (± S.E.M., $n = 4$) of total binding. The specific binding of ^{125}I-XBAL was displaced by buserelin with a K_i value of 0.22 ± 0.07 nM (± S.E.M., $n = 4$), similar to the value of 0.47 ± 0.14 nM (± S.E.M., $n = 6$) for displacement of ^{125}I-buserelin by buserelin.

Photoaffinity labelling

The receptor specificity of photolabelling will be determined in the first instance by the degree of specific binding of the iodinated ligand prior to photoactivation. In the

Fig. 2. Binding characteristics of the LHRH analogues

(*a*) Displacement of ^{125}I-buserelin by the photoaffinity analogue PBL (■), the LHRH analogue des-Gly^{10}-[D-Lys^6]LHRH ethylamide (◆) and the biotinyl-LHRH analogue XBAL (▲), from male rat anterior pituitary membrane preparations. Specific binding was 66 ± 5% (± S.E.M., $n = 8$) of total binding; non-specific binding was determined in the presence of 1 μM-LHRH. Values are the means ± S.E.M., $n = 4$–8. (*b*) Hofstee plot of PBL displacement of ^{125}I-buserelin from rat anterior pituitary membrane preparation. $K_d = 0.92 \pm 0.16$ nM (± S.E.M., $n = 4$). (*c*) Hofstee plot of des-Gly^{10}-[D-Lys^6]LHRH ethylamide displacement of ^{125}I-buserelin from rat anterior membrane preparation. $K_d = 0.098 \pm 0.01$ nM (± S.E.M., $n = 3$). (*d*) Hofstee plot of XBAL displacement of ^{125}I-buserelin from rat anterior pituitary membrane preparation. $K_d = 13.6 \pm 1.2$ pM (± S.E.M., $n = 6$).

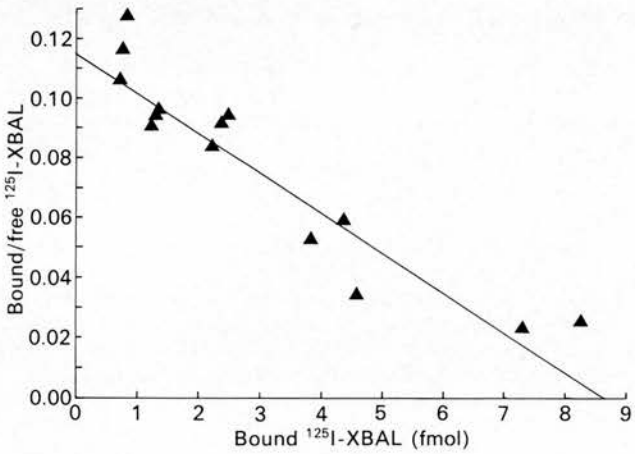


Fig. 3. Scatchard analysis of ^{125}I-XBAL binding

^{125}I-XBAL binding to rat anterior pituitary preparation is shown. All determinations were made in duplicate through the range of 5000 to 500000 counts of ^{125}I-XBAL added per assay tube. Non-specific binding was determined in the presence of 1 μM-LHRH; specific binding was 30–60% of total. $K_d = 131 \pm 16$ pM ($\pm$S.E.M., $n = 3$), $B_{max.}$ 63.2$\pm$5 fmol/anterior pituitary ($\pm$S.E.M., $n = 3$).

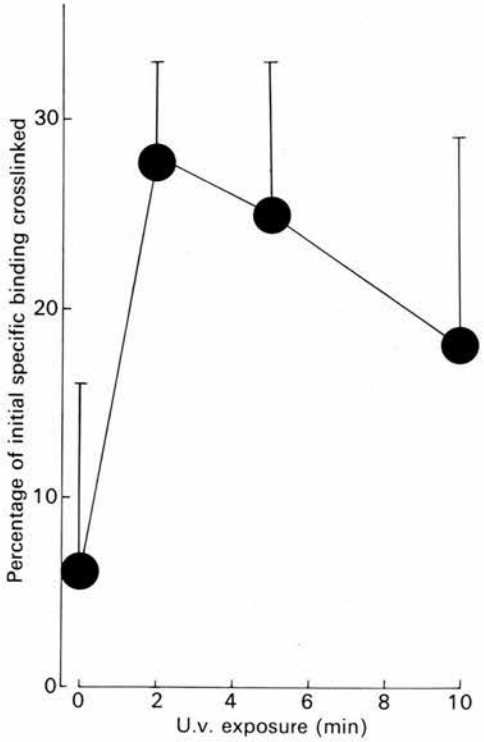


Fig. 4. Photoaffinity labelling of the LHRH receptor

The graph shows the percentage of the initial specific binding of ^{125}I-PBAL which had failed to dissociate after a period of 4 h at room temperature in the presence of 1 μM-LHRH (that is, the irreversible specific binding) plotted against exposure time to u.v. light. In samples unexposed to u.v. light, some 95$\pm$12% of initial specific ^{125}I-PBAL binding dissociated under these conditions. Initial specific binding was 10$\pm$3% of total binding; non-specific binding was determined in the presence of 1 μM-LHRH. Values are means$\pm$S.E.M., $n = 3$.

case of ^{125}I-PBL, the binding displaceable by 1 μM-LHRH (specific binding) was only 12$\pm$4% ($\pm$S.E.M., $n = 4$) of total binding [2$\pm$1% ($\pm$S.E.M., $n = 4$) of total added ligand)]. The photoaffinity analogue of des-Gly^{10}-[D-Lys^6]LHRH ethylamide, that is ^{125}I-PBAL, gave specific binding of only 10$\pm$3% ($\pm$S.E.M., $n = 3$) of total binding [3$\pm$1% ($\pm$S.E.M., $n = 3$) of total added ligand] and ^{125}I-XBAL-sulpho-SANPAH gave specific binding of only 15$\pm$4% ($\pm$S.E.M., $n = 4$) of total binding [2$\pm$1% ($\pm$S.E.M., $n = 4$) of total added ligand]. These values for ligands containing aryl azido photoreactive groups were all much lower than for the other ligands such as buserelin and XBAL. After activation (0–10 min) with u.v. light (366 nm), only minimal levels of specific irreversible labelling could be detected for either ^{125}I-PBL or ^{125}I-XBAL-sulpho-SANPAH. (Specific irreversible binding was defined as above as the excess binding in total above non-specific samples that was resistant to 4 h dissociation in the presence of excess LHRH.) ^{125}I-PBAL showed a small amount of labelling, reaching a maximum, after 2 min activation, of 28$\pm$5% ($\pm$S.E.M., $n = 3$) of initial specific binding (Fig. 4). Since however the initial specificity of ^{125}I-PBAL binding was low (10%), the specific irreversible binding represented only some 3% of initial total binding and with a poor specific:non-specific binding ratio.

Homobifunctional chemical crosslinkers

The success of specific chemical crosslinking was dependent on two factors: firstly, on the ability of the reagents to allow specific ligand binding to the receptor without detrimental effects, and secondly, on their efficiency at rendering specific binding irreversible (Table 2). Of the reagents used, both DSS and BSSS had detrimental effects on the initial level of specific binding; that is, measured immediately after the 30 min crosslinking incubation, they reduced the difference in bound counts between total and non-specific samples. Control values of specific binding were reduced to 58% in he presence of 0.2 mM-DSS, and to 55% in the presence of 0.5 mM-BSSS. Whilst DST had less effect on initial specific binding at low concentrations (0.1–0.5 mM), it too had profound inhibitory effects on specific binding at higher concentrations. However, in contrast, EGS had no significant effect on specific binding until the highest concentration tested, 5 mM, at which there was a small reduction in initial specific binding, to 79% (Table 2).

Concentrations above 1 mM-EGS were successful at crosslinking a proportion of the specific binding of ^{125}I-XBAL and rendering it resistant to dissociation. Concentration-dependent increases in irreversible specific binding were observed (Fig. 5) with a maximum of 23$\pm$3% ($\pm$S.E.M., $n = 3$) of the control level of initial specific binding being rendered irreversible after treatment with 5 mM-EGS. Both BSSS and DST also rendered significant proportions of specific binding irreversible but only at concentrations with profound inhibitory effects on specific binding. Under the present conditions, DSS appeared to be ineffective at crosslinking specific binding of XBAL to the LHRH receptor. The levels of non-specific irreversible binding were slightly increased (1.5–2-fold) by the highest concentrations of BSSS, DST and EGS, whereas DSS at concentrations of 1–3 mM caused large increases of greater than 10-fold. It appears therefore that all the reagents were active in these experiments, but that only EGS showed the required ability

Table 2. Crosslinking of ^{125}I-XBAL to rat anterior pituitary membranes using homobifunctional covalent crosslinkers

The effects of the crosslinking reagents DSS, BSSS, DST and EGS on the initial specific binding of ^{125}I-XBAL and their ability to irreversibly crosslink this specifically-bound ligand are shown at various concentrations. Rat anterior pituitary membrane preparations were equilibrated with ^{125}I-XBAL (90 min at 4 °C) prior to exposure to the crosslinkers or relevant solvent alone in the controls. The crosslinking reaction was quenched by the addition of excess glycine (67 mM). After centrifugation the membranes were resuspended into buffer containing 1 μM-LHRH in the presence of peptidase inhibitors and left at room temperature for 4 h. Meanwhile the initial specific binding (*a*) was determined as the difference between membrane counts in samples with or without 1 μM-LHRH present during the earlier equilibration with ^{125}I-XBAL and the crosslinking incubation. Results in (*a*) are expressed as percentages of control specific binding determined in the absence of any crosslinker (means ± S.E.M., $n = 4$–12). After 4 h dissociation of reversibly bound ligand, the same membrane samples were again centrifuged and the amount of specific binding remaining was determined. This irreversible specific binding (*b*) was expressed as a percentage of the previously measured (initial) specific binding in the presence of crosslinker which was resistant to dissociation (means ± S.E.M., $n = 4$–12). The irreversible specific binding gives an estimate of the amount of specific crosslinking of ^{125}I-XBAL to the LHRH receptor site achieved. In control sets of samples, routinely over 95% of the initial specifically-bound ligand had dissociated during the 4 h incubation. In the case of DSS, DST and EGS, their control experiments were carried out in the presence of 1% dimethyl sulphoxide (DMSO). The solvent had no deleterious effect on the initial specific binding, values in the presence of 1% dimethyl sulphoxide being 104 ± 6% (± S.E.M., $n = 12$) of solvent-free controls. The overall yield of specifically bound ligand crosslinked to receptor is dependent on both (*a*) and (*b*). In the case of 5 mM-EGS, this was 18 ± 3% (± S.E.M., $n = 5$), since there was 23 ± 3% (± S.E.M., $n = 5$) crosslinking of specifically bound ligand (*b*), and the available specific binding (*a*) was reduced to 79 ± 2% (± S.E.M., $n = 5$) by the disruptive effect of crosslinker on ligand binding. Since binding of ^{125}I-XBAL under these conditions was 44 ± 6% (± S.E.M., $n = 4$) specific, these figures represent specific crosslinking to the receptor of some 8% of the total binding and since specific binding was 22 ± 1% (± S.E.M., $n = 4$) of total added label, specific crosslinking of around 2% of total added label.

Crosslinker and reaction conditions	Concentration of crosslinker (mM)	(*a*) Initial specific binding (%)	(*b*) Irreversible specific binding (%)
DSS	0.025	96 ± 12	−6 ± 2
1% DMSO	0.2	58 ± 6	−3 ± 2
30 min on ice	0.3	35 ± 3	5 ± 5
	0.5	29 ± 8	1 ± 5
	1	23 ± 8	5 ± 11
	2	21 ± 10	4 ± 6
BSSS	0.5	55 ± 9	19 ± 6
No DMSO	1	56 ± 2	17 ± 4
30 min at	2	33 ± 1	24 ± 4
room temperature	5	42 ± 8	16 ± 5
DST	0.1	78 ± 9	−6 ± 4
1% DMSO	0.2	78 ± 13	−9 ± 3
30 min at	0.5	72 ± 14	17 ± 10
room temperature	1	54 ± 22	17 ± 7
	2	35 ± 8	30 ± 20
	5	23 ± 26	68 ± 25
EGS	0.1	99 ± 1	−1 ± 1
1% DMSO	0.2	100 ± 2	−2 ± 2
30 min on ice	0.5	93 ± 2	0 ± 4
	1	100 ± 4	3 ± 2
	2	94 ± 14	11 ± 3
	3	108 ± 4	17 ± 5
	5	79 ± 2	23 ± 3

to crosslink ligand bound specifically to the LHRH without disrupting the ligand–receptor interaction.

DISCUSSION

The present study describes the development of a novel approach for receptor purification. LHRH analogues incorporating biotin together with a covalent attachment site have been designed and synthesized, and methods for their covalent crosslinking to the LHRH receptor have been optimized.

Photoaffinity ligands may be expected to give a more specific labelling of receptor sites than the addition of non-specific bivalent crosslinkers, since the linking group is already localized to the ligand. Activation of the photoreactive aryl azido group gives rise to a transient aryl nitrene with a non-selective reactivity (Ji, 1976), and so receptor labelling will not be dependent on the local presence of a particular chemical group. In contrast, chemical crosslinking is dependent both on the presence of suitable functional groups on both ligand and receptor and on their appropriate spatial separation (Pilch & Czech, 1984), but generally occurs in higher yield (Strosberg, 1984). The present experiments employed a series of homobifunctional *N*-hydroxysuccinimide ester crosslinkers with selective reactivity for amino groups. Since the separation between the side-chain free amino group of ^{125}I-XBAL and hypothetical amino groups around the receptor binding site is unknown, we used four cross-

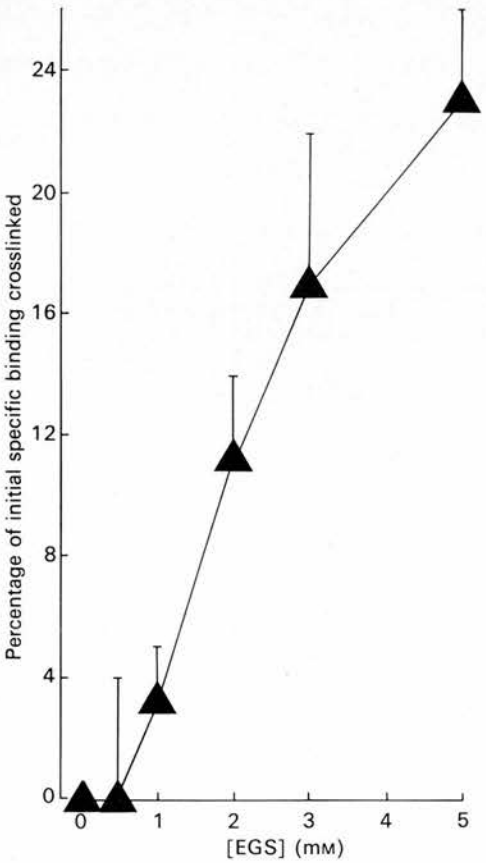


Fig. 5. EGS crosslinking of specific binding of ^{125}I-XBAL to anterior pituitary membranes

The graph shows the percentage of the initial specific binding of ^{125}I-XBAL which had failed to dissociate after a period of 4 h at room temperature in the presence of LHRH (that is, the irreversible specific binding) plotted against concentrations of the bivalent crosslinker EGS used. Initial specific binding was $44 \pm 6\%$ ($\pm$S.E.M., $n = 4$) of total binding; non-specific binding was determined in the presence of 1 μM-LHRH. Values are the means $\pm$ S.E.M., $n = 4$–12.

linkers of various bridge lengths. These range from DST at 0.5 nm (Smith *et al.*, 1978) to EGS at 1.51 nm (Baskin & Chung, 1980).

Initially, [D-Lys^6]LHRH was chosen as the parent LHRH ligand for the analogues. Attaching a biotin-photoreactive aryl azido group via the free amine on D-Lys^6 gave the PBL derivative which retained a high affinity for the LHRH receptor site, but a poor specific: non-specific binding ratio. As in previous reports (Perrin *et al.*, 1983), alteration of the analogue at the *C*-terminal to des-Gly^{10}-ethylamide gave better specific binding. However, the photoaffinity ligands ^{125}I-PBAL and ^{125}I-XBAL-sulpho-SANPAH gave much higher non-specific binding than the biotin-free amine derivative ^{125}I-XBAL. The presence of the aryl azido group seemed to increase the non-specific binding of the analogue – perhaps due to low-level activation of the photoreactive group outwith the u.v. illumination. Although care was taken to protect the analogues from exposure to light, low levels were necessary for safe handling of the iodinated compounds. None of the photoaffinity analogues tested therefore showed sufficient specificity for further use in receptor purification.

Of the bivalent crosslinkers used, EGS was the most successful in covalently crosslinking the ligand ^{125}I-XBAL to the receptor site. This is the longest of the crosslinking reagents tested, at 1.5 nm bridge length. Some 23% of the specific binding of ^{125}I-XBAL was rendered irreversible at the highest concentration of EGS tested, 5 mM. Both the appearance of an inhibitory effect on the ligand–receptor interaction and solubility problems prevented higher concentrations from being tested. This figure represents some 8% of the total binding (and 2% of added ligand) being specifically covalently attached to the receptor; sufficient to develop the strategy further for receptor purification.

Preliminary experiments examining displacement of [^{3}H]-biotin from avidin–sepharose showed that our biotinylated ligands retained a significant proportion of the potency of biotin even in the presence of detergent–salt solutions required for receptor solubilization (Ogier *et al.*, 1987). For example, PBL retained $45 \pm 5\%$ ($\pm$S.E.M., $n = 3$) of the potency of native biotin. Using solubilized membranes previously labelled with ^{125}I-PBL, we have further shown that labelled membrane proteins do bind to an avidin–Sepharose column and can be displaced by elution with 2 mM-biotin (Mitchell *et al.*, 1987). This procedure resulted in an approx. 700-fold increase in the specific activity of labelled proteins compared with the initial membrane preparation. However, the low specificity of ^{125}I-PBL binding made it unsuitable for receptor purification. Preliminary experiments showed that membrane proteins specifically labelled with ^{125}I-XBAL and then crosslinked with EGS can be similarly purified by affinity chromatography on streptavidin–agarose. The higher level of intrinsic specificity in ^{125}I-XBAL binding means that its use with covalent crosslinkers such as EGS should facilitate extensive purification of the LHRH receptor.

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