Activation of mitogen-activated protein (MAP) kinase by the luteinising hormone-releasing hormone (LHRH) receptor

by

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This thesis is dedicated to my maths teacher

Mr Neil McArthur

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I declare that the studies presented in this thesis are the result of my own independent investigation with the exceptions of [^{125}I]buserelin binding and PKC translocation studies which were carried out by Rory Mitchell and Eve Lutz, transfection of COS 7 and CHO cells with cDNA was also carried out by Eve Lutz and some of the anti-ERK immunoblots and PLA_2 assays which were carried out with the assistance of Bart Wolbers and Fiona Thompson respectively.

This work has not and is not currently being submitted for any other degree or professional qualification.

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ABSTRACT

The ability of the luteinising hormone-releasing hormone (LHRH) receptor to activate multiple signal transduction pathways (additional to its conventional activation of phospholipase C, PLC) was investigated. In particular the potential activation of the mitogen-activated protein (MAP) kinases was explored using an *in vitro* kinase activity assay, establishing that LHRH induces a marked and sustained increase in MAP kinase activity. Experiments with the Ca^{2+} ionophore, ionomycin and the phorbol ester, phorbol 12,13-dibutyrate (PDBu) were performed to assess whether the consequences of phosphoinositide hydrolysis evoked by the LHRH receptor such as Ca^{2+} mobilisation or PKC activation could mimic the LHRH-induced activation. This effect could be partially mimicked by PDBu, but not by, ionomycin. The role of PKC in LHRH-induced MAP kinase activity was further examined. The PKC inhibitors GF109203X, Ro-31 8220 and H7 or the downregulation of phorbol ester-sensitive PKC isoforms prevented the LHRH- and PDBu-induced responses. The LHRH-induced response was relatively resistant to H7, consistent with the possibility that the LHRH receptor may differentially activate one or more PKC species in gonadotrophs. The LHRH-induced response was additionally prevented by the tyrosine kinase (TK) inhibitors genistein and MDC or the tyrosine phosphatase inhibitor pervanadate. Phosphorylation of p42 and p44 MAP kinase was independently determined by anti-ERK 1 + 2 immunoblots of the electrophoretic mobility shift after SDS-PAGE separation of samples.

Pertussis toxin (PTx) also prevented the majority of the LHRH-induced MAP kinase activation, providing the first indication for $G\alpha_{i/o}$-mediated signal transduction by the LHRH-receptor. Mastoparan (which activates $G\alpha_{i/o}$ proteins) partially mimicked the effect of LHRH and was also sensitive to PTx. Further support for a role of $G\alpha_{i/o}$ in the LHRH action was provided by the demonstration that LHRH can inhibit in a dose-dependent manner the forskolin- and PACAP-stimulated cAMP formation in αT3-1 cells, an effect which was largely reversed by PTx and appears to be independent of PKC action or Ca^{2+} mobilisation.

The ability of the LHRH receptor to activate MAP kinase was compared to that of the 5-hydroxytryptamine $(5\text{-HT})_{2C}$ and the type 1 a metabotropic glutamate $(mGlu_{1a})$ receptors when transiently expressed in COS 7 cells. The LHRH receptor alone elicited a marked MAP kinase response whilst each receptor caused a large activation of [^{3}H]inositol phosphate production and translocation of [^{3}H]PDBu binding sites, suggesting that a component additional to PLC was necessary for MAP kinase activation. This was not attributable to the LHRH receptor containing an atypical motif for rhodopsin family members Asp instead of Asn at position 318 as a mutant LHRH receptor containing Asn 318 was still capable of yielding a MAP kinase response.

In anterior pituitary tissue from pro-oestrous rats, LHRH caused time- and concentration-dependent activation of MAP kinase. The magnitude of this response on various days of the oestrous cycle correlated with that of LHRH priming (the unique ability of LHRH to increase pituitary responsiveness to itself). The response to LHRH was mimicked by a phorbol ester but not by ionomycin and was blocked with high potency by GF109203X but not by H7 (in a similar manner to the PKC species that mediates LHRH priming). The functional significance of MAP kinase activation in gonadotrophs is considered with respect to LHRH priming. Possible steroidal influences on LHRH-induced MAP kinase activation in αT3-1 cells were also investigated, showing a facilitatory influence of oestrogen.

Finally LHRH-induced activation of the putative downstream target of MAP kinases, phospholipase A_2 (PLA_2) was assessed in αT3-1 cells. This response appeared to be mediated by a G-protein, probably $G\alpha_{12}$, and was unaffected by inhibitors of PKC or tyrosine kinases or by pertussis toxin. PLA_2 therefore does not appear to be a downstream target of the MAP kinases activated here in response to LHRH, despite the likely importance of both these enzymes as mediators in LHRH priming in the anterior pituitary.

ABBREVIATIONS

AA	arachidonic acid
cAMP	adenosine 3', 5'-cyclic monophosphate
ATP	adenosine triphosphate
ATPγS	adenosine-thiotrisphosphate
BSA	bovine serum albumin
CO_2	carbon dioxide
Ca^{2+}	calcium ion
CHO	Chinese hamster ovary
Ci	curies
CNS	central nervous system
CTx	cholera toxin
Da	daltons
DAG	diacylglycerol
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DNA	deoxtribonucleic acid
cDNA	complimentary DNA
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	sodium ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(b-aminoether) N,N,N',N'-tetraacetic acid
ERK	extracellular-regulated protein kinase
G-protein	guanosine-nucleotide-binding protein
GAP	GTP'ase activating protein
GDP	guanine diphosphate
GNRP	guanine-nucleotide releasing protein
GTP	guanine trisphosphate
dH_2O	distilled water
IP_3	inositol 1,3,4 triphosphate
IP	inositol phosphates
JNK	Jun-terminal protein kinase
LH	luteinising hormone
LHRH	luteinising hormone releasing-hormone
LPA	lysophosphatidic acid

LTP	long term potentiation
M	molar
M_x receptor	muscarinic receptor
mGlu	metabotropic glutamate
MAP Kinase	mitogen-activated protein kinase
MBP	myelin basic protein
MEK (MAPKK)	MAP or ERK kinase (MAP kinase kinase)
MEKK (MAPKKK)	MEK kinase
Mg^{2+}	magnesium ion
min	minute(s)
ml	millilitres
mM	millimolar
mMol	millimolar
Na^+	sodium ion
ng	nanogram
NGF	nerve growth factor
nrTK	non-receptor tyrosine kinase
PA	phosphatidic acid
PAF	platelet activating factor
PC	phosphatidylcholine
PDBu	phorbol 12,13-dibutyrate
PDGF	platelet derived growth factor
PH	pleckstrin homology
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PI3'K	phosphatidylinositol 3' kinase
PKA	cAMP-dependent protein kinase (protein kinase A)
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PTx	pertussis toxin
REKs	ras-activated MAP or ERK kinase kinase
rpm	revolutions per minute
SAPK	stress activated protein kinase
sec	second

Ser	serine
SRF	serum response factor
SH	Src homology
SOS	Son of sevenless
TCA	trichloracetic acid
TCF	ternary complex factors
TM D	transmembrane domain
TK	tyrosine kinase
TF	transcription factors
Tris	tris (hydroxymethyl) aminoethane
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume
5-HT	5-hydroxytryptophan
^{3}H	tritium
^{35}S	sulphur 35 radioisotope
^{125}I	iodine 125 radioisotope
B_{max}	maximum number of ligand binding sites /mg of protein
EC_{50}	concentration required to give 50% of the maximal response
IC_{50}	concentration required to inhibit 50% of the response
K_D	dissociation constant
pH	-log[H^+]

A	Ala	Alanine	N	Asn	Asparagine
C	Cys	Cysteine	Q	Gln	Glycine
D	Asp	Aspartic acid	R	Arg	Arginine
E	Glu	Glutamine	S	Ser	Serine
f	Phe	Phenylalanine	T	Thr	Threonine
G	Gly	Glycine	V	Val	Valine
h	His	Histadine	W	Trp	Tryptophan
I	Ile	Isoleucine	Y	Tyr	Tyrosine
K	Lys	Lysine	L	Leu	Leucine
M	Met	Methionine	Z	Glx	Glutamine /Glutamic acid

CHAPTER 1

INTRODUCTION

The transduction of signals from cell surface receptors to their effector systems in the cytoplasm and nucleus is most frequently mediated by a cascade of protein phosphorylation and dephosphorylation. This being achieved by the sequential activation of protein kinases and an array of adaptor proteins and guanyl-nucleotide exchange proteins. Most cellular phosphorylations occur at serine/threonine residues and since such events are initiated by growth factors via receptors which are tyrosine kinases they must in some way regulate serine/threonine-specific kinases or phosphatases. At the onset of the present study a wealth of evidence was emerging about a family of serine/threonine kinases known as the mitogen-activated protein (MAP) kinases or extracellular-regulated kinases (ERKs), which are rapidly activated in response to growth factor stimulation [Pelech & Sanghera, 1992; Sturgill, 1992; Thomas, 1992]. These agonist-dependent kinases which require to be phosphorylated on both a tyrosine and threonine residue for full activity [Anderson et al, 1990; Boulton et al, 1991], thus appeared to represent a crucial link between cell surface receptors and changes in a number of cellular responses, particularly gene expression. At this time the literature on MAP kinase signal transduction pathways essentially concerned growth factor regulation of these enzymes. However phorbol esters [reviewed in Cobb et al, 1991] and a G-protein activator fluoroaluminate [Anderson et al, 1991] had also been reported to activate MAP kinases, presenting the intriguing possibility that other agonists perhaps including those acting through G-protein coupled receptors might additionally activate MAP kinases.

1.1 AIMS OF THIS STUDY

The primary aim of this research project has been to determine if activation of the luteinising hormone-releasing hormone (LHRH) receptor, a seven transmembrane-spanning domain, G-protein-coupled receptor with no intrinsic tyrosine kinase activity, might result in the phosphorylation and activation of MAP kinase in a clonal gonadotroph cell line, αT3-1, which intrinsically expresses a large number of high

affinity LHRH receptors. Having established the occurrence of an LHRH-mediated MAP kinase activation the main questions addressed were:

(1) what are the main intermediary signalling components leading to LHRH-induced MAP kinase activation?

(2) is marked activation of MAP kinase an unusual property of the LHRH receptor, or is it common to all G-protein linked receptors?

(3) does activation of MAP kinase by LHRH occur in native gonadotrophs of the anterior pituitary gland and if so, is this an important regulatory event in the unique phenomenon of LHRH "self priming", (an effect observed in pro-oestrous rat anterior pituitary tissue which is crucial to coordinating the luteinising hormone surge prior to ovulation; see section 1.3)?

(4) is phospholipase A_2 (PLA_2) a cellular target for MAP kinase in αT3-1 cells?

1.2 THE αT3-1 CELL LINE

Studies of the molecular and cellular biology of anterior pituitary cells has depended for many years on primary cultures of pituitary cells or pituitary pieces *in vitro*, both which have limited viability and gradually lose their function over time. Furthermore, the anterior pituitary gland is composed of a number of different cell types including: gonadotrophs, which release luteinising hormone (LH) and follicle-stimulating hormone (FSH); thyrotrophs, which release thyroid-stimulating hormone (TSH); lactotrophs, which release prolactin (PRL); somatotrophs, which release growth hormone (GH); corticotrophs, which release adrenocorticotrophic hormone (ACTH) as well as non-endocrine endothelial cells, folliculo-stellate cells and stromal cells [Farquhar et al, 1975]. The cellular heterogeneity of the anterior pituitary gland therefore complicates any investigations regarding specific cell types, for example, gonadotrophs represent only approximately 10% of the anterior pituitary cell population. Consequently, unless a specific response such as LH or FSH release is measured, interpretation of results is problematic. An alternative approach to the use of pituitary pieces or primary cultures is to use clonal cell lines like the GH_3 and AtT-20 cell lines derived from pituitary tumours [Tashijan et al, 1968]. However until recently there was no clonal cell line of gonadotrophs. Accordingly an established cell line of gonadotroph lineage was developed to study regulation and expression of the gonadotrophins [Windle et al, 1990].

It is now possible to target expression of oncogenes to specific cell types in transgenic mice thus providing a method for immortalising rare cell types by directing expression of the simian virus-40 T-antigen (SV40 *Tag*) with the promoter and associated regulatory domains of a gene of interest [Jenkins & Copeland, 1989]. Expression of LH and FSH is a specialised function of gonadotrophs, therefore the genes encoding LH and FSH represented good candidate genes for this approach. LH and FSH are members of a larger family, the glycoprotein hormones. Each of

these hormones is a heterodimer sharing a common α-subunit, with a unique β-subunit [Pierce & Parsons, 1981]. A fusion gene containing ~ 1.8 kilobases of 5' flanking sequences of the human glycoprotein hormone α-subunit linked to the protein-coding sequences of SV40 T-antigen oncogene was used for *Tag*-driven tumorigenesis. Transgenic mice bearing this fusion gene heritably developed pituitary tumours, which were found to be anterior pituitary specific [Windle et al, 1990]. Tumour cells were dispersed then cultured and after serial dilution of the resulting stable cultures, several clonal cell lines were established. The αT3-1 cell line was maintained for two years without any change in phenotype and remained stably diploid when tested. This cell line was found to be expressing LH and FSH α-subunit, but not the specific β-subunits, consistent with these cells being precursors of gonadotrophs and/or thyrotrophs. Further characterisation of these cells revealed synthesis and secretion of mRNA for the α-subunit in response to an LHRH analog, nafarelin, in both a time- and concentration-dependent manner (consistent with the analog acting through specific LHRH receptors), whereas αT3-1 cells did not respond to TRH. Therefore these cells appear to be gonadotrophs due to the presence of LHRH (but not TRH) receptors. The lack of the β-subunit for LH or FSH may be explained if the transformed cells were not yet fully differentiated into gonadotrophs or thyrotrophs and were arrested at this stage.

1.3 LUTEINISING HORMONE-RELEASING HORMONE AND THE GONADOTROPHINS

Luteinising hormone-releasing hormone actions on gonadotrophin release

Hormones secreted (in a pulsatile manner) from the basophilic cells of the anterior pituitary gland, are responsible for regulating the function of many target tissues around the body including the gonads (luteinising hormone; LH and follicle-stimulating hormone; FSH), the thyroid (through thyroid-stimulating hormone; TSH) and the adrenals (through adrenocorticotrophic hormone). Other functions such as lactation (through prolactin; PRL) and growth (through the actions of growth hormone; GH) are also regulated by anterior pituitary hormones. Most of the hormones of the anterior pituitary are regulated by releasing hormones or factors secreted (again in a pulsatile manner), from the hypothalamus into the adenohypophyseal portal vasculature. Luteinising hormone-releasing hormone (LHRH, pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), also widely known as gonadotrophin-releasing hormone (GnRH) because it can stimulate the release of FSH as well, is the hypothalamic decapeptide, which is chiefly responsible for the production and secretion of the gonadotrophins LH and FSH. In brief, LH acts in females to induce ovulation and then maintain the secretory functions of the corpus luteum, whilst in males LH stimulates the Leydig cells in the testis to produce testosterone. FSH stimulates the development of ovarian follicles in the female and spermatogenesis and the production of androgen-binding protein in males. The actions of LHRH are therefore important for maintenance of the reproductive cycle in mammals. In addition to regulating the synthesis and secretion of LH and FSH, LHRH additionally regulates LHRH receptor numbers and significantly, it can increase gonadotroph responsiveness to itself, an effect known as LHRH self priming [Aiyer et al, 1974; Edwardson & Gilbert, 1976; Pickering & Fink, 1976; Pickering & Fink, 1979].

The LHRH self-priming effect and its role in the pre-ovulatory luteinising hormone surge

In the majority of mammals (including humans, sheep, monkeys and rats), ovulation is triggered by a spontaneous surge in LH which is triggered by a neural signal and mediated by a surge in LHRH, which in turn is evoked by a surge of oestradiol-17β (E_2) acting on the central nervous system [Everett, 1987; Fink, 1979; Fink, 1988a; Fink, 1988b]. However, the LHRH surge is too small on its own to cause the ovulatory LH surge and is dependent upon the LHRH self-priming effect, a property of LHRH that by direct action, massively increases (20-50 fold) the responsiveness of the gonadotroph to itself [Fink, 1979; Fink, 1988a; Fink, 1988b; Fink et al, 1976; Nett et al, 1984]. LHRH self-priming acts in co-ordination with the cyclic surge of LHRH in the hypophysial portal blood so that both events reach this peak capacity simultaneously, producing a massive and coherent peak of LH release, on a day of the cycle known as pro-oestrous.

A number of major differences have been detected between the priming and releasing action of LHRH (see Table 1.1). Notably in contrast to the releasing action of LHRH, priming: (1) cannot be mimicked by K^+ depolarisation or Ca^{2+} ionophores, (2) is independent on normal extracellular Ca^{2+} concentrations, (3) involves an elongation and change in the orientation of the microfilaments, (4) involves the movement of secretory granules towards a "marginal" zone close to the plasma membrane of immunoidentified gonadotrophs, (5) involves the synthesis of a new protein (other than additional hormone) with a relative molecular mass of 70 kDa, whose exact function in priming has yet to be determined, (6) involves post receptor changes which result in the potentiation of the IP_3 and Ca^{2+} mechanisms and may involve activation of an H7-resistant form of protein kinase C (PKC) or PKC-like kinase [briefly reviewed in Fink, 1995]. Although pulses of LHRH do increase LHRH receptor mRNA in pituitary cells [Kaiser et al, 1992], the LHRH priming effect is

unlikely to be attributed to an increase in LHRH receptors because over the time scale corresponding to the onset of priming (~1 h) the increase would be insignificant [Fink, 1995]. Furthermore in studies of LHRH priming carried out on pituitary slices *in vitro*, no detectable increase was observed in LHRH binding sites after a 60 min exposure to LHRH [Mitchell et al, 1988]. Whilst E_2 appears to be required to maintain the LH surge (in part due to an indirect effect on the LHRH-releasing neurones facilitating LHRH release [Fink, 1988c]), and whilst E_2 enhances the magnitude of the priming effect, steroids do not mediate the effect [Aiyer et al, 1974; Meiden et al, 1981]. Progesterone can also enhance LHRH responses, this effect is also dependent on a prior exposure to E_2 [Aiyer & Fink, 1974; Fink, 1988b; Turgeon & Waring, 1981]. Although the exact mechanisms of this effect are as yet unresolved, it is possible that some mechanisms of LHRH, E_2 and progesterone-induced increases in pituitary responsiveness may involve a common mechanism [Fink, 1995].

Table 1.1

Differences between the "releasing" and "priming" actions of LHRH in female pituitary gonadotrophs.

Comparison between the releasing and priming actions of LHRH in the pro-oesrtrous rat

(based on Fink 1986 and Thomson 1992)

	Releasing	**Priming**
Can be repeated frequently	yes	only once every 3h
Enhanced by oestrogen	yes	yes
Dependent on extracellular Ca^{2+}	yes	no
Mimicked by high K^{+}	yes	no
Mimicked by Ca^{2+} ionophores	yes	no
Dependent on protein synthesis	no	yes
Dependent on integrity of microfilaments	no	yes
Elicited in dispersed cells	yes	not for rat pituitary glands
Cyclic AMP acts as a mediator	no	no
$Ins(1,4,5)P_3$ acts as a mediator	yes	yes
PKC acts as a mediator	no (see text)	yes (but a potentially novel species)
AA acts as a mediator	no	yes

1.4 THE LHRH RECEPTOR

Cloning of the LHRH receptor

Luteinising hormone-releasing hormone (LHRH) exerts its effect on gonadotrophin synthesis and release via plasma membrane-bound receptors primarily found in the anterior pituitary gland [Clayton & Catt, 1981]. A number of studies demonstrated that anterior pituitary tissue was a potential source of mRNA for construction of cDNA libraries from which the LHRH receptor could be subsequently cloned [Eidne et al, 1981; Sealfon et al, 1990a; Sealfon et al, 1990b; Yoshida et al, 1989]. However the use of pituitary tissue had limited success as gonadotrophs account for ~10 % of the anterior pituitary cell population, therefore LHRH receptor mRNA as a percentage of the total anterior pituitary mRNA is also likely to be low. The development of the αT3-1 cell line provided a richer source of mRNA which greatly improved subsequent cloning attempts. This cell line has been shown to abundantly express the LHRH receptor [Windle et al, 1990], indeed when αT3-1 mRNA was injected into oocytes, these oocytes were shown to be twice as responsive to LHRH as those injected with anterior pituitary mRNA [Sealfon et al, 1990a]

The first cloning of the LHRH receptor was achieved by a homology screening strategy [Tsutsumi et al, 1992]. Sets of degenerate oligonucleotides to conserved transmembrane domains of G protein-coupled receptors were used to generate polymerase chain reaction (PCR) products using cDNA templates from the αT3-1 cell line. The products were cloned and sequenced. Complementary (antisense) oligonucleotides of promising sequences were co-injected with αT3-1 mRNA into oocytes and monitored for the hybrid arrest of receptor-mediated electrophysiological responses to LHRH [Tsutsumi et al, 1992]. The mouse LHRH receptor sequence was also identified by another group using a similar approach; RNA transcribed from αT3-1 cDNA clone pools (was also expressed in oocytes) and LHRH responses

were measured with a Ca^{2+}-sensitive photoprotein, aequorin [Reinhart et al, 1992]. This mouse sequence was identical to that reported by Tsutsumi and co-workers except for one single nucleotide, the methionine at residue position 250 is converted to a leucine. In another approach a mouse LHRH receptor cDNA clone was isolated by expression of a pool of cDNA clones in transformed COSM6 cells that were subsequently screened with an iodinated LHRH analogue [Perrin et al, 1993]. The cloned murine receptor exhibited appropriate ligand binding characteristics and LHRH-stimulated inositol phosphate (IP) production when expressed in COS 1 cells [Miller, 1993]. The cloning and sequencing of the mouse LHRH receptor was closely followed by the human [Chi et al, 1993; Kakar et al, 1992], rat [Eidne et al, 1992; Kaiser et al, 1992; Perrin et al, 1993] and sheep receptors [Brooks et al, 1993; Illing et al, 1993]. These were obtained by hybridisation with random-primed cDNA, labelled oligonucleotides or labelled PCR-generated probes, all based on the mouse sequence. Southern blot analyses with mouse and rat genomic DNA are consistent with the presence of a single gene for the LHRH receptor [Zhou et al, 1994]. Although several alternative transcripts have been identified, these appear only to encode truncated proteins [Stojilkovic et al, 1994].

Alignment of the mammalian LHRH receptors cloned to date has demonstrated a high degree of homology between species and has highlighted subtle amino acid changes which may account for the small differences in pharmacology observed between the species [Illing et al, 1993]. At the amino acid level any two of the LHRH receptors share greater than 85% amino acid sequence. The mouse and rat receptors share 97% identity, the human and sheep LHRH receptors have 89% identity with each other and both contain an additional (to the mouse and rat receptors) lysine residue in the second intracellular loop. The degree of homology rises to >90% in the transmembrane (TM) domains with TM2, TM3, TM5, TM6 and TM7 being most highly conserved. Extracellular (EC) loop EC1 is also well conserved.

Molecular structure of the LHRH receptor

The hydropathy plot of the amino acid sequence of the LHRH receptor has a predicted structure which is characteristic of the superfamily of G protein-coupled receptors (apart from a truncated carboxyl-terminus), consisting of a single polypeptide chain with seven hydrophobic transmembrane domains. Based on structural similarities to the extensively characterised proteins bacteriorhodopsin and rhodopsin, which included electron diffraction data [Henderson, 1990], TM domains 1-7 are predicted to be α-helical and to be arranged in a bundle with a central hydrophilic ligand binding pocket [Baldwin, 1993]. The TM domains are connected by hydrophilic extracellular and intracellular (IC) loops or domains (EC1-3 and IC1-3; Figure 1.1). In all species the cloned LHRH receptor has three consensus sequences for N-linked glycosylation sites located in EC1 and EC2. Previous studies on SDS-PAGE gel electrophoresis of solubilised native LHRH receptors reported an approximate molecular weight of 50,000-60,000 [Clayton, 1989]. The calculated molecular weight of the cloned mouse LHRH receptor sequence protein is 37, 684 [Reinhart et al, 1992; Tsutsumi et al, 1992], indicating that the LHRH receptor is indeed glycosylated. The LHRH receptor, in common with other G protein-coupled receptors, has many conserved residues within the transmembrane domains, including a number of proline residues in TM2, TM4, TM5, TM6 and TM7 [Baldwin, 1993]. These residues probably face inwards to the hydrophilic pocket of the receptor and are proposed to induce the kinks in the α-helices, and may also have a role in ligand-induced signal transduction [Davidson et al, 1994a] A pair of cysteine residues are found in EC1 and EC2 which are presumed to form a disulphide bridge, as in many G protein-coupled receptors. An alternative site for a disulphide bridge is additionally found in the LHRH receptor between a cysteine residue in EC2 and in the N-terminus. In TM6 there is a highly conserved Phe-X-X-Cys-Trp-X-Pro-Tyr motif [reviewed in [Davidson et al, 1994a]. Potential sites for phosphorylation by cAMP-dependent kinase and protein kinase C (PKC) have been

identified in IC1, for casein kinase II in IC2 and for PKC in IC3 [Tsutsumi, 1993]. Additionally, there is a tyrosine residue at position 238 but the surrounding residues do not match the consensus phosphorylation sequence for any known kinase. Other striking features of the LHRH receptor structure include an unusually long third intracellular loop which has a high content of basic amino acids and TMD 7 which is rich in phenylalanine residues.

UNUSUAL FEATURES OF THE LHRH RECEPTOR

Absence of a Carboxyl-terminal tail and desensitisation

The LHRH receptor is exceptional amongst G-protein coupled receptors by the absence of a carboxy-terminal tail, a region which has a functional significance in most other receptors in this super-family, being frequently implicated as a major phosphorylation site in rapid desensitisation and internalisation in response to continued exposure to the appropriate ligand and G-protein coupling [Dohlman et al, 1991; Inglese et al, 1993; Ostrowski, 1992] although other regions of the receptor may also be important. There is considerable evidence to support a role for phosphorylation in these events [Hausdorff, 1990]. The intracellular portions of all G-protein coupled receptors are rich in consensus sequences for phosphorylation by kinases including protein kinase A (PKA), protein kinase C (PKC) and receptor-specific kinases such as the β-adrenergic receptor kinase (βARK) [Hausdorff, 1990; Ostrowski, 1992].

The LHRH receptor is exceptional in that it is necessarily dependent on regions of the receptor other than the C-terminal tail for G-protein interactions, desensitisation or internalisation. In αT3-1 cells the formation of inositol phosphates (IP) responses is rapidly desensitised in response to TRH, but not after a 40-90 min exposure to LHRH [Davidson et al, 1994b; Sealfon et al, 1993; Mitchell et al, manuscript in preparation]. These results are consistent with a lack of direct receptor-targeted

desensitisation being due to an absence of an a C-terminal tail and would also suggest that the putative phosphorylation sites which are present on the LHRH receptor do not mediate rapid desensitisation (at least of the inositol phosphate response). Therefore it seems likely that other mechanisms must be employed by the LHRH receptor to achieve long term desensitised receptor numbers observed *in vivo* following continuous administration of LHRH [Clayton et al, 1989; Gorospe et al, 1987; Smith et al, 1981].

Atypical amino acid substitutions in TM2 and TM7 and the spatial relationship of these two domains

Another unusual characteristic of the LHRH receptor is the exchange of aspartate (Asp) and asparagine (Asn) residues that are present in TM2 and TM7 respectively in most other G protein-coupled receptors [Cook et al, 1993; Zhou et al, 1994]. The Asp at position 87 of TM2 (murine receptor) which is usually highly conserved (98%) and apparently essential for agonist binding in other G protein-coupled receptors, is replaced with an Asn in the LHRH receptor [Probst et al, 1992]. Whereas an Asp replaces the highly conserved Asn (95%) at position 318 in TM7 and may represent a natural reciprocal mutation [Zhou et al, 1994]. The NK 1 receptor has a Asn substitution at position in 87 in TMD II and the thrombin receptor has a Asp at position 318 in TMD VII, thus although other receptors may have one or other substitution, the LHRH receptor is unique amongst this family of receptors in that it contains both substitutions.

The implications of these substitutions is currently being investigated. Mutation of Asn87 to Asp has been found to impair ligand binding and subsequent signal transduction in the LHRH receptor [Cook et al, 1993; Sealfon et al, 1993; Zhou et al, 1994] consistent with findings in other receptors and further implying that this residue is crucial to ligand binding. One group also observed a loss of binding and signalling in the double mutant Asp87, Asn318 (a mutation which has effectively restored the

Asp and Asn residues to their typical motifs in other receptors of this family) [Cook et al, 1993]. However other workers who also observed a loss in binding due to the Asp87 mutation, have reported a moderate degree of high affinity ligand binding and a partial restoration of IP response in the double mutant [Probst et al, 1992; Sealfon et al, 1993; Zhou et al, 1994; Mitchell et al manuscript submitted; see Chapter 5]. This restoration of ligand binding indicates that these two specific residues in TM2 and TM7 may be adjacent in space and could act in a complementary manner to maintain the conformation of the receptor required for ligand binding [Zhou et al, 1994]. This has important implications on the three-dimensional structure of the receptor as it places a constraint on possible alignments between TM2 and TM7 which has been incorporated into a model [Zhou et al, 1994]. It is likely that TM2 and TM7 share a common microenvironment, a condition that could be fulfilled by direct hydrogen-bonding but which may also involve a more complex network of interacting side chains in the two helices. In the current model of the mutated LHRH receptor, Asp87 is assumed to be ionised and in a hydrogen bond interaction and could only be an electron acceptor, whilst asparagine can act as both an electron acceptor (C=O) and an electron donor (NH_2). Thus the Asp87 mutant side chains would electrostatically repel and impair binding through disruption of receptor structure, whilst the double mutant or a Asn318 mutant would allow favourable interaction such as hydrogen-bonding to occur. The observation that binding in the LHRH receptor is disrupted by a single Asp87 mutation but not a single Asn318 or the double mutant is consistent with this model [Zhou et al, 1994]. Recently it has been proposed that Asn87 and/or Asp318 are involved in the regulation of the LHRH receptor lifetime at the cell surface. Mutant receptors were prepared containing an Asn at 318 or the double mutation Asp87;Asn318, when these mutant receptors were stably expressed in GH_3 cells, the Asn318 and double mutation did not impair ligand binding as observed previously [Zhou et al, 1994], but this mutation abolished the initial down-

regulation that is observed by the wild type mouse and rat LHRH receptors [Awara et al, 1996].

Atypical substitutions in TM3

Many G protein-coupled receptors contain a conserved sequence of several amino acids at the end of TM3 and the beginning of the second cytoplasmic domain [Bairoch, 1991]. This sequence contains a Asp-Arg-Tyr triplet which has been implicated in G-protein coupling. In the LHRH receptor this sequence contains two substitutions most notably a serine at position 140 within this triplet in (Asp-Arg-Tyr > Asp-Arg-Ser) to form an additional potential phosphorylation site. However the functional significance of this substitution remains unclear as mutations within the triplet of the LHRH receptor had no effect on agonist binding or IP responses [Davidson et al, 1994b].

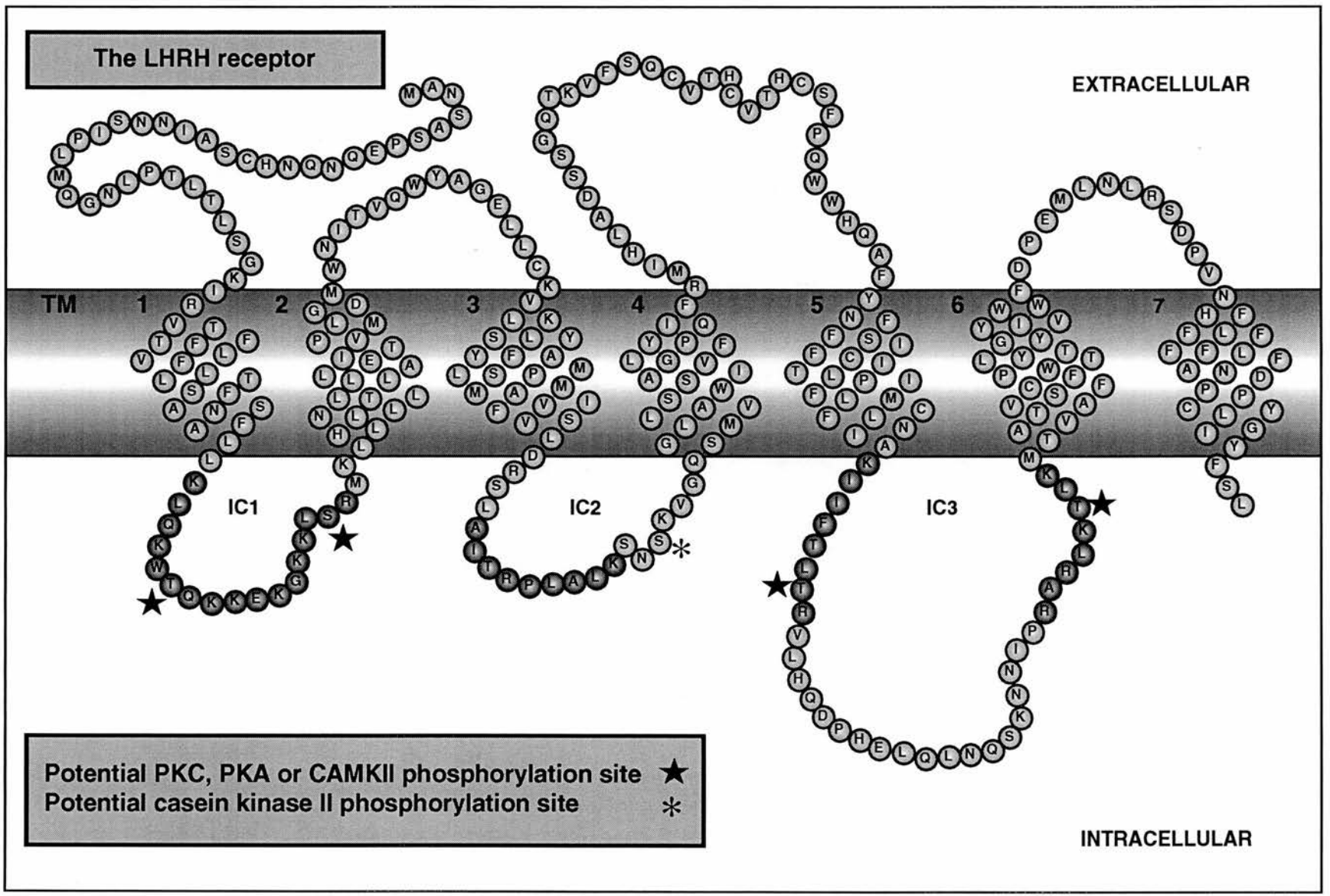
Tissue distribution and regulation of expression of luteinising hormone-releasing hormone (LHRH) receptors

LHRH receptors are found primarily in the anterior pituitary of several species and as shown in rat at least, LHRH receptors are almost exclusive to gonadotrophs [Childs, 1984; Naor & Childs, 1986]. High affinity LHRH receptors have also been identified in a number of other tissues, which include the hippocampus, lateral septal nucleus, anterior cingulate, cortex, subiculum and entorhinal cortex in the central nervous system (CNS) [Millan et al, 1985]. Consistent with their role in steroidogenesis and other cellular events in the gonads, high affinity LHRH receptors have also been identified in Leydig cells (but not Sertoli cells) of the testis, in granulosa and luteal cells of the ovary, in the placenta (although these are low-affinity LHRH binding sites which are probably a variant of those in the pituitary). Many tumorigenic tissues including pituitary adenomas, human breast cancer tissue and cell lines, human epithelial ovarian carcinomas and prostate tumours express LHRH receptors, where they exert potential antiproliferative effects. Indeed LHRH analogues have been

successfully employed as chemotherapeutic agent in some instances [Emons et al, 1993; Stojilkovic et al, 1994]. The availability of LHRH receptor cDNA has aided investigation of the tissue distribution of LHRH receptors and any potential variations in the levels of mRNAs. Both the mouse and rat receptor cDNAs have been used to probe a variety of tissues which has corroborated much of the ligand binding data [Stojilkovic et al, 1994].

Figure 1.1

The Human luteinising hormone-releasing hormone receptor


The LHRH receptor
EXTRACELLULAR
TM
1
2
3
4
5
6
7
IC1
IC2
IC3
Potential PKC, PKA or CAMKII phosphorylation site
Potential casein kinase II phosphorylation site
INTRACELLULAR

1.5 SIGNAL TRANSDUCTION MECHANISMS ASSOCIATED WITH THE LUTEINISING HORMONE-RELEASING HORMONE RECEPTOR

When LHRH binds to its receptor at the cell membrane it triggers an array of intracellular signal transduction pathways. The sequence and regulation of these mediators is not fully clarified. However the development of the αT3-1 clonal gonadotroph cell line and cloning of the LHRH receptor has led to an increase in our understanding of these mechanisms and is likely to facilitate future studies. The following mechanisms and components are postulated to be involved in LHRH actions downstream of the LHRH receptor.

G-proteins

Binding of agonists to the ligand binding pocket of seven transmembrane domain G-protein-coupled receptors such as the LHRH receptor, is thought to cause a conformational change in the receptor that leads to dissociation of heterotrimeric G-proteins into α- and βγ-subunits and the subsequent interactions of these subunits with the various effector systems. Several observations first indicated that LHRH receptor actions are mediated by G-proteins. These include the ability of GTP analogues to reduce the binding of LHRH agonists to bovine and rat pituitary LHRH receptors [Limor et al, 1989; Perrin et al, 1989], and to enhance inositol phosphate formation and gonadotrophin release in permeabilised pituitary cells [Andrews et al, 1986; Limor et al, 1989]. More recent studies have indicated that members of the pertussis toxin-insensitive, G_q subfamily of G-proteins, mediate control of phospholipase C (PLC) activity by LHRH [Hsieh & Martin, 1992]. In general, it appears that the two most studied members of the G_q family G_q and G_{11}, are equally effective in regulating PLC activity [Blank et al, 1991], and this is also likely to be the case in gonadotrophs where cellular levels of G_q and G_{11} are down regulated equally by sustained stimulation with LHRH [Shah & Milligan, 1994]. G-protein involvement

in the LHRH-induced PLC response was further investigated in the αT3-1 cell line. LHRH or a G-protein activator fluoroaluminate (AlF_4^-), caused a dose- and time-dependent IP accumulation [Anderson et al, 1993]. LHRH- or NaF-induced IP accumulation in αT3-1 cells was insensitive to pretreatment with pertussis toxin (an agent which inactivates G-proteins of the $G_{i/o}$ subclass of the G-protein family by ADP-ribosylating the α-subunit), suggesting that $G_{i/o}$-proteins are not involved in the PLC response, consistent with a role for $G_{q/11}$ proteins in this response. Moreover, immunoblot analysis of αT3-1 cell membranes revealed the presence of G_q [Anderson et al, 1993]. More recently the distribution and relative levels of expression of $G_{q/11}\alpha$ subunits was investigated in αT3-1 cells as part of a wider study of G-protein expression [Milligan, 1993]. Whilst G_q and G_{11} are both expressed, the relative level of these two G-proteins varies, with higher steady-state levels of immunoreactive $G_{11}\alpha$ than $G_q\alpha$.

Phospholipase C

Agonist binding to G-protein coupled, Ca^{2+}-mobilising receptors (for example the LHRH receptor) is associated with a rapid increase in phosphoinositide-specific phospholipase C (PLC) activity, leading to hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) and formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). There is now known to be a large family of at least 16 PLC isoforms that fall into three main types: PLC-β, PLC-γ and PLC-δ. All three types of PLC catalyse the hydrolysis of PIP_2. The α subunits of the G_q family specifically activate PLC-β1 *in vitro*, with a weak activation of this enzyme reported by βγ subunits, whilst PLC-β2 is only weakly activated by $G\alpha_q$ subunits and powerfully activated by βγ subunits, indicating a role for βγ subunits in mediating pertussis toxin-sensitive phosphoinositide (PI) hydrolysis by G-proteins of the $G_{i/o}$ class. PLC-β3 could underlie both pertussis toxin-insensitive (G_q α subunits) and -sensitive (βγ subunits) receptor linked PI hydrolysis, as it is activated by both α and βγ subunits.

PLC-γ1 and 2 are activated following activation of some receptor tyrosine kinases, or T-cell antigen receptors and some immunoglobulin receptors through activation of the Src family of intracellular tyrosine kinases. The cellular mechanism for control of PLC-δ1 and 2 is still unknown. All the PLCs require Ca^{2+} for catalytic activity and may additionally be regulated by PKC and PKA [Koch et al, 1991; Musacchio et al, 1992; Rhee & Choi, 1992; Schlesinger & Ullrich, 1992].

Activation of PLC is a general characteristic of LHRH receptor signalling, consistent with the role of the products of PLC activity in the cellular actions of LHRH in the gonadotroph. LHRH-induced PI turnover in pituitary cells was demonstrated some years ago [Naor & Catt, 1981] and PIP_2 has since been identified as the major substrate for PLC following LHRH receptor activation in gonadotrophs [Morgan et al, 1987; Naor & Childs, 1986]. LHRH causes a rapid and progressive increase in the formation of IP_3 and the higher phosphoinositols, inositol tetrakisphosphate and inositol pentakisphosphate [Mitchell et al, 1988; Morgan et al, 1987; Schrey, 1985]. Furthermore LHRH-induced IP_3 generation is biphasic, with an early peak within 10 sec and a subsequent slower rise and thus represents a primary and sustained response to agonist stimulation. These responses can be abolished by the addition of a specific LHRH receptor antagonist [Morgan et al, 1987]. Following PLC activation, IP_3 is catabolised by at least two pathways, dephosphorylation to $I(1,4)P_2$, I(4)P and inositol and phosphorylation to $I(1, 3, 4, 5)P_4$ and subsequent dephosphorylation to the inactive $I(1, 3, 4)P_3$ [Horn et al, 1991; Zheng & Guan, 1993].

Kinetic studies in αT3-1 cells have revealed that the time course of LHRH-induced PI hydrolysis is similar to that observed in native gonadotrophs, with an initial rapid peak followed by a sustained later phase [Anderson et al, 1993]. IP_3 catabolism has also been described in αT3-1 cells [Horn et al, 1991; Zheng & Guan, 1993]. The potency with which LHRH stimulates IP accumulation in αT3-1 cells is affected by

estradiol pretreatment, suggesting that ovarian steroids modulate the coupling of LHRH receptors to second messenger systems [McArdle et al, 1992; Mitchell et al, 1995a]. The cloned LHRH receptor can also induce PI hydrolysis when expressed in host cells. In COS-1 cells transfected with receptor cDNAs, LHRH increased total inositol phosphates [Chi et al, 1993; Eidne et al, 1992], whilst IP_3 formation, in particular, was also observed in COSM6 cells expressing mouse and rat cDNAs [Perrin et al, 1993].

LHRH action in pituitary gonadotrophs also evokes a prominent increase in DAG accumulation [Chang et al, 1987a], which associated with inositol phosphate (IP) formation, indicating that DAG formation is associated with PLC hydrolysis of phosphoinositides. DAG formation is biphasic with an initial spike within 45 s followed by a larger peak between 15-30 min of stimulation. The amplitude of the second sustained DAG peak in response to agonist is larger than the corresponding IP peak [Chang et al, 1987a], indicating that as in other cell types, a significant proportion of the DAG generated may also be derived from other pathways, such as the hydrolysis of phosphatidylcholine by phospholipases A_2 or D.

Ca^{2+} responses

Ca^{2+}-mobilising receptors release Ca^{2+} into the cytoplasm from vesicular stores as a consequence of IP_3 binding to intracellular receptors on the mitochondrial membranes and endoplasmic reticulum [Berridge & Irvine, 1989]. The cloned mouse, rat and human receptors mediated LHRH-induced increases in Ca^{2+} as measured by current responses in oocytes [Chi et al, 1993; Kaiser et al, 1992; Tsutsumi et al, 1992]. Ca^{2+} responses were additionally demonstrated for the mouse and human receptors by Fura-2 fluorescence in transfected COS 7 cells and by monitoring light emitted from aequorin-injected oocytes [Reinhart et al, 1992]. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements in pituitary and αT3-1 cells have confirmed that LHRH stimulates a rapid peak increase followed by a lower but sustained

elevation of $[Ca^{2+}]_i$. This matches the time scale of LHRH-induced IP_3 release in a time- and dose-dependent manner with an apparent EC_{50} similar to that observed for LH release. The initial phase of the $[Ca^{2+}]_i$ response is independent of extracellular Ca^{2+}, whilst the sustained phase is dependent on Ca^{2+} entry through voltage-sensitive and -insensitive Ca^{2+} channels [Johnson et al, 1993b; Merelli et al, 1992; Mitchell et al, 1988; Virmanni et al, 1990]. The temporal correlation between the second phase of IP_3 production and the extracellular Ca^{2+}-dependant elevation of $[Ca^{2+}]_i$ suggests that IPs may regulate the dihydropyridine-insensitive component of Ca^{2+} entry in gonadotrophs.

Protein kinase C

A key family of enzymes in LHRH-induced signal transduction is the protein kinase C (PKC) family of protein serine/threonine kinases. DAG formed from PI hydrolysis by PLC can elicit functional signals by activating this enzyme family. Briefly, all members of this family are a single polypeptide chains with an N-terminal regulatory domain and a C-terminal catalytic domain with several conserved and variable sequences. The catalytic domain contains the ATP- and substrate-binding sites and is subject to regulation by a pseudosubstrate sequence that is believed to bind to the catalytic domain and maintain the enzyme in an inactive form. The regulatory domain also contains the Ca^{2+}, Zn^{2+}, DAG/phorbol ester and phospholipid binding sites. Several of these cofactors or compounds are able to unmask the catalytic domain and cause activation of the enzymes [Nishizuka, 1992]. The PKC isozymes can be categorised into three groups: the 'classical' A group (α, βI, βII and γ) isoforms are responsive to Ca^{2+}, phospholipid (particularly phosphatidyl serine; PS), DAG and phorbol esters; the 'novel' group B (δ, ε, η and θ) isoforms lack the Ca^{2+}-binding domain and hence do not require Ca^{2+} for activation; the 'atypical' group C (ζ and λ) isoforms are sensitive to PS but are not affected by Ca^{2+}, DAG or phorbol esters. The α, βI, βII, δ, ε, ζ, λ and θ but not γ or η isoforms have been identified by

immunoblotting analysis in the anterior pituitary and GH_3 cells and the α, ε, ζ and θ are found in the αT3-1 cell line [Johnson et al, 1993a; Johnson et al, 1996; Naor, 1990a].

PKC regulates numerous intracellular events including: Ca^{2+} release by modulation of IP_3 receptor channel functions; Ca^{2+} entry through voltage-sensitive Ca^{2+} channels, down-regulation of receptors, modulation of Na^+, K^+ and glutamate channels, control of phospholipase activity and participation in exocytosis [Nishizuka, 1986]. PKC activity is modulated by an array of feedback regulation by a number of the products from these intracellular events which it regulates. PKC activation is also an essential element in the control of cell growth and differentiation, although it is not necessarily an absolute requirement for all of these actions [Nishizuka, 1986]. PKC also appears to be an important mediator of the LHRH priming effect. The priming-induced increment in LHRH-induced LH release *in vitro* is selectively blocked by a range of PKC inhibitors, without affecting unprimed or previously-primed responses and K^+- or ionomycin-induced LH release [Johnson et al, 1992; Thomson et al, 1993b]. However, the potency of the PKC inhibitor, H7, was very clearly less than other inhibitors on this and other PKC mediated responses [Johnson et al, 1992; Thomson et al, 1993b]. This initiated the hypothesis that the anterior pituitary may additionally express a distinct form or potentially novel species of PKC. This species of PKC is also involved in other LHRH receptor mediated responses including the facilitation of pituitary L-type Ca^{2+} channels [Johnson et al, 1993b; Mitchell et al, 1988; Mitchell & Johnson, 1987], PLA_2 (on pro-oestrous) [Thomson et al, 1994] and PLD activation [Fennell et al, 1993]. A phorbol-activated, Ca^{2+}-independent H7-resistant PKC has since been characterised following partial separation from other PKC isoforms by DEAE cellulose and on hydroxyapatite fractionation [Ison et al, 1993].

Phospholipase A_2

In addition to PLC, membrane phospholipids are the substrate for phospholipase A_2 (PLA_2). The action of PLA_2 liberates a number of metabolites including lysophospholipids and *cis*-unsaturated free fatty acids. Cytosolic PLA_2 selectively cleaves arachidonic acid (AA) from the sn-2 position which may act both as a biological second messengers in its own right [Nishizuka, 1986] and also serves as the precursor for lipoxygenases and epoxygenases (via the cyclooxygenase pathway) which are important inflammatory mediators [Irvine, 1982; Samuelsson et al, 1987]. PLA_2 activity is also regulated by PKC, based on the observation that phorbol esters and membrane permeant DAGs induce AA release [Gronich et al, 1988; Wijkander & Sundler, 1991]. PLA_2 activation has a role in the cellular actions of LHRH. Arachidonic acid and several of its metabolites stimulate gonadotrophin release in anterior pituitary pieces *in vitro*, in perifused cells, or static primary cultures of rat anterior pituitary cells and goldfish pituitary cells [Chang et al, 1986; Chang et al, 1987b; Kiesel & Catt, 1987; Naor et al, 1985; Thomson et al, 1994]. The effects on LHRH release can be mimicked by exogenous application of PLA_2 and is inhibited by several lipoxygenase inhibitors [Naor et al, 1985] or leukotriene antagonists [Kiesel & Catt, 1987]. In our laboratory it has been shown that PLA_2 inhibitors prevent LHRH priming but not unprimed or previously-primed LHRH-induced LH release [Thomson et al, 1994] and that the magnitude of phorbol ester-induced, PLA_2-mediated, LH release matches those days of the oestrous cycle where LHRH priming can occur [Thomson et al, 1994; Thomson et al, 1991]. On pro-oestrous, LHRH-induced [^{3}H]AA production is resistant to H7 but not to other PKC inhibitors, whilst the response to phorbol ester displays H7-sensitive and -resistant components [Thomson et al, 1994]. The magnitude of [^{3}H]AA responses also matches the ability of tissues to display LHRH priming [Thomson et al, 1994].

Phospholipase D

The initial rise in DAG production following LHRH receptor stimulation is usually followed by a sustained increase in DAG production [Liscovitch, 1992]. Activation of phospholipase D (PLD) promotes the hydrolysis of membrane phospholipids, primarily phosphatidylcholine (PC), into choline and phosphatidic acid (PA) which can be further metabolised to form DAG; thus activation of this pathway may represent an important contribution to maintaining a sustained DAG accumulation. In αT3-1 cells LHRH receptor activation results in a pronounced activation of PLD, as assessed by measurement of accumulation of phosphatidylethanol or phosphatidylbutanol (PEt or PBut), a specific product of PLD phosphatidyl transferase activity when ethanol/n-butanol is added as an artificial phosphatidyl group acceptor [Fennell et al, 1993; Netiv et al, 1991] This response occurs after a brief delay (~2 min), is blocked by an LHRH receptor antagonist and is not desensitised over a 120 min time course of exposure to LHRH [Fennell et al, 1993; Netiv et al, 1991]. PKC α and β_1 isoforms have been implicated in activation of PLD [Eldar et al, 1993; Pai et al, 1991] and phorbol esters have been shown to partially mimic the LHRH-induced response in αT3-1 cells [Fennell et al, 1993; Zheng et al, 1994]. Responses to LHRH and phorbol esters are additive [Fennell et al, 1993]. Whilst LHRH-induced PLD activity is sensitive to standard PKC inhibitors, it appears resistant to the inhibitory actions of H7 and the response to phorbol esters is inhibited with greater potency than that to LHRH. Phorbol ester down-regulation of phorbol-sensitive PKC isoforms diminished the response by 70% suggesting a possible involvement of a phorbol ester-insensitive PKC isoform such as PKC ζ. Interestingly a component of the PKC involvement in LHRH-induced PLD responses appears to show properties consistent with the novel species of PKC described in anterior pituitary PKC assays [Ison et al, 1993]. In addition LHRH-, but not phorbol ester-induced PLD activation appears to involve a tyrosine kinase, as LHRH-induced responses are sensitive to tyrosine kinase inhibitors [Fennell et al, 1993]. Also, two

PLC inhibitors U73122 and neomycin, reduced the amplitude of the PLD response to LHRH but did not effect phorbol ester-induced PEt accumulation [Zheng et al, 1994]. Thus it appears that PLD can be activated by two mechanisms in αT3-1 cells: one involving tyrosine kinases and another a species of PKC (which is relatively resistant to the PKC inhibitor H7); or by standard PKC species.

Tyrosine phosphorylation

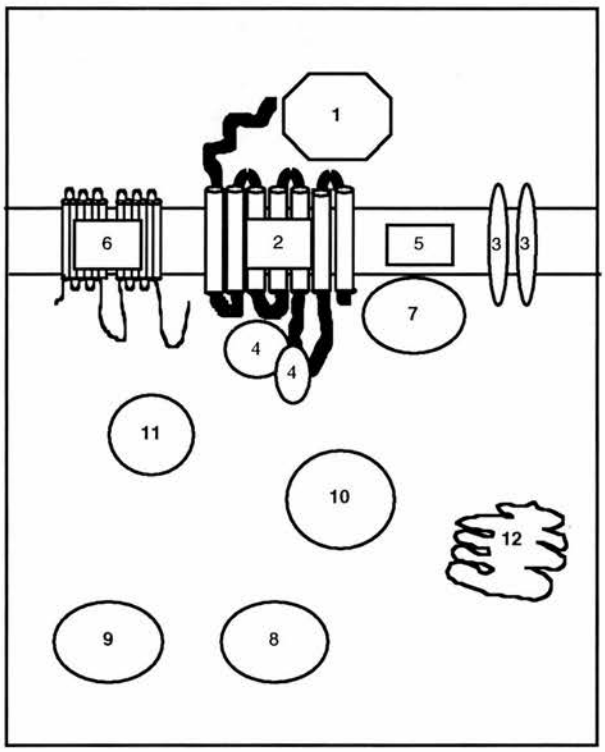
LHRH elicits tyrosine phosphorylation of a number of proteins in αT3-1 cells and in pro-oestrous pituitary cells (as assessed by antiphosphotyrosine immunoblots) [Fennell et al, 1993]. Some of these tyrosine phosphorylations can be mimicked by phorbol esters but not by ionomycin. The species of tyrosine kinases are not established but immunoreactivity for *src* and *fyn* but not *lyn*, *hck*, *yes* or *fgr* has been identified in these cells [Fennell et al, 1993]. PLC-dependent activation of tyrosine kinases by LHRH has been described in αT3-1 cells, furthermore it appears likely that this is an important event in the priming of inositol phosphate production which underlies LHRH self priming [Mitchell et al, 1995a; Mitchell et al, 1995b]. Nevertheless the regulation of tyrosine phosphorylation in cellular responses to LHRH is likely to be complex since PKC can activate tyrosine dephosphorylation [Brautigan & Pinault, 1991] and LHRH-induced tyrosine phosphorylations have been described [Liebow et al, 1991]. In pro-oestrous pituitary tissue LHRH-, but not phorbol ester- or ionomycin-induced LH release is inhibited by tyrosine kinase inhibitors but without a specific blockade of priming [Johnson et al, 1995]. One possible functional role of tyrosine phosphorylation, might be the selective release of LH over FSH (especially important for regulating the different time courses of the LH and FSH surge *in vivo*), as LHRH-induced FSH release from pro-oestrous tissue appeared to be relatively less sensitive to tyrosine kinase inhibitors [Johnson et al, 1995].

cAMP and cGMP signalling

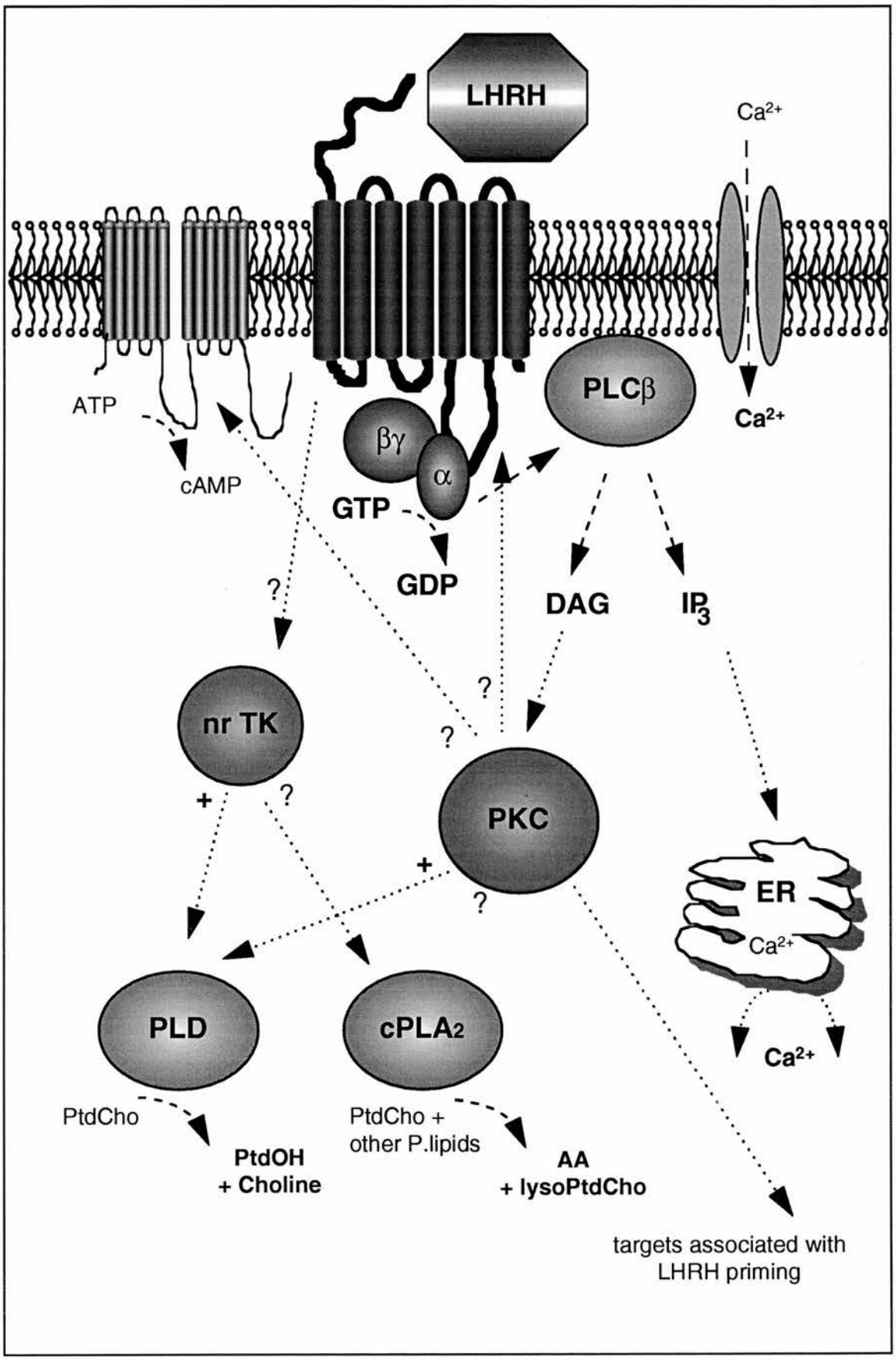
During prolonged stimulation of cultured pituitary cells, LHRH can induce a moderate increase in both cAMP and cGMP [Liu & Jackson, 1981; Naor et al, 1975; Naor et al, 1978]. It is possible this is of importance in LHRH-induced gonadotrophin synthesis but these second messengers do not appear to be involved in LHRH-induced exocytosis of gonadotrophins [Conn et al, 1979; Naor & Catt, 1980]. It is notable that LHRH-induced cAMP accumulation has not been observed in αT3-1 cells [Horn et al, 1991], and it is possible therefore that increases in cyclic nucleotides may result from paracrine effects within the subpopulations of pituitary cells in primary culture.

Figure 1.3

Schematic representation of the signal transduction mechanisms associated with the LHRH receptor



1. Luteinising hormone releasing-hormone peptide
2. The luteinising hormone releasing-hormone receptor
3. Voltage-gated and receptor operated Ca^{2+} channe
4. Heterotrimeric G-protein ($G_{q/11}$)
5. Plasma membrane containing phospholipids
6. Adenylyl cyclase
7. Phospholipase C β
8. Phospholipase A_2
9. Phospholipase D
10. protein kinase C
11. non-receptor tyrosine kinases
12. Endoplasmic reticulum(with IP_3 receptors)


LHRH
Ca2+
ATP
cAMP
βγ
α
GTP
GDP
PLCβ
Ca2+
?
DAG
IP3
nr TK
?
?
+
?
PKC
+
?
ER
Ca2+
PLD
cPLA2
Ca2+
PtdCho
PtdOH
+ Choline
PtdCho +
other P.lipids
AA
+ lysoPtdCho
targets associated with
LHRH priming

1.6 MITOGEN-ACTIVATED PROTEIN (MAP) KINASES

Background

In the late 1980s Sturgill and Ray first described a 42 kDa serine/threonine specific protein kinase which was rapidly phosphorylated on both tyrosine and threonine in response to stimulatory agents [Ray & Sturgill, 1987; Ray & Sturgill, 1988] This enzyme was named microtubule-associated protein kinase 2 or MAP2 kinase, because it utilised MAP 2 as a substrate *in vitro* [Ray & Sturgill, 1988] Over the next few years this protein kinase acquired a number of names and acronyms from independent research groups, but is now commonly referred to as mitogen-activated protein (MAP) kinase, reflecting its well-documented role in cell cycle control and activation in response to a wide array of diverse mitogens such as insulin, growth factors (e.g. epidermal growth factor, platelet-derived growth factor; fibroblast growth factor, insulin-like growth factor 1), phorbol esters and AlF_4^- [for early review see: [Sturgill & Wu, 1991; Cobb et al, 1991; Thomas, 1992]. MAP kinase requires phosphorylation at both tyrosine and threonine residues for full activity; removal of phosphate either from threonine by phosphatase 2A or from tyrosine by CD45 results in inactivation of these enzymes [Anderson et al, 1990; Boulton et al, 1991]. Both these phosphorylations have been localised within a short tryptic peptide in subdomain VIII [Payne et al, 1991] just upstream of the conserved Ala-Pro-Glu motif found in all serine/threonine kinases [Hanks et al, 1988] and were assigned to Thr183 and Tyr185 by alignment of the tryptic peptide with the cDNA sequence of p42 MAP kinase [Boulton et al, 1991; Her et al, 1991]. The requirement for dual phosphorylation to gain full activation of this enzyme led to the hypothesis that MAP kinases represented a convergence point for signalling input from multiple pathways [Anderson et al, 1990]. Furthermore serine/threonine-specific protein kinases have been identified as regulatory effectors in numerous cellular activities, but often the cell surface receptors that initiate these changes are protein tyrosine kinases [Cobb

et al, 1991], therefore the discovery of a tyrosine-regulated serine/threonine kinase which could effectively "switch" these signals prompted immense interest. However MAP kinases are no longer regarded as "switch kinases", as it has since been shown that these proteins are not directly activated by tyrosine kinase receptors. In a remarkably short period since the discovery of MAP kinases, our understanding of these enzymes, their regulation, cellular targets and expression has increased dramatically.

The MAP kinase family

Much of our current knowledge of MAP kinase family and its regulators was uncovered by homology screening for components of MAP kinase pathways in the budding yeast *Saccharomyces cerevisiae* [recently reviewed in [Levin & Errede, 1995; Waskiewicz & Cooper, 1995]. MAP kinases are broad specificity, proline-directed serine/threonine kinases which function in at least five, physiologically distinct, signalling pathways in this yeast including the mating pheromone pathway (Fus3/Kss1), pseudohyphal development and haploid invasive cell growth (unnamed), cell integrity (Mpk1), sporulation (Smk1) and response to high osmolarity (Hog1). Of these, the mating response pathway has most in common with mammalian growth factor and G-protein receptor signalling. Following the discovery of multiple, related but distinct, MAP kinase pathways in yeast, recent work has highlighted the emergence of related MAP kinase pathways in mammalian cells. Whereas mitogens, growth factors and a growing array of other agents, many of which act through G-protein coupled receptors with no intrinsic tyrosine kinase activity, lead to the activation of the ERK (extracellular signal-regulated kinase) family [Cobb et al, 1991], many forms of cellular stress including cycloheximide treatment, ultraviolet radiation (UV), tumour necrosis factor (TNFα) interleukin-1 and heat shock lead to the activation of two related signalling pathways: the stress-activated protein kinase (SAPK), also known as Jun N-terminal kinase (JNK),

because they phosphorylate c-Jun at Ser63 and Ser73 to functionally activate it as a transcription factor and p38 or CSBP/RK which is the mammalian counterpart of the HOG1 high osmoregularity pathway in yeast [Davis, 1994; Kyriakis et al, 1994; Pombo et al, 1994; Sanchez et al, 1994]. Although the activators of these two kinases overlap, there are distinct differences in the upstream activators and downstream targets of SAPK and p38 [Zanke et al, 1996].

At present there are 5 members of the ERK family. ERKs 1 and 2 are by far the most abundant and studied isoforms. ERK1 (44 kDa) and ERK2 (42 kDa) are the products of two distinct genes but are highly related with 83% homology between the two enzymes [Boulton et al, 1991]. They are most closely related to the cdc2 family of kinases which are also regulated by dual phosphorylation and are between 37% and 56% homologous to Fus3/Kss1. ERK3 (62.6 kDa) encodes a more distantly related kinase with an additional 180 amino acid C-terminal extension, which is most closely related to ERK1 (~50% within the catalytic domain) [Boulton et al, 1991; Robbins et al, 1993], however within certain subdomains of the ERKs, these enzymes are more highly conserved (e.g. Subdomain V, VI, VII, IX and XI) [Hanks et al, 1988]. ERK 4 (45 kDa) was also identified by cross reactivity with ERK1 and ERK2 antiserum [Boulton et al, 1991], and has since been characterised in PC12 cells, demonstrating similar characteristics of response to growth factors, as those shown by ERKs 1 and 2 [Peng et al, 1996]. ERK5 is the largest of the ERK family at 815 amino acids; the C-terminus of ERK5 has sequences which suggest it may be targeted to the cytoskeleton [Zhou et al, 1995]. ERKs 1 and 2 are uniformly distributed but are especially prolific in the brain and peripheral nervous system. ERK3 is found especially in hindbrain, the nervous system and skeletal muscle [Boulton et al, 1991]. ERK5 is expressed in many adult tissues and is abundant in the heart and skeletal muscle [Zhou et al, 1995]. The distribution pattern of ERK expression suggests that each of these isoforms may have distinct functions in the tissues and cell lines examined [Boulton et al, 1991; Cobb et al, 1991].

Molecular cloning revealed at least eight known members of the JNK/SAPK family [Davis, 1994; Derijard et al, 1994; Kyriakis et al, 1994]. These are derived by differential splicing from three or more genes (α, β and γ) which range in size from 45-54 kDa and are 43-44% identical to the catalytic domains of ERK1 and ERK2. JNK1 and JNK2 correspond to p46SAPKγ and p54SAPKαII whilst a brain specific isoform $p49^{3F12}$ has been isolated which corresponds to p54SAPKβ [Mohitt et al, 1995]. Like the ERK family, the SAPK/JNKs require phosphorylation on a closely spaced tyrosine and threonine within subdomain VIII. However members of this family of proteins contain a proline between the phosphorylation sites (TPY), whereas the ERKs contain glutamine (TEY) and p38s contain glutamic acid in this position (TGY) [Davis, 1994; Kyriakis et al, 1994; Pombo et al, 1994; Sanchez et al, 1994]. Transcripts for this kinase are widely expressed, especially in skeletal muscle. Also activated by stress, but differing in substrate specificity is p38, the mammalian counterpart of the yeast HOG1 [Han et al, 1994].

Activation and upstream regulation of MAP kinases

Between the cell surface receptors and MAP kinase lies a complex, hierarchical signalling system including adaptor proteins, GTPases and many protein kinases. Up to six tiers in this cascade contribute to the amplification and specificity of signal transduction which eventually leads to the activation of several signalling molecules in the cytoplasm and nucleus. Of particular note, the tyrosine and threonine phosphorylations required for full MAP kinase activity are achieved by one enzyme from a family of dual-specificity tyrosine/threonine kinases [Crews, 1991; Gomez & Cohen, 1991; Matsuda et al, 1992; Seger et al, 1992; Zheng & Guan, 1993]. Thus a diverse range of external stimuli which lead to MAP kinase activation converge upstream of MAP kinase by activating sequential cascades of highly related protein kinases which form a core module consisting of a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MEK) and finally MAP kinase

(MAPK). The known components of the MAP kinase pathways in yeast and mammals is shown in Figure 1.3. This organisation provides multiple sites for signal modulation as well as the potential for multiple sites for integration of input signals and divergent output signals [Seger & Krebs, 1995; Waskiewicz & Cooper, 1995].

Pheromone response pathway in *S. Cerevisiae* - Fus3/Kss1

The mating response intracellular signalling pathway comprises a heterotrimeric G-protein, small Ras-like GTPases and several sequentially acting protein kinases [Errede & Levin, 1993]. Binding of the mating factor to the pheromone receptor triggers a dissociation of the G-protein. The free $\beta\gamma$ subunit stimulates a MAP kinase module in a mechanism which additionally involves the serine/threonine kinase Ste20. The Ste11 protein (the MEKK of this module) phosphorylates and activates Ste7 (the MEK) which in turn phosphorylates and activates Fus3 and Kss1 (the MAPKs). More recently it was revealed that Ste11, Ste7, Fus3 and Kss1 associate independently with the protein Ste5 and similarly with each other [Choi et al, 1994; Marcus et al, 1994; Printen & Sprague, 1994]. These observations are consistent with Ste5 acting as a scaffold for the components of the MAP kinase module, which has been suggested as a potential mechanism for minimising cross-interaction between kinases involved in other similar paths in order to maintain the fidelity of signal transmission in cells expressing components of multiple signalling pathways.

Mammalian mitogen/hormone responses - ERKs 1 and 2

At present the best understood of the mammalian MAP kinase pathways is that leading to the activation of ERKs 1 and 2. Most stimuli which activate ERKs involve activation of the Ras family of plasma membrane-anchored, small molecular weight G-proteins. Growth factor- and cytokine-induced stimulation of tyrosine kinase-receptors and associated tyrosine kinases, recruits many proteins to the plasma membrane including Ras-specific guanine nucleotide releasing proteins (GNRPs;

predominantly Sos) [Marshall, 1995]. Under the influence of a GNRP, Ras proteins bind GTP resulting in the activation of the Ras signal. Increased Ras-GTP loading by GNRPs is achieved through interaction of an adaptor protein Grb2 which contains a "Src homology" (SH) 2 domain flanked by two SH3 domains. The SH2 and SH3 regions are binding sites for tyrosine phosphoproteins (SH2) and specific proline rich motifs (SH3) respectively [Koch et al, 1991; Mussachio et al, 1992]. On one hand Grb2 can bind Sos through its two SH3 groups on the other it can bind to tyrosine kinase substrates (either the autophosphorylated tyrosine kinase receptor or Shc, a substrate of Src kinases) [Egan et al, 1993; McCormick, 1993; Rozakis-Adcock et al, 1993]. MAP kinases can also be activated by seven transmembrane-domain spanning G-protein -coupled receptors. As G-protein receptor-mediated activation of MAP kinase is of most relevance to this particular study, this area will be covered in more details in the discussions of the relevant chapters. However it appears that G-protein coupled receptors can intercept the growth factor signalling cascade at an early stage of the signalling cascade, often at the level of stimulating increased Ras-GTP loading [Van Biesen et al, 1995; Van Corven et al, 1993, Davis, 1994]. The precise mechanisms of this action are as yet still unresolved but a general trend is becoming apparent. Receptors which couple primarily to the $G_{i/o}$ family of G-proteins such as the LPA and α_2 adrenergic receptor, appear to activate ERKs 1 and 2 through a $\beta\gamma$-subunit-dependent activation of Ras and is independent of the $\alpha_{i/o}$ subunit or protein kinase C [Hawes et al, 1995, Faure et al 1995, Van Biesen et al, 1995, Luttrell et al, 1995]. Furthermore recently it has been proposed that this activation involves interaction of pleckstrin homology domains within there signalling cascade components [Luttrell et al 1995]. In contrast, it appears that receptors which couple primarily to $G_{q/11}$ proteins such as the M_2 muscarinic receptor activate Ras or a downstream kinase Raf-1 in a mechanism which involes $\alpha_{q/11}$ activation of PLCβ and consequently protein kinase C but does not involve the $\beta\gamma$ subunits [Hawes et al 1995; Buhl et al 1995]. Ras-GTP binds directly to the serine/threonine kinase Raf-1

forming a transient membrane-anchoring signal [Avruch et al, 1994; Leevers et al, 1994]. Thus growth factor and G-protein coupled receptor signalling appear to converge at Raf-1. In addition a number of Ras effectors including Ras-GTPase-activating protein (GAP), phosphatidyl inositol 3-kinase, PKCζ and MEKK1 have recently been identified [Marshall, 1996; Marshall, 1995].

Raf-1 is the MAPKKK or MEK kinase in this module, also analogous to Ste11 in the yeast cascade. The native structure of Raf-1 is a large multisubunit protein complex [Wartmann & Davis, 1994]. Analysis of the structure of the Raf complex demonstrates that it contains a single Raf protein kinase with the molecular chaperones hsp90 and p50 and changes of the complex are not required or observed in activated cells for activation of Raf-1 [Wartmann & Davis, 1994]. Localisation of Raf-1 to the plasma membrane is insufficient for full activation, as membrane associated Raf-1 (by the addition of a constitutive membrane localisation tag or by binding to Ras, *in vitro*) did not result in full activity [Leevers et al, 1994; Stokoe et al, 1994]. Furthermore membrane associated Raf-1 is activated 8-10 fold by the EGF receptor in a Ras-dependent manner, suggesting the presence of additional factors [Leevers et al, 1994; Stokoe et al, 1994]. It is now known that the Ras-induced translocation of inactive Raf, permits the multi-site phosphorylation and phosphotransferase activity of Raf-1 by a variety of membrane associated kinases [Morrison, 1995; Waskiewicz & Cooper, 1995]. The Src family of non-receptor tyrosine kinases activate Raf-1 by phosphorylating Tyr340 and Tyr341 [Marais et al, 1995]. Phorbol esters can also activate Raf-1 by activating PKC, which phosphorylates and activates (*in vitro*) Raf-1 at residues Ser497, Ser499 and Ser619 [Van Marquadt et al, 1994; Van Renterghem et al, 1994]. Interestingly (as mentioned previously), the zeta isoform of PKC (PKCζ) is a downstream target of Ras, perhaps by a direct interaction of Ras and PKCζ, or by activation of the PI-3 kinase [Berra et al, 1995]. Another family of kinases which cause a striking activation of Raf-1 are the ceramide-activated kinases (CAKs); CAKs stimulate Raf-1

phosphotransferase activity by phosphorylation of Thr268 and Thr269 residues [Yao et al, 1995].

The downstream target of Raf-1 is MEK 1 or 2 (MAPK or ERK kinase; also known as MKK), the kinase equivalent to Ste7 in the yeast cascade. MEK was first identified as a 45 kDa protein threonine/tyrosine kinase and was the first such enzyme identified which showed dual specificity towards both tyrosine and threonine residues [Crews, 1991; Gomez & Cohen, 1991; Matsuda et al, 1992; Seger et al, 1992; Zheng & Guan, 1993]. MEKs 1 and 2 are activated following phosphorylation on Ser residues 218 and 222 [Alessi et al, 1994; Pham et al, 1994; Yan & Templeton, 1994; Zheng & Guan, 1994]. The importance of phosphorylation of these residues was further demonstrated. Substitution of Ser residues with Asn residues at 218 and 222 mimics the effect of Ser phosphorylation in MEK; expression of these mutant MEK proteins has been shown them to be constitutively active *in vitro* and oncogenic *in vivo* [Cowley et al, 1994; Mansour et al, 1994]. MEK 1 is phosphorylated and activated *in vitro*, by all three members of the Raf-1 family (A-Raf-1, B-Raf-1 and c-Raf-1) [Jelinek et al, 1994; Wu et al, 1996]. Furthermore MEK1 is found to be bound to the Raf-1 enzymes in inactive complexes in the cytoplasm [Jelinek et al, 1994; Wu et al, 1996]. MEK 1 and 2 are the product of a distinct genes [Zheng & Guan, 1993]. MEK 1 is the isoform reported to be favoured by Ras activation [Jelinek et al, 1994], however MEK2 (which is 7 amino acid longer and 81% homologous to MEK1), has been suggested to be the most potent ERK activator *in vitro*. Mammalian MEKs 1 and 2 contain a proline-rich sequence which appears unique to Raf-1-activated MEKs, as it is absent in the equivalent enzymes of the stress-activated MAP kinase pathways and the yeast homologues [Catling et al, 1995]. MEK1 additionally contains an important site of phosphorylation within this proline-rich sequence which is required for the sustained activation of MEK1 in response to serum stimulation and is absent in MEK2 (perhaps explaining the transient serum-induced activation of MEK2 in Rat-1 fibroblasts) [Catling et al, 1995]. In contrast to ERK, where tested

MEK appears to localise exclusively in the cytoplasm [Zheng & Guan, 1994]. MEK 1 is additionally phosphorylated on Ser residues 218 and 222 by a number of other kinases including: Mos a proto-oncogene-encoded 37 kDa serine/threonine kinase isolated in *Xenopus laevis* oocytes [Papin, 1996; Pham et al, 1995]; a Ras-activated kinase also from *Xenopus* oocyte cytosol, called REKS [Kuroda et al, 1995]; an insulin-activated 56 kDa kinase in rat adipocytes, called MEKK-1; a murine Ste11-related kinase known as MEKK1, which may also be directly activated by Ras [Russell et al, 1995] and Ras-activated PKCζ [Berra et al, 1995]. The mechanism by which PKCζ activates MEK requires further clarification, however may also involve Raf-1 or other kinases. Recently 12 kDa protein has been reported, which is proposed to enhance the rate at which ERK 2 is phosphorylated by MEK 1 and 2, but this has yet to be characterised [Scott et al, 1995].

Recently a new Raf-like protein has been described; Ksr1 for kinase suppresser of Ras, because its loss can compensate for the expression of activated Ras to restore normal signalling in genetic studies of *Drosophila melanogaster* and *Caenorhabditis elegans* [Kornfeld et al, 1995; Sundara & Han, 1995; Therrien et al, 1995]. Murine and human forms have also been described [Therrien et al, 1995]. Ksr1 is about 35-41% homologous to Raf-1 within the catalytic domain and contains some other conserved sequences found in Raf-1. A notable difference to Raf-1 is the lack of the Ras binding domain although Ksr1 contains a cysteine-rich zinc finger that is found in other kinases such as PKC and Raf-1. Additionally Ksr1 contains a serine and threonine-rich region and a motif found in Raf-1; FXFPXXS/T, as well as a putative class II SH3 domain (PXXPXR/K). It is still to be clarified as to where Ksr1 fits into our current understanding of the ERK 1 and 2 signalling pathway. Of interest is a putative role as a MEK2 activator as MEK2 has never been found in complex with any of the Raf family and is not activated *in vitro* by A-Raf, although it is by Raf-1 and B-Raf [Wu et al, 1996]. At present, yeast two-hybrid systems have failed to detect any interactions between Ksr1 and Ras, Raf or MEK 1 and 2 and it seems likely that

this kinase functions upstream of Raf or on a parallel pathway which is partly redundant with the Raf-MEK1 route.

Activated MEK 1 and 2 phosphorylate ERKs 1 and 2 at residues Thr183 and Tyr185 of the TEY motif in subdomain VIII. ERKs 1 and 2 are the only known substrates of MEK1 (45 kDa) and MEK2 (46 kDa). MEK3 can form a complex with ERK1 *in vitro*, however this is totally inactive as an ERK activator [Zheng & Guan, 1993].

Mammalian stress response pathways - SAPK/JNK and p38

The SAPK/JNK pathway has many parallels with the ERK pathway. This pathway is activated by the Rho family of GTPases, Cdc42 and Rac1, analogous to activation of ERKs by Ras [Coso et al, 1995; Minden et al, 1995; Olson et al, 1995]. Expressing activated versions of Rac1 or Cdc42 mutants, or their exchange factors, in COS-7 cells is sufficient to increase JNK1 activity 5-10 fold without increasing ERK activity [Coso et al, 1995; Minden et al, 1995; Olson et al, 1995]. However, the membrane localised Ras exchange factor Sos, which stimulates ERK1 and 2 does not affect JNK activity [Waskiewicz & Cooper, 1995]. The downstream targets of Cdc42 or Rac1 are not fully defined, although several candidate effectors have been suggested including the p21 Cdc42- or Rac-activated serine/threonine kinases (PAKs) [Bagrodia et al, 1995; Waskiewicz & Cooper, 1995]. The mammalian PAKs are homologous to Ste20 [Marcus et al, 1995] and are activated upon the interaction of their amino-terminal regulatory domains with the GTPases [Bagrodia et al, 1995; Brown et al, 1996; Cahill et al, 1996]. Another more distantly related Ste20 homologue is the germinal centre kinase (GCK) which has also been shown to activate the SAPK pathway *in vitro* [Pombo et al, 1995], further illustrating the conservation of signalling pathways between yeasts and mammalian cells. The MAP kinase module which leads to activation of JNKs consists of MEKK (homologous to Ste11) which phosphorylates and activates SEK1 (SAPK or ERK kinase, also known as MKK4 and JNKK). SEK doubly phosphorylates JNK in the TPY motif. SEK1

shares 45% homology with MEK 1 and 2 but lacks the proline rich sequence found in other members of this family [Lin et al, 1995; Sanchez et al, 1994].

There is at least 3 isoforms of MEKK cloned to date [Blank et al, 1996; Minden et al, 1995]. MEKK1 can cause phosphorylation of MEK 1 and 2 *in vitro*, and was first thought to encode an activator of MEK in G-protein activated pathways [Minden et al, 1994]. However inducible expression of MEKK1 in NIH 3T3 cells activates JNK1 activity at least 4-fold, whereas ERK2 activity remains mostly unchanged [Minden et al, 1995; Yan et al, 1994]. Furthermore expression of activated MEKK resulted in cell death in Swiss 3T3 and REF52 fibroblasts and enhanced UV-induced apoptosis [Johnson et al, 1996]. In contrast Raf-1 kinase, which selectively activates ERKs, did not induce any of the cellular changes associated with MEKK expression and cell death. Thus the SAPK/JNK family appear likely to selectively regulate pathways in apoptotic and cellular stress responses [Xu et al, 1995; Johnson et al, 1996]. Activation of MEKK1 may be possible by direct phosphorylation by Rac1, or may be dependant on a second signal in addition to PAK phosphorylation [Waskiewicz & Cooper, 1995]. Another MAPKKK-related protein termed MUK, has been identified and characterised recently [Hirai et al, 1996]. MUK also appears to be a selective activator of the SAPK/JNK pathway.

p38 (also named as CSBP or RK, reactivating kinase) is another MAP kinase activated by a similar cascade of phosphorylation. p38 is dual phorphorylated on tyrosine and threonine within a TGY motif by the upstream activators MKK3 and MKK6 [Han et al, 1996; Raingeaud et al, 1996]. Little is known at present about the upstream activators of MKK3 and MKK6, although more recently it has been suggested that the PAK isoforms might also regulate p38 cascades [Cahill et al, 1996; Pelech, 1996]. Overexpression of MEKK1 failed to activate p38, whist raising JNK activity within the same experiment [Derijard et al, 1995]. Similarly when overexpressed SEK1 (MKK4) is also capable of causing activation of p38 but this is

unlikely to be the physiological activator *in vivo* [Derijard et al, 1995; Lin et al, 1995]. In view of the similarity of the MAP kinases cascades, it is likely that a MEKK1 type enzyme will be characterised which connects PAK (or other undiscovered protein) signals with MKK3 and MKK6. The detection of p38 in the nucleus of activated cells, suggests that p38 can mediate signalling to the nucleus [Raingeaud et al, 1996]. Similar to SAPK/JNKs, the p38 family may also have a role in apoptosis [Pelech, 1996].

MAP kinase inactivation by phosphatases

The down-regulation or inactivation of a response is of an equal importance as the activation and amplification of the signal. ERKs 1 and 2 may be dephosphorylated and hence inactivated by the actions of an array of MAP kinase-specific serine/threonine and tyrosine phosphatases and broad range phosphatases [Alessi et al, 1993; Anderson et al, 1990; Sun et al, 1993]. These phosphatases also play an important role in maintaining MAP kinases at a low basal catalytic activity prior to activation. However, although a number of phosphatases have been demonstrated to dephosphorylate MAP kinase *in vitro*, these may not necessarily be of as major physiologically relevance *in vivo*.

MKP1 (also referred to as CL100 or 3CH134) was initially identified as a murine serine/threonine/tyrosine phosphatase which specifically dephosphorylates both regulatory threonine and tyrosine residues in ERKs 1 and 2 *in vitro* [Sun et al, 1993; Duff et al, 1984; Alessi et al, 1993]. Interestingly, MKP1 does not dephosphorylate JNK1, therefore distinct MAP kinase pathways might be differentially regulated. Recently a related phosphatase, MKP2 has been characterised in PC12 cells [Misrapress et al, 1995]. A number of related phosphatases have also been identified including: PAC (phosphatase of activated cells)-1 [Ward et al, 1994] and HVH2 a novel dual specificity phophatase [Guan & Butch, 1995]. MPK1 and its relations are immediate early gene products which are strictly localised to the

nucleus [Alessi et al, 1993; Guan, 1995; Ishibashi et al, 1994; Ward et al, 1994]. However whilst induction of MKP1/PAC1 might explain the slow dephosphorylation which is observed in the nucleus other phosphatases must be present to account for the rapid dephosphorylation of ERKs 1 and 2 observed in the cytoplasm [Wu et al, 1994].

The other predominant MAP kinase phosphatase is PP2A, one member of a family of broad specificity protein serine/threonine phosphatases [Alessi et al, 1995b]. MEK can also be inactivated by PP2A (but not by tyrosine phosphatase CD45 [Gomez & Cohen, 1991] Following growth factor stimulation, PP2A becomes tyrosyl phosphorylated (possibly by the *src* family); this temporarily inhibits it ability to dephosphorylate substrates [Chen & Murakami, 1992]. Protein tyrosine specific phosphatases have been identified in mammalian cells; in *Xenopus* oocytes a phosphatase specific for tyrosine residues was found to be activated following phosphorylation by MEK and has been suggested to dephosphorylate MAP kinase *in vivo* [Sarcevic et al, 1993].

Phosphatases may regulate the MAP kinase cascade upstream of the MAP kinase enzyme. In insulin signalling, Syp a tyrosine phosphatase is activated which is reported to act immediately downstream of Ras [Sawada et al, 1995]. In yeast several serine/threonine specific and tyrosyl phosphatases have been shown genetically to participate in MAP kinase signalling. The overexpression of either SLN1, PTP2 (a tyrosine phosphatase) or the PP2C genes PTC1 and PTC3 can overcome a lethal mutation involving an inhibitor (SLN1) of the Hog1 MAP kinase pathway [Maeda et al, 1994]. In fission yeast deletion of the three PP2C genes is lethal [Shiozaki & Russell, 1995]. Also identified in *S. cerevisiae* is Msg5, a phosphotyrosyl phosphatase which genetically opposes the actions of Fus3; Msg5 is related to MKP1 and has been shown to inactivate Fus3 *in vitro* [Doi et al, 1994]. From these observations and others, it appears that MAP kinase is both co-

ordinately regulated and spatially distributed with an increasing array of protein phosphatases, as would be expected of such a key signalling protein.

Cellular targets of MAP kinases

Activated MAP kinases have been shown to phosphorylate numerous cellular proteins. Downstream targets of ERKs 1 and 2 include another kinase called Rsk (p90 ribosomal S6 kinase) [Sturgill & Wu, 1992] cytoplasmic phospholipase A_2 (PLA_2) [Lin et al, 1993], the tail of the EGF receptor [Takishimi, 1991] and the microtubule associated protein MAP2 [Ray & Sturgill, 1988]. A downstream target of p38 is the protein kinase, MAPK-activated kinase 2 (MAPKAPK2). MAPKAPK2 was originally described as a kinase that was phosphorylated and activated by ERK2 [Stokoe et al, 1992]. This kinase may be responsible for the phosphorylation of heat shock proteins including Hsp25 and Hsp27 in response to various stress [Rouse et al, 1994; Stokoe et al, 1992]. Very recently a novel enzyme termed MAPKAPK3 has been identified in a yeast two hybrid system [McLaughlin et al, 1996]. MAPKAPK3 shares 70% amino acid identity with MAPKAPK2 and has been shown *in vitro* and *in vivo* to be another physiological target of p38 [McLaughlin et al, 1996]. A number of upstream kinases such as Raf-1 and MEK are also targets for MAP kinase, thus providing the opportunity for feedback regulation of the MAP kinase cascades [Blenis, 1993; Davis, 1993].

In addition, activated MAP kinases can translocate to the nucleus, the location of many transcription factors (TFs) and ternary complex factors (TCFs), these are proteins which once phosphorylated bind in dimers to sequences in DNA to regulate transcription [reviewed in Davis, 1995; Treisman, 1996]. The functional consequences of transcription factor phosphorylation include, stabilising the transcription factor protein, regulation of DNA binding, potentiation of transcriptional activation, regulation of transcriptional repression or a combination of these effects which effectively links transcriptional responses to mitogenic and stress stimuli

[Treisman, 1996]. Downstream targets of ERK 1 and 2 include multiple serine/threonine residues in the carboxy terminus of the TCFs Elk-1, SAP-1 and possibly SAP-2 as well as Ets-1 and Ets-2 (all of the ETs domain-containing family), these can form ternary complexes with a serum response factor (SRF) at the c-Fos serum response element [Treisman, 1996]. Activation of the transcription factor c-Jun is potentiated following phosphorylation by JNK/SAPK, this discovery led to the isolation and characterisation of this group of MAP kinases [Minden et al, 1994]. The mammalian bZIP transcription factor ATF-2 forms a transcriptional complex with c-Jun and is also a target for JNK/SAPK, p38 [Treisman, 1996]. It is likely that many more MAP kinase phosphorylation sites exist, both in the nucleus and cytoplasm, which will become increasingly obvious as our understanding of the genetic and biochemical pathways increases.

In summary, MAP kinases including ERKs 1 and 2 are activated in a sequential cascade by a range of extracellular stimuli including growth factors acting through tyrosine kinase receptors. Activated MAP kinases phosphorylate a number of downstream signalling components and transcription factors and are thus imortant to many cellular processes like growth, differentiation and environmental stress and cell division. It is now apparent that G-protein-coupled receptors can switch into this cascade by activating Ras and Raf by means of the $\beta\gamma$ subunits or activating upstream kinases such as Src or PKC, which gives the potential for G-protein coupled receptor ligands to usurp these cascades for their actions and potentially for mitogenic roles or altering cellular responsiveness in differentiated cell types. Here we explored the potential ability of the LHRH receptor to activate ERKs in partially (αT3-1) and wholly (pituitary cells) differentiated gonadotroph cells in the context of the known ability to alter cellular respnsiveness.

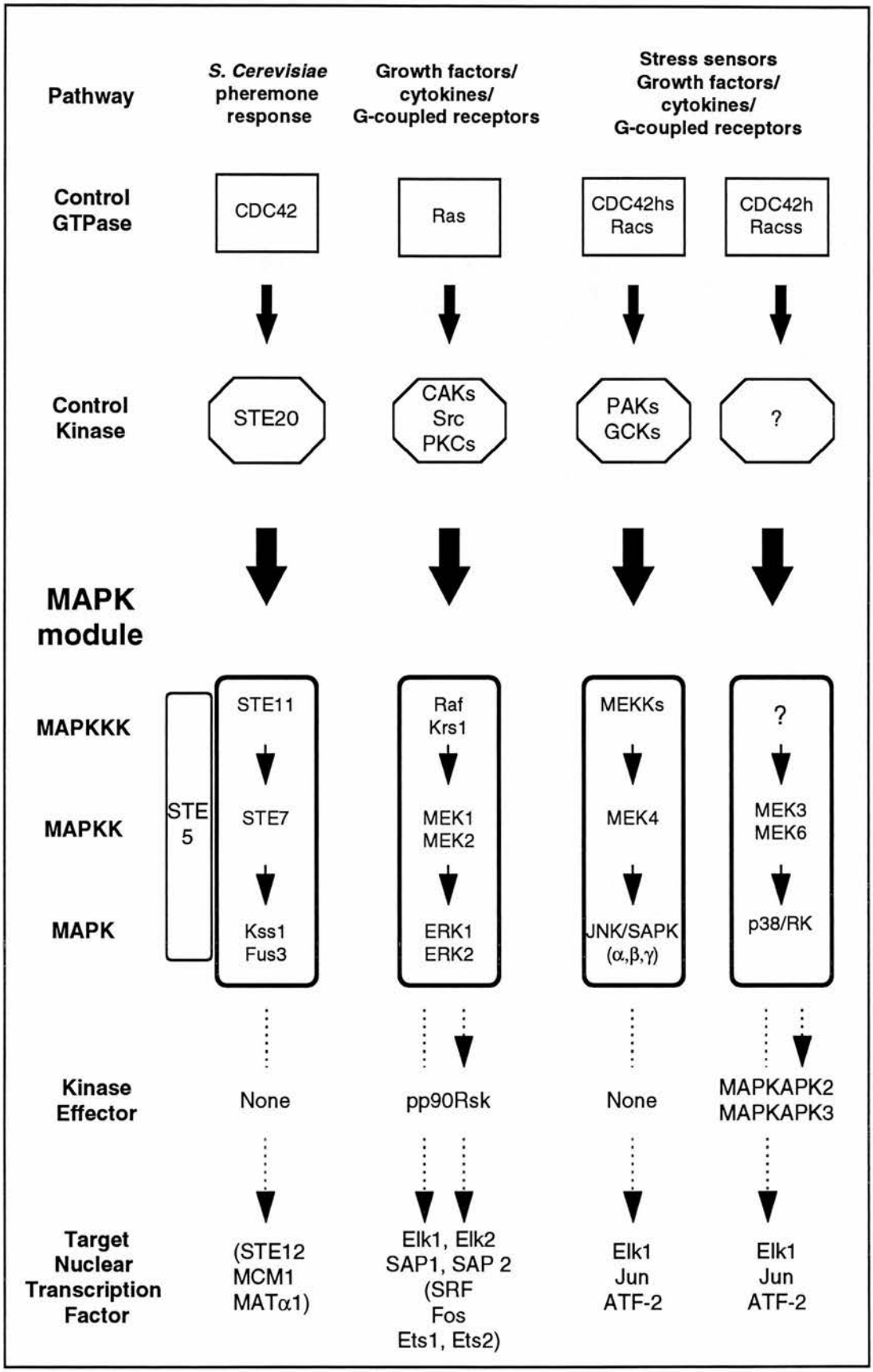

Pathway
S. Cerevisiae pheremone response
Growth factors/ cytokines/ G-coupled receptors
Stress sensors Growth factors/ cytokines/ G-coupled receptors
Control GTPase
CDC42
Ras
CDC42hs Racs
CDC42h Racss
Control Kinase
STE20
CAKs Src PKCs
PAKs GCKs
?
MAPK module
MAPKKK
MAPKK
MAPK
STE 5
STE11
STE7
Kss1 Fus3
Raf Krs1
MEK1 MEK2
ERK1 ERK2
MEKKs
MEK4
JNK/SAPK (α,β,γ)
?
MEK3 MEK6
p38/RK
Kinase Effector
None
pp90Rsk
None
MAPKAPK2 MAPKAPK3
Target Nuclear Transcription Factor
(STE12 MCM1 MATα1)
Elk1, Elk2 SAP1, SAP 2 (SRF Fos Ets1, Ets2)
Elk1 Jun ATF-2
Elk1 Jun ATF-2

Figure 1.3

MAP kinase pathways in *S.cerevisiae* and in mammalian cells.

Structurally related components of the various pathways are shown with information flow from top to bottom. Each MAP kinase module is shown in one long rounded rectangle (shaded grey) containing the MAPKKK, MAPKK and MAPK. Pathways may contain more than one MAPKKK, MAPKK or MAPK, which may exhibit variable functional redundancy. Links to receptors and sensor molecules have been omitted for simplicity. The activity of several of the pathways is controlled by GTPases of the Ras superfamily, shown as open rectangles. The majority of MAP kinase modules is controlled by additional upstream kinases shown in squares. These kinases are members of different subfamilies of kinase (and they are not necessarily homologous to the Ste20 protein in yeasts).

At least five MAPK modules have been characterised in yeast and other less well characterised MAPK modules exist in mammalian cells, such as the MEK5-ERK5 module.

CHAPTER 2

MATERIALS AND METHODS

2.1 BIOCHEMICALS

Inhibitors: working stocks of inhibitors were made up at 10^{-2} M in dH_20 or dimethylformamide (DMF) and stored at -20°C or 4°C where appropriate before dilution in the relevant medium.

PKC inhibitors: staurosporine (*Streptomyces sp.*), K252a (*Norcardiopsis sp.*) Bisindolylmaleimide 1 (GF109203X; 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl] 3-(1H-indol-3-yl)-maleimide) quercetin and chelerythrine chloride, all from Calbiochem-Novabiochem, Nottingham, UK. Ro-31 8220 {[1-[3-(Amidoinothio)propyl-1H-indoyl-3-(1-methyl-1H-indoyl-3-yl)maleimide-methane sulphate} - gift from Roche Products Ltd., Welwyn, Garden City, Herts, UK.

1-(5-Isoquinolinesulphonyl)-R series: –piperazine (C1/HA100), –homopiperazine (HA 1077), –2,5-dimethylpiperazine,–N-1-(methylamino)ethyl) (H8),–N-(2-aminoethyl) (H9) –N-(2-aminoethyl)-N-n-hexyl) from Cookson Chemical Co., –N-(2-guanidinoethyl) (HA 1004), –2 methylpiperazine (H7) and –3-methylpiperazine (isoH7) from Sigma,

tyrosine kinase inhibitors: tyrphostin A-47 (AG 213; α-cyano-(3,4-dihydroxy)thiocinnamide), lavendustin A, {5-amino-[(N-2,5-dihydroxybenzyl)-N'-2-hydroxybenzyl]-salicylic acid, lavendustin B, [5-amino-(N,N'-bis-2-hydroxybenzyl)-salicylic acid], ST 271 (3-(3', 5'-di-isoprpyl-4'hydroxycinnamide), genistein (4',5,7-trihydroxyisoflavone), geldanamycin, RG-13022 [α-(3'-pyridyl)-(3,4-dimethoxy) cinnamonitrile] all from Calbiochem

tyrosine phosphatase inhibitor: Pervanadate was prepared by the addition of sodium orthovanadate (Sigma) to a divalent cation-free Earles balanced salt solution containing Hepes (30 mM) to a final concentration of 5 mM. The pH was adjusted to pH 7.4 with NaOH, then H_2O_2 (1 M) was added to a final concentration of 1 mM, for

15 min at room temperature before the addition of catalase (4 mg; Sigma) to remove excess (*Streptomyces hygroscopicus*) H_2O_2 [Grinstein et al, 1990].

Phospholipase inhibitors: (PLA_2 inhibitors) aristolochic acid sodium salt, from Biomol Research Laboratories, c/o Semat, St. Albans, Herts, UK. Manoalide (*Luffariella variabillis*) and Isotetrandine from Calbiochem. 4-(4-octadecyl)-4-oxobenzene-butenoic acid from Cookson Chemicals; benzenesulphonamide 4 was a gift from Dr Mike Clark, USA. (PLC inhibitors) U-73122, {1-6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione} and U-73343, {1-6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrolidine-dione} both gifts from Upjohn Pharmaceuticals, Kalamazoo, USA.

Activators/agonists LHRH and the antagonist peptide ([Ac-D-p-Cl-$Phe^{1,2}$, D-Trp^3, D-Arg^6, D-Ala^{10}]-LHRH, epidermal growth factor (EGF), mastoparan (*Vespula lewisii*), forskolin (*coleus forskohli*-7β-acetoxy-8,β-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one) phorbol 12,13-dibutyrate (4α– and 4β–isomers), ionomycin (free acid, *Streptomyces conglobatus*), 5-hydroxytryptophan (5-HT) and Cholera toxin (*Vibrio cholerae*) were obtained from Sigma Chemical Co., Poole, Dorset, UK. Pertussis toxin (*Bordetella pertussis*) and pertussis toxin B-oligomer were both obtained from Calbiochem. (1S,3R)-aminocyclopentane dicarboxyclic acid (ACPD) was obtained from Tocris Cookson, Bristol, UK. Buserelin (D-t-butyl-Ser^6, des-Gly^{10}, LHRH ethylamide) was a gift from Hoeschst, AG.

Radiochemicals: Adenosine 5'-[γ–^{35}S-thio]trisphosphate (ATP-γ–^{35}S) specific activity approximately 250 Ci/mmol, and [5, 6, 8, 9, 11, 12, 14, 25-^{3}H(N)]-arachidonic acid specific activity approximately 250 Ci/mMol) were obtained from NEN DuPont, Germany, Na ^{125}I from ICN Radiochemicals, Irvine, CA, USA and [^{3}H]-inositol from Amersham International plc., Aylesbury, Bucks, UK,.

Anti-sera: Primary antisera: mouse monoclonal antibodies against ERK 1+2 (Zymed ZO33, Zymed Laboratories Inc., San Francisco, USA, affinity-purified rabbit polyclonal antibodies to $G_{\alpha q/11}$ (C1-19) raised against a peptide corresponding to amino acids 341-359 mapping within a domain common to $G_{\alpha q}$ and $G_{\alpha 11}$ of mouse cell origin and $G_{\alpha 12}$ (S-20) were purchased from Santa Cruz Biotechnology, Inc., anti-β tubulin antibody (Amersham N357), was from Amersham International plc, UK. Rabbit anti-cAMP antiserum (RIB7) was a generous gift from Dr. I. Gow, Dept. of Physiology, University of Edinburgh.

All secondary antisera and non-immune rabbit serum were gifted from the Scottish Antibody Production Unit (SAPU), Carluke Lanarkshire, UK.

Oligonucleotides: mRNA antisense oligonucleotides corresponding to regions around the initiation codon for ERK 2 (5'-CGC CAT GTT GGC TGC ACA GCC GCC-3'), and $G_{\alpha q/11}$ (5'-CGC CAT (GC)AT GGA CTC CAG AGT-3') and $G\alpha_{12}$ (5'-GGG TCC GCA CCC CGG ACA TGG-3') (murine sequences) were custom synthesised and HPLC purified by Oswel DNA, University of Southampton, Southampton, UK.

Tissue Culture reagents: All tissue culture media, sterile glucose supplements, Lipofectamine™ and "UltroSer G" was obtained from Gibco-BRL. Foetal calf serum was obtained from Sera-Lab, Crawley Down, Sussex UK. and after batch testing for sustained growth and standard assay results a batch was reserved for subsequent use. Penicillin, streptomycin, L-glutamine and trypsin were obtained from Sigma. N-[1-(2,3-dioleoyloxy) propyl]-N, N, N-trimethyl ammoniummethylsulphate (DOTAP) was from Boehringer Mannheim and DEAE-Dextran was from Promega, Southampton UK. Tissue culture plastics were obtained either from Costar, UK Ltd, Bucks UK, or Greiner Laboritechnik Ltd, UK.

Miscellaneous: General biochemicals were from BDH and were all Analar grade. MAP kinase selective peptide substrate Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg

(APRTPGGRR) was synthesised by Dr J Keyte, Department of Biochemistry, University of Nottingham, Nottingham, UK. Bovine serum albumin (fraction V and essentially fatty acid free), isobutylmethylxanthine (IBMX), dithiothreitol (DTT), N-tris(hydroxymethyl)methylglycine (Tricine), [4-(aminoethyl)benzenesulphonyl fluoride (AEBSF), pepstatin, aprotinin, sodium orthovanadate (Na_3VO_4), β-glycerophosphate, leupeptin, trans-epoxysuccinyl-L-leucylamido-(4-guanido)-butane (E64), 2-mercaptoethanol (EtSH) and sodium fluoride (NaF) were all obtained from Sigma. Okadaic acid, ammonium salt was obtained from Calbiochem. Sagatal™, sodium pentabarbitone; May and Baker, Dagenham, Essex, P81 phosphocellulose paper from Whatman International limited Kent, UK, Coomassie® Plus protein assay reagent and BSA standard solution from Pierce, Chester, UK; Immobilon-P™, Millipore, Watford, Herts; Enhanced Chemi Luminescence (ECL) detection system, Amersham International plc, Bucks, UK, Sep-Pak C_{18} cartridges, Waters Chromatography, Watford, Hertfordshire, UK

2.2 ANIMALS

COB-Wistar rats were obtained from Charles River UK Ltd, Margate Kent UK or were derived from a colony bred in this department from a stock originally obtained from Charles River. Adult female (~200g) or male (~250g) rats were maintained under controlled lighting (lights on from 05.00 to 19.00h) and temperature (22°C) with free access to food pellets and tap water.

Female rats were assessed for a minimum of two 4 day oestrous cycles (as determined by vaginal lavage). The cytological characteristics of the vaginal smears in a 4-day cyclic rats were as follows;

Metoestrus: large numbers of leucocytes, epithelial cells and cornified epithelial cells.

Dioestrus: leucocytes, epithelial cells and cornified epithelial cells, but in smaller numbers than metoestrus animals.

Pro-oestrus: mainly nucleated epithelial cells.

Oestrus: predominantly cornified epithelial cells.

To prepare OVX rats, female rats at various stages in of the oestrous cycle, were bilaterally ovariectomised under halothane anaesthesia and left over a period of 4 weeks before use as with normal cycling animals.

Prior to experimentation rats were anaesthetised with "Sagatal" (30 mg/kg body weight) by 11.00-12.00 am on the appropriate day of the cycle and killed by cervical dislocation

2.3 CELL CULTURE

All cells were grown and maintained under a humidified atmosphere of 95% air/5% CO_2 at 37°C and received fresh medium every 3-4 days. Cells were harvested either by trypsin digestion or using Hanks buffered saline (HBSS, without calcium, magnesium or phenol red) containing 0.1% w/v EDTA. Medium was removed and replaced with 1.5 ml of a 0.25% (v/v) trypsin solution or HBSS/EDTA and left for 5-10 min. After pelleting at 200 *x g* for 10 min, cells were resuspended in an appropriate volume and split into fresh flasks at a ratio of 1:3 for 75 cm^2 flasks, 1:8 for 25 cm^2 or were seeded into 12, 24 or 48 well plates where appropriate for assay purposes.

αT3-1 mouse gonadotroph cells

αT3-1 cells (gift from Dr Pamela Mellon, the Salk Institute, USA.) were grown and sub-cultured in Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate (110 mg/l) containing 10% foetal calf serum (FCS) 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were passaged after reaching ~100% confluency in 75 cm^2 flasks every 5-7 days.

COS 7 fibroblast cell line

COS 7 cells (gift from Dr. Janet Allen, Glasgow University, UK), used for transient transfection procedures, were grown in DMEM supplemented with 10% new-born calf serum and 100 U/ml each of penicillin and streptomycin and were passaged every 3-4 days.

Chinese Hamster ovary (CHO) cells

CHO cells, used to produce stably transfected cell lines, were grown and maintained in Ham's F12 medium with 100 U/ml each of penicillin and streptomycin and were passaged every 5-7 days when ~60-80% confluent in 75 cm^2 flasks.

2.4 METHODS

Cytosolic MAP kinase assay

Cells were grown to confluency in 25 cm^2 (αT3-1) or 75 cm^2 (transfected COS 7 (tCOS 7) or transfected CHO (tCHO)) flasks and then the medium replaced with serum free medium for ~24h. Rat anterior pituitary glands were removed immediately following decapitation. Hemisected pituitaries were randomly distributed into 25 ml conical flasks containing 2 ml of pre-gassed and pre-warmed (37°C) Hepes-buffered minimal essential medium with Earles salts (MEM). The hemipituitary pieces were pre-incubated for 30 min in a shaking water bath at 37°C under an atmosphere of 95% O_2 : 5% $C0_2$ after which the medium was discarded and replaced with fresh medium. Where required inhibitory drugs were added directly to the flasks from 100X concentration for 5 min before addition of the agonist for 10 min (unless otherwise stated). When appropriate dimethylformamide (DMF) vehicle was present in controls at 0.03% (v/v). This concentration of vehicle was shown to have no effect on MAP kinase activity.

After incubation with the pharmacological agent, αT3-1 cells, tCOS 7 and tCHO cells or hemipituitaries were thoroughly aspirated and rapidly scraped from the flask and homogenised into 200 µl of ice-cold homogenisation medium: 20 mmol Tris/l; 12 mM EDTA/l, 50 mM 2-mercaptoethanol (EtSH)/l, 1 mM [4-(aminoethyl) benzenesulphonyl fluoride (AEBSF)/l, pH 7.4 with HCl, containing 0.01% (w/v) leupeptin, 20 µM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane/l, 2 mg aprotinin/l, 1 µM pepstatin/l, 2.5 mM Na_3VO_4/l, 62.5 mM β-glycerophosphate/l and 200 nM okadaic acid. The supernatant fraction was collected after centrifugation, 6000 x *g*, 45 min. The 25 µl assay finally contained 20 mM Tris/l, 0.5 mM EGTA/l, 2.4 mM EDTA/l, 20 mM $MgCl_2$/l, pH 7.5 with HCl, with 50 µM NaF/l, 50 mM EtSH/l and 200 nM okadaic acid/l as well as the other peptidase and phosphatase inhibitors diluted 1 in 5 from their concentrations in the 5 µl aliquot of cytosolic extract added to the assay. Substrate peptide (APRTPGGRR; Clark-Lewis et al 1991) was used at 2 mmol/l. Substrate-free blanks were determined in all cases. Assays were started with 100 µM of ATP-γ[^{35}S]/l (containing 0.58 µCi ATP-γ[^{35}S]/tube) and brief centrifugation.

Tubes were incubated for 40 min at 30°C (within the linear range of the assay). Incubations were stopped by addition of 25 µl ice-cold trichloroacetic acid (120 g/l) and 10 µl bovine serum albumin (20 g/l). After 15 min on ice, samples were centrifuged (12000 x *g* for 5 min at 4°C), then 30 µl aliquots were spotted onto 4 cm^2 pieces of P81 phosphocellulose paper. Papers were washed for 3 x 2 min in ~10 ml of H_3PO_4 (75 mM) and dried before scintillation counting. Specific MAP kinase activity was defined by subtracting the substrate-free blanks which were generally less than 25% of values with substrate and were unaltered by any of the present treatments.

Immunoprecipitation of ERK 1 and 2

The specificity of this assay was monitored by immunoprecipitation using a monoclonal anti-p42 /p44 antibody (Zymed Z033) conjugated to protein G-sepharose

4B beads. The protein G-sepharose-4B (100 µl packed gel) was washed and resuspended in 140 µl 25 mmol Tris/l, 150 mmol NaCl/l, 0.04% (v/v) Nonidet-P40, 0.25% (w/v) gelatin, pH 7.4 with HCl. MAP kinase antibody (Zymed Z033, specific for p44 and p42 species; 50 µg), or a control anti-β tubulin antibody (Amersham N357) was added to the gel and incubated for 2 h at room temperature with rolling. The derivatised gel was washed twice and resuspended in homogenization buffer to a final volume of 140 µl. Fifty µl aliquots, together with 50 µl cytosolic extract were incubated for 18 h at 4°C with rolling. After brief centrifugation, the supernatant was removed, the gel washed once and resuspended to 100 µl. Aliquots (10 µl) of supernatant, of resuspended gel and of cytosolic extract (diluted 1:1) were assayed. As seen in Figure 2.2, in cytosolic extracts for αT3-1 cells treated with either nothing, LHRH (100 nM, 10 min) or phorbol 12,13-dibutyrate (PDBu, 1 µM , 8 min), 71 ± 12%, 74 ± 10% and 67 ± 9% of the recovered activity was associated with the beads rather than the supernatant. Corresponding values using the control anti-β tubulin antibody of the same IgG subclass were 12 ± 8%, 4 ± 8% and 23 ± 13% respectively (means ± SEM, n=3).

Map kinase immunoblots

Phosphorylation of p42 and p44 MAP kinase was determined by the electrophoretic mobility shift assay. αT3-1 cells were grown as before in 12 well plates and serum starved for 24 h prior to experimentation. Incubation with pharmacological agents as appropriate was as described for the MAP kinase assay. After the incubation period the medium was aspirated and the cells washed once in 1 ml ice cold Hanks balanced salt solution (HBSS), and the cells scraped immediately into 500 µl SDS buffer ; 50 mM Tris base, 5% β- mercaptoethanol, 2% sodium dodecyl sulphate, pH 7.2, heated to 100°C for 5 min and stored at -20°C.

Four µl of each sample was subjected to electrophoresis on a 7.5% homogenous phast-gel, 30 min (10 mA, not exceeding 250 V). After separation by

electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P). Electroblotting was performed at 20°C for 15 min in 25 mM Tris base, 192 mM glycine, 20% methanol, pH 8.3. A non-specific binding of antibody was prevented by incubating the blots in 5% BSA/PBS overnight. Both SDS-electrophoresis and electroblotting were performed on a Phast-system apparatus (Pharmacia Biotech, Milton Keynes, Bucks).

The blots were washed 5 X 5 min at 20°C in PBS/0.1% Tween 20. Phosphorylated MAP kinases were then identified by incubating with mouse monoclonal anti-MAP kinase antibody (Zymed ZO33), which recognises both p42 and p44 species of MAP kinase, for 1-3 h in 0.25% BSA/PBS (diluted 1:1200) followed by extensive washing in PBS/0.1% Tween 20. The blots were subsequently incubated with horseradish peroxidase (HRP)-anti mouse IgG in 0.25% BSA/PBS/Tween 80 (diluted 1:5000). After washing, immunostained proteins were visualised using the Enhanced Chemi Luminescence (ECL) detection system.

Radioimmunoassay for cAMP

Prior to agonist stimulation, quiescent αT3-1 cells were washed extensively in MEM-BSA (0.25%) and pre-incubated in medium containing 0.5 mM isobutylmethylxanthine (IBMX) for 15 min. Stimulations were terminated by aspirating the medium and addition of 0.1 N HCl to the cells (intracellular cAMP) or addition of an equal volume of 0.2 N HCl (total cAMP). After freeze thawing the plates to aid membrane breakdown the cells were homogenised by trituration in the 0.1 N HCl and stored at -40°C.

Duplicate 50 µl samples of homogenate in 0.1 N HCl were assayed for immunoreactive cAMP using a double antibody precipitation radioimmunoassay (Dufau et al 1973) after appropriate dilution in 50 mM sodium acetate buffer, pH 6.0, 0.1% BSA (fraction V), 0.1% sodium azide, using antiserum RIB7 at a final dilution of

1:200,000. All assays were performed in a final volume of 275 µl in polypropylene tubes (Alpha-Labs). After overnight incubation with ~10K cpm of ^{125}I-cAMP, donkey anti-rabbit IgG and non-immune rabbit serum were added to a final titre of 1:40 and 1:400 respectively and incubated at 4°C. After 3 h, 700 µl of ice-cold PEG-6000 (6%) was applied and the bound label separated from free by centrifugation at 1,500 x *g* for 25 min at 4°C. Non specific binding was typically <10% and <5% respectively. Standard curves were generated in the range of 25 to 512 pmole/tube using cAMP as standard. Assayed cAMP content was determined by interpolation from the % Bound counts versus log cAMP concentration curve or its logit transformation, generated using the respective algorithm of the Packard Cobra autogamma analysis package. Specific bound counts were typically 35% of total counts applied. Percent bound counts (%B) is defined as:

$$\frac{X\text{-}NSB}{B_o\text{-}NSB} \times 100\%$$

Where X = cpm for standard/unknown sample, B_0 = cpm in absence of cAMP, and NSB is the non-specific cpm.

Logit % B is defined as:

$$\log\frac{\%B}{100\text{-}\%B}$$

[^{3}H]-Arachidonic acid release measurements

The release of [^{3}H]-AA from pre-labelled cells in culture was measured by reverse phase liquid chromatography on octadecyl silica cartridges (ODS) (Sep-Pak C_{18} cartridges) using the solvent system described by Powell (1982). αT3-1 cells were grown and sub-cultured as previously described The culture medium was removed and replaced with fresh pre-warmed MEM containing 0.5 µCi of [5, 6, 8, 9, 11, 12, 14, 25-^{3}H(N)]-arachidonic acid (specific activity approximately 250 Ci/mmol). The cells were incubated for 18 hours at 37°C under a humidified atmosphere of 95%

air/5% CO_2. The cells were then washed three times in MEM containing 1% bovine serum albumin (BSA; essentially fatty acid free).

After washing, the cells were incubated in MEM + 0.5% BSA (as a trap for released [^{3}H]-AA). Where required inhibitory drugs were added directly to the plate from 100X concentration stocks for 10 min before the medium was aspirated and replaced with fresh medium containing either no drug (basal) or LHRH (100 nM unless otherwise stated) and the relevant inhibitors. When appropriate dimethylformamide was present at 0.03% (v/v). This concentration of vehicle was shown to have no effect on arachidonic acid release. After incubation for 15 min with the pharmacological agents, the incubation medium was removed and placed in 2 ml ice-cold ethanol and 3.7 ml of distilled H_2O was added (to give a final ethanol concentration of 30%) and this was retained for [^{3}H]-AA determination. The cells were washed twice in pre-warmed and pre-gassed MEM + 1% BSA, then a final aliquot of pre-warmed and pre-gassed MEM + 0.5% BSA was added to each. The cells were scraped into the medium and added to 2 ml of ice-cold ethanol and was then diluted with distilled water to give a final ethanol concentration of 30%.

Both the cell homogenates and the medium were centrifuged (10', 4°C, 3000 *x g*) and the supernatant from each was retained and acidified to pH 3 with 1 M HCl. To measure medium and tissue [^{3}H]-AA levels, a 4 ml aliquot of the acidified supernatant from each was loaded onto an ODS column (pre-washed with 5 ml ethanol followed by 5 ml of dH_2O). Solvents were then passed through the columns in the following order:

(a) 30% ethanol (20 ml) to elute polar substances such as polar lipids

(b) distilled H_2O (20 ml) to remove the ethanol

(c) petroleum ether [BP 60-80°C](10 ml) to remove the water

(d) petroleum ether: $CHCl_3$ (1:1, 20 ml) to elute fatty acids

(e) methyl formate (10 ml) to elute prostaglandins and leukotrienes.

A 4 ml aliquot of the petroleum ether: $CHCl_3$ fraction was allowed to evaporate off overnight, then the radioactivity in the radioactivity in each sample was determined by liquid scintillation counting. The radioactivity in 200 µl samples of the acidified cell homogenate and of the incubation medium supernatants was also determined to give a total amount of label incorporated into the cells. The amount of [^{3}H]-AA released could then be expressed as a percentage of the total amount incorporated into the cells thus: dpm in 200 µl supernatant + 200 µl cell homogenate (X 20) = dpm in 4 ml (total radioactivity applied to the column); dpm in 4 ml of the petroleum ether: $CHCl_3$ fraction (X5) = dpm in 20 ml eluted from column (total radioactivity eluted from column).

$$\frac{\text{total radioactivity applied to column}}{\text{total radioactivity eluted from column}} \text{ X } 100\%$$

Experiments with addition of authentic [^{3}H]AA after cell homogenisation showed that >80% of [^{3}H]-AA detected was found to be in fraction (d) (the petroleum ether: $CHCl_3$ fraction) as shown in Figure 2.7a This correlates with observations made by Powell (1982) who showed that, whereas the more polar phospholipids were probably passed through the column without being retained, fatty acids, such as [^{3}H]-AA, and monohydroxy fatty acids were eluted by mixtures of petroleum ether and $CHCl_3$. Methyl formate (fraction e) can elute prostaglandins and thromboxanes from the column. There was some evidence (Figure 2.7) that LHRH treatment of αT3-1 cells additionally increases formation of these AA metabolites. LHRH treatment did not appear to significantly increase the levels of radioactivity in the solvent fractions of the cell homogenates (Figure 2.7 b). However the results must be used with restricted interpretation as it is unclear as to how measurements of total cellular [^{3}H]-AA levels reflect changes in pools relevant to receptor-operated PLA_2. Internal positive controls were incorporated in each experimental design.

[^{3}H]inositol phosphate measurement

αT3-1 cells were grown and sub-cultured as before in 12-well plates, then normal growth medium was removed and replaced with 0.5 ml/well Earles Balanced Salt solution (EBSS) containing 10 mM glucose and 0.2% w/v BSA with myo-[^{3}H]inositol 2 μCi/well and incubated for 16 h under 95/5% O_2/CO_2. After washing, 10 mM LiCl was added for 15 min before stimulation with agonist for 30 min. Incubations were stopped by aspiration and the addition of 700 μl cold 1.34 M trichloracetic acid before assaying. Following centrifugation of cell homogenates at 12,000 x *g* for 5 min at 4°C, aliquots of supernatant were transferred to tubes containing 50 μl EDTA (pH 7.0) and 500 μl 1:1 1, 1, 2-trichloro-trifluoro-ethane: tri-N-octylamine for solvent extraction of labelled phospholipids. After extensive vortexing and centrifugation at 12,000 x *g* for 5 min (4°C), 300 μl aliquots of the upper aqueous phase were added to 200 μl 1M $NaHCO_3$ with Universal indicator and adjusted to pH 8. Labelled hydrophilic inositol-containing compounds were separated on 1 ml columns of Dowex 1 x 8 (formate, mesh size 200-400).The columns were washed extensively with water before sample application and then 20 column volumes of water, 5 column volumes of 1 M ammonium formate, 0.1 M formic acid were applied to elute unmodified inositol phosphates respectively.

Translocation of PKC (monitored as [^{3}H]PDBu binding sites)

Cytosolic [^{3}H]PDBu binding was performed in a method similar to that described by Leach et al 1983. After stimulation for 15 min in culture flasks, the medium was aspirated and cells were scraped into 0.5 ml of ice-cold 'Kuo' buffer (20 mM Tris-HCl (pH 7.5) 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM [4-(aminoethyl) benzenesulphonyl fluoride] (AEBSF) containing 0.01% leupeptin and 20 μM transepoxysuccinyl-L-leucylamido-(4-guanido) butane (E 64) and homogenised. Samples were centrifuged (38,000 x *g* , 20 min, 4°C). The supernatant and pellet were carefully separated and the pellet rehomogenised in 0.5 ml 'Tris-BSA' (50 mM

Tris-HCl, pH 7.4), 4 mg/ml essentially fatty acid-free bovine serum albumin. While membrane binding was carried out in Tris-BSA, cytosolic binding assays additionally contained 1 mM $CaCl_2$ and 75 mM magnesium acetate and 1.25 mg/ml sonicated phosphatidyl serine, sodium salt (Lipid products Ltd, Nuffield, UK) [Leach et al, 1983]. Assays were conducted in a total volume of 250 µl (30 min, 37·C), with 5 nM [^{3}H]PDBu (approximately 0.03 µCi per assay tube) and DMF (0.5% final concentration) or 10 µM PDBu in DMF for total and non-specific binding measurements, respectively. Tissue samples gave a total binding of less than 10% of total radioactivity present. Protein was precipitated at 4°C by the addition of 100 µl of 12 mg/ml bovine γ-globulin and 300 µl of 24% polyethyleneglycol 8000 in 50 mM Tris-HCl (pH 7.4). After 20 min, assay tubes were centrifuged (12,000 x *g*, 5 min, 4°C), the supernatant aspirated and the ^{3}H radioactivity in each pellet determined after solubilisation.

[^{125}I]buserelin binding measurement

The equilibrium binding assay uses the LHRH agonist [^{125}I]buserelin and is carried out essentially as described in Mitchell et al 1988. Cells from each 75cm^2 culture flask were scraped into 20 ml ice-cold 25 mM Tris HCl pH 7.4, homogenised and centrifuged (38,000 x *g* for 20 min at 4°C). The membrane pellet was resuspended in ice-cold 25 mM Tris HCl pH 7.4 containing a final concentration of 0.1% BSA. Aliquots of 100 µl were assayed using approximately 130,000 dpm of [^{125}I]buserelin per assay tube. The ligand specific activity was approximately 200-300 Ci/mmol. After 90 min on ice, membranes were pelleted by centrifugation (14,000 *x g* for 10 min at 4°C) and the supernatant carefully aspirated. Non-specific binding (in presence of 3 µM LHRH) was in the range of 7,000-11,000 dpm per assay tube. The affinity of the binding sites for [^{125}I]buserelin (expressed as IC_{50} values) was estimated in a self-displacement assay using unlabelled buserelin in the range of 0.03-30 nM. Data were fitted by a non-linear error-weighted procedure (P-fit,

Elsevier Biosoft) to give best-fit IC_{50} values and then the B_{max} values were calculated by the method of Swillens [Swillens, 1992].

Transfections

Preparation of cDNA

The mouse LHRH receptor cDNA [Tsutsumi et al, 1992] was inserted into the expression vector pcDNA neomycin (clone SCS-56). The mutant LHRH receptors (generated in pSelect by oligonucleotide-directed mutagenesis; [Zhou et al, 1994]) were prepared in pcDNA/AMP (Invitrogen, San Diego, USA). The 5-HT_{2C} receptor cDNA (PMVR7SRIc, a gift from Dr D Julius [Julius et al, 1988] was prepared as in Lutz et al 1993. The metabotropic glutamate receptor (mGlu 1a) cDNA, pmGRI, a gift from Professor S Nakianishi [Masu et al, 1991] was digested with BamH1 and Not1 and the 4.65 Kb insert excised from agarose gel using the Pharmacia Sephaglas kit. This was subsequently subcloned into pcDNA1 (InVitrogen). All cDNA used for expression in host cells was purified first through CsCl density gradients.

DEAE dextran (method for transient transfection of cells)

COS 7 cells were grown and maintained in DMEM supplemented with 10% newborn calf serum and penicillin/streptomycin. Cells were trypsinised and replated at ~50% confluency into 75 cm^2 flasks on the afternoon prior to transfection.

During the morning of transfection, the cells were washed 2 x 5 min with pre-warmed OptiMEM supplemented with 100 U/ml each of streptomycin and penicillin at 37°C before exposure to transfecting medium for 4 h. The transfecting medium consisted of OptiMEM/streptomycin/penicillin, 400 µg/ml DEAE dextran (Promega), 100 µM chloroquine phosphate (Sigma) and 10-20 µg of plasmid containing pcDNA, with a 1.2 Kb EcoR1/Xho1 fragment of the mouse GnRH Receptor cDNA, full coding

sequence, per flask. This was replaced with 10% DMSO in PBS for 2 min, then DMEM/2% UltroSer G/penicillin/streptomycin. Cells were grown for 24 h, then trypsinised and re-plated. Cells were harvested 48 h later.

DOTAP method for transfection

In order to produce a stable cell line expressing functional LHRH receptors, CHO cells were grown and maintained as described previously until reaching 60-80% confluency prior to transfection. The cells were incubated overnight in the transfection medium containing; OptiMEM streptomycin/penicillin, DOTAP, 20 µg/dish pcDNA, incorporating a 1.2 Kb EcoR1/Xho1 fragment of the mouse GnRH Receptor cDNA, and a neomycin resistance gene. The following day the medium was replaced with Hams F12 supplemented with 2% UltroSer G and streptomycin/penicillin (100 U each) for ~24 h, when the medium was again replaced with fresh medium also containing the neomycin analogue, geneticin (500 µg/ml). Cells were selected one month later by placing cloning rings over individual colonies in order to remove and replate them. Finally the cells were assessed for expression of functional LHRH receptors by equilibrium binding of [^{125}I]buserelin (an LHRH receptor agonist), according to previously described procedures [Mitchell et al, 1988].

Transfection of antisense oligonucleotides using Lipofectamine™

Antisense oligonucleotides were usually supplied as a lyophilised powder and were resuspended in sterile dH_2O according to the manufacturer's instructions then aliquoted and stored at -20°C. Any further dilutions before use were in sterile OptiMEM. Approximately 24 h prior to transfection αT3-1 cells were split and re-seeded into 12 well plates (~15,000 cells/ml) in normal growth medium and allowed to reach 60-70% confluency. Solutions were prepared for transfection in 200 µl/ml OptiMEM (Gibco) with 20 µl lipofectamine™ reagent (Gibco) ± 100 µl of the oligonucleotide solution (10 µM). Liposomes were allowed to form for 45 min at

room temperature with gentle agitation then made up to 1 ml with warm OptiMEM. During the morning of transfection the αT3-1 cells were washed 2 x 5 min with pre-warmed OptiMEM at 37°C before exposure to transfecting medium for 14-24 h. The transfection medium was aspirated and the cells washed 2 x 5 min with OptiMEM then incubated in normal growth medium for a further 24-48 h at 37°C before being assayed or harvested for immunoblotting.

Protein assays

Where protein content was required to be determined in the following studies a Pierce protein assay kit (Pierce, Chester, UK) was used. This method uses an assay reagent based on the Bradford method (1976) consisting of Coomassie Blue G-250, phosphoric acid, methanol, water and solubilising agents. When Coomassie Blue binds to proteins in an acidic solution, an absorbance shift from 465 to 595 nM occurs [Davis, 1988]. A standard curve for protein concentration determinations was constructed in microtiter plates in a total volume of 300 µl within the range of 75-1500 µg/ml with a standard solution of BSA (essentially fatty acid free, 2 mg/ml, Pierce) in the appropriate assay medium, which was consistently linear with the standard protein concentrations used.

APPENDIX 1

Effects of various agents (added during the assay) on constitutive MAP kinase activity from rat hippocampus cytosol extract

Table 1

Effects of various protein kinase inhibitor directly on MAP kinase activity from rat hippocampus

The hippocampus was immediately removed from decapitated rats and homogenised in 2-3 vol of MAP kinase homogenisation buffer: 20 mmol Tris/l; 12 mM EDTA/l, 50 mM 2-mercaptoethanol (EtSH)/l, 1 mM [4-(aminoethyl)benzenesulphonyl fluoride (AEBSF)/l, pH 7.4 with HCl, containing 0.01% (w/v) leupeptin, 20 µM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane/l, 2 mg aprotinin/l, 1 µM pepstatin/l, 2.5 mM Na_3VO_4/l, 62.5 mM β-glycerophosphate/l and 200 nM okadaic acid. The supernatant fraction was collected after centrifugation, 6000 x *g*, 45 min. The 25 µl assay finally contained 20 mM Tris/l, 0.5 mM EGTA/l, 2.4 mM EDTA/l, 20 mM $MgCl_2$/l, pH 7.5 with HCl, with 50 µM NaF/l, 50 mM EtSH/l and 200 nM okadaic acid/l as well as the other peptidase and phosphatase inhibitors diluted 1 in 5 from their concentrations in the 5 µl aliquot of cytosolic extract added to the assay. Substrate peptide (APRTPGGRR; Clark-Lewis *et al.* 1991) was used at 2 mmol/l. Substrate-free blanks were determined in all cases. Assays were started with 100 µM of ATP-γ[^{35}S]/l (containing 0.58 µCi ATP-γ[^{35}S]/tube) and brief centrifugation.

Tubes were incubated for 40 min at 30°C (within the linear range of the assay). Incubations were stopped by addition of 25 µl ice-cold trichloroacetic acid (120 g/l) and 10 µl bovine serum albumin (20 g/l). After 15 min on ice, samples were centrifuged (12000 x *g* for 5 min at 4°C), then 30 µl aliquots were spotted onto 4 cm^2 pieces of P81 phosphocellulose paper. Papers were washed for 3 x 2 min in ~10 ml

of H_3PO_4 (75 mM) and dried before scintillation counting. Specific MAP kinase activity was defined by subtracting the substrate-free blanks which were generally less than 25% of values with substrate and were unaltered by any of the present treatments. All determinations are means ± SEM where n=>6.

Table 2

Effects of phorbol esters and a related activator of PKC (added during the assay) on constitutive MAP kinase activity from rat hippocampal cytosol

To determine whether phorbol esters or a related compound could directly activate MAP kinase *in vitro*, the hippocampus was immediately removed from decapitated rats and a crude cytosolic preparation was prepared as described above. Aliquots of the cytosolic fraction were included in the MAP kinase activity assay described above with the inclusion where appropriate of 4α-phorbol 12,13-dibutyrate (4α-PDBu), 4β-phorbol 12,13-dibutyrate (4β-PDBu), mezerein and phorbol 12-myristate 13-acetate (PMA) at 1 or 10 μM for 40 min, substrate-free blanks were determined in all cases. Thereafter the reaction was terminated as above. All determinations are means ± SEM where n=>6.

Table 3

Effects of phosphatase treatment on the constitutively-active MAP kinase activity from rat hippocampal cytosol

To determine that MAP kinase activity was dependent on phosphorylation of the enzyme, aliquots of the crude cytosolic fraction of rat hippocampus were prepared as above, and then assayed after a 20 min incubation, at 37°C with either no addition, 0.3 U sweet potato acid phosphatase per 200 μl aliquot, or phosphatase in the presence of 10 mM sodium pyrophosphate (more than 10 X the K_i for sweet potato acid phosphatase). Pyrophosphate was added to the same concentration in the other samples prior to assay. Reactions were terminated and MAP kinase activity determined as before. All determinations are means ± SEM where n=>6.

TABLE 2

Treatment	Concentration	specific [^{35}S]thiophosphorylation of APRTPGGRR (as % control)
4 α-PDBu	1 μM	109 ± 14
	10 μM	118 ± 12
4 β-PDBu	1 μM	83 ± 15
	10 μM	99 ± 11
mezerein	1 μM	88 ± 11
	10 μM	74 ± 5
PMA	1 μM	104 ± 5
	10 μM	91 ± 6

TABLE 3

Preincubation	specific [^{35}S]thiophosphorylation of APRTPGGRR (dpm per assay)
control	1440 ± 152
0.3U sweet potato acid phosphatase	388 ± 226
phosphatase + 10 mM pPi	1517 ± 215

APPENDIX 2

Preparation of stripped foetal calf serum

Removal of thyroid hormones

Dowex 1 x8-400 resin (chloride form) was washed extensively in distilled water, then passed through a 0.22 μm filter. The resin was then incubated with heat-inactivated foetal calf serum (100 mg wet wt/ml serum) for 5 h on a rotor. The resin was removed by centrifugation (1000 x g, 10 min). Fresh resin was added to the supernatant (100 mg wet wt/ml serum) then incubated for 15-18 h at room temperature. The resin was removed by centrifugation (1000 x g, 10 min) followed by passing the supernatant through a 0.8 μm filter.

Removal of steroids

Activated charcoal was mixed with distilled H_20 and centrifuged (3000 x *g*) for 20 min. The water was discarded and the charcoal was incubated with the thyroid hormone-stripped foetal calf serum (0.02 g wet wt/ml serum), initially at 37°C for 40 min followed by 30 min at 55°C. The charcoal was removed by centrifugation (10 min, 300 x *g*) followed by passage through filters of 0.8 μm, 0.45 μm and 0.22 μm in succession.

Steroid-free DMEM

Phenol red free DMEM was obtained from Gibco BRL. This was additionally supplemented with sucrose (also from Gibco BRL) to give a final concentration of 4500 g/l which is equivalent to the sucrose concentration in the standard DMEM preparation . Penicillin and streptomycin were added to give a final concentration of 100 U/l and 0.1 mg/ml respectively. Stripped foetal calf serum was added to give a 10 % final concentration. All additions were under aseptic conditions.

APPENDIX 3

Iodination of cAMP using the Iodogen™ method

Labelling of cAMP with ^{125}Iodine for use in radioimmunoassay was performed using the Iodogen™ method essentially as described by Salacinski, McLean, Sykes et al (1981). Iodogen™ coated polypropylene 1.5 ml conical eppendorf centrifuge tubes (Sarstedt) were prepared by adding 50 µl of 0.04 mg/ml Iodogen (Sigma) in dichloromethane which was evaporated to dryness in a 37°C water bath in a fan extraction fume hood. 2'-0-monosuccinyl cAMP tyrosyl methyl ester (Sigma, cAMP) was stored in aliquots at 25 µM in dH_20 at -70°C before use.

Five microlitres of Na ^{125}I (ICN Radiochemicals, Irvine, CA, USA) containing 0.5 mCi (~0.25 nmol Na ^{125}I) were incubated with 0.25 nmol of cAMP-TME in a total volume of 50 µl in 0.5 M sodium phosphate buffer pH 7 in an Iodogen™ coated tube. After 12 min at room temperature the reaction was terminated by the addition of 1 ml 0.1% trifluoroacetic acid (TFA) and applied to a Sep-Pak C_{18} octadensilyl (ODS) cartridge. The cartridge was washed with three 2 ml aliquots of 0.1% TFA followed by 2 ml each of a stepwise gradient of methanol (10-80%) containing 0.1% TFA. Free iodine elutes in the initial wash, the 20% fraction was used in the cAMP radioimmunoassay.

Figure 2.1

Flow diagram of the method used to determine cytosolic MAP kinase activity in αT3-1, COS 7 or CHO cells and anterior pituitary pieces

Figure 2.1

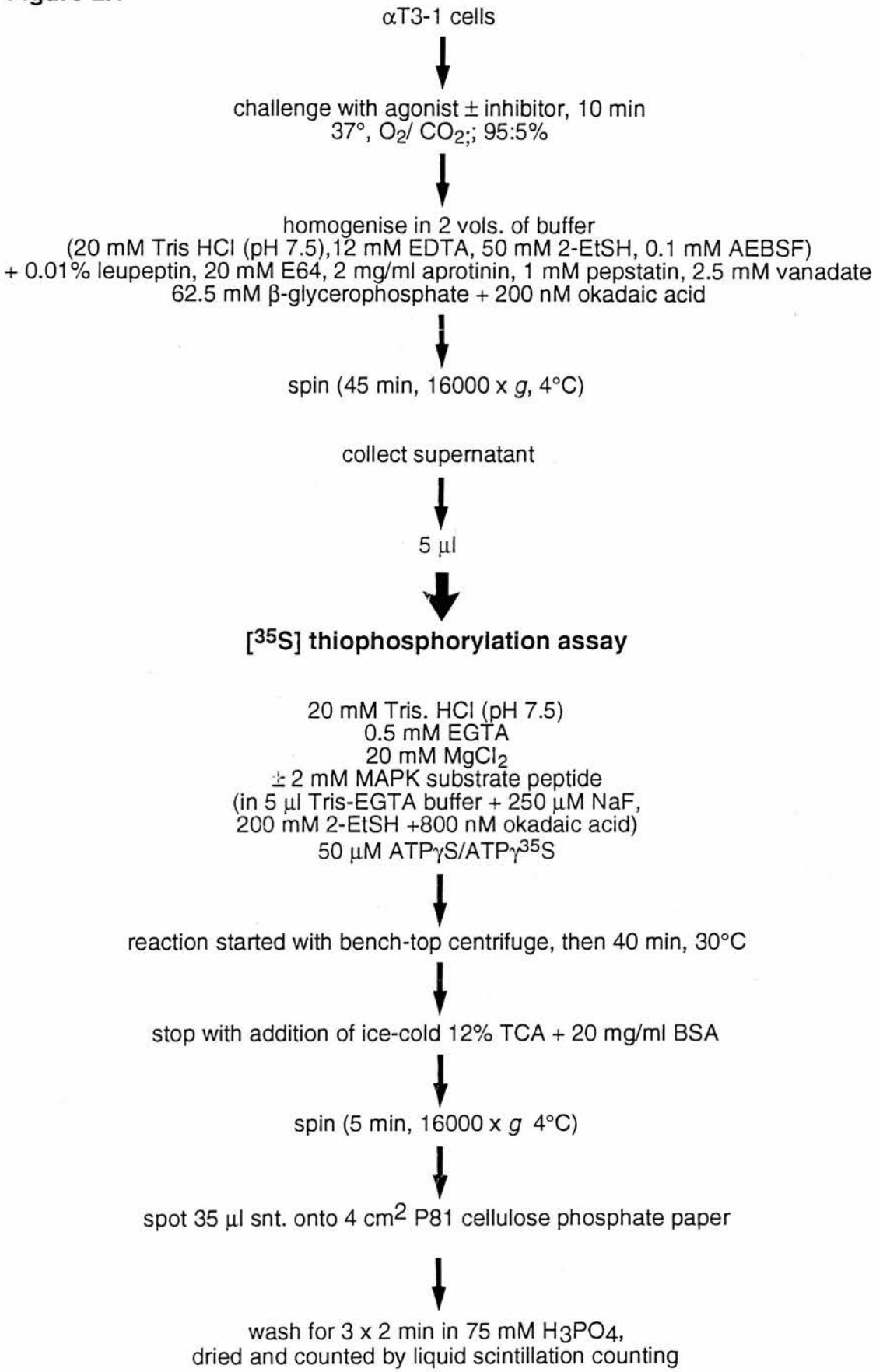

αT3-1 cells
challenge with agonist ± inhibitor, 10 min
37°, O_2/ CO_2;; 95:5%
homogenise in 2 vols. of buffer
(20 mM Tris HCl (pH 7.5),12 mM EDTA, 50 mM 2-EtSH, 0.1 mM AEBSF)
+ 0.01% leupeptin, 20 mM E64, 2 mg/ml aprotinin, 1 mM pepstatin, 2.5 mM vanadate
62.5 mM β-glycerophosphate + 200 nM okadaic acid
spin (45 min, 16000 x g, 4°C)
collect supernatant
5 µl
[35S] thiophosphorylation assay
20 mM Tris. HCl (pH 7.5)
0.5 mM EGTA
20 mM $MgCl_2$
± 2 mM MAPK substrate peptide
(in 5 µl Tris-EGTA buffer + 250 µM NaF,
200 mM 2-EtSH +800 nM okadaic acid)
50 µM ATPγS/ATPγ35S
reaction started with bench-top centrifuge, then 40 min, 30°C
stop with addition of ice-cold 12% TCA + 20 mg/ml BSA
spin (5 min, 16000 x g 4°C)
spot 35 µl snt. onto 4 cm^2 P81 cellulose phosphate paper
wash for 3 x 2 min in 75 mM H_3PO_4,
dried and counted by liquid scintillation counting

Figure 2.2

Cytosolic MAP kinase activity in pellet and supernatant fractions of αT3-1 cells treated with nil, LHRH or PDBu after immunoprecipitation with anti ERK (1 and 2) or anti β-tubulin antisera

αT3-1 cells were treated as in protocol for anti MAP kinase immunoprecipitation then pellet (P) and supernatant (S) fractions were included in a cytosolic MAP kinase assay

Figure 2.2

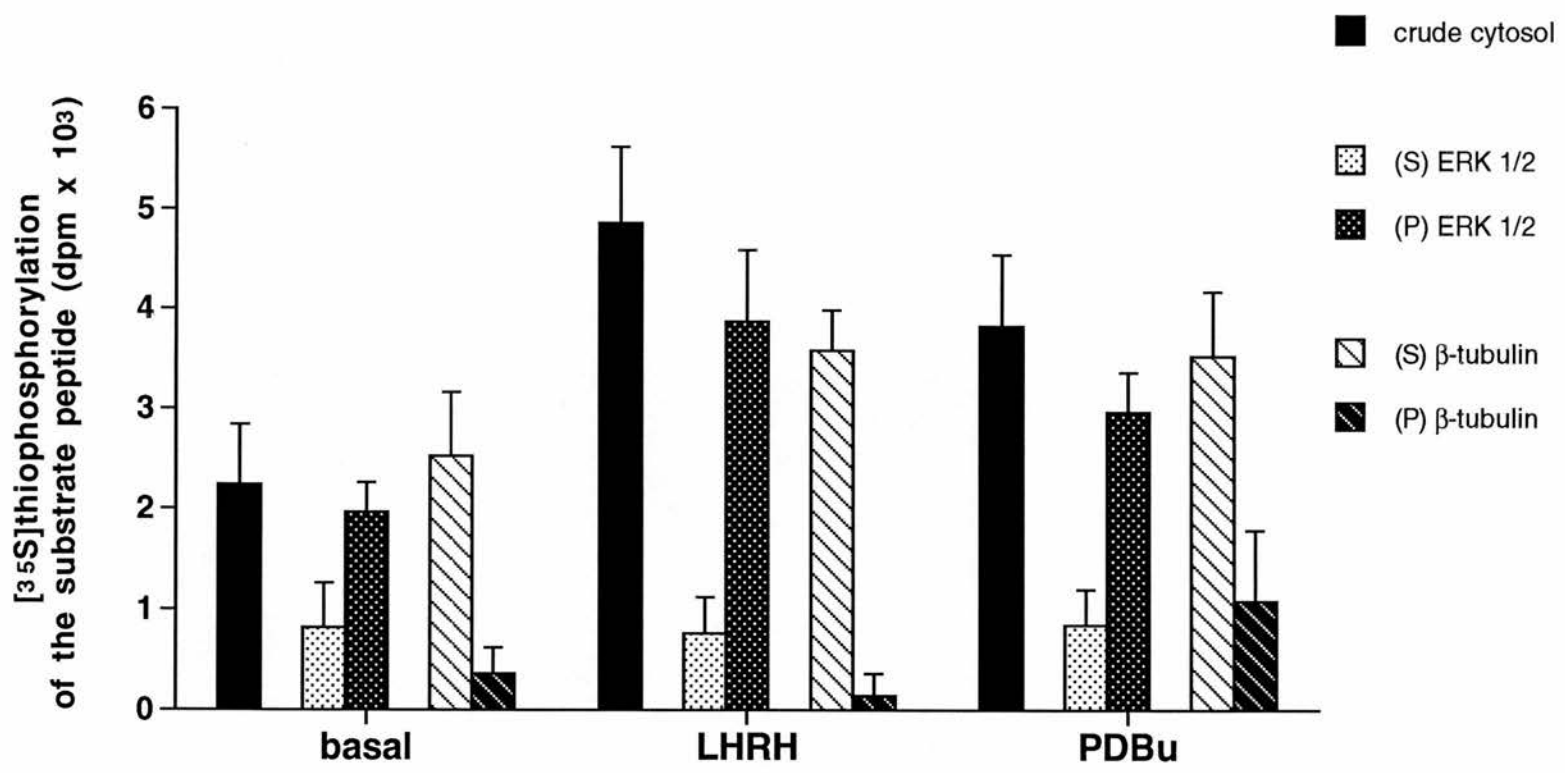

crude cytosol
(S) ERK 1/2
(P) ERK 1/2
(S) β-tubulin
(P) β-tubulin
[35S]thiophosphorylation of the substrate peptide (dpm x 10³)
0
1
2
3
4
5
6
basal
LHRH
PDBu

Figure 2.3

Flow diagram of methodology for the cAMP radioimmunoassay

Figure 2.3

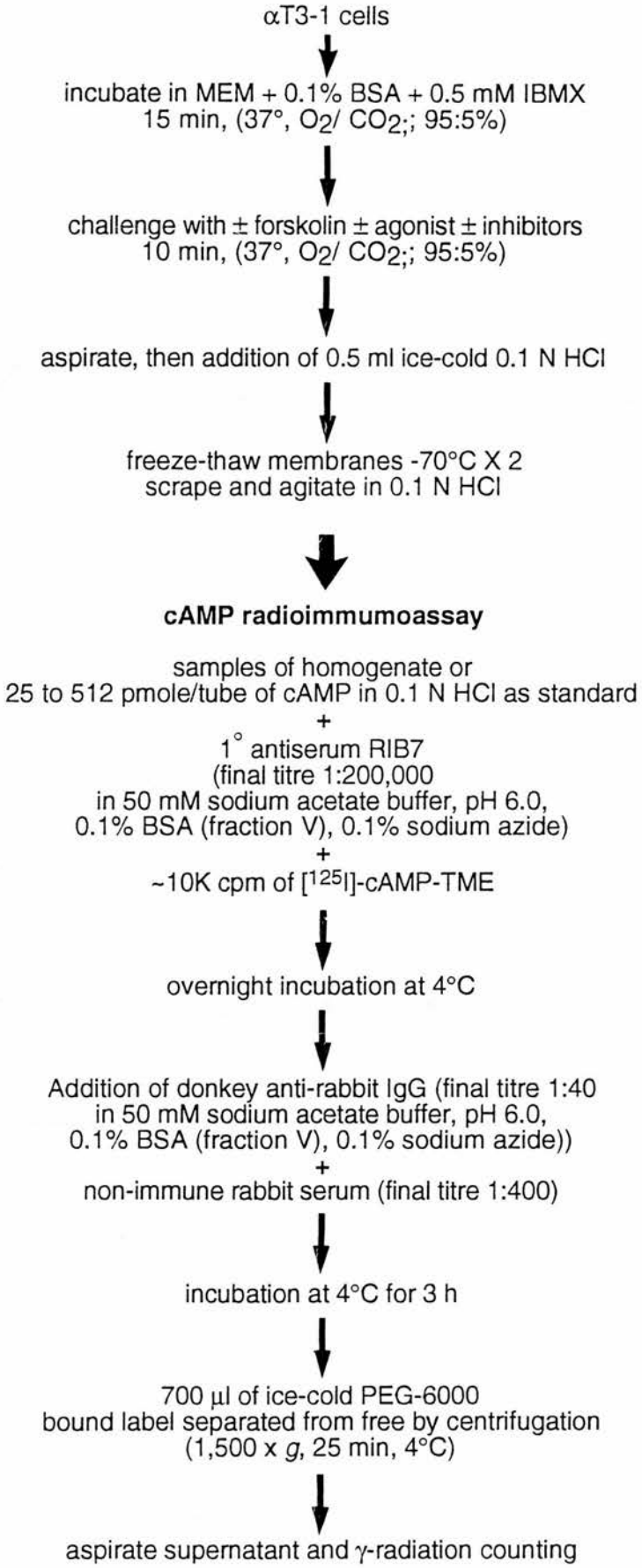

αT3-1 cells
incubate in MEM + 0.1% BSA + 0.5 mM IBMX
15 min, (37°, O_2/ CO_2;; 95:5%)
challenge with ± forskolin ± agonist ± inhibitors
10 min, (37°, O_2/ CO_2;; 95:5%)
aspirate, then addition of 0.5 ml ice-cold 0.1 N HCl
freeze-thaw membranes -70°C X 2
scrape and agitate in 0.1 N HCl
cAMP radioimmumoassay
samples of homogenate or
25 to 512 pmole/tube of cAMP in 0.1 N HCl as standard
+
1° antiserum RIB7
(final titre 1:200,000
in 50 mM sodium acetate buffer, pH 6.0,
0.1% BSA (fraction V), 0.1% sodium azide)
+
~10K cpm of [^{125}I]-cAMP-TME
overnight incubation at 4°C
Addition of donkey anti-rabbit IgG (final titre 1:40
in 50 mM sodium acetate buffer, pH 6.0,
0.1% BSA (fraction V), 0.1% sodium azide))
+
non-immune rabbit serum (final titre 1:400)
incubation at 4°C for 3 h
700 µl of ice-cold PEG-6000
bound label separated from free by centrifugation
(1,500 x *g*, 25 min, 4°C)
aspirate supernatant and γ-radiation counting

Figure 2.4

Characterisation of primary antisera (RIB 7) used in cAMP radioimmunoassay

(a) cAMP primary antibody (RIB 7) dilution curve

Linear relationship of total bound counts with increasing RIB 7 dilution. Total applied cpm = 13500 cpm and non specific binding was 350 cpm

(b) cAMP standard curve

cAMP concentration standard curve using RIB 7 at a final dilution of 1:200,00.

Both curves were generated using a 100 µl assay as described in methods section.

Figure 2.4

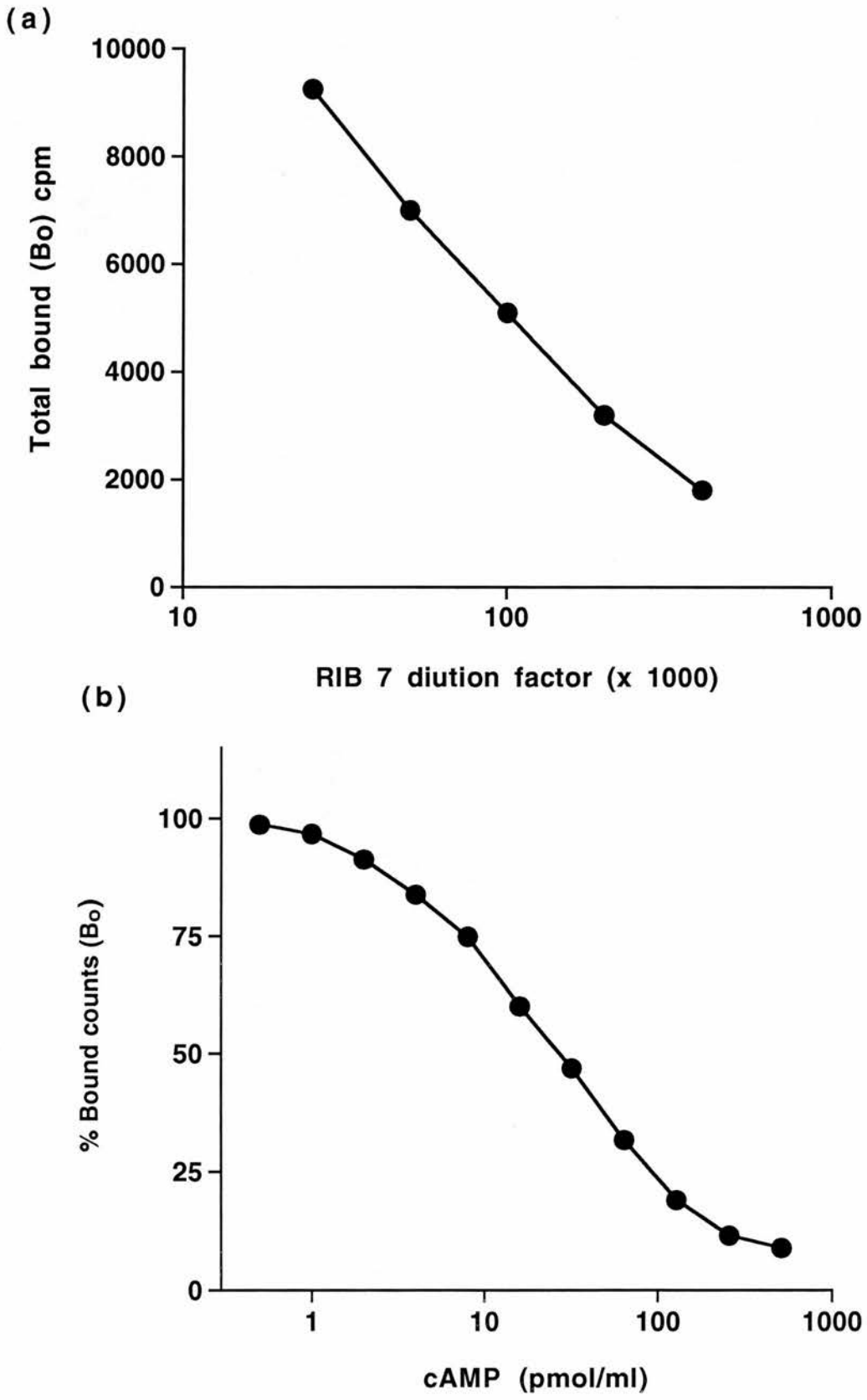

(a)
Total bound (Bo) cpm
10000
8000
6000
4000
2000
0
10
100
1000
RIB 7 diution factor (x 1000)
(b)
% Bound counts (Bo)
100
75
50
25
0
1
10
100
1000
cAMP (pmol/ml)

Figure 2.5

Flow diagram of the methods used to measure [^{3}H]arachidonic acid release from αT3-1 cells.

Figure 2.5

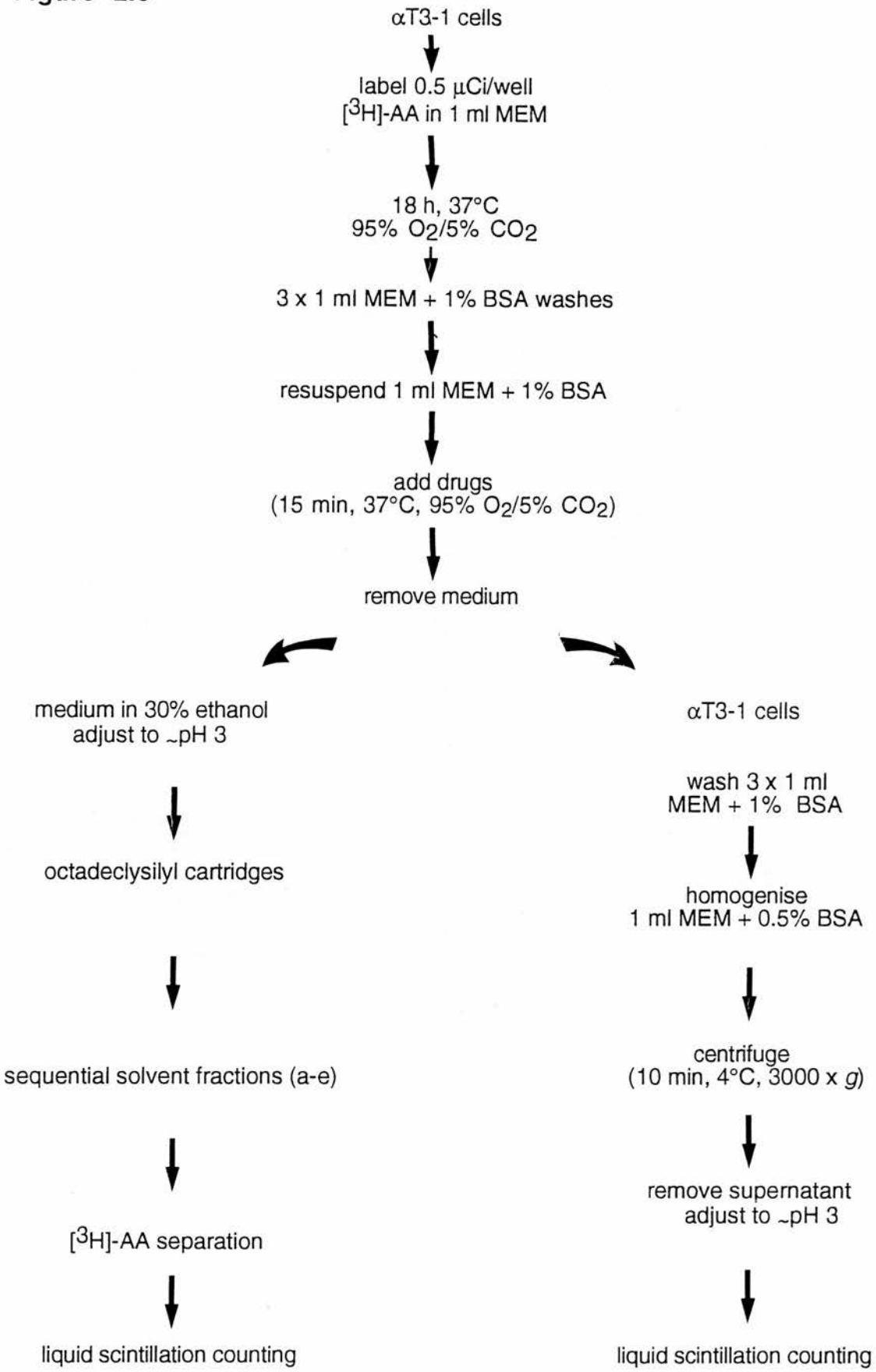
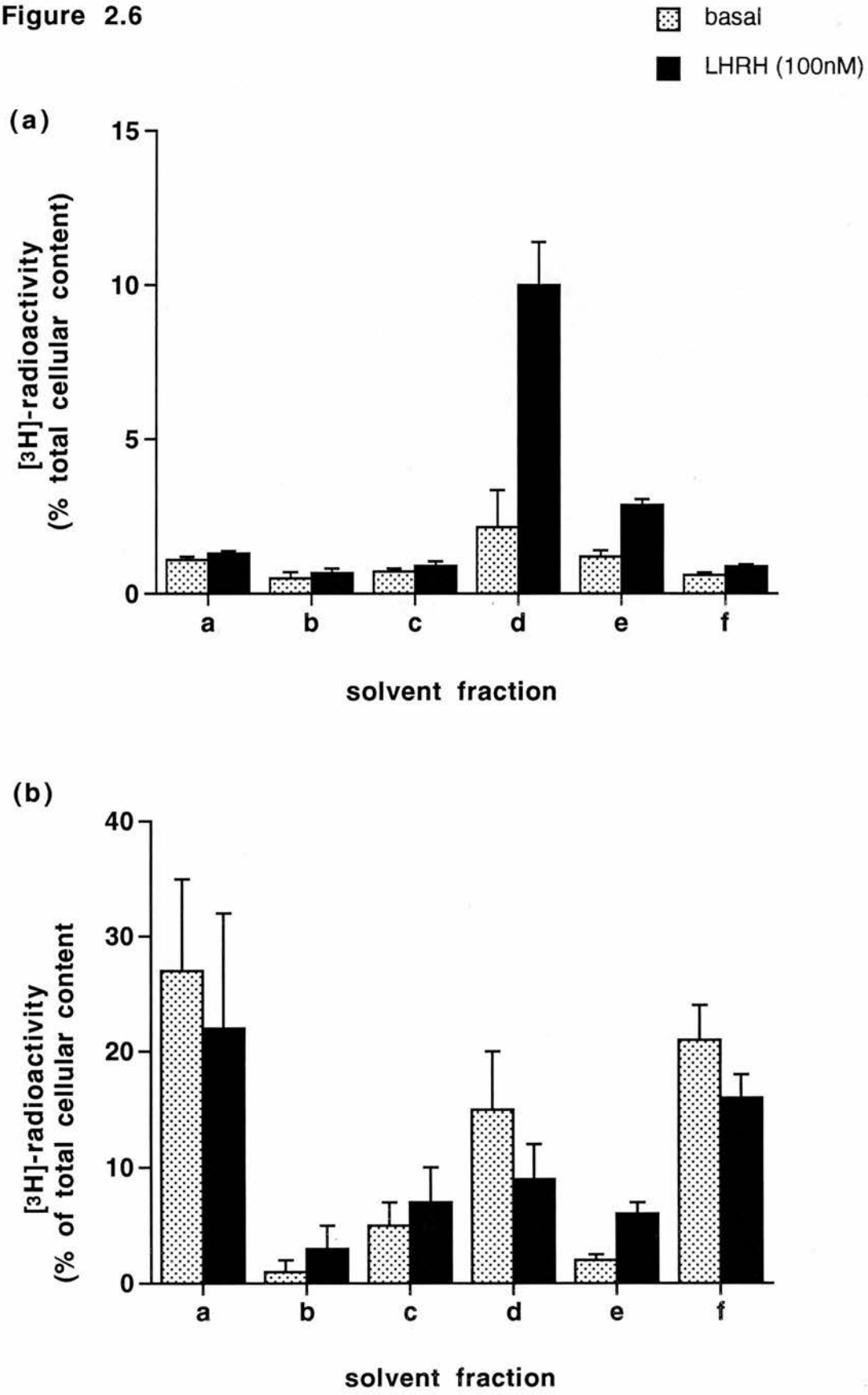

αT3-1 cells
label 0.5 µCi/well
[3H]-AA in 1 ml MEM
18 h, 37°C
95% O2/5% CO2
3 x 1 ml MEM + 1% BSA washes
resuspend 1 ml MEM + 1% BSA
add drugs
(15 min, 37°C, 95% O2/5% CO2)
remove medium
medium in 30% ethanol
adjust to ~pH 3
octadeclysilyl cartridges
sequential solvent fractions (a-e)
[3H]-AA separation
liquid scintillation counting
αT3-1 cells
wash 3 x 1 ml
MEM + 1% BSA
homogenise
1 ml MEM + 0.5% BSA
centrifuge
(10 min, 4°C, 3000 x g)
remove supernatant
adjust to ~pH 3
liquid scintillation counting

Figure 2.6

Typical example of the levels of radioactivity in solvent fractions from (a) the medium and (b) the cell homogenate from control and LHRH treated αT3-1 cells

αT3-1 cells were incubated in medium containing either no drug (basal) or LHRH (100 nM) for 15 min. The radioactivity in the solvent fractions of the medium (a) and cell homogenate (b) from each treatment was determined. The fractions are as follows; **a:** distilled H_2O; **b**: 30% ethanol; **c:** petroleum ether; **d**: petroleum ether:$CHCl_3$ (1:1); **e**: methyl formate; **f**: 100 % ethanol. Values are means ± SEM for 4 determinations.

Figure 2.6


basal
LHRH (100nM)
(a)
[3H]-radioactivity
(% total cellular content)
15
10
5
0
a
b
c
d
e
f
solvent fraction
(b)
[3H]-radioactivity
(% of total cellular content
40
30
20
10
0
a
b
c
d
e
f
solvent fraction

CHAPTER 3

ACTIVATION OF MITOGEN-ACTIVATED PROTEIN (MAP) KINASE BY THE LHRH RECEPTOR IN αT3-1 CELLS

3.1 INTRODUCTION

Mitogen-activated protein kinase appears to be expressed ubiquitously and represents a common point of convergence for a wide range of signals originating from numerous cell surface receptors [Sturgill & Wu, 1991]. The transduction of a mitogenic signal from the membrane to the nucleus by growth factor receptors (eg the epidermal growth factor (EGF) receptor) which have intrinsic tyrosine kinase activity is now well defined (as examined at greater length in the main introduction) and involves the formation of a membrane-associated multi-protein complex which catalyses the GTP-exchange and resulting activation of a low molecular weight G-protein Ras [Aronheim et al, 1994]. Functional activation of GTP-liganded Ras seems to be required for activation of Raf-1 kinase activity [Dent et al, 1992; Howe et al, 1992; Kyriakas et al, 1992] which in turn phosphorylates and activates the direct upstream activator of MAP kinase MEK-1, [Zheng & Guan, 1993]. Activation of Ras has been postulated to bring Raf-1 into the proximity of the membrane for a second regulatory event [Leevers et al, 1994]. Alternatively activators of PKC (such as phorbol esters) [Kazlauskas & Cooper, 1988; Nori et al, 1992] or a direct G-protein activator, fluoroaluminate [Anderson et al, 1991], have been reported to activate MAP kinase. More recently it has been reported that a number of receptors coupled to heterotrimeric G-proteins can stimulate MAP kinase activity. The signalling steps involved at present are less clearly defined than that of growth factor receptors and were unpublished at the onset of this study, hence this area will be considered in more depth in the discussion of this chapter.

Although the LHRH receptor is not commonly associated with mitogenesis [Batticane et al, 1991], in light of the diversity of events which MAP kinase regulates and the ability of agents which activate PKC or G-proteins to stimulate MAP kinase, it is possible that MAP kinase may participate in this receptors downstream signalling (as activation of the LHRH receptor results in phosphoinositide hydrolysis, Ca^{2+}

mobilisation and activation of PKC [Stojilkovic et al, 1994]). Furthermore there is also a few reports that MAP kinase is involved in secretion [Kanda et al, 1994; Offermans et al, 1994a; Ohmichi et al, 1994; Williams, 1995], which is the main endpoint of LHRH action on pituitary cells. In order to monitor stimulus-induced MAP kinase activity we have developed an *in vitro* assay measuring [^{35}S] thiophosphorylation of a selective peptide substrate containing the target motif characteristic of MAP kinases [Mitchell et al, 1994; Mitchell et al, 1993; Sim et al, 1994] and used an electrophoretic mobility shift assay to determine phosphorylation of p42 and p44 MAP kinase. Using a number of pharmacological agents we have determined that the LHRH receptor is capable of both phosphorylating and activating MAP kinase and have attempted to further resolve this novel signalling cascade.

3.2 RESULTS

Employing the [^{35}S]thiophosphorylation assays described in Chapter 2, the potential activation of MAP kinase in the αT3-1 gonadotroph cell line was investigated over a time course of 0-120 min with 100 nM LHRH (Figure 3.1). After a short lag of approximately 2 min, MAP kinase activity increased rapidly, reaching a peak by 10 min that represented an increase to 245 ± 9% of basal activity (mean ± SEM, n=6). This increased activity remained elevated for approximately 40 min, gradually declining thereafter leaving a significant residual activation at 120 min. LHRH-induced MAP kinase activity was concentration-dependent (Figure 3.2a), being nearly maximal by 100 nM LHRH and showing an apparent EC_{50} value (the concentration required for 50% of maximal activation) of 3.4 ± 0.4 nM. This value was comparable to that for LHRH-induced inositol phosphate formation in this cell line which was 2.8 ± 0.3 nM [Sim et al, 1995]. LHRH-induced activity was competitively inhibited by a highly selective LHRH antagonist [Ac-D-p-Cl-Phe^{1,2}, D-Trp^{3}, D-Arg^{6}, D-Ala^{10}]LHRH [Humphries, 1978], over a concentration range of 0.3 - 3000 nM with an IC_{50} of 38.2 ± 13 nM

antagonist (Figure 3.2b), confirming that the evoked activity was a specific result of LHRH receptor activation. The antagonist alone had no effect on MAP kinase activity.

A protein kinase C (PKC) activator 4β-phorbol-12,13-dibutyrate (PDBu, 1μM) could partially mimic the LHRH-induced increase in activity (Figure 3.1). The effect of PDBu peaked slightly earlier than that of LHRH to 197 ± 13% of basal activity between 5-8 min (mean ± SEM, n=6) but had returned to basal levels by 60 min in contrast to the LHRH-stimulated activity. This effect was also concentration-dependent (50 ± 8 nM, 8 min; mean ± SEM, n=6) being nearly maximal at 1 μM PDBu and was again shown to be specific as it was not mimicked by the inactive isomer 4α-PDBu; 0-10 μM; 8 min; maximum activity 110 ± 9% of basal, n=6 (Figure 3.3a). With the exception of epidermal growth factor (EGF), no other potential activator tested including sphingosine and ceramide was seen to significantly increase MAP kinase activity in these cells (results not shown). Basal (unstimulated) levels of MAP kinase activity in these cells remained constant throughout an equivalent time course measured for all agonists and were on average a mean value of 4000 dpm per assay tube compared to stimulated counts in the region of 4500-13000 dpm per assay tube. Non-specific counts were never more than 1500 dpm per assay tube. For all subsequent experiments using αT3-1 cells, MAP kinase activity was elicited by 100 nM LHRH and 1μM PDBu at 10 and 8 min respectively. The specificity of this assay for MAP kinase activity was monitored by immunoprecipitation with a monoclonal anti-p42/p44 antibody and is described in full in Chapter 2.

Having firmly established an LHRH-induced activation of MAP kinase a number of other approaches were used to further determine elements of this signalling cascade. The effect of number of selective PKC inhibitors was studied. LHRH-elevated activation appears to be dependent on PKC since the response was

effectively inhibited by all the PKC inhibitors examined (Figure 3.4a). IC_{50} values (the concentration required to inhibit 50% of the maximal response) for inhibition by GF109203X [Toullec et al, 1991], Ro 31-8220 [Davis et al, 1989] and H7 [Nakadate et al, 1989], of LHRH-induced MAP kinase activity were 1.8 ± 0.12, 0.64 ± 0.08 and 168 ± 12 μM (mean ± SEM, n=6) respectively. These values are consistent with other known PKC mediated responses, especially in anterior pituitary tissue [Johnson et al, 1992]. In addition, when compared to a control set of cells, 92 ± 10% (mean ± SEM, n=4) of the LHRH-induced activity was inhibited when phorbol ester-sensitive isoforms of PKC were down-regulated by an 18h exposure to 300 nM PDBu. Control cells were exposed to vehicle (0.3% DMF) over the same time period (Figure 3.3b).

As anticipated, GF109203X, Ro 31-8220 and H7, also strongly inhibited PDBu-induced MAP kinase activity and this data is shown in Figure 3.4b. The IC_{50} values are 0.26 ± 0.02, 0.57 ± 0.03 and 43.5 ± 5.1 μM respectively (mean ± SEM, n=6). All inhibitors were incubated with the cells for 2 min before addition of LHRH and where appropriate vehicle alone was added to a control flask. At nearly maximal concentrations (5 and 1 and 300μM for GF109203X, Ro-31 8220 and H7 respectively) none of the inhibitors had any effect on basal MAP kinase activity in αT3-1 cells and had previously been shown to have no direct effect on MAP kinase when included together with a crude cytosolic preparation of hippocampus tissue in an *in vitro* activity assay of MAP kinase (see Appendix 1).

A potential involvement of tyrosine kinases was also investigated. The tyrosine kinase inhibitors genistein [Akiyama & Ogawara, 1991] and N-methyl 2, 5-dihydroxycinnamate (MDC) [Isshiki et al, 1987] inhibited the LHRH-induced MAP kinase response with apparent IC_{50} values of 15 ± 0.6 μM and 33 ± 7 μM respectively.

Pre-treatment with pertussis toxin has been shown to inactivate G-proteins of the $G_{i/o}$ class by catalysing the ADP-ribosylation of a specific cysteine residue in the α-subunit of the heterotrimer thus preventing receptor interaction and the consequent GDP/GTP exchange [Milligan, 1988]. Preincubation for 15-18 h has been shown to be fully adequate for down-regulation of any pertussis toxin-sensitive G-proteins in this way [Milligan, 1988]. In Figure 3.6a, treatment of αT3-1 cells for 18 h with pertussis toxin in the range of 3-300 ng/ml clearly inhibited LHRH-induced MAP kinase activity. A maximum inhibition of approximately 75% was seen at the highest concentration used and the IC_{50} was 32 ± 4 ng/ml (mean ± SEM, n=8). This effect was shown to be specific to the active holotoxin and not some non-specific consequence such as membrane perturbation (Figure 3.6b) as under similar conditions, treatment of the cells with either the inactive B-subunit of pertussis toxin [O'Neill et al, 1992] or N-ethyl maleimide-inactivated holotoxin [Banga et al, 1987] at a concentration of 100 ng/ml, had no significant effect on LHRH-induced MAP kinase activity (104 ± 10%, 95 ± 8% of control LHRH responses respectively. Also shown in Figure 3.4b none of the pertussis toxin treatments had any effect on basal (unstimulated) activity.

Mastoparan is a peptide activator of G-proteins with reported selectivity for $G_{i/o}$ over other G-proteins and hence can be used to monitor $G_{i/o}$ action without the requirement for receptor activation [Gil et al, 1991; Higashijima et al, 1988] Mastoparan however is known to be toxic at high concentrations and has reported membrane perturbing effects [Higashijima et al, 1988]. In αT3-1 cells mastoparan, like LHRH, caused a marked increase in MAP kinase activity at 10 min. This was maximal at 10 μM and represented an increase of 190 ± 9% of basal activity (mean ± SEM, n=6). Mastoparan had no effect on inositol phosphate formation in these cells as seen in Figure 3.7a at concentrations up to 12.5 μM. The concentrations used here are similar to those used by other

laboratories in apparently $G_{i/o}$-specific responses [Gil et al, 1991; Higashijima et al, 1988].

Mastoparan-induced MAP kinase activity was substantially inhibited by pre-treatment with pertussis toxin (60 ± 4%; 100 ng/ml) but not by the PKC inhibitor GF109203X (12 ± 0.9%, 3μM, mean ± SEM, n=4) as shown in Figure 5.3b Figure 3.5b. In contrast, PDBu-induced activity was essentially prevented by GF109203X as expected and was unaffected by pertussis toxin (85 ± 7% and 10 ± 2% respectively; mean ± SEM, n=6). These data would suggest that the PKC activation necessary for LHRH action was not downstream of $G_{i/o}$ derived βγ subunits activating PLC β2 or 3, but more likely a result of $G_{q/11}$-mediated stimulation of PLC β1. It would also precludes the possibility that the pertussis toxin sensitive component was executed downstream of PKC rather than by direct receptor interaction. The combination of PDBu and mastoparan caused greater MAP kinase activation than either alone (Figure 3.7b). From the data obtained under these conditions it is not possible to determine whether the interaction between the two effects is cumulative or additive although no evidence for synergy was observed.

In order to confirm the results with the MAP kinase activity assay an independent technique was applied, MAP kinases are activated by phosphorylation on both a tyrosine and threonine residue by dual specificity kinases, therefore the phosphorylation-induced gel mobility shift in immunoreactive p42 and p44 MAP kinases can be monitored as an index of their activation [deVries et al, 1992]. Figure 3.8 shows both p42 and (to a lesser extent) p44 species displayed a component of reduced electrophoretic mobility after LHRH stimulation for 10 min. This effect was concentration-dependant with a shift already detectable at 1 nM, being maximal at 10-100 nM LHRH Figure 3.8a). The increase in LHRH-induced MAP kinase phosphorylation is detectable after 2 min, reached a maximum at 5-10 min and

remains elevated for at least 60 minutes (Figure 3.8b). Treatment of the cells with PDBu (1μM) also resulted in phosphorylation of p42 and p44 MAP kinase Figure 3.8c). It is apparent that the entire pool of MAP kinase present in αT3-1 cells was not completely phosphorylated to the active form in these conditions. Confirming the thiophosphorylation data, both the PKC inhibitor GF109203X (3μM; Figure 3.9) and since we shown with the *in vitro* kinase assay a potential role for a tyrosine kinase involvement during LHRH action in αT3-1 cells, this was further investigated. The tyrosine kinase inhibitors genistein and to a lesser extent piceatannol [Gaehlen and McLaughlin, 1989] inhibited the LHRH-induced shift in mobility of immunoreactive MAP kinase (Fig. 3.9). Piceatannol and genistein also inhibited PDBu-induced tyrosine phosphorylations and MAP kinase activation, suggesting a putative role for tyrosine kinases downstream of PKC (data not shown). The tyrosine phosphatase inhibitor pervanadate [Grinstein et al 1990] also stimulated MAP kinase phosphorylation and hence reduction in mobility (although not to the extent of LHRH or PDBu), further implying a requirement for tyrosine phosphorylation in MAP kinase activation. This effect was also more prominent in the presence of LHRH (100 nM) which may also imply that LHRH-induced phosphorylation and activation is dependent on dual signalling input.

Pertussis toxin inhibited LHRH-induced phosphorylation and prevented the band shift (Figure 3.10). U73122 inhibits activation by interfering with G-protein coupling, because it prevents agonist- but not ionomycin-induced inositol phosphate responses in neuroblastoma cells [Thompson et al, 1991]. On the other hand, neomycin binds to phosphatidylinositol 4,5-bisphosphate and interferes with its hydrolysis by agonist-activated PLC [Slivka & Insel, 1988]. These two agents prevented the band shift implying a requirement for the products of PI hydrolysis (Fig. 3.10). Taken together these data indicate some involvement by both $G_{i/o}$ and $G_{q/11}$ proteins.

3.3 DISCUSSION

The evidence presented in Chapter 3 clearly demonstrates a marked and sustained phosphorylation and activation of p42 and p44 MAP kinase by the LHRH receptor. This response was highly dependent on PKC action and could be partially mimicked by phorbol ester. Tyrosine phosphorylation was also required. Inhibition of this response by pertussis toxin and partial mimicry by mastoparan (in a pertussis sensitive manner) provided evidence for $G_{i/o}$-mediated signal transduction by the LHRH receptor in addition to the well characterised hydrolysis of phosphoinositide by PLCβ-1 and activation of PKC, mediated by $G_{q/11}$ [Anderson et al, 1993; Hsieh & Martin, 1992; Shah & Milligan, 1994; Mitchell et al submitted 1995]. The LHRH receptor is an unusual member of the rhodopsin family of seven transmembrane-domain G-protein linked receptors. Recent sequence data have revealed a number of atypical sequence motifs and the complete lack of a C-terminal tail and hence the loss of some key potential phosphorylation sites implicated in desensitisation [Tsutsumi et al, 1992]. This may account for its characteristic resistance to rapid homologous desensitisation [Davidson et al, 1994b; Fennell et al, 1993] and as shown here, its ability to interact not only with $G_{q/11}$ but also $G_{i/o}$ and sustain a relatively long-lasting activation of MAP kinase.

The present results strongly support a hypothesis that the LHRH receptor in αT3-1 cells can interact with multiple G-protein families. MAP kinase phosphorylation was prevented by two PLCβ inhibitors neomycin and U73122 [Bleasdale et al, 1990; Smith et al, 1990] and pertussis toxin. Mastoparan or pertussis toxin had no effect on LHRH-induced inositol phosphate production in αT3-1 cells [Sim et al, 1995] dismissing the possibility that PKC activation arises from βγ subunits released from $G_{i/o}$ leading to the activation of PLCβ 2 or 3. Notably, until this study, there has been no clear evidence that the LHRH receptor can interact with any other G-protein other than $G_{q/11}$ (Figure 3.5) [Stojilkovic et al, 1994]. Only one isolated report has

described partial sensitivity to pertussis toxin of LHRH-induced [^{3}H] inositol phosphate formation, but that was in the absence of any corresponding changes in gonadotrophin secretion [Hawes et al, 1993]. However pertussis toxin can demonstrate some anomalous effects, independent of the attenuation of $G_{i/o}$ activation [Banga et al, 1987; Burch et al, 1988; O'Neill et al, 1992]. Like the blockade of LHRH-induced MAP kinase activation here, authentic ADP-ribosylation of $G_{i/o}$ is seen only with pertussis holotoxin and not with N-ethylmaleimide-treated toxin or the toxin B-oligomer [Banga et al, 1987; O'Neill et al, 1992].

The present results provide strong evidence for an obligatory role of PKC in MAP kinase activation, since the response was greatly attenuated by PKC depletion and was fully blocked by several PKC inhibitors. The use of phorbol ester down-regulation as a means of assessing PKC involvement nevertheless should be treated with a degree of caution. Long term treatment with phorbol esters will not only cause intense activation of phorbol ester-sensitive PKC isoforms which may lead to numerous secondary effects within the cells, but many other signalling proteins such as Raf-1, myosin heavy chain kinase, DAG-kinase and a low molecular weight G-protein Vav amongst others [reviewed in Liscovitch & Cantley, 1994] contain cysteine-rich motifs which are target sequences for phorbol ester interaction hence may therefore be affected by this treatment. αT3-1 cells are known to express the α, ε and ζ isoforms of PKC at high levels [Johnson et al, 1993a]. Each of these isoforms displays somewhat varying pharmacological, and probably also targeting specificities [Martiny-Baron et al, 1993]. However in this present study no attempt was made to assess the relative contributions of individual isoforms to MAP kinase activation. On examination of the IC_{50} values for inhibition by two members of the bisindolylameide series of PKC inhibitors, GF 109203X and Ro-31 8220, on the PKC-mediated, LHRH stimulation of MAP kinase activity, in comparison with their inhibition of the various known isoforms in a cell free assay system, the profile would suggest involvement of a Ca^{2+}-independent isoform or isoforms, for example PKC-ε

[Martiny-Baron et al, 1993; Toullec et al, 1991]. Compared to various PKC isoforms in cell free assays [Schaap & Parker, 1990], LHRH stimulated MAP kinase activity displayed a relative resistance to the inhibitor H7. The IC_{50} value, 168 ± 12 μM, for this response is nonetheless consistent with a recently characterised Ca^{2+}-independent PKC isoform found in the anterior pituitary gland of rats [Ison et al, 1993]. It is likely that at the relatively high concentration of PDBu used in these experiments, uniform activation of all PDBu-sensitive PKCs will occur. Thus a different PKC isoform or sub-set of isoforms to that of LHRH may well be activated in these cells which would account for the appreciable differences observed between the IC_{50} values for these agents between LHRH- and PDBu-stimulated MAP kinase activity. Such differences were also observed between PDBu and LHRH in phospholipase D (PLD) activity also in αT3-1 cells [Fennell et al, 1993] and in the LHRH or PDBu-induced LH release from anterior pituitaries *in vitro* [Johnson et al, 1992; Thomson et al, 1993b; Thomson & Mitchell, 1993]. It is also possible that if PKC is only one of several possible components leading to LHRH-induced MAP kinase response that the LHRH response may be somewhat less sensitive to PKC inhibitors than the MAP kinase activation elicited by a PKC-specific stimulus. Long term exposure to phorbol esters will downregulate the phorbol ester-sensitive PKC isoforms present in the cells [Hug & Sarre, 1993; Huwiler et al, 1991]. Using this regime, PKC α, and ε have been shown to be fully downregulated in αT3-1 by 18 hours [Johnson et al, 1993a]. The atypical Ca^{2+}-independent isoform, PKC-ζ only has one cysteine-rich zinc finger domain and is therefore not downregulated by phorbol esters which require two zinc finger domains for their molecular recognition [Chen & Murakami, 1992; Ways et al, 1992]. This would make PKC-ζ a poor candidate for the PKC action. However a phorbol sensitive PKC-ζ isomer may be involved since it has been reported that PKC-ζ translocates to the membrane as a result of PMA treatment in human platelets [Crabos et al, 1992] and has been shown to be downregulated as a result of PDBu-treatment in αT3-1 cells [Johnson et al,

1993a] Although PKC-ζ has been proposed as an essential effector for Ras function [Dominguez et al, 1992], conflicting reports of PKC ζ's role in MAP kinase activation exist. In *Xenopus* oocytes, PKC ζ is associated with activation of the MEK-MAPK cascade [Carnero et al, 1995; Diaz-Meco et al, 1994], and this may be required for proliferation [Diaz-Meco et al, 1994]. However it has been proposed that PKC ζ does not likely mediate Ras-induced signal transduction as PKC ζ-mediated activation of downstream pathways was sensitive to cyclohexamide (CHX), whilst Ras-induced pathways such as activation of GVDB, MAP kinase or S6 kinase II were insensitive to CHX [Carnero et al, 1995]. Furthermore in NIH 3T3 cells overexpression of PKC ζ did not alter the growth characteristics or transform cells or potentiate Ras-mediated effects [Crespo et al, 1995b]. The concentrations of agents that were effective in the present experiments are in line with concentrations used in other laboratories [Buhl et al, 1995; Cook et al, 1992; Purkiss & Boarder, 1992; Toullec et al, 1991] and taken together the data indicate an assured role for PKC, but of course do not conclusively preclude the possibility that some other kinase may be involved in MAP kinase activation.

The present results suggest a role of tyrosine phosphorylation but at what stage this is required is unresolved. Immunoblotting experiments showed that αT3-1 cells contain *src* and *fyn* (but not *fgr*, *hck*, *lyn* or *yes*) [Fennell et al, 1993]. The inhibitor genistein is known to inhibit several classes of protein-tyrosine kinases including *src* and *fyn* [Akiyama & Ogawara, 1991]. Genistein has now also been shown to inhibit at higher concentrations, some serine/threonine kinases (ie PKC) [Hollenberg, 1994] but MDC and piceatannol, which are structurally unrelated to genistein, also prevented MAP kinase activation. Stimulation of αT3-1 cells with LHRH results in tyrosine phosphorylation of a number of proteins with molecular weights of 65-75, 125 and 130 kDa in addition to p42 and p44 MAP kinase [Wolbers et al, 1995]. Interestingly this corresponds closely to the weights of a number of proteins which are tyrosine phosphorylated downstream of the lysophosphatidic acid (LPA) [Hordijk

et al, 1994]. These tyrosine phosphorylations are probably not all involved in MAP kinase activation and their function remains unclear at present. An involvement of both PKC and as yet unidentified tyrosine kinases is similar to the LHRH-induced PLD activation in αT3-1 cells [Fennell et al, 1993] and to platelet-activating factor (PAF)-induced PLD activation in CHO cells [Liu et al, 1994]. In the latter case the possibility that MAP kinase may be involved in PLD activation was assessed. It was concluded that these enzymes are activated through a common pathway which acts in a parallel but not linear sequence.

It is becoming increasingly obvious that MAP kinase can be activated by a wide array of G-protein linked receptors. These include amongst others, receptors which are predominantly linked to $G_{q/11}$ such as M_1 and M_3 muscarinic, α_{1B} adrenergic, endothelin, angiotensin II, bombesin, TRH, histamine, P_{2U} nucleotide receptors, and cholecystokinin (CCK)/gastrin receptors [Bogoyevitch et al, 1993; Cazaubon et al, 1993; Crespo et al, 1995a; Duan & Williams, 1994; Ely et al, 1990; Faure et al, 1994; Hawes et al, 1995; Ohmichi et al, 1994; Pang et al, 1993; Seufferlein et al, 1995; Wang et al, 1992; Wheeler-Jones & Pearson, 1995]; those that are predominantly linked to $G_{i/o}$ such as the α_{C10} adrenergic, M_2 muscarinic, D_2 dopaminergic and α_1 adenosine receptors, chemoattractant C5 and C3 receptors [Albas et al, 1993; Buhl et al, 1995; Crespo et al, 1994; Faure et al, 1994; Grinstein et al, 1994] and those that readily interact with both families of G-proteins such as LPA, thrombin and PAF [Honda et al, 1994; Hordijk et al, 1994; Kahan et al, 1992b].

Receptors that couple to the heterotrimeric G-proteins G_q or G_i can stimulate phosphoinositide (PI) hydrolysis through the interaction of the α_q or G_i-$\beta\gamma$ subunits with the β-isoforms of the effector enzyme PI-PLC. PI hydrolysis produces both inositol 1,4,5-trisphosphate and diacylglycerol, leading to activation of protein kinase C (PKC), which can also stimulate MAP kinase activity in some cases. However it now also seems apparent that distinct pathways exist for activation of MAP kinase by

these different classes of G-protein and these pathways can be distinguished by a number of characteristics. It now appears that $G_{q/11}$ signalling to MAP kinase require α subunit activation of PI-PLCβ, are PKC-dependent and are in some instances Ras independent. In contrast $G_{i/o}$ receptor signalling is conveyed by $\beta\gamma$ subunits to Ras with a likely involvement of tyrosine phosphorylation [Hawes et al, 1995].

The responses leading to phosphorylation and activation of MAP kinase following $G_{q/11}$ and especially $G_{i/o}$-linked receptors activation often are accompanied by an increased GTP loading of Ras [Albas et al, 1993; Duan et al, 1995; Hawes et al, 1995; Hordijk et al, 1994; Ohmichi et al, 1994] and are severely attenuated by overexpression of dominant negative mutant Ras [Crespo et al, 1994; Hawes et al, 1995]. However Ras-independent paths also exist [LaMorte et al, 1994; Van Renterghem et al, 1994]. This suggests that these $G_{q/11}$ and $G_{i/o}$-mediated responses are predominantly utilising a conventional Ras-Raf cascade of activation [Avruch et al, 1994]. Proposed alternatives to Raf include: MEK kinase [Lange-Carter et al, 1993], now known to be involved in the signalling cascades which lead to activation of the Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) a family of serine-threonine kinases which are closely related to ERKs [Coso et al, 1995; Minden et al, 1994; Xu et al, 1995] although MEKK can activate ERKs by overexpression [Minden et al, 1994]; or Mos a Ras-independent pathway observed in *Xenopus* oocytes [Posada et al, 1993]. The role of Ras in PKC-dependent MAP kinase activity is complicated. There have been reports of PKC acting both upstream and downstream of Ras [De Vries-Smits et al, 1992; Downward et al, 1990; Harwood & Cambier, 1993; Nori et al, 1992]. Correspondingly, PKC may inactivate the Ras GTPase-activating protein GAP [Downward et al, 1990], potentially cause activation of GTP exchange factors [Feig & Schaffhausen, 1994], and in the case of PKC α and β, may directly phosphorylate the Ras target Raf-1 [Kolch et al, 1993; Sözeri et al, 1992]. It is not clear, however, that PKC action on

Raf-1 actually increases its functional activation of MAP kinase kinase (MEK) [Macdonald et al, 1993]. It has been proposed that the activation of Ca^{2+}-independent PKC isoforms (such as ε) by the products of phosphatidyl choline-specific phospholipase C may be capable of Ras-independent stimulation of Raf-1 [Daum et al, 1994]. Although TRH promotes both GTP loading of Ras and phosphorylation of Raf-1, these effects are reported to be PKC-independent but accompanied by tyrosine phosphorylation of a potential director of Ras activation, *Shc* [Ohmichi et al, 1994]. The precise mechanism by which PKC activates Raf-1 is as yet unresolved and under investigation.

G_i-linked receptor activation seems to be mediated not by the α subunit but by βγ subunits [Faure et al, 1994; Hawes et al, 1995; Luttrell et al, 1995; Van Biesen et al, 1995]. When GTPase deficient α_s, α_i, α_{11} subunits (which are rendered constitutively activate by this mutation) and βγ subunits were expressed in HEK 293 cells, only the βγ subunits induced phosphorylation of MAP kinase [Ito et al, 1995]. Furthermore, in COS and CHO cells transfected with α_{2A} adrenergic receptor (AR), stimulation of MAP kinase activity was blocked by expression of the carboxy terminus-peptide from the β-adrenergic receptor (βARK ct), which sequesters βγ subunits, or dominant negative mutants of Ras (Ras N17) and/or Raf-1(N*Raf) but was unaffected by PKC depletion by downregulation. In contrast under similar conditions the G_q-linked receptors, $\alpha 1_B$ AR or M_1 were inhibited by downregulation and expression of N*Raf but unaffected by expression of βARKct or RasN17 [Hawes et al, 1995]. Also in COS 7 cells, overexpression of βγ subunits or PLCβ2 (which is predominantly activated by βγ), as well as constitutively active α_q subunits could increase MAP kinase activity whereas mutationally activated α_i was without effect [Faure et al 1994]. Stimulation of MAP kinase by β-adrenergic receptors which are typical of those that act through G_s may also involve βγ subunits. Exposure of COS 7 cells to a β-adrenergic agonist increases MAP kinase activity, which was not mimicked by expression of an GTPase-deficient, constitutively activated mutant α_s or

by PKA stimulating agents [Crespo et al, 1995a]. Furthermore activation was not inhibited by depletion of PKC but was completely abolished by expression of βARK ct peptide (including the βγ binding domain) or Ras-inhibiting molecules [Crespo et al, 1995a]. Although now widely accepted that G_q-linked receptors signal via α subunits, some studies of the G-protein subunit responsible for mediating G_q-coupled receptor-stimulated MAP kinase activation have shown conflicting results [Crespo et al, 1994; Faure et al, 1994; Koch et al, 1994]. αT, the Gα subunit of transducin, which sequesters free Gβγ subunits, inhibited M_1 receptor-stimulated MAP kinase activation [Crespo et al, 1994], suggesting that G_q-coupled receptor activation was mediated by βγ subunits, although another study reported that neither M_1- or bombesin receptor-stimulated MAP kinase activation was attenuated by αT [Faure et al, 1994].

A partial PKC-dependence of M_1 muscarinic receptor-mediated MAP kinase activation has been observed in both CCL 39 and COS 1 host cells [Kahan et al 1992b; Qian et al 1993], whereas the M_3 receptor (but not the epidermal growth factor (EGF) receptor response in SH-SY5Y cells was completely prevented by PKC downregulation or by the PKC inhibitor, calphostin C [Offermans et al, 1993]. Activation of MAP kinase by endothelin-1 (ET-1) in renal mesangial cells, cardiomyocytes and astrocytes is substantially inhibited by PKC downregulation and is unaffected by pertussis toxin [Bogoyevitch et al, 1993; Cazaubon et al, 1993; Wang et al, 1992]. Similarly, in CHO host cells both the M_1 receptor- and α_{1B} adrenergic receptor-mediated MAP kinase activation are completely abolished by PKC depletion [Hawes et al, 1995]. Activation of MAP kinase by bombesin, but not EGF, in Swiss 3T3 cells was inhibited over 60% after PKC downregulation or by the inhibitor, H7 [Pang et al, 1993]. In GH_3 cells, TRH-induced phosphorylation/activation of MAP kinase was decreased by approximately 60% after PKC down regulation [Ohmichi et al, 1994]. It is not yet clear whether the different degrees of PKC dependence displayed by these G_q-linked receptors reflect

different signal transduction abilities by the receptors themselves or differences in the pathway components in the cells where they are expressed (for example the complement of PKC isoforms present in the cell or the target proteins).

$G_{i/o}$ receptor-mediated MAP kinase activation appears not to be dependent on PKC, since the pertussis toxin-sensitive MAP kinase response to α_2C10 receptors in Rat-1 fibroblasts is unaccompanied by any phosphoinositide hydrolysis and cannot be reliably mimicked by phorbol esters [Albas et al, 1993; Hordijk et al, 1994]. In accordance with this, $\beta\gamma$-mediated MAP kinase responses which were induced by either activation of the transfected α_2C10 receptor or overexpression of $\beta\gamma$ subunits in COS 7 cells, were unaffected by PKC depletion [Hawes et al, 1995]. Furthermore, a direct comparison of M_1 and M_2 muscarinic receptors expressed in COS cells confirmed a partial inhibition of M_1 but not M_2 receptor responses by PKC-down-regulation, while responses to EGF were little affected [Crespo et al, 1994]. M_2 receptor responses were prevented by pertussis toxin while M_1 receptor responses were virtually unaffected. Correspondingly, the activation of MAP kinase by overexpression of a GTPase-deficient mutant α_{i2} subunit (in Rat-1 but not COS 7 cells) is unaltered by PKC down-regulation [Gupta et al, 1992].

In the case of thrombin and LPA receptors, MAP kinase activation is pertussis toxin-sensitive whilst other response such as phosphoinositide hydrolysis and tyrosine phosphorylation, as well as control response to basic fibroblast growth factor (FGF) and EGF are unaffected [Hordijk et al, 1994; Hung et al, 1992; Kahan et al, 1992b]. Interestingly, thrombin-induced tyrosine phosphorylation but not MAP kinase activation is required for DNA synthesis [Rao et al, 1994]. The MAP kinase activation caused by LPA in Rat-1 cells cannot be mimicked by endothelin, despite the ability of endothelin to elicit phosphoinositide hydrolysis and tyrosine phosphorylation in these cells [Hordijk et al, 1994]. As found with the receptors signalling singly through $G_{i/o}$, MAP kinase responses to LPA and thrombin probably

depend little on PKC. In Rat-1 cells, the MAP kinase response to LPA was not mimicked by phorbol esters (although it was inhibited by a general kinase inhibitor staurosporine) [Hordijk et al, 1994] and the response to thrombin was unaffected by PKC-down regulation [Gupta et al, 1992]. In umbilical vein endothelial cells or CCL 39 cells however, thrombin-evoked MAP kinase activation was attenuated by a selective PKC-inhibitor, Ro 31-8220, or by PKC downregulation, although surprisingly in the latter case the response to FGF was similarly inhibited [Kahan et al, 1992b; Wheeler-Jones & Pearson, 1995]. Another receptor which uses multiple G-proteins to transmit signals is the platelet-activating factor (PAF) receptor. As with thrombin and LPA, PAF stimulates MAP kinase activation and phosphoinositide hydrolysis. This cascade leading to MAP kinase activation is partially sensitive to pertussis toxin (approximately 60%) whereas PI hydrolysis is completely PTx-insensitive. These observations are very similar to our findings with the LHRH receptor in αT3-1 cells. Of some note is that in contrast to work from other groups suggesting a requirement of Ras in G_i protein activation of MAP kinase, this group found no evidence for increased Ras-GTP loading [Honda et al, 1994].

The role of tyrosine phosphorylation in MAP kinase activation has also been investigated. LPA stimulated MAP kinase activation in Rat-1 cells involves a tyrosine kinase-dependent step which appears to lie downstream of $G_{i/o}$ but upstream of Ras, based on the sensitivity of LPA-induced MAP kinase activation to the tyrosine kinase inhibitor, genistein [Hordijk et al, 1994]. However tyrosine phosphorylation of a number of proteins, induced by LPA and stimulation of the human cholecystokinin/gastrin receptor, including the focal adhesion kinase p125 (FAK) were independent of MAP kinase activation [Hordijk et al, 1994; Seufferlein et al, 1995]. In COS 7 host cells the Src type non-receptor tyrosine kinases have been implicated in MAP kinase activation [Hawes et al, 1995]. Further investigation of βγ-mediated signals by overexpression of βγ subunits or the expression of the G_i-linked α_2C10 receptor revealed the phosphorylation of two proteins (~50KDa) which co-

immunoprecipitated with the adaptor protein Shc [Touhara et al, 1995]. Beta-gamma subunit-stimulated phosphorylation of Shc has been suggested as a mechanism by which βγ transduces signals to Ras. Shc phosphorylation was inhibited by two tyrosine kinase inhibitors genistein and herbimycin A and an inhibitor of phosphatidylinositol 3-kinase (PI3K), wortmannin, (suggesting that Shc can be phosphorylated by multiple kinases) as well as expression of the βARKct peptide which acts as an antagonist to βγ subunit-mediated signals but not by expression of a dominant negative Ras [Touhara et al, 1995]. Additionally tyrosine phosphorylation of Shc in COS 7 cells has also been shown to lead to an increased association of Shc with the adaptor protein Grb-2 and the Ras-GTP exchange factor Sos, providing evidence that βγ subunits do not interact directly with Sos. Moreover disruption of the Shc-Grb-2-Sos complex prevents βγ-mediated MAP kinase activation indicating a requirement for Shc interaction in this activation mechanism. These observations (and others) suggest that G_i-coupled receptors and receptor tyrosine kinases share a common pathway from a very early stage [Touhara et al, 1995; Van Biesen et al, 1995]. The manner in which Shc is recruited to the membrane in βγ–mediated signal transduction is still unresolved, however it is possible that interactions between βγ subunits and pleckstrin homology (PH) domains may have a significant role [Luttrell et al, 1995]. The PH domain is a sequence of approximately 120 amino acids which is homologous to a region in pleckstrin (the major substrate of PKC in platelets), they are found in a variety of signalling molecules. By analogy with SH2 and SH3 (Src homology) domains, it is suggested that these domains mediate protein-protein interactions in signalling processes [Koch et al, 1991; Lemmon et al, 1996]. PH domains bind βγ subunits with varying affinities. Expression of plasmid minigene constructs containing βARK, PLCγ, IRS-1, Ras-GRF and Ras-GAP PH domains in COS 7 host cells significantly inhibited both inositol phosphate formation and MAP kinase activation by the G_i-linked α_2C10 receptor. In contrast only the PLCγ-PH domain peptide had any effect on a G_q-linked M_1 receptor responses suggesting that

the inhibitory effect of most of the PH domains was βγ subunit-specific [Luttrell et al, 1995]. Thus these peptides behave as antagonists for βγ subunit-mediated signals and taken together with the data presented earlier from Hawes, Van Biesen and co-workers provide strong evidence for βγ subunit-mediated signalling downstream of G_i- but not G_q-coupled receptors. Furthermore these data imply that PH domains may have a valid role in protein-protein interactions required for intracellular signalling cascades (particularly leading to MAP kinase activation). An involvement of tyrosine kinases in G_q-mediated signalling is also observed. In cardiac myocytes and neonatal fibroblasts angiotensin II stimulated tyrosine phosphorylation of a number of proteins with molecular weights of 42-,44-,75-to 80- and 120- to 130 kDa [Sadoshima et al, 1995]. This appeared to be downstream of PKC activation but in this instance Ca^{2+} release seemed more important than tyrosine phosphorylation to MAP kinase activation [Sadoshima et al, 1995]. The LHRH receptor is therefore unusual in that it displays aspects of both $G_{i/o}$ and $G_{q/11}$ signalling cascades. The participation of Ras or Raf-1 has not been investigated as yet. Certainly this would help to clarify any ambiguity in this signalling cascade, as would providing evidence that βγ subunits released from $G_{i/o}$ were necessary. This could presumably be achieved using antisense cDNA to βγ subunits DNA sequences or by expression of the βARKct peptide to sequester the βγ subunits, although the latter could only be performed in a host cell system such as COS7 cells with co-expression of the mouse LHRH receptor. It appears from the data with mastoparan, pervanadate and phorbol that either G_i or PKC can elicit a modest MAP kinase activation. However to observe the maximal effect, "dual input" from both these cascades is essential. From these results alone it is still unclear precisely how these signals integrate. One explanation is that concurrent paths are triggered from both G-proteins and that these converge additively either at Ras by increasing GTP loading or at the level of Raf-1. Another possibility is that PKC or a tyrosine kinase, perhaps acting downstream of PKC, may exert some facilitatory action on the $G_{i/o}$-mediated signal. Alternatively PKC may

represent the predominant signalling cascade mediated by the LHRH receptor but its ability to interact with multiple G-proteins, possibly only under certain circumstances, may allow the cells/receptor to control the level of MAP kinase response and so only triggers a downstream event once a threshold level of MAP kinase activation has been achieved. This is an enticing hypothesis if MAP kinase activity was only increased in response to LHRH in gonadotrophs at certain stages in the oestrous cycle for example LHRH 'self priming' effect [Mitchell et al, 1994] or possibly in some circumstances, mitogenesis [Batticane et al, 1991; Lewis et al, 1986; Linkie et al, 1981; Van Bael et al, 1994]. Another receptor which has displayed some sensitivity to both pertussis toxin and PKC is another rhodopsin family member, the thrombin receptor [Kahan et al, 1992b]. Its sustained activation by this dual mechanism has likewise been associated with mitogenicity [Kahan et al, 1992b], although other signals from the thrombin receptor desensitise rapidly [Vouret-Craviari & Van Obberghen-Schilling, 1992]. Furthermore, the thrombin receptor in common with the LHRH receptor has a D for N substitution in the conserved NPXXY motif in transmembrane domain seven. It is possible that this or another of the non-canonical sequence motifs may account for a sustained activation of MAP kinase by this unusual dual mechanism of MAP kinase activation.

It seems likely that dual input mechanisms are more common than first thought. Aspects of both thrombin, ATP and PAF receptor activation of phospholipase A_2 (PLA_2) in CHO cells, although incompletely defined, are reported to require the interaction of pathways of two G-proteins G_{i2} and G_q [Honda et al, 1994; Murray-Whelan et al, 1995]. Activation of MAP kinase following stimulation of the endothelin B receptor with ET-1 in cultured astrocytes was mediated by two independent signalling pathways, a PKC-dependent and a pertussis toxin-sensitive G-protein mediated pathway, whereas ET-1 stimulation of phosphoinositides is insensitive to PTx treatment [Kasuya et al, 1994]. Recently it has been reported that maximum MAP kinase activation from G-protein coupled anaphylatoxin C5a receptors

expressed in transfected HEK 293 cells requires two independent regulatory signals [Buhl et al, 1995]. Stimulation of the C5a receptor resulted in a minimal activation of MAP kinase which was pertussis toxin sensitive and unaffected by PKC inhibitors. In contrast co-expression of the C5a receptor with the α subunit of G_{16} (a homologue of G_q, [Amatruda et al 1991]), resulted in both a robust stimulation of PLCβ and MAP kinase activity which was inhibited by approximately 60% by either GF109203X or pertussis toxin. It was further shown that α_{16} was a necessity for the maximal MAP kinase response as it could not be reconstituted by overexpression of a combination of $\beta\gamma$ subunits and PLCβ. Moreover there is considered to be insufficient PLCβ 2 or 3 in these cells for a wholly $\beta\gamma$ mediated PLC response of a large size [Buhl et al, 1995]. The requirement for additional signal input has also been suggested in other cases. Down regulation of PKC in 3T3-L1 cells attenuated but not fully blocked the MAP kinase activation by leukaemia inhibitory factor (LIF). On the contrary EGF activation of MAP kinase in these cells was unaffected by down-regulation implying again that full activation of MAP kinase by LIF required multiple signalling pathways [Schiemann & Nathanson, 1994]. Phorbol ester or stimulation of the M_1 receptor expressed in COS cells could activate MAP kinase however expression of a GTPase-deficient α_q mutant (α_q Q209L) could persistently stimulate PI hydrolysis but not MAP kinase activity, implying an additional effector must be involved in M_1 mediated MAP kinase signalling pathway [Qian et al, 1993]. Furthermore a two signal model is proposed for activation of Raf-1. Although both Src and Ras can independently activate the autokinase activity of Raf-1 to a limited extent, both are required to fully activate Raf-1. Of the two signals it is suggested that the Ras signal is indirect and an intermediate such as a kinase or a lipase is involved [Williams et al, 1992; Marshall, 1996]. This type of regulation is observed in many examples in neurobiology where an enzyme for example adenylyl cyclase or a receptor such as the NMDA receptor serve as a "coincidence detector" to recognise and integrate two

or more distinct signals and modify the amplitude of the output response [Bourne & Nicoll, 1993].

The ability of MAP kinase or its immediate upstream activators Raf-1 and MEK to be regulated by multiple signal inputs therefore provides the potential for a graded response and tremendous diversity for regulation. Furthermore it is evident from a number of recent reports, that there is a much greater degree of crosstalk between differing classes of receptors such as receptor-tyrosine kinases, seven transmembrane domain G-protein-coupled receptors and ion channel receptors, rather than these receptor activating exclusive pathways with unique signalling components as was originally anticipated. For example, it is proposed that G-protein coupled receptors such as endothelin, LPA and thrombin utilise the EGF receptor as a downstream mediator in a transactivation mechanism to bring about activation of MAP kinase and hence gene expression and DNA synthesis in Rat-1 cells [Daub et al, 1996]. The EGF receptor (EGFR) and the *neu* oncoprotein became rapidly tyrosine-phosphorylated in response to thrombin, LPA and endothelin-1. Inhibition of EGFR function by tyrphostin AG1478 or expression of a dominant negative mutant of the EGFR prevented MAP kinase activation in response to the GPCRs or EGF but had no effect on a control PDGF-stimulated activation. This would indicate that these mitogens (and possibly others) mediate cell proliferation through a ligand-independent induction of tyrosine phosphorylation of the receptor-tyrosine kinases EGFR and $p185^{neu}$ [Daub et al, 1996], however this report failed to address whether this was a specific property of the EGF receptor or whether all receptor tyrosine kinases could be substituted in this role. Furthermore if the PDGF receptor was present in these cells as shown by the use of PDGF to monitor MAP kinase activation as a control response it presumably could not substitute as the link for GPCR action. In another report it is proposed that a pertussis toxin-sensitive G-protein (probably G_i) is required (as an additional signal to tyrosine phosphorylation) for activation of PLCγ and the consequent EGF-induced phosphoinositide turnover in

rat hepatocytes [Yang et al, 1991; Yang et al, 1993]. In yet another article, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signalling was found to involve a pertussis toxin sensitive G-protein, coupled to a protein kinase cascade [Wang & Durkin, 1995]. This is particularly surprising as AMPA is an ionotrope (channel) receptor and would not usually be expected to interact with G-proteins. AMPA, but not N-methyl-D-aspartate (NMDA) which can also stimulate MAP kinase in rat cortical neurones by Ca^{2+} influx, was shown to cause an association of the $\beta\gamma$ subunit to a Ras protein complex and hence activation of MAP kinase in a Ca^{2+}-influx-dependent manner [Wang & Durkin, 1995]. The proposed mechanism is similar to that proposed for TMD VII domain G_i-coupled receptors such as the α_2 adrenergic receptor [Hawes et al, 1995], where the $\beta\gamma$ subunits interact and activate Ras following ligand-induced dissociation from the α_i subunit. This opens up the possibility of even greater diversity as synergistic parallel pathways may be put to use in a cell or receptor specific manner. It is not surprising that a key signalling molecule such as MAP kinase would command such varied and complex control. Ultimately the magnitude of the response will depend on multiple factors such as the ability of a receptor to interact with one or more G-proteins, or the complement of signalling machinery present in that cell.

Figure 3.1

Time course of LHRH and PDBu-induced MAP kinase activation in αT3-1 cells.

Figure 3.1 shows the specific [^{35}S]thiophosphorylation of MAP kinase substrate peptide after different times of incubation of αT3-1 cells with luteinising hormone releasing-hormone (LHRH, (●), 100 nM) or phorbol 12,13-dibutyrate; (PDBu, (▲)), 1μM; vehicle, (■)). Each value is the mean ± SEM from 4-6 separate determinations. Basal levels of MAP kinase activity remained constant throughout the time course measured.

Figure 3.1

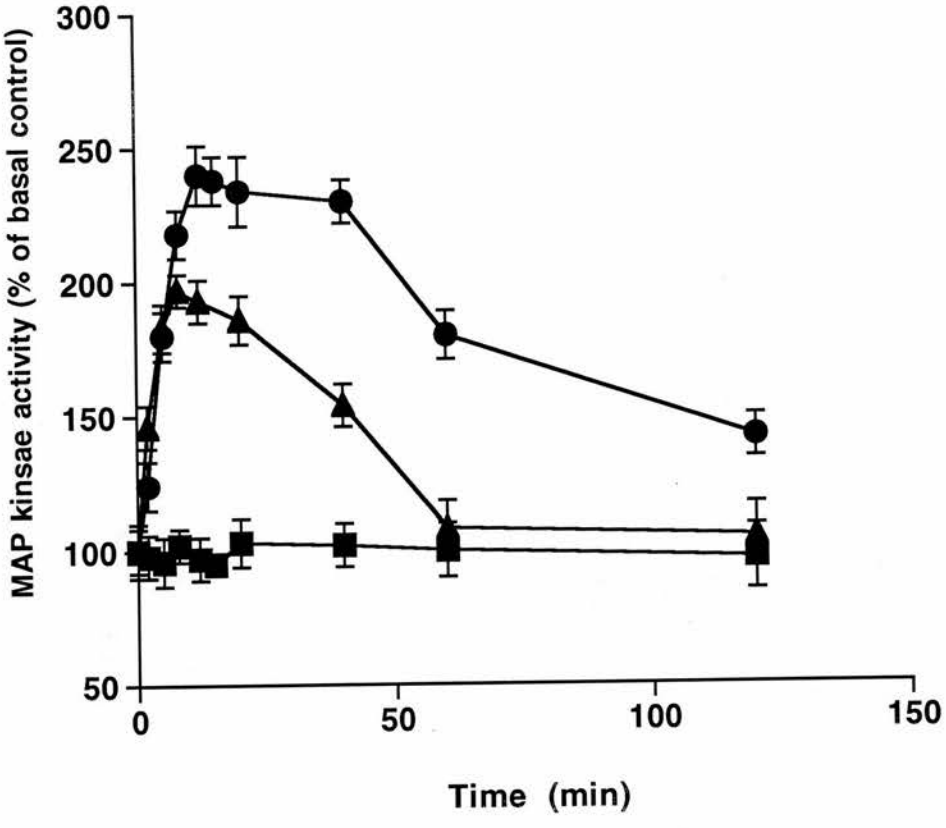

MAP kinsae activity (% of basal control)
300
250
200
150
100
50
0
50
100
150
Time (min)

Figure 3.2

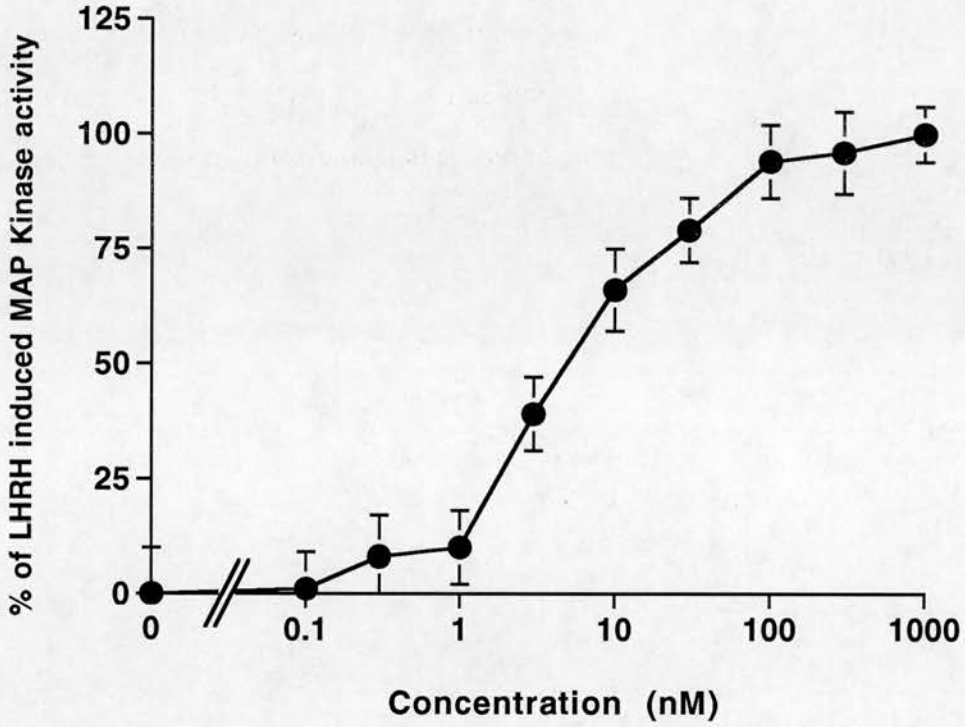
Concentration response curves for MAP kinase activity in αT3-1 cells

The specific [^{35}S]thiophosphorylation of MAP kinase substrate peptide was measured at various concentrations of agonist or antagonist. Each value is the mean ± SEM from 6-8 determinations. Baseline activity in unstimulated cells was subtracted and the agonist-evoked responses were normalised to the activity obtained with the maximally effective dose. Curve fitting and derivation of the EC_{50} or IC_{50} values was then conducted as described in Chapter 2.

The Concentration-response curves for LHRH (●) is shown in (a) with EC_{50} values of 3.4 ± 0.4 nM. The pharmacological specificity of LHRH was tested by the addition of increasing concentrations of an LHRH antagonist [Ac-D-p-Cl-Phe^{1,2}, D-Trp^{3}, D-Arg^{6}, D-Ala^{10}] (○) which inhibited activity induced by 100 nM LHRH with an IC_{50} of 38.2 ±13 nM (b).

Figure 3.2

(a)


% of LHRH induced MAP Kinase activity
125
100
75
50
25
0
0
0.1
1
10
100
1000
Concentration (nM)

(b)

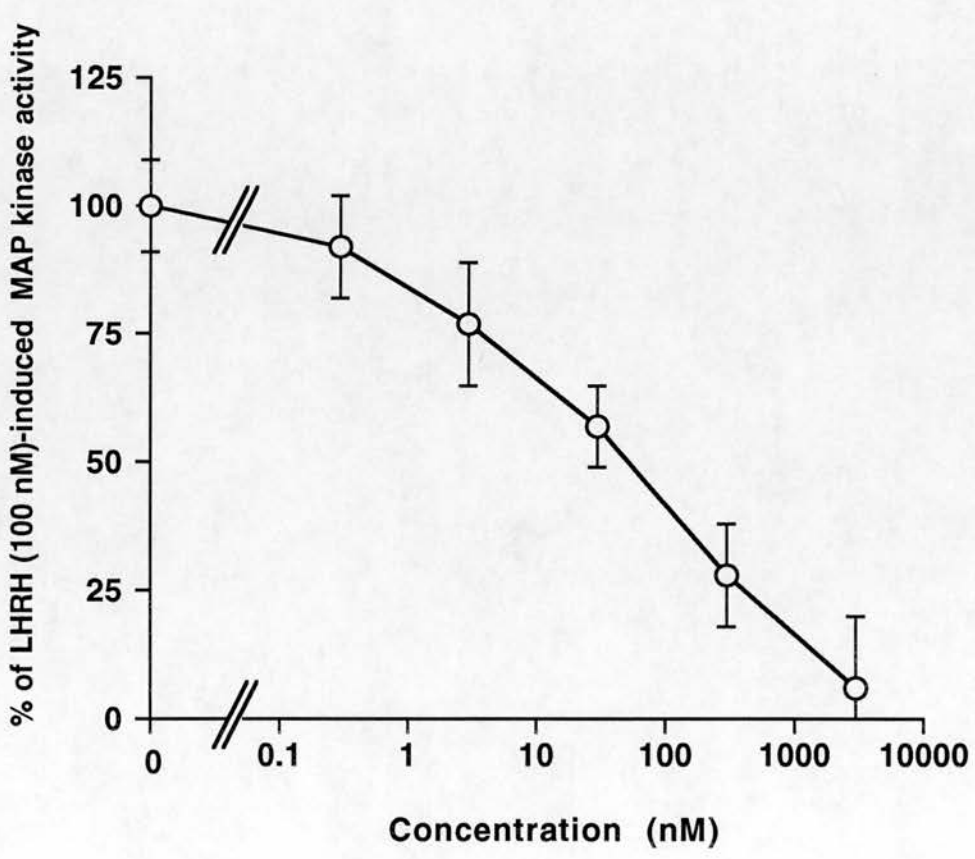

% of LHRH (100 nM)-induced MAP kinase activity
125
100
75
50
25
0
0
0.1
1
10
100
1000
10000
Concentration (nM)

Figure 3.3

Concentration response curves for MAP kinase activity in αT3-1 cells

The Concentration-response curves for 4β-phorbol 12,13-dibutyrate; 4β-PDBu (■) is shown in (a) with EC_{50} values of 50 ± 8 nM. The pharmacological specificity of this reagents was tested by replacing 4β-PDBu with the essentially inactive stereoisomer 4α-PDBu (❑) which had a maximum effect of 110 ± 9% at concentrations up to 10 μM.

(b) Down-regulation of phorbol-sensitive PKC isoforms and the effect on LHRH-induced MAP kinase activity.

LHRH (100 nM)-induced MAP kinase activity after 10 min was monitored in αT3-1 cells which had previously been exposed to 300 nM PDBu or vehicle (≤0.3% DMF) for 18 h. Results are shown as fold of basal activity and are the means (± SEM) of at least 3 separate experiments.

Figure 3.3

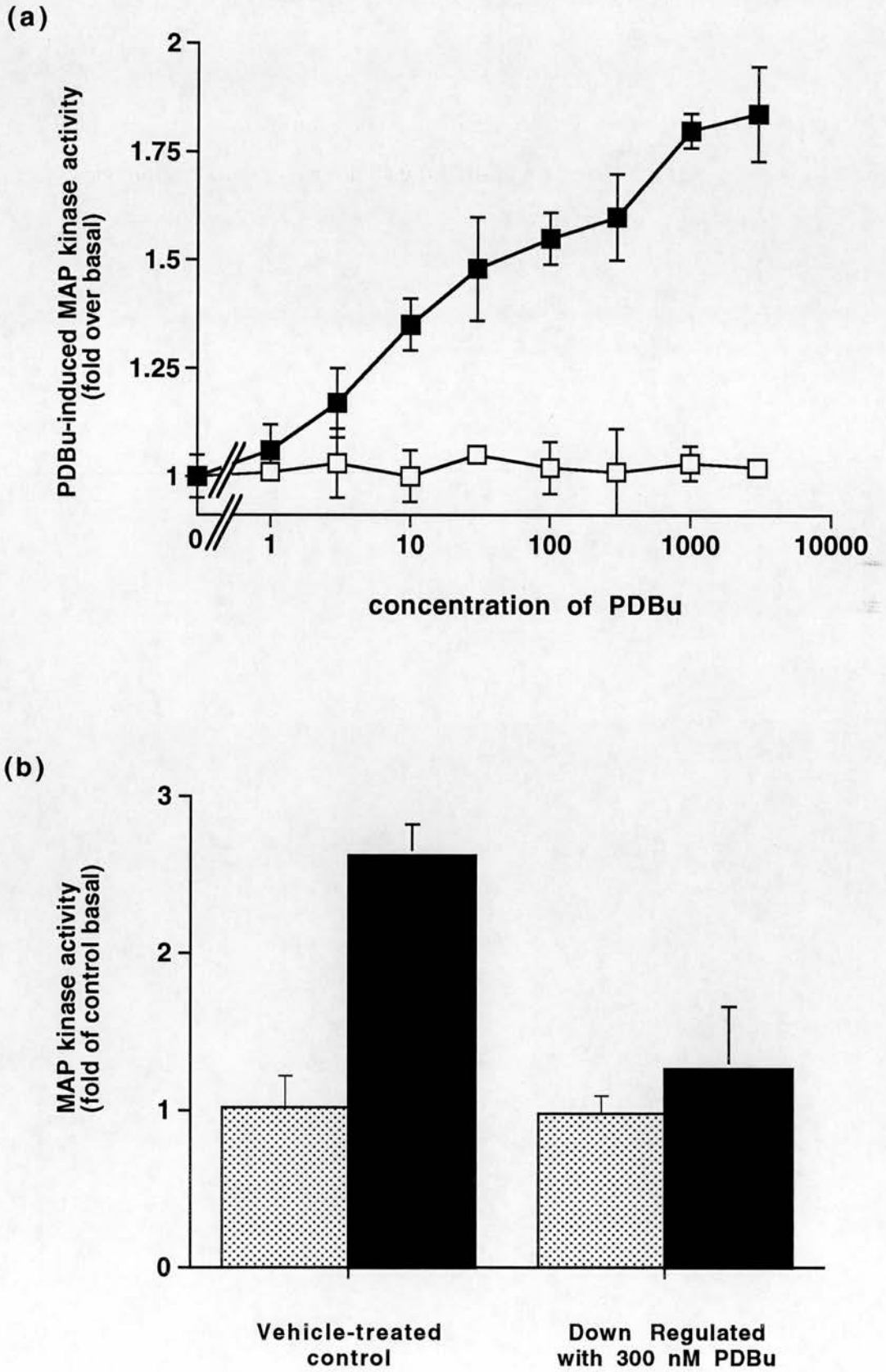

(a)
PDBu-induced MAP kinase activity
(fold over basal)
2
1.75
1.5
1.25
1
0
1
10
100
1000
10000
concentration of PDBu
(b)
MAP kinase activity
(fold of control basal)
3
2
1
0
Vehicle-treated
control
Down Regulated
with 300 nM PDBu

Figure 3.4

Inhibition of MAP kinase activity by various PKC inhibitors.

The concentration-dependence of effects on (a)LHRH (100 nM, 10 min) and (b) PDBu (1μM, 8 min)-induced MAP kinase substrate peptide [^{35}S]thiophosphorylation is shown in Figure 3.3. All inhibitors were added for 2 min prior to agonist and where required vehicle alone was added to control flasks. All data are expressed as a percentage of the LHRH- or PDBu-induced activity and are means ± SEM (n = 6-8).

After curve fitting as described previously the IC_{50} values obtained for GF109203X (▲), Ro 31-8220 (■) and H7(●) were 1.8 ± 0.12 μM, 0.64 ± 0.08 μM and 168 ± 12 μM respectively for LHRH (a) and 0.26 ± 0.02 μM, 0.57 ± 0.03 μM and 43.5 ± 5.1 μM respectively for PDBu (b).

Figure 3.4

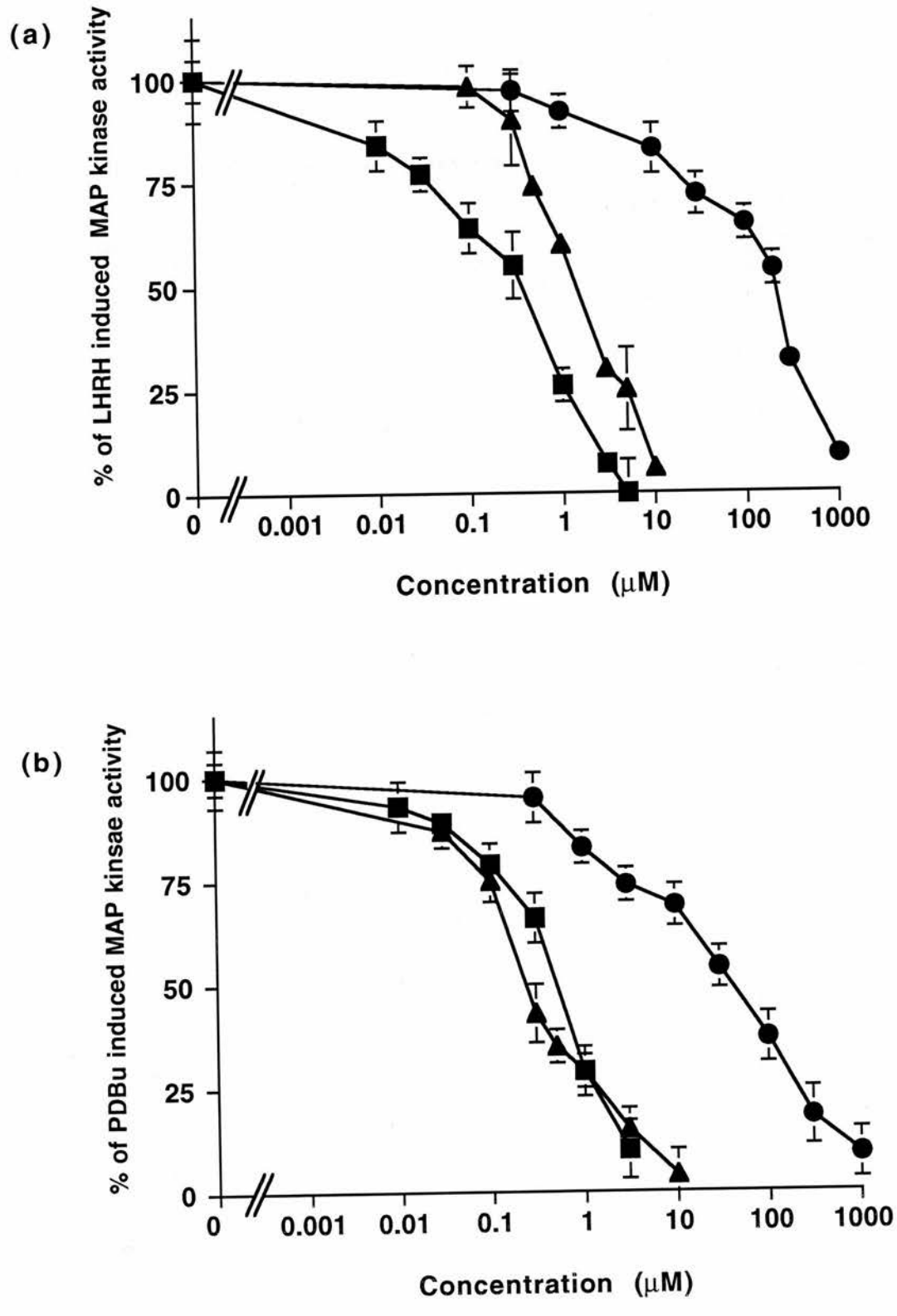

(a)
% of LHRH induced MAP kinase activity
100
75
50
25
0
0
0.001
0.01
0.1
1
10
100
1000
Concentration (μM)
(b)
% of PDBu induced MAP kinsae activity
100
75
50
25
0
0
0.001
0.01
0.1
1
10
100
1000
Concentration (μM)

Figure 3.5

Inhibition of LHRH-induced MAP kinase activity by tyrosine kinase inhibitors.

The concentration-dependence of effects of tyrosine kinase inhibitors on LHRH (100 nM, 10 min)-induced MAP kinase substrate peptide [^{35}S]thiophosphorylation is shown in Figure 3.5. All inhibitors were added for 2 min prior to agonist and where required vehicle alone was added to control flasks. All data are expressed as a percentage of the LHRH-induced activity and are means ± SEM (n = 4).

After curve fitting as described previously, IC_{50} values obtained for genistein (●) and N-methyl 2,5-dihydroxy cinnamate (MDC, (▲)) were 15 ± 0.6μM and 33 ±7 μM respectively.

Figure 3.5

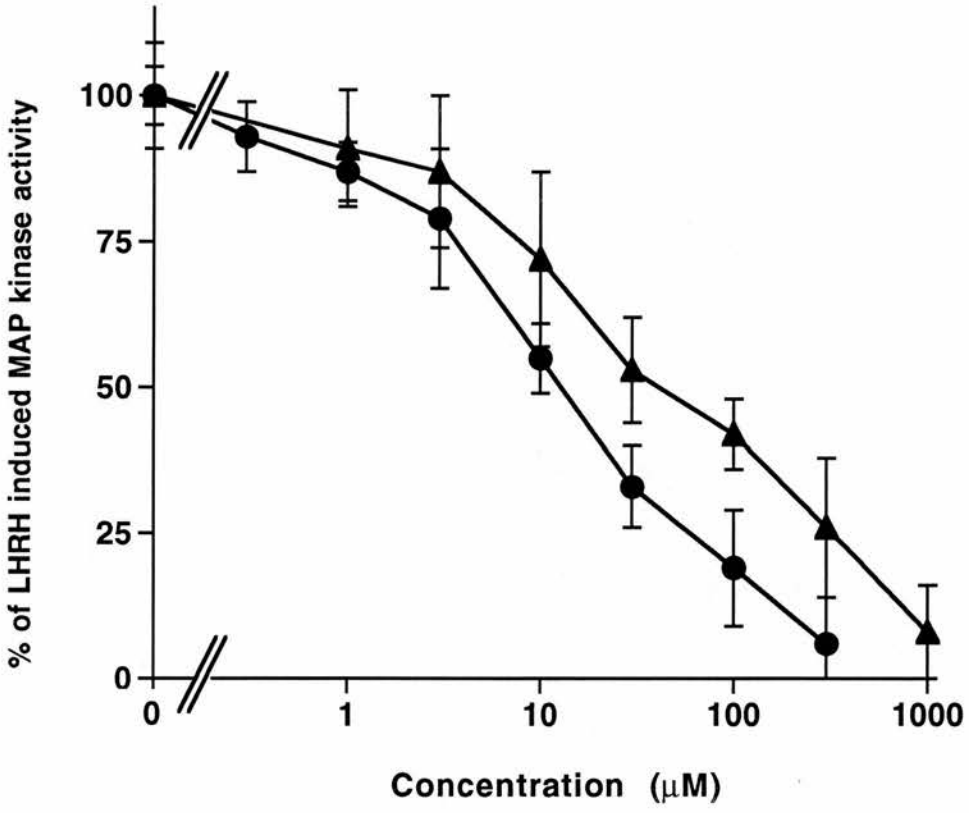

% of LHRH induced MAP kinase activity
100
75
50
25
0
0
1
10
100
1000
Concentration (μM)

Figure 3.6

Inhibition of LHRH-induced MAP kinase activity in αT3-1 cells by pertussis toxin.

Figure 3.6 (a) shows the concentration-dependence of inhibition of MAP kinase substrate peptide [^{35}S]thiophosphorylation in αT3-1 cells when preincubated for 18 h with pertussis toxin (PTx). The IC_{50} value for this effect was 32 ± 4 ng/ml with a maximum inhibition of ~75% at 300 ng/ml. Inhibition of MAP kinase responses by ≥ 10 ng/ml pertussis toxin was statistically significant ($p < 0.05$, Mann-Whitney U-test).

This effect was shown to be a specific effect of an active holotoxin as under the same conditions neither the B-subunit or N-ethyl maleimide (NEM)-inactivated holotoxin had any significant effect on MAP kinase substrate peptide [^{35}S]thiophosphorylation. None of these treatments had any significant effect on basal MAP kinase activity (mean ± SEM, n=4).

Figure 3.6

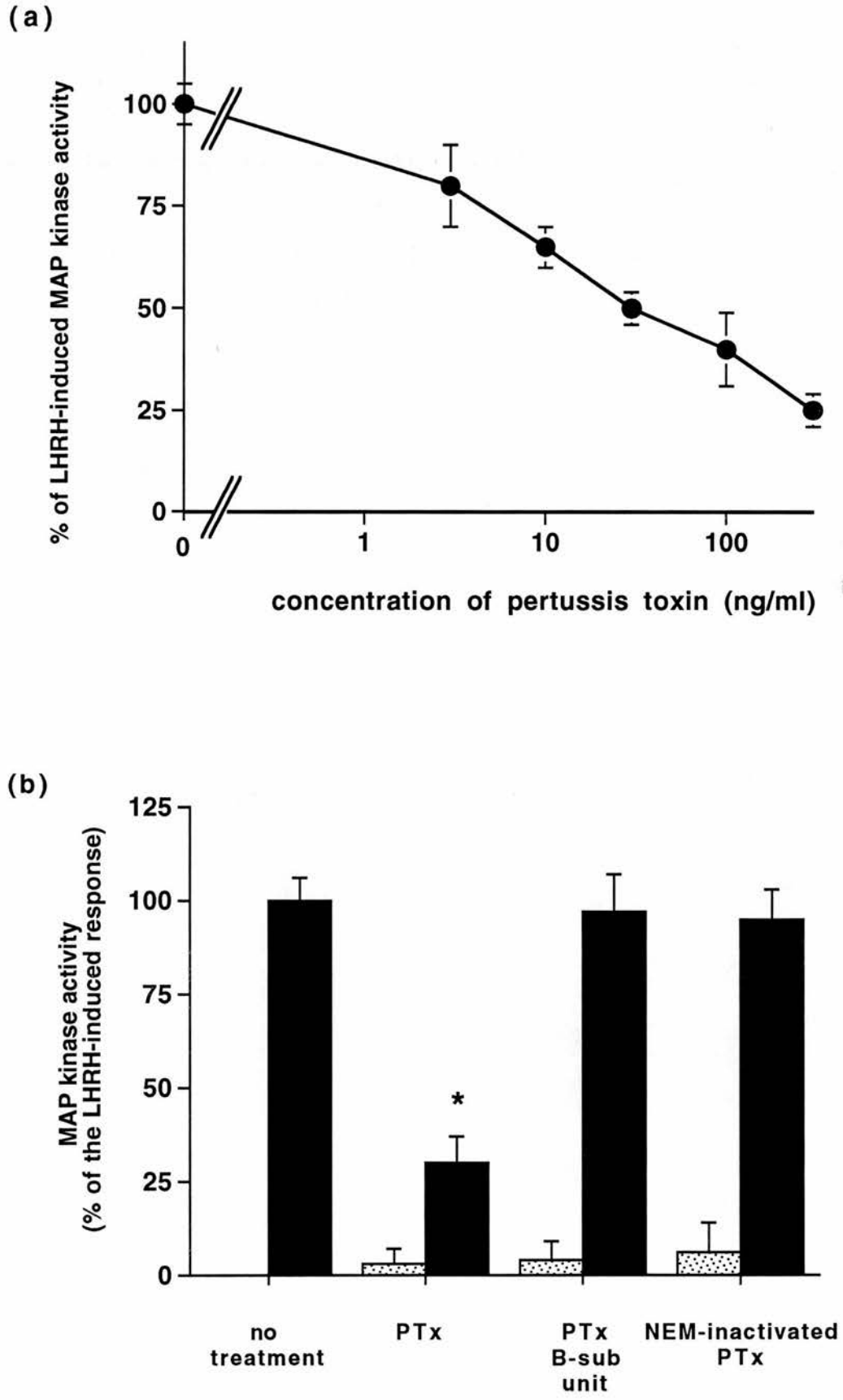

(a)
% of LHRH-induced MAP kinase activity
100
75
50
25
0
0
1
10
100
concentration of pertussis toxin (ng/ml)
(b)
MAP kinase activity
(% of the LHRH-induced response)
125
100
75
50
25
0
*
no treatment
PTx
PTx B-sub unit
NEM-inactivated PTx

Figure 3.7

Characteristics of mastoparan- and phorbol-ester-induced MAP kinase activation

(a) Shows the concentration-dependence of the effects of mastoparan (10 min incubation) on MAP kinase substrate peptide [^{35}S]thiophosphorylation (●) and [^{3}H]inositol phosphate formation (▲). Values are the means ± SEM from 6 and 4 separate determinations respectively. (b) Shows the activation of MAP kinase induced by 10 min incubations with mastoparan (10 μM), phorbol 12,14-dibutyrate (PDBu; 1μM) and the combination, as well as the effects thereon of GF109203X (GF; 3 μM) and pertussis toxin (PTx; 100 ng/ml for 18 h). Values are the means ± SEM from 6-9 separate determinations. (✳) Indicates statistically significant inhibition of responses ($p<0.05$, Mann-Whitney U-test).

Figure 3.7

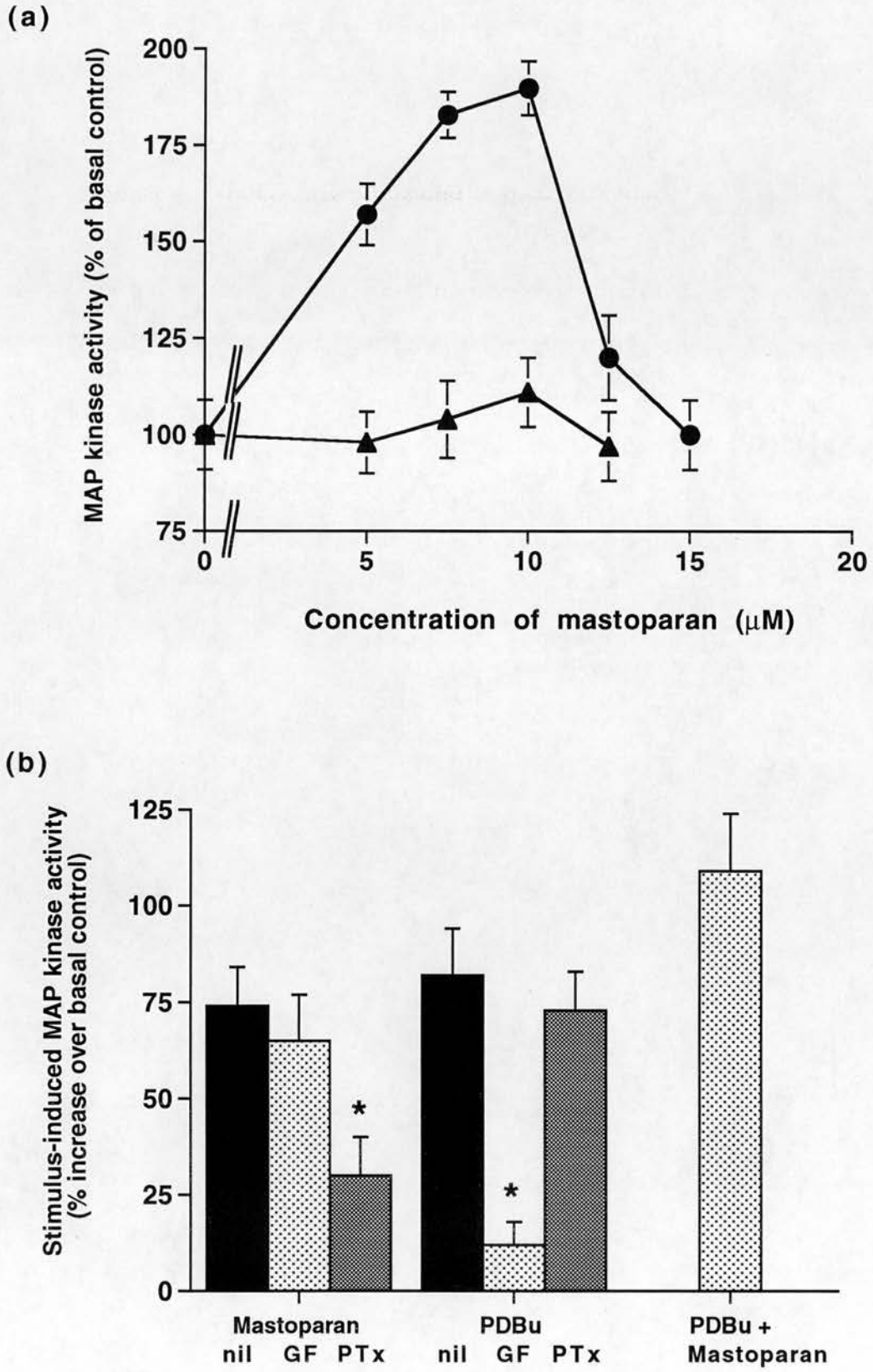

(a)
MAP kinase activity (% of basal control)
200
175
150
125
100
75
0
5
10
15
20
Concentration of mastoparan (μM)
(b)
Stimulus-induced MAP kinase activity
(% increase over basal control)
125
100
75
50
25
0
*
*
Mastoparan
nil GF PTx
PDBu
nil GF PTx
PDBu +
Mastoparan

Figure 3.8

Anti-MAP kinase immunoblots showing dose- and time-dependent relationship of LHRH-induced phosphorylation of MAP kinase.

Phosphorylation of p42 and p44 MAP Kinase was determined by the electrophoretic mobility shift assay. In response to agonist, MAP kinases are activated by phosphorylation on both a tyrosine and threonine residue, therefore the phosphorylation-induced gel mobility shift in immunoreactive p42 and p44 MAP kinases can be monitored (deVries et al., 1992). In all cases, no other distinct staining was detected in the lanes. Each observation was replicated in the experiment and was typical of three separate experiments.

Anti-p42/p44 MAP kinase immunoblots were carried out after protein separation by electrophoresis as described in Chapter 2. (a) Shows the concentration-dependence of LHRH-induced reduction in mobility of p42 (and to a lesser extent p44). Lanes 1, 2, 3, 4, 5 and 6 show results from 10 min incubations with 0, 1 nM, 3 nM, 10 nM, 30 nM and 100 nM LHRH respectively.

(b) shows the time course of LHRH-induced phosphorylation of p42 and p44 MAP kinase. Lanes 1, 2, 3, 4, 5 show results from 0, 5, 10, 20, 40 and 60 min incubations with LHRH (100 nM). A reduction in mobility was apparent within 5 min as seen in lane 2 and subsequent lanes. Unstimulated αT3-1 cells showed no change in phosphorylation over the 60 min time course (not shown).

(c) shows the PDBu (1μM)-induced phosphorylation of p42 and p44 MAP kinase after 10 min.

Figure 3.8

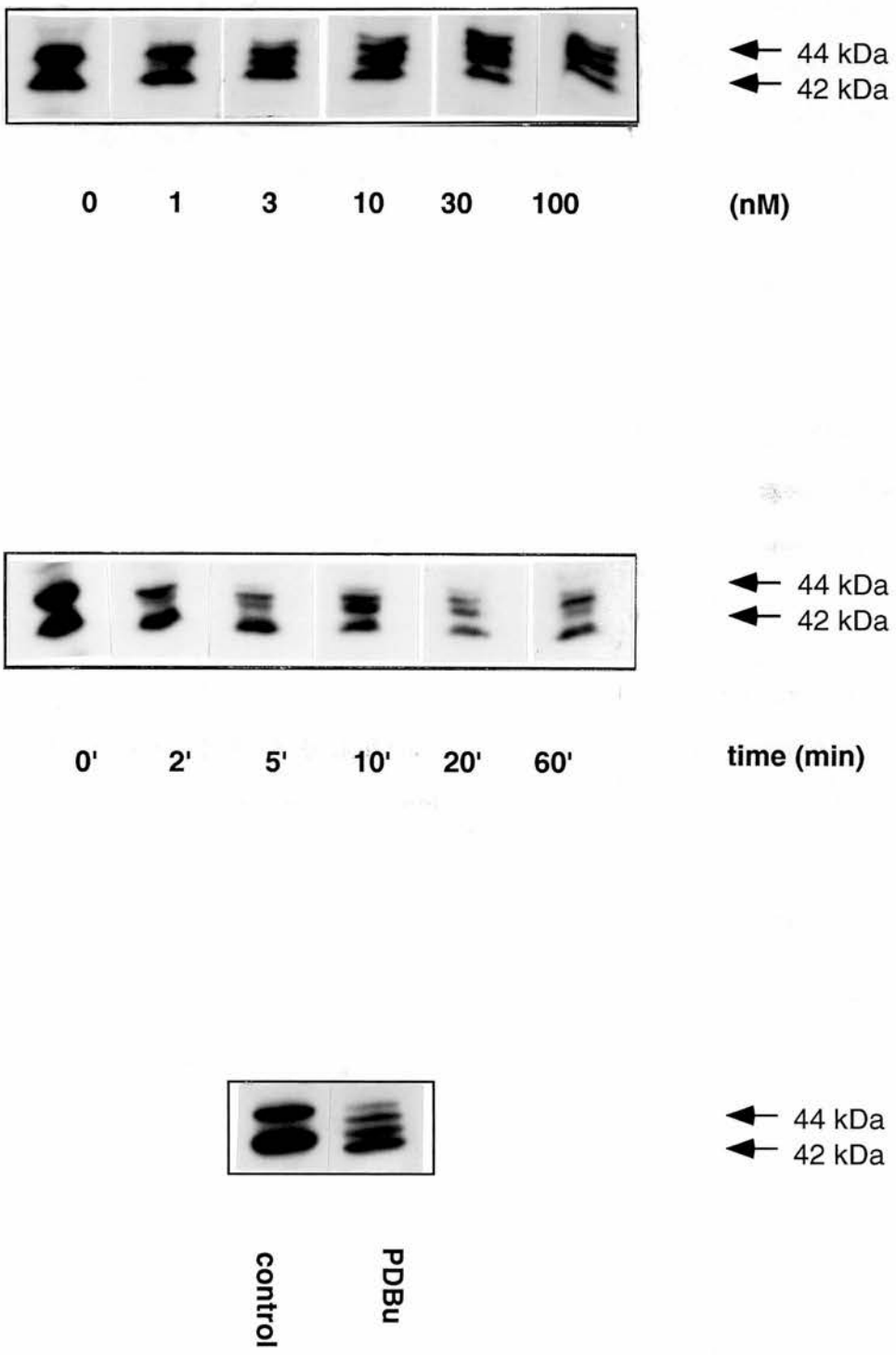

44 kDa
42 kDa
0
1
3
10
30
100
(nM)
44 kDa
42 kDa
0'
2'
5'
10'
20'
60'
time (min)
44 kDa
42 kDa
control
PDBu

Figure 3.9

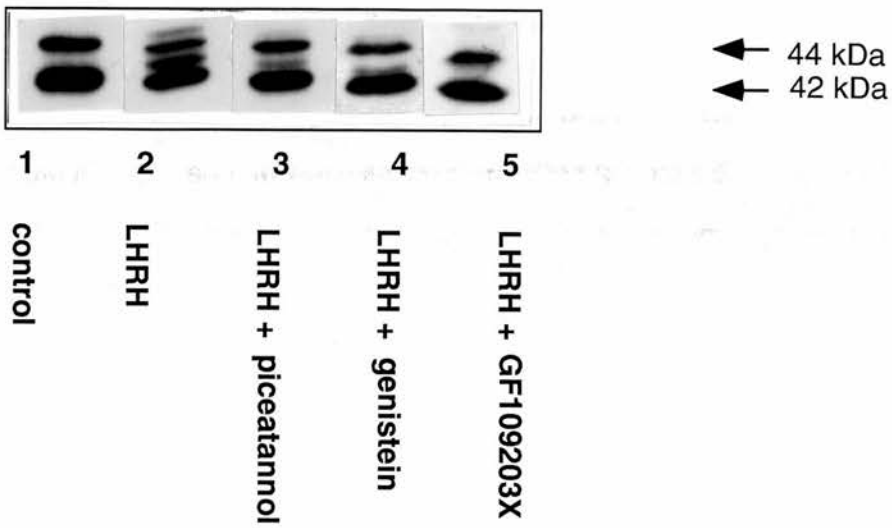
Effects of various inhibitors on LHRH-induced phosphorylation of p42 and p44 MAP kinase.

Anti-MAP kinase immunoblots showing LHRH-induced mobility shifts and the observed effects of several different inhibitors of a number of signalling cascade components. In all cases, no other distinct staining was detected in the lanes. Each observation was replicated in the experiment and was typical of three separate experiments.

In (a), the effects of piceatannol, genistein (inhibitors of tyrosine kinases) and GF109203X (a PKC inhibitor) on the LHRH-induced mobility shift are shown. Lanes 1, 2, 3, 4 and 5 show control, 100 nM LHRH, 100 nM LHRH with 30 μM piceatannol, 100 nM LHRH with 100 μM genistein and 100 nM LHRH with 3 μM GF109203X respectively.

In (b) the effects of a tyrosine phosphatase inhibitor, pervanadate (see Chapter 2 for preparation details) are shown. αT3-1 cells were preincubated with pervanadate for 15 min before the addition of 100 nM LHRH for 15 min. Lanes 1, 2, 3 and 4 show control, 100 nM LHRH, 1 mM pervanadate and 100 nM LHRH with 1 mM pervanadate respectively.

Figure 3.9


44 kDa
42 kDa
1
control
2
LHRH
3
LHRH + piceatannol
4
LHRH + genistein
5
LHRH + GF109203X

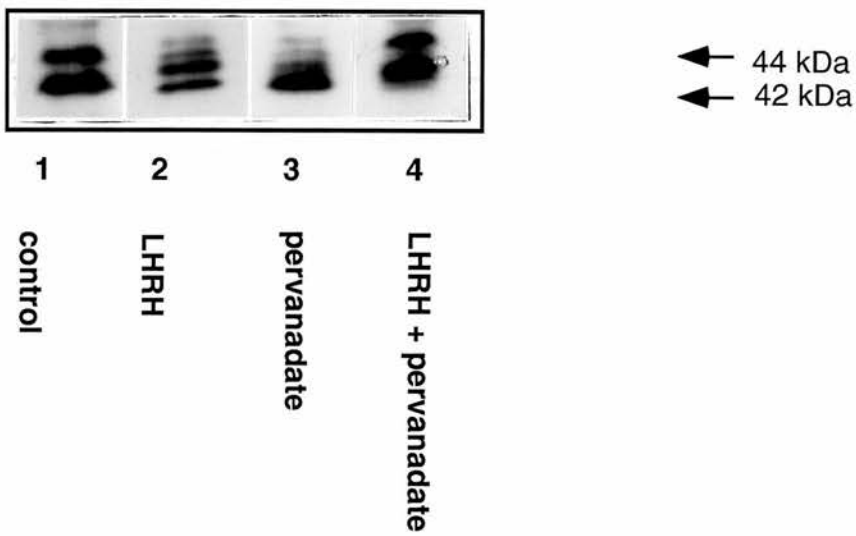

44 kDa
42 kDa
1
control
2
LHRH
3
pervanadate
4
LHRH + pervanadate

Figure 3.10

The effects of PLC inhibitors and pertussis toxin on LHRH-induced phosphorylation of p42 and p44 MAP kinase

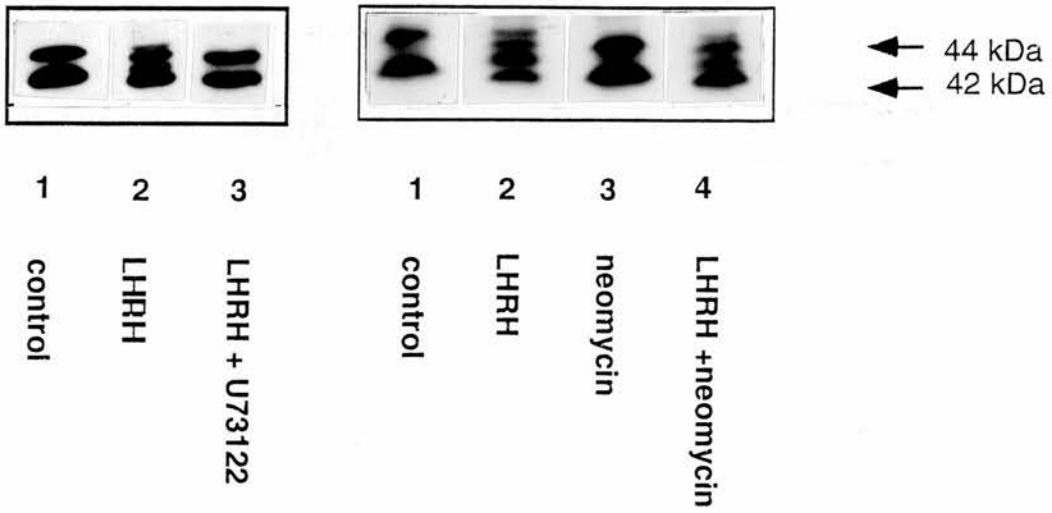
Anti-MAP kinase immunoblots showing LHRH-induced mobility shifts and the observed effects of several different inhibitors which act at the level of G-proteins. In all cases, no other distinct staining was detected in the lanes. Each observation was replicated in the experiment and was typical of three separate experiments.

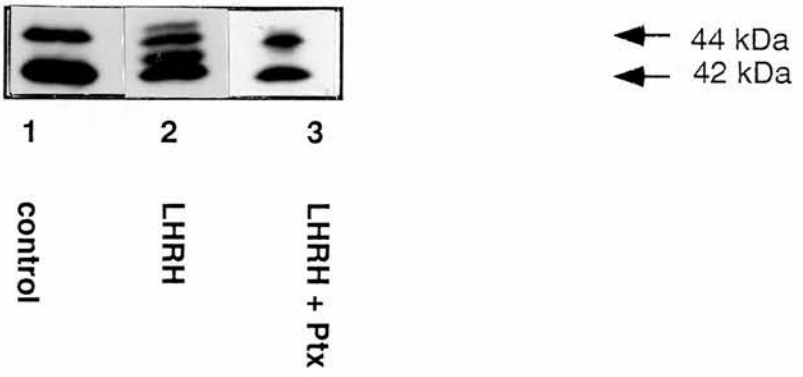
(a) displays the effects of U73122 a PLC inhibitor. Lanes 1, 2 and 3 show respectively, control, 100 nM LHRH and 100 nM LHRH with 2μM U73122.

(b) shows the effects of another PLC inhibitor neomycin. Lanes 1, 2, 3 and 4 show respectively control, 100 nM LHRH, 5 mM neomycin and 100 nM LHRH with 5 mM neomycin.

(c) illustrates the effect on LHRH-induced phosphorylation of p42 and p44 MAP kinase of pertussis toxin (a $G_{i/0}$ inhibitor). Lane 1, 2 and 3 show respectively control, 100 nM LHRH and 100 nM LHRH after 100 ng/ml pertussis toxin for 18 h.

Figure 3.10


44 kDa
42 kDa
1 control
2 LHRH
3 LHRH + U73122
1 control
2 LHRH
3 neomycin
4 LHRH +neomycin


44 kDa
42 kDa
1 control
2 LHRH
3 LHRH + Ptx

CHAPTER 4

FURTHER EVIDENCE FOR AN INTERACTION OF THE LHRH RECEPTOR WITH THE $G_{I/O}$ FAMILY OF G-PROTEINS

4.1 INTRODUCTION

During the course of investigations into the upstream regulation of LHRH-induced MAP kinase activation described in Chapter 3, it became apparent that there was a pertussis toxin-sensitive component to this LHRH-induced activity. Furthermore the LHRH-induced activity was partially mimicked by a G-protein activating peptide mastoparan, which has been reported to selectively activate G_i proteins *in vitro* [Gil et al, 1991; Higashijima et al, 1988]. Together these observations formed the first suggestion that the LHRH receptor did not exclusively interact with $G_{q/11}$ but may functionally interact with multiple G-proteins. This phenomenon is not uncommon, many receptors can interact with multiple effector systems through the activation of multiple G-proteins [Milligan, 1993; Offermans & Schultz, 1994]. For example, heterologous expression of the α_2C10 adrenergic receptor in Rat-1 fibroblast cells has been noted to result in the activation of phospholipase D as well as inhibition of adenylyl cyclase [MacNulty et al, 1992]. Furthermore in these cells the single defined receptor was shown to activate two separate G proteins, G_{i2} and G_{i3}, and adenylyl cyclase regulation at least, was attributable to G_{i2} [Milligan et al, 1991]. Additionally following high level expression of α_2C10 adrenergic receptors in CHO cells both inhibition and stimulation of adenylyl cyclase has been observed and immunoprecipitates of this receptor with an α_2C10 adrenoreceptor antibody contained both G_i and G_s [Eason et al, 1992]. Similarly, thrombin receptor activation can stimulate PLC activity as well as inhibiting adenylyl cyclase, and PLC activity is not prevented by pertussis toxin treatment, suggesting PLC activation is mediated by $G_{q/11}$ as opposed to G_i $\beta\gamma$ subunits [Hung et al, 1992]. Another example of receptor-multiple G-protein interaction is the thyrotrophin-releasing hormone (TRH) receptor. In GH_3 cells ligand-induced activation of PLCβ1 was attenuated by immunoprecipitation of $G_{q/11}$ antibodies [Hsieh & Martin, 1992], whilst TRH-evoked adenylyl cyclase activation in GH_3 cells was prevented by treatment with anti-Gα_s

antiserum [Paulssen et al, 1992]. Activation of the thyrotrophin (TSH) receptor also results in stimulation of adenylyl cyclase and PLC when expressed in COS 7 or CHO cells [Van Sande et al, 1990] suggesting interaction with both $G_{q/11}$ and G_s. In addition it is widely appreciated that cells might receive signalling input through numerous receptors at any one time which may consequently activate multiple intracellular signalling systems further propagating the diversity of the outcome to the initial stimulus. The integration of these signals and the sum of potential cross-talk between these effector systems will ultimately regulate the magnitude and length of any one receptor-mediated response.

In this light, it follows that if the LHRH receptor is capable of stimulating G-proteins other than $G_{q/11}$ then LHRH may act not only to stimulate the release of LH but may modulate other responses in its target tissue or indeed these auxiliary pathways may modulate responses to itself. It was therefore important to further examine the basis for pertussis toxin-sensitivity of LHRH-induced MAP kinase activation. The G-proteins designated as G_i ($G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$) are substrates for pertussis toxin. The discovery and nomenclature of the G_i family of G-proteins resulted from the inhibitory effect these oligomers exerted on intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) formation (from adenosine triphosphate, ATP) catalysed by adenylyl cyclase [Taussig et al, 1993; Wong et al, 1991]. It therefore seemed logical to determine the potential effects of LHRH on cAMP formation in αT3-1 cells as a simple assay for LHRH mediated G_i activation with a presumed LHRH-induced inhibition of cAMP formation resulting if this hypothesis was correct.

To date nine full length cDNA clones for adenylyl cyclases have been identified [Bakalyar & Reed, 1990; Cali et al, 1994; Feinstein et al, 1991; Gao & Gilman, 1991; Ishikawa et al, 1992; Katsushika et al, 1992; Krupinski et al, 1989; Paterson et al, 1995; Premont et al, 1992] all of which have between 1064-1248 amino acid residues. Adenylyl cyclases have a predicted topology within the membrane

consisting of a short cytoplasmic amino terminus followed by a cassette of six putative transmembrane spans (M_1) and a large cytoplasmic domain (C_1). This motif is repeated forming the M_2 and C_2 domains. This structure is similar to certain ion channels and to ATP-dependent transporters, particularly the P-glycoprotein and the cystic fibrosis transmembrane conductance regulator [Cooper et al, 1995; Taussig & Gilman, 1995]. Two regions in particular designated C_{1a} and C_{2a} are highly conserved (~93%) amongst the isoforms and it is these regions that are considered to be the catalytic domains. Notably these regions are highly homologous to each other and to the catalytic domains of both cytosolic and membrane bound guanyl cyclases. Although it is still unresolved whether both domains are of equal importance in catalysis. Other regions of interest include the conserved peptide sequences spanning amino acids 425-444 and 495-522 in C_1, representing a putative internal inhibitory domain [Kawabe et al, 1994] and the site of Ca^{2+}/calmodulin interaction [Vorherr et al, 1993; Wu et al, 1993] respectively. As a result of the recent cloning and expression of adenylyl cyclases a wealth of information now exists on their regulation. All nine isoforms are commonly activated by either $G\alpha_s$ or by forskolin, a diterpene which acts directly on adenylyl cyclase catalytic subunits and conversely all AC are inhibited by P-site inhibitors such as 3'-AMP, adenosine and related analogues [Tang & Gilman, 1992]. However it is now apparent that each isoform is uniquely regulated by a variety of influences and consequently the traditional view that $G\alpha_i$ inhibits all adenylyl cyclase has been reassessed. In addition to G-protein α subunits, $\beta\gamma$ dimers [Katada et al, 1987; Smigel, 1986; Tang & Gilman, 1991], phosphorylation [Dohlman et al, 1991; Jacobowitz et al, 1993; Kawabe et al, 1994] and Ca^{2+} [Boyajian et al, 1991; Debernardi et al, 1993; Ishikawa et al, 1992; Katsushika et al, 1992; Tang et al, 1991; Yoshimura & Cooper, 1992] all exert either facilitatory or inhibitory influences on adenylyl cyclases. Furthermore cell-specific differences in the regulation of specific adenylyl cyclase isoforms have also been described [Taussig & Gilman,

1995], for example Type II adenylyl cyclase was reported to be inhibited by $G_{\alpha i}$ subunits when expressed in COS 7 cells [Chen & Ivengar, 1993], however no such regulation was observed when this isoform was expressed in Sf9 membranes [Taussig & Gilman, 1995], or HEK 293 cells [Federman et al, 1992]. Most importantly, differences in adenylyl cyclase isotype profiles in a cell type may produce opposite outcomes, with respect to cAMP regulation, to the effects of G_i or G_q coupled receptors stimulation. Therefore caution should be applied to the interpretation of results measuring adenylyl cyclase activity or cAMP formation in whole cell situations and generalisations regarding receptor regulation of a particular species of adenylyl cyclase cannot be made unless a fully defined system is available.

In this Chapter we have examined the potential effects of LHRH on cAMP formation induced by forskolin and pituitary adenylyl cyclase-activating peptide (PACAP). Type I PACAP receptors belong to the seven transmembrane spanning G-protein-coupled superfamily of receptors [Hashimoto et al, 1993; Pisegna & Wank, 1993; Spengler et al, 1993]. Occupancy of these receptors has been shown to activate adenylyl cyclase presumably via $G_{\alpha s}$, as well as PLC in αT3-1 cells and PC12 cells [Deutsch & Sun, 1992; Schomerus et al, 1993]. The experiments in this chapter were performed with the aim of highlighting any further pertussis toxin-sensitive responses to LHRH which would corroborate our earlier data rather than attempting to be a definitive study of the enzymes involved.

4.2 RESULTS

Cyclic AMP formation stimulated in response to forskolin and PACAP-38 was assessed in αT3-1 cells in the presence of the broad spectrum phosphodiesterase inhibitor [Beavo, 1970] isobutyl-1-methylxanthine (IBMX; 0.5 mM). Extracellular, intracellular and total cAMP measurements were performed over a time course of 90 min. However only minimal cAMP was detected in the extracellular medium during

the first 45 min and responses were near maximal within 30 min (data not shown), therefore all subsequent measurements were performed after a 30 min incubation and represent the intracellular cAMP concentrations.

Forskolin stimulated a massive concentration-dependent increase in cAMP formation with responses to 1 μM forskolin typically being in the order of 70-120 fold of the basal cAMP concentrations measured. PACAP-38 stimulated a concentration-dependent increase in cAMP (not shown) which was maximal at 100 nM and represented an approximately 40 fold increase over the basal concentrations measured. Near maximal doses were used in all subsequent experiments. LHRH clearly reduced the stimulatory effects of both forskolin and PACAP-38 in a concentration-dependent manner (Figure 4.1) although approximately 45% of either the forskolin- or PACAP-38-stimulated activity remained uninhibited by LHRH at the highest concentration tested (1 μM). LHRH had no effect on basal levels of cAMP (Table 4.2).

Subsequent studies concerned the likely mechanism underlying the effect of LHRH. Since we have shown that the LHRH-mediated MAP kinase response is attenuated by pertussis toxin [Sim et al, 1995; Chapter 3+5], we investigated if the LHRH receptor was resulting in the inhibition of cAMP formation through the interaction of $G\alpha_{i/o}$ with adenylyl cyclase. Pertussis toxin reversed the majority of the LHRH-mediated inhibition of forskolin- and PACAP 38- stimulated cAMP formation in a dose-dependent manner (Figure 4.2). This effect was specific to active holotoxin as it was not observed with NEM-inactivated holotoxin or B-subunit alone. However PTx (200 ng/ml) was unable to fully restore the forskolin (1 μM)- stimulated cAMP concentration with a portion of this response in the region of 17 ± 5% (mean ± SEM, n=8) still inhibited in the presence of LHRH (300 nM). This effect was not as apparent with PACAP-38 where 92 ± 4% (mean ± SEM, n=6) of the PACAP-38 (100

nM)- stimulated cAMP concentration restored. PTx alone did not significantly alter basal cAMP concentrations (Table 4.2).

Since the LHRH receptor stimulates PKC activity in the anterior pituitary gland [Ison et al, 1993; Johnson et al, 1992] and in the αT3-1 gonadotroph cell line [Horn et al, 1991; Johnson et al, 1995], we investigated whether activation of PKC by LHRH could possibly explain this inhibitory action. The effect of the PKC activator PDBu (1 μM) was compared to that of LHRH on forskolin (100 nM)-stimulated cAMP formation (Figure 4.3a). PDBu displayed a tendency to be very slightly elevated above basal cAMP formation however this did just achieve a statistical significance (Wilcoxon signed rank test). Cyclic AMP production in response to PDBu in combination with forskolin was also slightly elevated in contrast to the effect of LHRH on forskolin-evoked cAMP formation (still 55 ± 7% inhibition within the same experiment). In response to the combination of forskolin + LHRH + PDBu cAMP formation was again slightly elevated compared to forskolin + LHRH, suggesting that PDBu was acting to slightly elevate cAMP formation in all conditions but had no specific effect on cAMP formation. The effects of the general kinase inhibitor staurosporine and the PKC inhibitor GF109203X and the tyrosine kinase inhibitors genistein and RG-13022 on these responses were also assessed (Table 4.1). None of the kinase inhibitors had any effect on basal cAMP formation or that in the presence of LHRH or PTx (Table 4.2). However GF109203X and staurosporine displayed a consistent trend to a small increase in cAMP formation in the presence of forskolin, forskolin + LHRH and forskolin + LHRH + PTx (Table 4.1). This did not reach statistical significance under the present conditions. Genistein and RG-13022 had no apparent effects except for a slight inhibition of the forskolin-stimulated response. This was a consistently observed effect but again failed to reach a statistical significance. The role of PKC was further investigated by long term exposure to PDBu (300 nM) to downregulate phorbol ester-sensitive PKC isoforms. Under these conditions the responses to all treatments (forskolin (F), forskolin +

LHRH (F+L), and forskolin + LHRH + pertussis toxin (F+L+PTx)) were all reduced in comparison to control cells which had not been exposed to the phorbol ester (Figure 4.3b). Downregulation caused a marked effect on the F and F+L+PTx treatments, where the cAMP responses to these agents were reduced by 45 ± 6% and 55 ± 7% (mean ± SEM, n=6) compared to the responses in cells treated with vehicle. The response in the presence of F+L was only slightly reduced 20 ± 4% (mean ± SEM, n=6), in the down-regulated cells. LHRH inhibited the forskolin-stimulated cAMP formation by 25 ± 7% (compared to the usual 55 ± 6%; mean ± SEM, n=8), this 25% inhibition was also largely reversed by PTx. These results were shown to be statistically significant using the Wilcoxon signed rank test. Again it appears that this phorbol ester, PKC downregulation protocol is acting non-specifically to dampen down the whole cAMP formation system.

Since LHRH also elevates intracellular Ca^{2+} levels in its target cells (including αT3-1 cells) [Anderson et al, 1993; Merelli et al, 1992; Mitchell et al, 1988], we investigated the possibility that Ca^{2+} may participate in mediating inhibition of cAMP accumulation. Experiments were carried out to determine the Ca^{2+}-dependence of forskolin-evoked cAMP production and its inhibition by LHRH effect (Figure 4.4). Elevation of intracellular Ca^{2+} by ionomycin had no effect on cAMP formation either alone or in combination with forskolin (Figure 4.4a). Reduction of cytosolic Ca^{2+} by incubation of αT3-1 cells in MEM supplemented with 5 mM EGTA did however cause a large reduction in stimulated cAMP formation (Figure 4.4b). This effect was most prominent on the response to forskolin alone which had decreased by 55 ± 10% (mean ± SEM, n=6), with F+L+PTx decreased by 44 ± 8% (mean ± SEM, n=6) with respect to responses in the absence of EGTA. In the presence of EGTA, LHRH had no inhibitory effect on forskolin-evoked cAMP production, in contrast to the situation in normal medium LHRH appeared to facilitate the lower response to forskolin that occurred in the presence of EGTA and displayed a small increase in basal or forskolin-evoked cAMP formation. Whilst the combination of PDBu and forskolin

displayed a trend towards an additive response using the Wilcoxon signed rank test this was not found to reach statistical significance

4.3 DISCUSSION

We investigated the influence of LHRH on forskolin- and PACAP-induced cAMP formation. LHRH clearly had an inhibitory effect on both forskolin- and PACAP-stimulated responses and this was susceptible to the $G_{i/o}$ antagonist pertussis toxin (Figure 4.1). Taking into account earlier studies too, it is now possible to infer that activation of the LHRH receptor consequently results in activation of $G\alpha_{q/11}$, $G\alpha_{i/o}$, the release of $\beta\gamma$ subunits, raised intracellular Ca^{2+} and activation of intracellular protein kinases, primarily PKC, all of which have the potential to regulate adenylyl cyclases and cAMP formation. Intracellular cAMP levels may also be regulated by phosphodiesterases [Beavo, 1995] which are often activated in response to Ca^{2+}/calmodulin [Sharma et al, 1988]. Furthermore, each of the nine cDNA clones for mammalian AC which have been identified to date, have been shown to be regulated in a distinctive manner by these mediators probably reflecting the diverse structural differences in the clones [Cooper et al, 1995; Tang & Gilman, 1992; Taussig & Gilman, 1995]. Clearly LHRH may regulate cAMP formation in numerous ways with the capacity for multiple sites of cross-talk between these regulatory mechanisms.

The activation of $G_{i/o}$ appears to be a significant event in LHRH-mediated inhibition of cAMP formation as PTx reversed the majority of LHRH-mediated inhibition of this response (Figure 4.2). This observation further adds to the data concerning a role for $G_{i/o}$ proteins in LHRH receptor signalling [Sim et al, 1995; Chapter 3+5]. PTx treatment will inactivate $\alpha_{i/o}$ subunits through ADP-ribosylation, thus preventing an inhibitory interaction of this subunit with AC. An alternative possibility is that the parallel release of $\beta\gamma$ subunits from the heterotrimer is also prevented, which in some circumstances also act in an inhibitory manner on G_s-evoked AC activation [Tang et

al, 1991]. PTx was however unable to fully restore the agonist stimulated levels of cAMP formation suggesting additional regulatory events. This action may be indicative of a more widespread role for LHRH as a modulator of other concurrent stimuli for example PACAP, although the extent to which such interactions occur in gonadotrophs is as yet undetermined.

LHRH-induced activation of PKC in target cells is well documented including αT3-1 cells [Horn et al, 1991; Johnson et al, 1994; Stojilkovic et al, 1994]. PKC in particular is known to play a pivotal role in the crosstalk between activators of AC which stimulate $G\alpha_s$ and phosphoinositide hydrolysis [Cooper et al, 1995; Tang & Gilman, 1992]. PKC (or indeed other kinases potentially activated in response to LHRH) therefore were strong candidates for mediators of the LHRH inhibitory effect. Brief phorbol ester stimulation of PKC in αT3-1 cells (20 min) was unable to mimic the LHRH-induced inhibition of forskolin-stimulated cAMP accumulation [Figure 4.3]. Furthermore although not achieving statistical significance, PDBu displayed a trend to increase basal or forskolin- and forskolin + LHRH-evoked cAMP formation. The protein kinase inhibitors GF109203X, staurosporine, genistein and RG-13022 were ineffective against the LHRH inhibition of forskolin-stimulated responses [Table 4.1], for example LHRH inhibition in the absence of inhibitors is usually found to be 53 ± 3 %, and in the presence of GF109203X, staurosporine, genistein and RG-13022 were 47 ± 4%, 42 ± 4%, 55 ± 7% and 56 ± 5% respectively, further evidence that a kinase does not seem to be mediating the LHRH effect. GF109203X and staurosporine, when included with forskolin and LHRH and PTx, appeared to increase the capacity for PTx reversal of the LHRH inhibition of this response. However in the presence of these inhibitors, there is a small increase in all the cAMP formation responses. Chronic treatment of αT3-1 cells with PDBu also had a considerable effect on cellular cAMP formation. Under these conditions all forskolin-stimulated responses were significantly reduced and the proportional inhibitory effect of LHRH was reduced (ie. 32 ± 8% inhibition, compared to approximately 55 ± 6% in untreated cell). In PKC-

downregulated cells LHRH inhibition of forskolin-stimulated cAMP accumulation was still partially PTx sensitive (ie LHRH + PTx now gave 82 ± 6% inhibition). However with reduced LHRH effects and the present number of replications the effect of PTx in these conditions did not reach statistical significance. Therefore PKC does not seem to be required for the acute effect of LHRH on forskolin-evoked cAMP accumulation, although it is still possible that activation of PKC leads to some increase and some decrease in AC responses which is impossible to discern in the whole cell assay used here. The forskolin-evoked activation of ACs however does seem to be dependent on a longer term PKC presence for expression of a full magnitude response. Taken together these results imply that there is no critical role of PKC (or other kinase sensitive to these inhibitors) in the LHRH-mediated inhibition of forskolin-evoked cAMP formation. One previous study of LHRH inhibition of PACAP-stimulated cAMP formation suggested that PKC was responsible for mediating the LHRH inhibition [McArdle et al, 1994]. In this report they concluded that this PKC action was exerted at an early stage of PACAP receptor signalling and was not intrinsically at the level of G-proteins, however their only evidence of a PKC involvement was by an acute treatment with phorbol ester and despite the additional attempts to clarify the role of PKC with kinase inhibitors or PKC downregulation no evidence in agreement with their observations was found. This study also failed to observe a LHRH inhibition of either forskolin- or $G\alpha_s$-induced cAMP accumulation or a PTx-sensitive component to the LHRH inhibition of PACAP-stimulated cAMP formation, however was in agreement with the Ca^{2+} data (concerning both the ionophore and EGTA). At present it remains unclear as to why our data is different, it is possible that alternative cell culturing regimes and a different passage number may effect the results as slight changes in αT3-1 cells become apparent as the passage number increases. Alternatively it is known that different serum sources can affect the growth and responses of αT3-1 cells [P. Mellon; L. Anderson, personal

communication] and all our experiments were carried out after at least 18 h in serum free medium.

LHRH increases cytosolic Ca^{2+} levels in gonadotrophs [Johnson et al, 1993b; Mitchell et al, 1988] and αT3-1 cells [Anderson et al, 1993; Johnson et al, 1993b; Merelli et al, 1992]. From these results it seems unlikely that cAMP inhibition is a reflection of raised Ca^{2+} perhaps inhibiting AC as the Ca^{2+} ionophore, ionomycin, was unable to mimic this inhibitory effect and had a very slight stimulatory effect on cAMP formation alone (Figure 4.4). LHRH inhibition of forskolin- and PACAP-stimulated cAMP accumulation occurred in the presence of a phosphodiesterase inhibitor IBMX therefore is unlikely to be acting by activating a Ca^{2+}/calmodulin-dependent phosphodiesterase. Reduction of cytosolic Ca^{2+} with EGTA had a profound effect on cellular cAMP formation. This treatment prevented LHRH inhibition of forskolin-evoked cAMP accumulation. However in these conditions the forskolin-stimulated response was greatly reduced (Figure 4.4). This profile could be consistent with a high proportion of Ca^{2+}/calmodulin-dependent AC isoforms in these cells which are rendered ineffective by the absence of Ca^{2+}. The increased cAMP formation in the presence of LHRH could possibly be explained if subset of AC present in αT3-1 cells were unaffected by Ca^{2+}/calmodulin but were stimulated by βγ subunits. This would require the βγ subunits to be derived from G-proteins other than $G_{i/o}$ as PTx treatment did not decrease the cAMP accumulation any further. An alternative possibility exists if certain Ca^{2+}-independent AC isoforms were more responsive following phosphorylation induced by LHRH receptor activation, but this seems unlikely as the PKC inhibitor GF109203X would prevent such an action on cAMP accumulation. Alternatively if LHRH is activating cytosolic phosphatases, a tonic inhibitory influence such as phosphorylation of AC or a G-protein may be removed as a consequence of LHRH action which could in turn increase cAMP formation. Alternatively it could be not only those AC isotypes that are indifferent to Ca^{2+}, but also Ca^{2+}-inhibited isotypes whose response is now exposed and

consequently as they would be more active would account for a bigger percentage of the EGTA-reduced response.

The cloning of AC has facilitated the investigations of isoform specific regulation [Cooper et al, 1995; Tang & Gilman, 1992; Taussig & Gilman, 1995]. G-protein regulation of adenylyl cyclases can be mediated both by α and $\beta\gamma$ subunits. As previously stated, all the isoforms are stimulated by $G\alpha_s$. Transfection of cells with cDNAs encoding constitutively activate $G\alpha_i$ subunits lowered intracellular concentrations of cAMP suggesting that these proteins could inhibit AC [Wong et al, 1991]. When tested on expressed Type I, V and Type VI AC isoforms, $G\alpha_i$ displayed prominent inhibition of $G\alpha_s$- and forskolin-stimulated activity, although this effect was less prominent on Type I AC and the inhibition was largely confined to the forskolin-stimulated response [Taussig & Gilman, 1995; Taussig et al, 1993]. Type II AC has also been reported to be inhibited by $G\alpha_{i/o}$ [Chen & Ivengar, 1993]. However this has been contested and there appears to be more evidence favouring activation of this isoform by $\beta\gamma$ subunits liberated from the $G_{i/o}$ heterotrimer [Federman et al, 1992; Tang & Gilman, 1991; Taussig & Gilman, 1995]. To date only Type I AC has been shown to be inhibited by $\beta\gamma$ subunits [Tang & Gilman, 1991]. In contrast $\beta\gamma$ subunits are stimulatory for Type II [Tang & Gilman, 1991] and Type IV [Gao & Gilman, 1991] AC (in the presence of activated $G\alpha_s$). Changes in Ca^{2+} can have pronounced effects on cellular cAMP concentrations depending on the AC isoforms present. Types I, VIII and to a lesser extent Type III are all markedly stimulated by Ca^{2+}/calmodulin. The other isoforms are insensitive to Ca^{2+}/calmodulin. In comparison, Types V and VI AC are inhibited by low micromolar concentrations of Ca^{2+}, but are insensitive to calmodulin therefore this seem to be a direct interaction of the Ca^{2+} ion with the cyclase [Ishikawa et al, 1992; Katsushika et al, 1992; Yoshimura & Cooper, 1992].

Cellular cAMP concentrations are likely to be highly dependent on the state of phosphorylation in a cell as many receptors which stimulate or inhibit AC are the substrate for kinases including PKA, PKC and β–adrenergic receptor kinase, consequently phosphorylation often results in desensitisation or down regulation of these receptors [Dohlman et al, 1991]. Alternatively kinases may phosphorylate components of the AC signalling system. In one case a mutational swap approach has been used which has implicated the C2b domain as the region required for PKC regulation of AC II [Taussig & Gilman, 1995; Cooper et al, 1995]. Phosphorylation of AC isoforms as a consequence of ligand-induced kinase activation, has been suggested to potentially regulate AC activity. Whilst this is acknowledged as a valid regulatory mechanism, isotype specific effects appear to be a contentious area of research with many conflicting reports in press [reviewed in [Taussig & Gilman, 1995]. Activity of Type II AC is reported to be substantially augmented by phosphorylation by activated PKC [Jacobowitz et al, 1993; Lustig et al, 1993; Yoshimura & Cooper, 1993]. This effect may be indirect or involve a specific isoform of PKC, however not all laboratories have been able to mimic this effect [Taussig & Gilman, 1995]. These differences might be explained as Taussig et al have expressed Type II AC in Sf9 membranes and added activated protein kinase C, whereas the other groups have transfected the Type II AC cDNA into a host cell expression system and it is likely that cell specific differences may be acting to promote this phosphorylation response. Type V AC activity is enhanced *in vitro* specifically by PKC α and δ [Kawabe et al, 1994]. As PKC α is often activated in response to $G_{q/11}$-mediated pathways and PKC δ has been reported to be activated following growth factor receptor activation, a potential for cross-talk between $G_{q/11}$ and tyrosine kinase regulated signalling pathways and this AC has been suggested [Kawabe et al, 1994], although the physiological relevance of this observation is disputed as only a weak effects have been noted *in vivo* [Jacobowitz et al, 1993]. Reports of Type I AC phosphorylation are again disputed, nonetheless there are

reports that phosphorylation enhances forskolin- [Choi et al, 1993] and Ca^{2+}/calmodulin- [Jacobowitz et al, 1993] stimulated activity. At present there are few reports of regulation of adenylyl cyclase activity by phosphorylation at the level of G-proteins [Carlson et al, 1989; Daniel-Issakani et al, 1989]. Nevertheless $G\alpha_s$ has been reported to be phosphorylated by both protein kinase C and protein kinase A *in vitro* [Pyne et al, 1992] and G-proteins, including $G\alpha_s$, are reported to be a substrate for the protein tyrosine kinase Src [Hausdorff et al, 1992]. Furthermore phosphorylation of $G\alpha_i$ by PKC has been suggested as a mechanism for phosphorylation-mediated inhibition of Ca^{2+} mobilisation in human platelets [Yatomi et al, 1992]. It is therefore possible that G-protein phosphorylation, as well as AC phosphorylation, may become more apparent as a form of regulation in the future.

In summary through the interaction of the α_i (inhibitory) or $\beta\gamma$ subunits (which can stimulate or inhibit AC activity) with AC isoforms and or α_q interaction with other effector systems which might consequently raise Ca^{2+} or activate PKC (which can stimulate or inhibit AC activity) we find that three distinct patterns of G-proteins regulation of AC exist: G_q and G_i both stimulate AC activity; G_q and Gi both inhibit; G_i inhibits/G_q stimulates. All the isoforms are positively regulated by $G\alpha_s$ subunits. It is therefore apparent that AC isoforms have evolved such that there is little apparent redundancy amongst the isoforms with respect to the precise profile of regulation. Amongst the isoforms isolated to date there exists a large potential for cross-talk and integration of signals mediating from at least G_s, $G_{q/11}$ and $G_{i/o}$ linked receptors and possibly growth factor tyrosine kinase receptors.

Information concerning isoform-specific regulation of AC's is obviously important especially to the understanding of cAMP formation in the brain where individual isoforms are specifically expressed in discrete regions [reviewed in Cooper et al, 1995]. Expression of AC isoforms in tissues such as the anterior pituitary gland however remains largely undescribed. At present there is no published information

on the AC isoforms present in αT3-1 cells. From data presented in this chapter, it appears that multiple isoforms are being expressed as the profile of regulation demonstrated does not obviously correspond to any one isoform based on our current understanding of this field. The present evidence therefore suggests that LHRH is indeed functionally capable of activating the $G_{i/o}$ family of G-proteins. Recent experiments in our laboratory have demonstrated agonist-regulated co-immunoprecipitation of $G_{i\alpha 1/2}$ together with the LHRH receptor [Mitchell et al manuscript in preparation], which clearly authenticates our inferences from the MAP kinase and cAMP assay data. The LHRH receptor now joins an increasing number of receptors which are promiscuous in their interactions with G-proteins.

Table 4.1

Effects of protein kinase inhibitors on forskolin-stimulated cAMP responses with LHRH and pertussis toxin in αT3-1 cells

Where appropriate pertussis toxin (PTx; 100 ng/ml) was added to the normal growth medium to a final concentration of 100 ng/ml for ~18 h. Thereafter αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 mM) for 15 min at 37°C before the addition of forskolin (1 μM) and LHRH (300 nM) as indicated for 30 min. All inhibitors were added 5 min prior to stimuli. The table shows the mean values ± SEM of data from at least 6-8 determinations. Data were first expressed as a % of the forskolin-stimulated response in each individual experiment so that the normalised forskolin-stimulated value applies to all incubations. Typical values for basal, LHRH, PTx and forskolin were in the region of 0.8-1.2, 0.6-1.0, 0.6-1.1 and 70-120 nmoles/50 μl respectively. None of the inhibitors had any significant effect on basal cAMP accumulation or that in the presence of LHRH or PTx (data shown in Table 4.2).

Table 4.1

inhibitor	Effect of inhibitors on condition (% of forskolin-stimulated cAMP formation)		
	forskolin	forskolin + LHRH	forskolin + LHRH + PTx
nil	100	47 ± 3	88 ±10
GF109203X (5 μM)	110 ± 9	53 ± 4	95 ± 8
staurosporine (100 nM)	115 ± 15	58 ± 4	96 ± 8
genistein (30 μM)	94 ± 13	45 ± 7	80 ± 12
RG-13022 (10 μM)	97 ± 3	46 ± 5	90 ± 11

Table 4.2

Effects of protein kinase inhibitors on basal cAMP responses with LHRH and pertussis toxin in αT3-1 cells

Where appropriate pertussis toxin (PTx; 100 ng/ml) was added to the normal growth medium to a final concentration of 100 ng/ml for ~18 h. Thereafter αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 mM) for 15 min at 37°C before the addition of forskolin (1 μM) and LHRH (300 nM) as indicated for 30 min. All inhibitors were added 5 min prior to stimuli. The table shows the mean values ± SEM of data from at least 6-8 determinations. Data were first expressed as a % of the basal response in each individual experiment so that the normalised basal value applies to all incubations. Typical values for basal, LHRH, PTx were in the region of 0.8-1.2, 0.6-1.0, 0.6-1.1 nmoles/50 μl respectively. None of the inhibitors had any significant effect on basal cAMP accumulation or that in the presence of LHRH or PTx.

Table 4.2

inhibitor	Effect of inhibitors on condition (% of basal cAMP formation)		
	basal	LHRH	PTx
nil	100	95 ± 7	106 ± 5
GF109203X (5 μM)	106 ± 9	97 ± 3	108 ± 5
staurosporine (100 nM)	105 ± 5	98 ± 6	104 ± 6
genistein (30 μM)	94 ± 3	96 ± 7	105 ± 4
RG-13022 (10 μM)	97 ± 3	98 ± 5	103 ± 5

Figure 4.1

Effect of LHRH on forskolin- and PACAP-stimulated cAMP formation in αT3-1 cells.

αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 mM) for 15 min at 37°C before the addition of forskolin (1 μM), PACAP (10 μM) and LHRH (0.3-1000 nM) as indicated for 30 min. Values are means ± SEM of data from at least 6-8 determinations. Data is expressed as the concentration of cAMP (nmoles) in a 50 μl sample from each individual well.

Figure 4.1

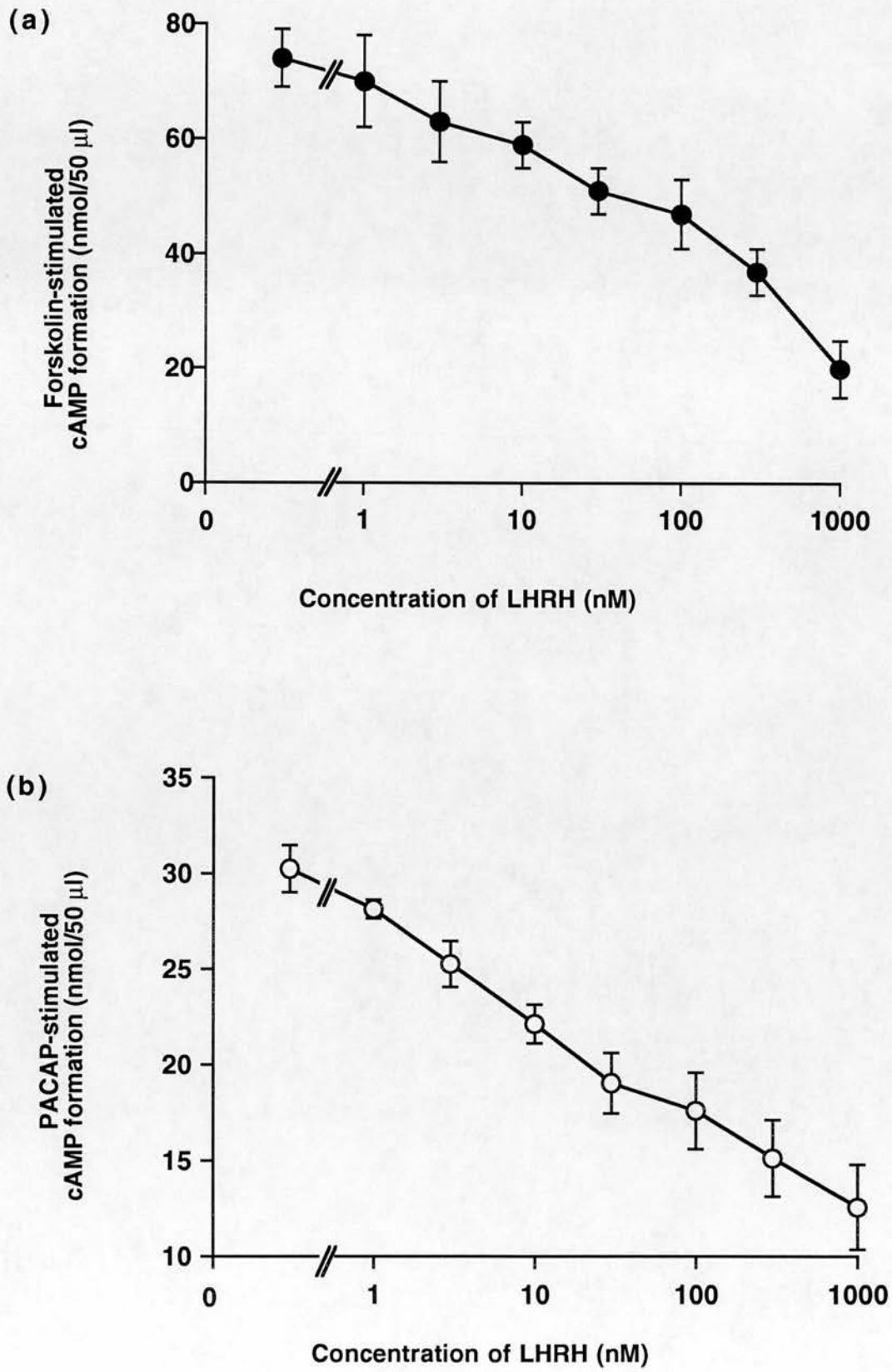

(a)
Forskolin-stimulated cAMP formation (nmol/50 µl)
80
60
40
20
0
0
1
10
100
1000
Concentration of LHRH (nM)
(b)
PACAP-stimulated cAMP formation (nmol/50 µl)
35
30
25
20
15
10
0
1
10
100
1000
Concentration of LHRH (nM)

Figure 4.2

Effect of pertussis toxin on LHRH inhibition of forskolin- and PACAP-stimulated cAMP formation in αT3-1 cells.

Where appropriate pertussis toxin (PTx) was added to the normal growth medium to a final concentration of 3-300 ng/ml for ~18 h. Thereafter αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 mM) for 15 min at 37°C before the addition of forskolin (1 μM), PACAP (10 nM) and LHRH (300 nM) as indicated for 30 min. Values are means ± SEM of data from at least 6-8 determinations. Data is expressed as the concentration of cAMP (nmoles) in a 50 μl sample from each individual well.

Figure 4.2

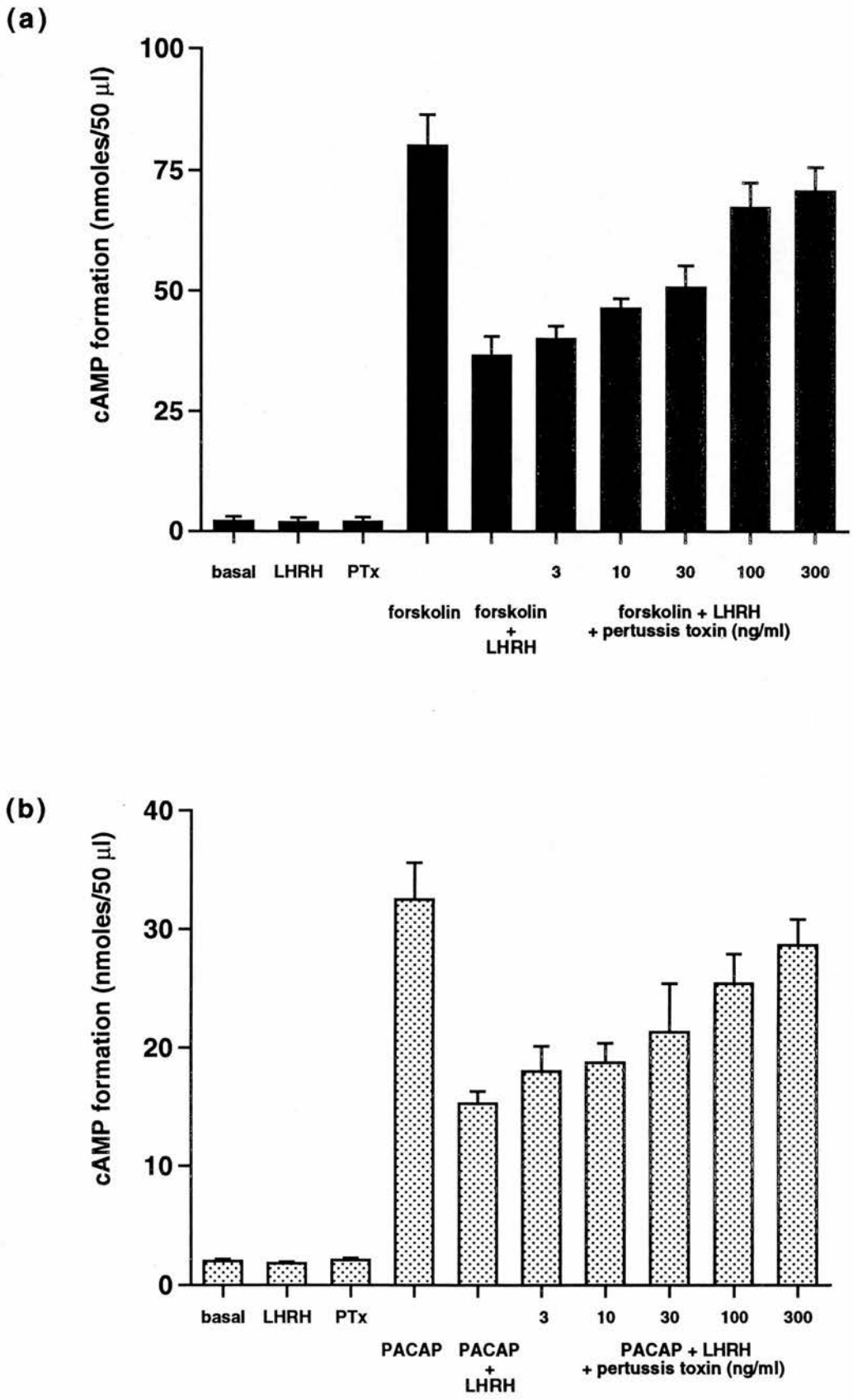

(a)
cAMP formation (nmoles/50 µl)
100
75
50
25
0
basal
LHRH
PTx
forskolin
forskolin + LHRH
3
10
30
100
300
forskolin + LHRH + pertussis toxin (ng/ml)
(b)
cAMP formation (nmoles/50 µl)
40
30
20
10
0
basal
LHRH
PTx
PACAP
PACAP + LHRH
3
10
30
100
300
PACAP + LHRH + pertussis toxin (ng/ml)

Figure 4.3

Effects of acute and chronic PDBu treatment on forskolin-stimulated cAMP formation with LHRH and pertussis toxin in αT3-1 cells.

Figure 4.3a shows the acute effects of PDBu on cAMP formation. αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 mM) for 15 min at 37°C before the addition of forskolin (1 μM), PDBu (1 μM) and LHRH (300 nM) as indicated for 30 min.

Figure 4.3b shows the effects of PDBu down regulation of phorbol ester-sensitive PKC isoforms on cAMP formation in αT3-1 cells. PDBu or pertussis toxin (PTx) was added to the normal growth medium to a final concentration of 300 nM or 100 ng/ml respectively for ~18 h where indicated. αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 M) for 15 min at 37°C before the addition of forskolin (1 μM) and LHRH (300 nM) as indicated for 30 min.

Values are means ± SEM of data from at least 3-6 determinations. Data is expressed as the concentration of cAMP (nmoles) in a 50 μl sample from each individual well.

Figure 4.3

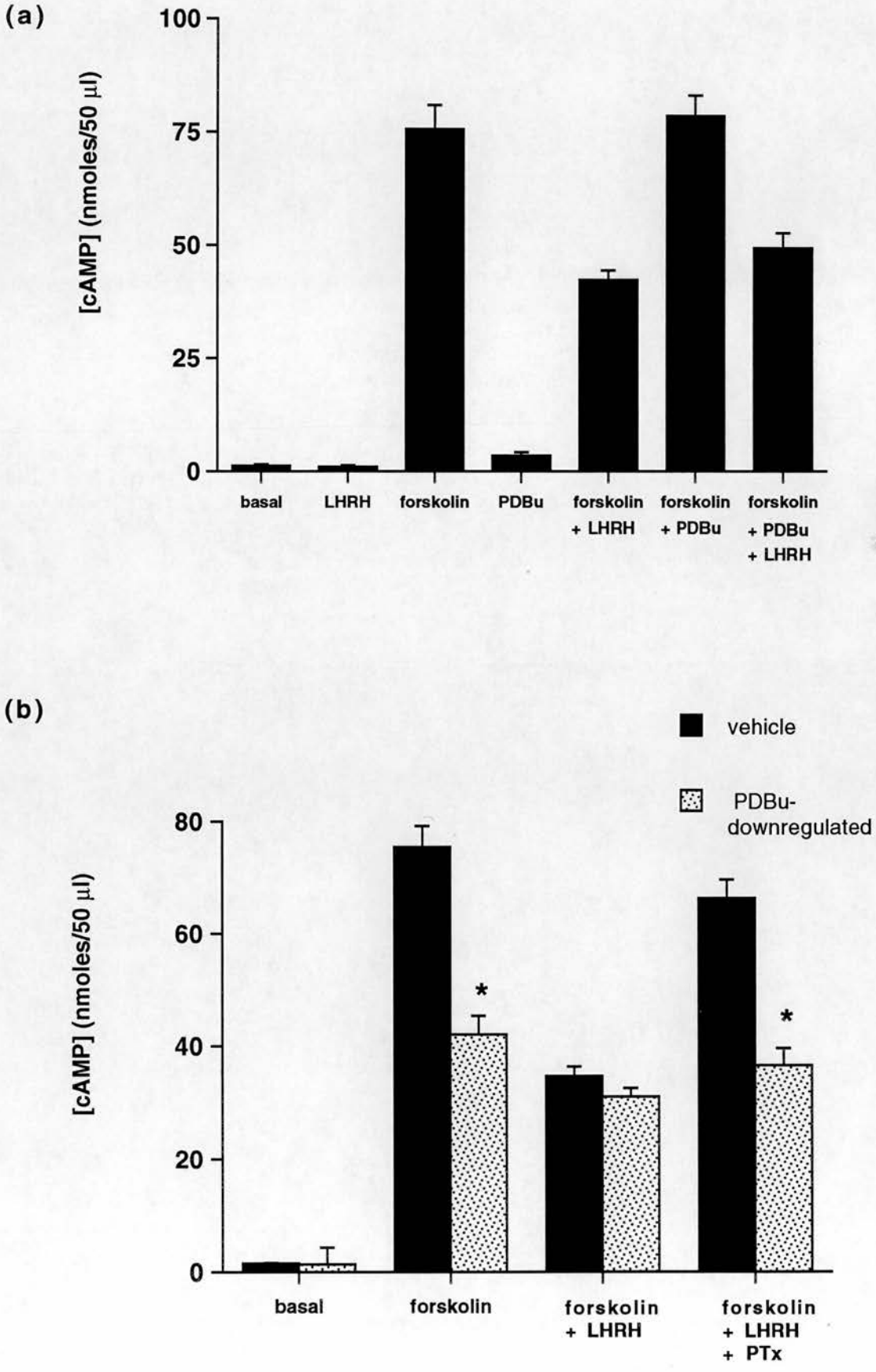

(a)
[cAMP] (nmoles/50 µl)
100
75
50
25
0
basal
LHRH
forskolin
PDBu
forskolin + LHRH
forskolin + PDBu
forskolin + PDBu + LHRH
(b)
vehicle
PDBu-downregulated
[cAMP] (nmoles/50 µl)
80
60
40
20
0
*
*
basal
forskolin
forskolin + LHRH
forskolin + LHRH + PTx

Figure 4.4

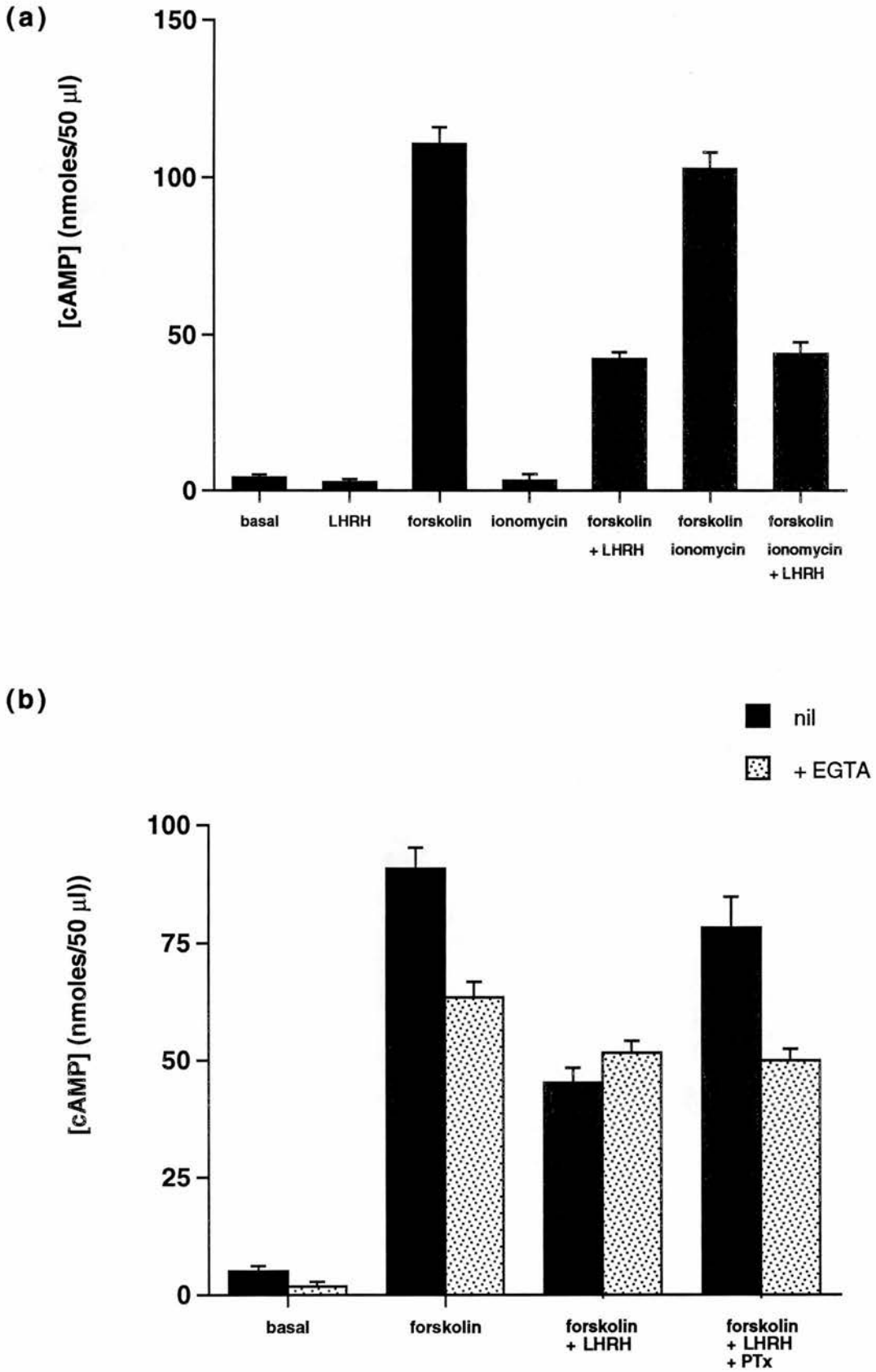
Effects of ionomycin and EGTA (agents which affect Ca^{2+} mobilisation) on forskolin-stimulated cAMP formation with LHRH and pertussis toxin in αT3-1 cells.

Figure 4.4a shows the effects of the Ca^{2+} ionophore, ionomycin, on cAMP formation. αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 mM) for 15 min at 37°C before the addition of forskolin (1 μM), ionomycin (30 μM) and LHRH (300 nM) as indicated for 30 min.

Figure 4.4b shows the effects of the Ca^{2+} chelator EGTA on cAMP formation in αT3-1 cells. Pertussis toxin was added to the normal growth medium to a final concentration of 100 ng/ml for ~18 h and αT3-1 cells were preincubated in MEM/BSA (0.1%) with IBMX (0.5 M) ± EGTA (5 mM) for 45 min at 37°C before the addition of forskolin (1 μM) and LHRH (300 nM) as indicated for 30 min.

Values are means ± SEM of data from at least 3-6 determinations. Data is expressed as the concentration of cAMP (nmoles) in a 50 μl sample from each individual well.

Figure 4.4


(a)
[cAMP] (nmoles/50 µl)
150
100
50
0
basal
LHRH
forskolin
ionomycin
forskolin + LHRH
forskolin ionomycin
forskolin ionomycin + LHRH
(b)
nil
+ EGTA
[cAMP] (nmoles/50 µl))
100
75
50
25
0
basal
forskolin
forskolin + LHRH
forskolin + LHRH + PTx

CHAPTER 5

DIFFERENTIAL ACTIVATION OF MAP KINASE BY PHOSPHOLIPASE C-COUPLED RECEPTORS

5.1 INTRODUCTION

We have previously demonstrated that stimulation of the native LHRH receptor in αT3-1 cells results in the phosphorylation and activation of MAP kinase [Sim & Mitchell, 1995a; Sim et al, 1994; Sim et al, 1995; Wolbers et al, 1995]. This novel signalling cascade appears to be dependent on two signalling inputs, both PKC activation via G_q stimulation of PLC and a pertussis toxin-sensitive G-protein [Sim et al, 1995 and Chapter 3]. Since this work was first undertaken it has become apparent that many G-protein coupled receptors can activate this important family of enzymes, especially those receptors that are coupled to $G_{i/o}$-proteins [Crespo et al, 1994; Faure et al, 1994; Johnson et al, 1994; Koch et al, 1994]. The mechanisms involved are not fully clarified but it seems that for $G_{i/o}$-receptors, the signal is transduced to MAP kinase by the βγ subunits, tyrosine phosphorylation and Ras [Hawes et al, 1995]. $G_{q/11}$ coupled receptor signalling is less clearly defined but would appear to involve phosphoinositide (PI) hydrolysis and PKC activation [Hawes et al, 1995; Koch et al, 1994], although whilst in some cases PKC activation is sufficient to activate Raf-1 and hence MAP kinase, other G_q-mediated signalling systems require additional signal input such as Ras activation and/or tyrosine phosphorylation [Buhl et al, 1995; Qian et al, 1994; Seufferlein & Rozengurt, 1995].

In this Chapter we have transiently expressed (in COS 7 cells) several PLC-coupled receptors of structurally-dissimilar subfamilies, in order to assess whether or not their ability to activate PLC and cause phosphoinositide hydrolysis correlates with an ability to activate MAP kinase. The receptors investigated are the LHRH receptor (an atypical rhodopsin family member), the 5-hydroxytryptamine $(5\text{-HT})_{2C}$ receptor (a more conventional rhodopsin family member) and the mGlu 1a metabotropic glutamate receptor (from a distinct family with large extracellular domains and lacking many of the structural motifs conserved in the rhodopsin family).

In a few cases, for example the thrombin receptor in CCL 39 cells, the spingosylphosphorylcholine receptor (SPC) in Swiss 3T3 cells and the platelet activating factor (PAF) receptor when transfected in CHO cells evidence for "dual activation" of MAP kinase by both PKC-dependent and pertussis toxin-sensitive paths has been reported [Honda et al, 1994; Kahan et al, 1992b; Seufferlein & Rozengurt, 1995]. Interestingly these receptors have all been reported to interact with more than one G-protein [Offermans & Schultz, 1994; Sim et al, 1995], notably the thrombin receptor, another member of the rhodopsin family of G-protein coupled receptors, which displays an unusual Asp for Asn substitution in the seventh transmembrane domain (TMD VII) [Vu et al, 1991]. This is similar to the LHRH receptor which displays a double reciprocal mutation of Asn for Asp87 in TMD II and Asp for Asn318 in TMD VII [Tsutsumi et al, 1992]. Thus it is possible that the TMD VII Asp-containing motif of the LHRH receptor is critical to its ability to activate MAP kinase. Using site-directed mutagenesis, two mutants of the murine LHRH receptor have been produced, Asp318Asn and Asn87Asp; Asp318Asn. Both these mutants retained the ability to bind ligand with high affinity and activate PLC in response to specific agonists [Mitchell et al, 1996; Zhou et al, 1994]. The ligand binding characteristics and PLC responses as well as MAP kinase activation were monitored for both mutants and the wild type receptor after transient expression in COS 7 host cells, in order to assess whether these atypical sequence motifs are important to MAP kinase activation by the LHRH receptor.

5.2 RESULTS

When transiently expressed in COS cells, the LHRH, 5-HT_{2C} and the mGlu 1a receptor showed a marked and concentration dependent increase in [^{3}H]inositol phosphate formation in response to LHRH, 5-hydroxytryptophan (5-HT) and (1S, 3R)-aminocyclopentane dicarboxyclic acid (ACPD) respectively (Figure 5.1a). Under these conditions, the maximum responses observed for the LHRH and 5-HT_{2C}

receptors were similar and that for the mGlu 1a receptor was slightly less representing a 4.5 ± 0.4, 3.7 ± 0.3 and 2.9 ± 0.4 fold increases over basal activity respectively (means ± SEM, n=4). The EC_{50} values for inositol phosphate formation by the respective agonists were 11 ± 6 nM, 118 ± 81 nM and 7.0 ± 5.4 μM for LHRH, 5-HT and ACPD. None of these agonists stimulated [^{3}H]inositol phosphate formation in any of the cells expressing the other receptors or in untransfected COS 7 cells and basal levels of [^{3}H] inositol phosphates were not significantly different between cells transfected with the different cDNAs.

The ability of these receptors (expressed in COS 7 host cells) to activate MAP kinase in response to LHRH, 5-HT and ACPD was also examined. The transfected LHRH receptor mediated a prominent concentration-dependent MAP kinase response to LHRH (Figure 5.1b), which reached a maximum of 119 ± 9% increase over basal activity at 10 min exposure to LHRH. The EC_{50} value for this response was 34 ± 2 nM (means ± SEM, n=7). Also seen in Figure 5.1b the 5-HT_{2C} and mGlu 1a receptors were only able to elicit a minimal activation of MAP kinase with maximum responses of 14 ± 7% and 21 ± 9% over basal following stimulation for 10 min with 20 μM 5-HT and 100 μM ACPD respectively (means ± SEM, n=5). These experiments with 5-HT and ACPD were repeated over a time course of 0-30 min to address the possibility that these receptors were activated only transiently or after a significant delay. No greater response was observed over this period. In contrast [^{3}H]inositol phosphates continued to accumulate throughout a 30 min time course in response to LHRH, 5-HT and ACPD with only a slight reduction after 3-4 min (data not shown), demonstrating that functional coupling of the receptor was still apparent during this period.

In Figure 5.2, LHRH-induced inositol phosphate formation (a), and MAP kinase activation (b), are compared in three cell lines to demonstrate that this phenomenon is not restricted to COS 7 cells. These include COS 7 cells where the receptor is

transiently expressed, a Chinese Hamster ovary (CHO) cell line, CHO 7.5, which stably expresses the LHRH receptor, and the αT3-1 cell line where the LHRH receptor is endogenously expressed. In αT3-1 cells and CHO 7.5 cells, 100 nM LHRH caused a 5.8 ± 0.6 and a 6.2 ± 0.7 fold increase over basal in [^{3}H]inositol phosphate formation with EC_{50} values for this response of 2.8 ± 0.3 nM and 3.1 ± 0.2 nM respectively. This compares with mean values of 4.5 fold and 11 nM in COS 7 host cells (above). MAP kinase activation in these cell lines was concentration dependent and reached a maximum of 245 ± 9% and 198 ± 11% of basal activity with corresponding EC_{50} values of 3.4 ± 0.6 nM and 97 ± 11 nM respectively. Corresponding values in COS 7 host cells were 219 ± 8% and 34 nM (above).

Experiments were carried out to investigate the possible involvement of PKC in MAP kinase activation by the LHRH receptor expressed in COS 7 and CHO 7.5 cells (Table 5.1) and are compared to data obtained by this receptor in αT3-1 cells. The MAP kinase response to LHRH (100 nM for 10 min) was mimicked by the PKC activator, PDBu (1 μM for 10 min) in both cases. The responses to LHRH and PDBu were inhibited by the highly selective PKC inhibitor GF109203X [Toullec et al, 1991], with very similar IC_{50} values, 1.4 ± 0.4 μM and 1.3 ± 0.3 μM respectively for COS 7 cells (Figure 5.3) and 1.8 ± 0.6 μM and 0.8 ± 0.2 μM respectively for CHO 7.5 cells (means ± SEM, n=4-6). This pattern was also observed for two other PKC inhibitors: Ro-31 8220 [Davis et al, 1989] (0.36 ± 0.12 nM and 0.27 ± 0.11 nM; for LHRH and PDBu responses in CHO cells) and H7 [Hidaka et al, 1984] (69.8 ± 24 nM and 30.6 ± 13 nM for corresponding responses in COS 7 cells; 36.7 ± 13 nM and 32.9 ± 10 nM in CHO 7.5 cells).

The ability of the LHRH receptor to activate MAP kinase evidently appears to rely on PKC. However the other PLC-stimulating receptors tested here do not seem to have the ability to evoke such a response, within the limits of detection of the present assay. Therefore the ability of these receptors to activate PKC was examined to see

assay. Therefore the ability of these receptors to activate PKC was examined to see if any differences were apparent [Lutz et al, 1993]. The translocation of [^{3}H]PDBu binding sites from the cytosol to membrane fractions is a critical step in activation of conventional PKC isoforms (ie phorbol ester-sensitive PKC isoforms) [Bazzi & Nelsestuen, 1987]. It can be seen in Table 5.2 that in agreement with the data showing a similar ability of the LHRH, 5-HT_{2C} and mGlu 1a receptors to elicit [^{3}H]inositol phosphate formation all these receptors achieved a comparable translocation/activation of PKC (with on average 33, 39 and 31% of the [^{3}H]PDBu binding sites being recovered in the membrane compared to 12, 14 and 11% in unstimulated cells). Control responses of these cells to PDBu (300 nM) resulted in a stronger translocation/activation with on average translocation of 60-80% of the [^{3}H]PDBu binding sites to the membrane [Lutz et al, 1993]. This treatment also caused translocation/activation of PKC in CHO 7.5 cells with 49 ± 6% of the [^{3}H]PDBu binding sites being recovered in the membrane compared to 12 ± 0.8% in untreated cells. PKC α is the only conventional PKC isoform present in COS 7 cells [Kosaka et al, 1988]. It has previously been shown that translocation to the membrane of PKC α in response to 100 nM LHRH was largely prevented as a result of removing free Ca^{2+} from the medium by the addition of 3 mM EGTA (14 ± 6% incremental translocation to the membrane compared to 33 ± 8% in normal medium; means ± SEM, n=5) [Lutz et al, 1993].

It is possible that MAP kinase activation by the LHRH receptor in transfected cells may be an inappropriate consequence of overexpression of the receptor protein. To address this possibility, the affinity and number of LHRH receptors was determined by equilibrium binding with the LHRH agonist [^{125}]buserelin. In samples of the transfections used for the previous experiments, the K_D and B_{max} of specific [^{125}]buserelin binding sites were 1.0 ± 0.4 nM and 0.93 ± 0.3 pmol/mg protein for the LHRH receptor expressed in COS 7 cells and 0.4 ± 0.3 and 0.59 ± 0.26 pmol/mg protein for the receptor expressed in CHO 7.5 cells. αT3-1 cells expressed 1.27 ±

0.29 pmol/mg protein of [^{125}]buserelin binding sites which had a K_D of 0.6 ± 0.3 nM. These corresponding values would indicate that both the number and affinity of LHRH receptors expressed in these host cells equates with LHRH receptor numbers in a cell line which naturally express the receptor and the ability of this receptor to activate MAP kinase is unlikely to be an artefact of overexpression of the receptor protein.

The uncommon pattern of PKC dependence and pertussis toxin sensitivity for LHRH-induced MAP kinase activation in αT3-1 cell [Sim et al, 1995; Chapter 3] has also been reported for the thrombin receptor [Kahan et al, 1992b]. The thrombin receptor also displays an atypical Asp substitution for the generally conserved Asn in TMD VII, therefore it is possible that (in addition to PKC activation) the ability of the LHRH receptor to activate MAP kinase relies in some way upon this unusual characteristic of the receptor. MAP kinase activation by the wild type receptor was therefore compared to two mutant receptors developed by site-directed mutagenesis and containing a Asp318Asn substitution and a double reciprocal Asn87Asp;Asp318Asn substitution. The Asn87Asp single mutant receptor displayed no ligand binding and was therefore discarded from subsequent studies. Table 5.3 shows the ligand binding characteristics and, [^{3}H]inositol phosphate responses and MAP kinase activation for these experiments. Both the Asp318Asn mutant and the Asn87Asp;Asp318Asn double mutant displayed less than 50% (47% and 29% respectively) of the wild type [^{125}]buserelin binding sites/mg of protein after transfection with equal amounts (25 μg) of cDNA in COS 7 cells, indicating expression of a substantially reduced number of functional receptors. However the K_D value of 1.1 ± 0.3 and 0.8 ± 0.3 nM for the mutant and double mutant were comparable to a K_D value of 1.0 ± 0.6 nM for the wild type receptor suggesting that the functional receptors present have a similar affinity for ligand. This pattern is mimicked by the [^{3}H]inositol phosphate formation and MAP kinase responses where the EC_{50} values for each activity are similar with respect to the wild type receptor but

both mutants displayed greatly reduced maximum responses. Therefore it is apparent that where functional mutant receptors are present they are equally capable of activating PLC and MAP kinase. Furthermore it is improbable that the atypical amino acid substitutions in TMD VII (and TMD II) confer the especially powerful ability of the LHRH receptor to activate MAP kinase.

The effects of pertussis toxin on MAP kinase activation by the wild type and mutant LHRH receptors was also assessed in COS 7 cells to determine if it reflected the inhibition detected in αT3-1 cells [Sim et al, 1995; Chapter 3]. Control LHRH-induced MAP kinase activity (100 nM, 10 min) represented an increase of 95 ± 7%, 42 ± 6% and 19 ± 9% of basal MAP kinase activity for the wild type, Asp318Asn mutant and the Asn87Asp;Asp318Asn double mutant respectively. After a 18 h pretreatment with pertussis toxin, LHRH-induced activity was reduced to 38 ± 6% and 19 ± 5% for the wild type and Asp318Asn mutant. The reductions caused by pertussis toxin were statistically significant in both cases ($p \leq 0.05$, Mann-Whitney U-test). Under the present experimental conditions, the MAP kinase response to LHRH in the Asn87Asp;Asp318Asn double mutant was considered too small to accurately determine any inhibition by pertussis toxin. Pertussis toxin treatment had no effect on [^{3}H]inositol phosphate formation by any of these receptors (Figure 5.4b) and was shown to be specific to the pertussis toxin holotoxin, as neither the B-subunit [O'Neill et al, 1992] or N-ethyl maleimide (NEM)-inactivated holotoxin had any significant effect on LHRH-mediated MAP kinase activity.

5.3 DISCUSSION

When the LHRH, 5-HT_{2C} and mGlu 1a receptors were transiently expressed in COS 7 host cells after transfection of their cDNAs, all three receptors mediated both a strong [^{3}H]inositol phosphate formation (Figure 5.1) and the translocation of PKC from the cytosol to the membrane (Table 5.2). Amongst $G_{q/11}$-protein coupled receptors that are reported to cause activation of MAP kinase it is proposed that a

functional coupling to PLC by the Gα subunits and a PKC-dependent step is involved [Faure et al, 1994; Hawes et al, 1995]. The G_q-linked receptors, $\alpha 1_B$ AR or M_1 (when expressed in COS 7 cells) were inhibited by downregulation or PKC inhibition with GF109203X and expression of mutationally inactive Raf, but were unaffected by expression of β–adrenergic receptor kinase carboxy terminus peptide (which sequesters βγ subunits) or mutationally inactive Ras [Hawes et al, 1995]. Also in COS 7 cells, overexpression of βγ subunits or PLCβ2 (which is predominantly activated by βγ), as well as constitutively active α_q subunits could increase MAP kinase activity whereas mutationally activated α_i was without effect [Faure et al, 1994]. However in COS 7 cells, demonstration of a functional coupling to PLC by measurement of [^{3}H]inositol phosphate formation and activation of PKC by showing a shift in the [^{3}H]PDBu binding sites from the cytosol to the membrane fraction was insufficient to cause a significant MAP kinase activation by the 5-HT and mGlu 1a receptors. In contrast the LHRH receptor elicited a substantial and sustained activation of MAP kinase in addition to [^{3}H]IP formation and PKC translocation. This closely paralleled the activation of MAP kinase previously demonstrated in the αT3-1 gonadotroph cell line [Sim et al, 1995; Chapter 3] and was further shown to have this property in a stably transfected CHO cell line containing the LHRH receptor. In all cell lines MAP kinase activation was achieved by the phorbol ester, 12,13 phorbol dibutyrate (PDBu) showing a greater response than the LHRH-mediated response in COS 7 and CHO cells and a weaker response in αT3-1 cells. In all cases both LHRH- and PDBu-induced MAP kinase activation was substantially attenuated by inclusion of a number of specific PKC inhibitors. It is interesting to note from the data concerning the IC_{50} values in Table 5.1 that in the host cell lines containing the transfected receptors the IC_{50} for inhibition of LHRH- and PDBu-induced activity were of a similar potency for GF109203X and Ro-31 8220 and H7 suggesting that it is largely a similar species of PKC isoform that is involved in the transduction of both these signals to MAP kinase. In contrast, in αT3-1 cells there is a marked difference

between the potency of inhibition of LHRH- and PDBu-stimulated responses especially with respect to inhibition with H7. COS 7 and CHO cells are reported to only contain α and ζ isoforms [Kosaka et al, 1988; Ways et al, 1992]. These observations are in agreement with earlier investigations form our laboratory; only PKC α and PKC ζ were present in COS 7 cells [Johnson et al, 1995] whilst in CHO cells the only one strongly staining band was present corresponding to PKC ζ, although no clear staining was observed there was some evidence for very low levels of PKC α and PKC θ in these cells which was below the reasonable levels of detection of the current experimental conditions (not shown) [Simpson, personal communication]. The αT3-1 cell line is known to contain at least the α, ε and ζ isoforms at high levels and additionally an apparently novel isoform or modified variant of a PKC isoform which has recently been characterised in the anterior pituitary [Ison et al, 1993]. The pharmacological characteristics of this PKC appear to be similar to the PKC involved in the LHRH-induced activation of MAP kinase activation in the αT3-1 cell line [Sim et al, 1995; Chapter 3] and anterior pituitary tissue [Mitchell et al 1994; Chapter 6]. The differences in PKC isoforms available in the cells are likely to be reflected in the variances observed in the IC_{50} values obtained for these reagents. The isozyme-specific potency of two compounds which are like the PKC inhibitors GF109203X and Ro-31 8220 (Go 6850 and Go 6976) has been investigated using recombinant PKC isoforms in an *in vitro* kinase assay [Martiny-Baron et al, 1993]. This study found that the IC_{50}s varied considerably, especially between the Ca^{2+} dependent and independent isoforms. Furthermore that the compound identical to GF109203X, inhibited the PKC isozymes with a rank order of $\alpha > \beta_1 > \varepsilon > \delta > \zeta$. Our data would strongly suggest a requisite role for PKC and PLC in this signalling cascade, however it would appear that these alone are insufficient to cause activation of MAP kinase by all receptors. The 5-HT and mGlu 1a receptors both caused translocation of PKC and [^{3}H]IP formation to the same extent as the LHRH receptor after transfection with identical amounts of cDNA (25 μg). It therefore

seems unlikely (unless these receptors require a lower functional occupancy to elicit PLC activation than the LHRH receptor) that the inability of these two receptors to activate MAP kinase is due to lower expression of receptors. It was confirmed that the LHRH receptor was not being expressed in excess of physiological levels by measurement of the [^{125}I]buserelin binding to the LHRH receptor expressed in COS 7 cells. At the time of these experiments were undertaken, no good agonist was available for binding studies on the mGlu 1a receptor or the 5-HT_{2C} receptor so the question that lower receptor numbers were expressed cannot be entirely ruled out. However this would only be crucial if the receptor number as well as level of IP formation and activation of PKC is important.

From these results, it would therefore seem likely that some secondary signal input must be additionally required. Alternatively the structure of the receptor may somehow allow the receptor to interact with the appropriate intracellular machinery to activate MAP kinase. The LHRH receptor is an unusual member of the rhodopsin superfamily of seven transmembrane-domain (TMD VII) G-protein-coupled receptors. Sequence analysis has revealed a number of atypical amino acid substitutions in TMD II and VII including an Asn for Asp at residue 87 and an Asp for Asn at residue 318 [Tsutsumi et al, 1992]. These residues lie in close proximity to each other in the three dimensional structure and are probably joined by a hydrogen bond between the two residues [Zhou et al, 1994]. The substitution in TMD VII occurs within a generally conserved Asn-Pro-X-X-Tyr motif in rhodopsin family members [Tsutsumi et al, 1992; Zhou et al, 1994]. An identical substitution in this conserved motif is also found in another member of this family, the thrombin receptor [Vu et al, 1991]. Correspondingly the thrombin receptor is also capable of activating MAP kinase [Kahan et al, 1992b]. Remarkably the thrombin receptor, but not the M_1 muscarinic or 5-HT_{2c} (all PLC-coupled receptors), in CCL 39 hamster fibroblasts was able to cause mitogenesis which may be attributed to its ability to activate MAP kinase [Kahan et al, 1992a; Seuwen et al, 1990]. Using site-directed mutagenesis of the

murine LHRH receptor, three mutant receptors were generated (Asp318Asn, Asn87Asp and Asn87Asp;Asp318Asn) to assess the effects on intracellular signalling of these substitutions by re-instating the typical conserved sequence [Zhou et al, 1994]. The mutation of Asp(318) to Asn is able to maintain the hydrogen bond between TMD II and TMD VII and it appears that Asn is able to act as both donor or acceptor in this site [Zhou et al, 1994]. The double reciprocal mutation, or a single Asn for Asp(318) should therefore be able to maintain the structural integrity of the receptor and ligand binding characteristics. Interestingly the single mutation of Asn87 to Asp resulted in a complete loss of agonist binding [Zhou et al, 1994] and was therefore not used in further studies. The two mutants were both shown to elicit significant [^{3}H]inositol phosphate formation and MAP kinase activation albeit with greatly reduced responses compared to the maximal measured response of the wild type LHRH receptor. Similar observations have previously been made [Zhou et al, 1994]. Measurement of LHRH binding characteristics of these receptors with the superagonist [^{125}I]buserelin, revealed a vastly reduced number of high affinity ligand binding sites in cells transfected with identical amounts of cDNA for the mutant and wild type receptors. However, all three variants bound the ligand with similar affinity which may suggest that these mutations in some way interfere with protein folding, insertion or targeting to the plasma membrane, rather than an impairment of functional coupling to PLC and MAP kinase. The reduction of maximal [^{3}H]inositol phosphate formation and MAP kinase responses in each case reflect a reduced population of functional LHRH receptors as the EC_{50} values for the responses are all similar to those obtained for the wild type receptor. Furthermore the maximal responses for PLC and MAP kinase achieved by the mutants reflected quite closely the B_{max} value from the ligand binding. The findings from a parallel study of the corresponding mutants of the rat LHRH receptor were contradictory [Cook et al, 1993]. It was suggested that the mutants were unresponsive with respect to [^{3}H]inositol phosphate formation and furthermore that the double mutant did not bind

ligand. However it is possible that the sensitivity of the assay used may be insufficient at the low levels at which the mutant receptors are expressed.

These results indicate that the atypical substitution in TMD VII does not alone permit the LHRH receptor to cause activation of MAP kinase. An alternative possibility may be that the Asp Pro X X Tyr motif is important to the ability of the receptor to interact with multiple G-proteins, particularly $G_{i/o}$. Many of the G-protein coupled receptors that are capable of activating MAP kinase are either coupled to pertussis toxin-sensitive $G_{i/o}$ proteins such as the M_2 muscarinic receptor [Crespo et al, 1994] and the α_{2A}-adrenergic receptor [Albas et al, 1993; Hawes et al, 1995] or to multiple G-proteins such as the LPA receptor [Hordijk et al, 1994], PAF receptor [Honda et al, 1994], endothelin receptor [Bogoyevitch et al, 1995; Kasuya et al, 1994] thrombin receptor [Winitz et al, 1994] (although the signals generated from multiple G-proteins are not necessarily involved in activation of MAP kinase). It has previously been demonstrated for the LHRH receptor that agonist-induced MAP kinase response are sensitive to pertussis toxin [Sim et al, 1995; Wolbers et al, 1995]. The response of the double mutant was considered too small for accurate investigation but assessment of the effect of 100 ng/ml pertussis toxin on LHRH-induced MAP kinase by the Asp318Asn receptor was shown to be similar to the wild type receptor resulting in a significant attenuation of the response and indicating that the Asp 318 motif was not critical to interaction with $G_{i/o}$ proteins. Although the atypical motif in TMD VII appears inconsequential to the interaction of the LHRH receptor with $G_{i/o}$ proteins [Sim & Mitchell, 1995a; Wolbers et al, 1995], the ability of the LHRH receptor to interact with multiple G-proteins and generate multiple signal inputs is still likely to be pivotal to the ability of this receptor (and perhaps many others) to activate MAP kinase .

Another unusual aspect of the LHRH receptor is the absence of the carboxyl terminal tail which is characteristic of all receptors of the TMD VII G-protein linked

superfamily. The carboxy-terminal sequence of most receptors contain many serine/threonine residues which are potential phosphorylation sites for kinases, such as β-adrenergic receptor kinase (βARK) and PKC and PKA [Hausdorff, 1990; Ostrowski, 1992]. These sites (and potentially others in the third intracellular loop) may be phosphorylated in response to chronic exposure to a receptor agonist and are involved in rapid homologous desensitisation of these receptors [Probst et al, 1992; Wojcikiewicz et al, 1993]. Many of the responses induced by the wild type LHRH receptor do not appear to desensitise, including [^{3}H]inositol phosphate formation in either COS 7, GH_3 or αT3-1 cells [Davidson et al, 1994b; Mitchell et al unpublished observation] and PLD [Fennell et al, 1993; Naor, 1990b; Stojilkovic et al, 1994]. Curiously the Asp318Asn and Asn87Asp;Asp318Asn mutants of the LHRH receptor displayed a rapid and transient PLD (but not PLC) response to LHRH, despite a reported involvement of PLC activity upstream of PLD in αT3-1 cells [Stojilkovic et al, 1994] and COS 7 and CHO cells transfected with wild type LHRH receptors [Mitchell et al, unpublished observation]. Thus these residues (or at least Asn 318) must somehow allow this response to become susceptible to desensitisation. MAP kinase activity observed in response to LHRH is relatively long lasting being sustained at high levels for 40 min and remaining significantly above basal for over 2 h [Sim et al 1994; Chapter 3]. The capacity of the LHRH receptor to sustain the upstream signalling responses may perhaps be mandatory to the ability to activate MAP kinase. In support of this, a truncated mutant of the platelet activating factor (PAF) receptor is reported to show a non-desensitising [^{3}H]inositol phosphate formation and increased MAP kinase activity in response to PAF when expressed in host cells [Honda et al, 1994; Takano et al, 1994]. A sustained activation of MAP kinase is also seen for the thrombin receptor in quiescent hamster CCL 39 cells, whilst the M_1 muscarinic receptor elicited only a transient activation in these cells [Kahan et al, 1992b]. However this could not be assigned to the capacity of the thrombin receptor, but not the M_1 receptor, to sustain non-desensitising

signalling responses, as some components of the M_1 and M_3 muscarinic receptors PLC activation are resistant to rapid desensitisation [Hu et al, 1991; Wojcikiewicz et al, 1993]. Moreover in contrast to PLC responses by the LHRH receptor [Davidson et al, 1994b], intracellular responses of the thrombin receptor such as PLD activation [Nieto et al, 1994] and phosphoinositide hydrolysis [Paris et al, 1988], desensitise rapidly. Interestingly the thrombin receptor, in contrast to the M_1 muscarinic and 5-HT_{2C} receptors (which also cause marked PLC activation), was able to cause mitogenesis which may in part be a reflection of its ability to cause a sustained activation of MAP kinase. Furthermore of the receptors we investigated, the ability to activate MAP kinase cannot all be attributable to sustained PLC activation, as whilst the mGlu 1a receptors PLC responses appear to desensitise rapidly [Aramori & Nakanishi, 1992], the 5-HT_{2C} receptor in some cases, for example agonist-induced phosphoinositide turnover in the choroid plexus, is resistant to inactivation [Conn & Sanders-Bush, 1986]. Taken together, it therefore seems that no obvious correlation between desensitisation of PLC coupling and the ability to activate MAP kinase exists.

One further possibility which was not investigated here as to why the LHRH receptor but not the mGlu or 5-HT_{2C} receptors can activate MAP kinase is the ability of receptors to cause tyrosine phosphorylation. This unexpected property of G-protein linked receptors is reported for the LPA [Hordijk et al, 1994], thrombin [Rao et al, 1994], PAF [Honda et al 1995; Lui et al 1995], endothelin [Cazaubon et al 1993], and LHRH [Johnson et al, 1995; Wolbers et al, 1995] receptors. Additionally the LHRH receptor has a tyrosine residue at position 323 in close proximity to the carboxyl terminus. Phosphorylation of this tyrosine residue may somehow allow an interaction with intracellular binding proteins which could in principle enable the LHRH receptor to activate additional cascades traditionally utilised by the growth factor receptors. Alternatively, increasing evidence is being reported on the importance of Src homology (SH) 2 and SH3 binding domains or pleckstrin homology

(PH) domains in interactions between adaptor proteins and downstream signalling components such as the activation of Ras by βγ subunits [Luttrell et al, 1995] or the phosphorylation-dependent attraction/activation of guanosine nucleotide exchange factors via adaptor proteins [Honda et al, 1994; Liu et al, 1994]. Therefore it is possible that the LHRH receptor, but not all PLC-coupled receptors has the ability to utilise such mechanisms. Furthermore there is now accumulating evidence of cross-talk between receptor tyrosine kinases cascades and G-protein-coupled receptor signalling cascades [Daub et al, 1996; Yang et al, 1991]. Evidence for this hypothesis has centred on the EGF receptor. It was first suggested that stimulation of the EGF receptor resulted in increased tyrosine kinase-dependent activation of PLCγ via a pertussis toxin-sensitive G-protein (G_i), furthermore G_i was coimmunoprecipitated with the EGF receptor following exposure to EGF [Yang et al, 1991]. Further research by this group has confirmed these observations and additionally shown a requirement for activation of the tyrosine kinase domain of the EGF receptor [Yang et al, 1991]. More recently, it has been suggested that G-protein linked receptors activate MAP kinase by a transactivation mechanism whereby G-proteins can intercept signalling pathways usually utilised by tyrosine kinase growth factor receptors [Daub et al, 1996]. At present there still appears to be more evidence supporting the more conventional idea that G-protein coupled receptors can activate MAP kinase on there own merits, however it is possible that these isolated examples may hint at underlying mechanisms still to be unravelled.

In conclusion, it is still unclear why activation of some, but not all PLC-coupled receptors can result in an increased MAP kinase activity. The factors which determine this ability are as yet undetermined but are likely to be dependent on many circumstances such as the ability to activate specific kinases or intracellular signalling cascade components although from the evidence presented in this Chapter it appears that rather than the ability to cause sustained activation of PLC and PKC translocation, the ability to interact with multiple G-proteins may be

important, although this must be largely due to domains other than the TMD VII Asp (or Asn)-containing motif investigated here.

Table 5.1

Effects of GF109203X, Ro-31 8220 and H7 on LHRH- and PDBu-stimulated MAP kinase activity in host COS 7 and CHO cells and αT3-1 cells

COS 7 and CHO cells previously transfected with plasmids containing cDNA for the wild type LHRH receptor or αT3-1 cells were treated as indicated for 10 min. Inhibitors were present for 2-5 min prior to agonist stimulation and where required vehicle alone was added to a control flask. All data are expressed as a percentage of the LHRH- or PDBu-induced activity and are means ± SEM (n=4-6). Curve fitting was carried out as described previously.

Table 5.1

Effects of GF109203X, Ro-31 8220 and H7 on LHRH- and PDBu-induced MAP kinase activity in host and native cells

Inhibitor	Treatment	IC_{50} (μM) for inhibitors in host cells or αT3-1 cells		
		αT3-1	COS 7	CHO
GF109203X	LHRH (100 nM)	1.8 ± 0.12	1.4 ± 0.4	1.8 ± 0.6
	PDBu (1 μM)	0.28 ± 0.02	1.3 ± 0.4	0.8 ± 0.2
Ro-31 8220	LHRH (100 nM)	0.64 ± 0.08	NA	0.36 ± 0.12
	PDBu (1 μM)	0.57 ± 0.03	NA	0.27 ± 0.11
H7	LHRH (100 nM)	168 ±12	69.8 ± 24	36.7 ± 13
	PDBu (1 μM)	43.5 ± 5.1	30.6 ± 13	32.9 ± 10

Table 5.2

Agonist-induced translocation of PKC in cells expressing wild type LHRH, 5-HT_{2C} or mGlu 1a receptors

COS 7 cells previously transfected with plasmids containing cDNA for the mouse wild type LHRH, 5-HT_{2C} and mGlu 1a receptors were treated as indicated for 15 min, then immediately homogenised. The values represent specific equilibrium binding of [^{3}H]PDBu as dpm per tube in cytosol and membrane fractions prepared from the COS 7 cells. Non-specific binding was in the range of 500-950 dpm per tube for cytosol and 260-400 dpm for membranes. Results are the means ± SEM from 4-6 experiments. Values marked *, showed a statistically significant change from control values by the Student's t-test ($p < 0.05$)

Table 5.2

Agonist-evoked translocation of PKC in cells expressing LHRH, 5-HT_{2C} or mGlu 1a receptors.

Expressed receptor	Treatment	Cytosol	Membrane	Mean % recovered in membrane
		Specific [^{3}H]PDBu binding (dpm per assay tube)		
LHRH	Control	2927 ± 457	417 ± 85	12
	LHRH (100 nM)	1734 ± 250	846 ± 102	33
	PDBu (300 nM)	539 ± 181	897 ± 110	62
5-HT_{2C}	Control	2573 ± 309	429 ± 175	14
	5-HT (20 μM)	1544 ± 277	996 ± 166	39
	PDBu (300 nM)	608 ± 102	951 ± 123	61
mGlu 1a	Control	3886 ± 495	523 ± 202	11
	ACPD (100 μM)	2419 ± 366	1102 ± 223	31
	PDBu (300 nM)	377 ± 172	1198 ± 210	76

Table 5.3

Ligand binding, inositol phosphate and MAP kinase responses of wild type and mutant LHRH receptors

COS 7 cells were previously transfected with plasmids containing cDNA for the wild type and mutant mouse(Asp318Asn and Asn87Asp;Asp318Asn) LHRH receptors.

Concentration-response data for [^{3}H]inositol phosphate formation and MAP kinase activity were determined over a range of 0-1000 nM LHRH as described previously. Each value is the mean ± SEM from 4-6 determinations. Basal inositol phosphate formation was in the range of 400-800 dpm and basal [^{35}S]thiophosphorylation of the MAP kinase substrate peptide was usually 1200-1800 dpm. Baseline activity in unstimulated cells was subtracted and the agonist-induced curves were normalised to the activity obtained with the maximally effective dose. Curve fitting was then conducted as described in Chapter 2 and the EC_{50} values determined.

Non-specific [^{125}I]buserelin binding (in the presence of 3 μM LHRH) was in the range of 7000-11000 dpm per tube while specific binding ranged from 5000-114000 dpm per tube. The affinity of the binding sites for [^{125}I]buserelin was expressed as IC_{50} values, which were estimated by a self-displacement assay using unlabelled buserelin (0.03-30 nM). A non-linear error-weighted program (P. fit, Elsevier Biosoft) was used to calculate IC_{50} values and B_{max} values were determined by the method of Swillens [Swillens, 1992].

Table 5.3

Ligand binding, inositol phosphate and MAP kinase responses of wild type and mutant LHRH receptors.

Response measured	LHRH receptor structure		
	wild type	Asp318Asn	Asn87Asp; Asp318Asn
specific [^{125}I]buserelin binding			
K_D	1.6 ± 0.4	1.1 ± 0.3	0.8 ± 0.3
B_{max} pmol/mg protein	0.61 ± 0.11	0.29 ± 0.07	0.18 ± 0.06
(% of wild type value)	100%	47%	29%
LHRH-induced [^{3}H]inositol phosphate formation			
EC_{50} (nM)	13 ± 5	18 ± 5	9 ± 7
relative maximal response	100%	38 ± 4%	20 ± 6%
LHRH-induced MAP kinase activation			
EC_{50} (nM)	33 ± 7	20 ± 6	11 ± 8
relative maximal response	100%	33 ± 6%	21 ± 9%

Figure 5.1

Agonist-induced [^{3}H]inositol phosphate formation and MAP kinase activation in COS 7 cells

Figure 5.1 shows concentration-response curves for agonist-induced [^{3}H]inositol phosphate formation (a) and MAP kinase activation (b), in COS 7 cells expressing the LHRH, 5-HT_{2C} or mGlu 1a receptors. The agonists used were LHRH (1-1000 nM; ●) for the LHRH receptor, 5-HT (1-300 μM; ■) for the 5-HT_{2C} receptor and ACPD (1-300 μM; ▲) for the mGlu 1a receptor. Each value is the mean ± SEM from 4-6 separate determinations.

In (a), all the transfected cells were preincubated for 10 min with 10 mM LiCl and represents the total [^{3}H]inositol phosphate accumulation in 30 min incubations with the appropriate agonists in the presence of 10 mM LiCl. Basal levels of inositol phosphate accumulations were similar in all transfections and were in the range of 400-700 dpm per assay.

In (b), the specific [^{35}S]thiophosphorylation of MAP kinase substrate peptide was measured at various concentrations of agonist. Each value is the mean ± SEM from 4-6 determinations. Baseline activity in unstimulated cells was subtracted and the agonist-induced curves were normalised to the activity obtained with the maximally effective dose. Curve fitting was then conducted as described in Chapter 2. Basal unstimulated levels of MAP kinase activity remained very similar between transfections and untransfected cells and were in the range of 1200-1800 dpm per assay. In each transfection a sample was stimulated with PDBu (1μM) as a positive control for the assay and additionally samples were shown to evoke [^{3}H]inositol phosphate responses to the appropriate ligand.

Figure 5.1

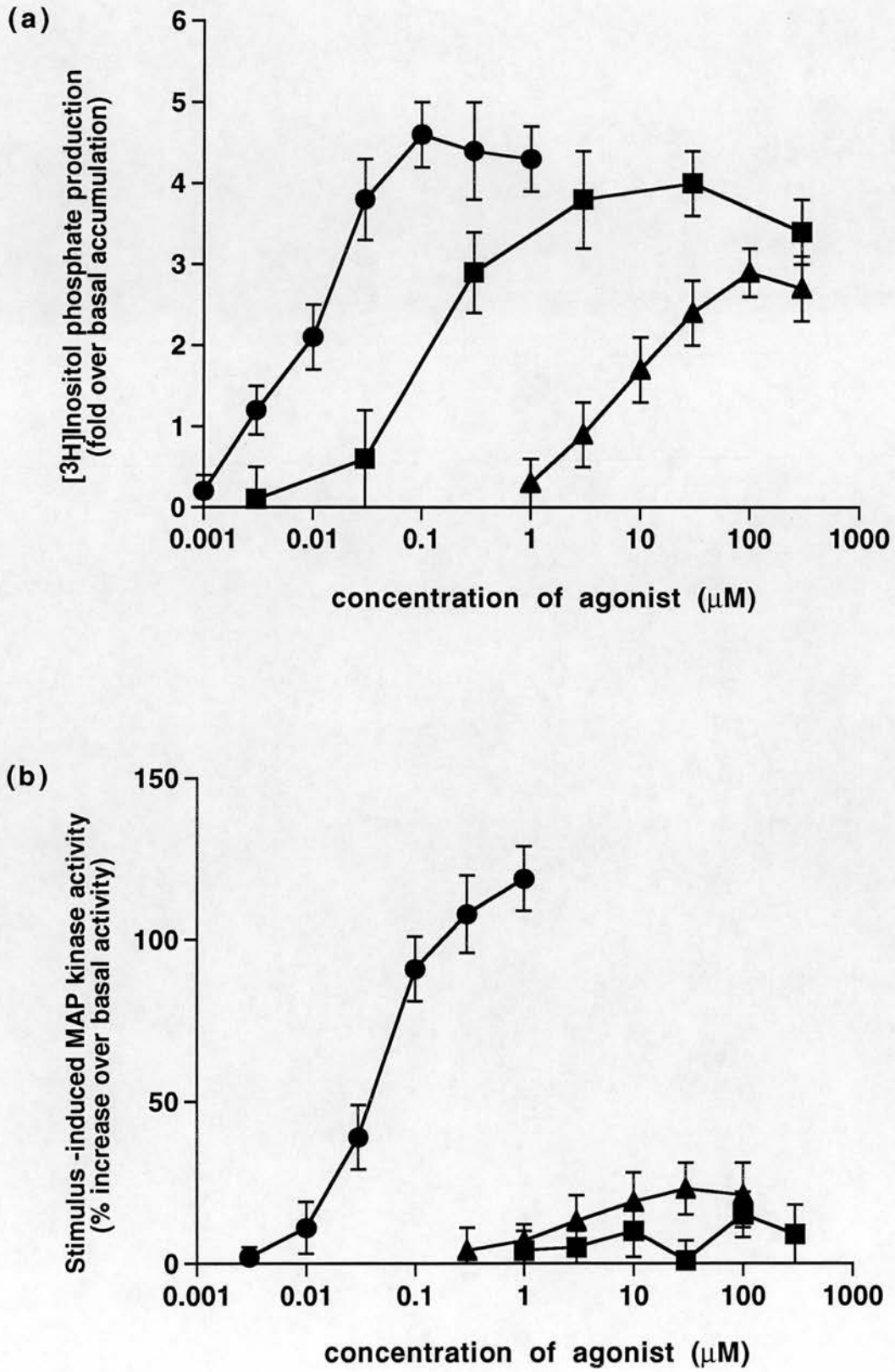

(a)
[3H]Inositol phosphate production
(fold over basal accumulation)
0
1
2
3
4
5
6
0.001
0.01
0.1
1
10
100
1000
concentration of agonist (μM)
(b)
Stimulus -induced MAP kinase activity
(% increase over basal activity)
0
50
100
150
0.001
0.01
0.1
1
10
100
1000
concentration of agonist (μM)

Figure 5.2

LHRH-induced [^{3}H]inositol phosphate formation and MAP kinase activation in COS 7, CHO and αT3-1 cells

Figure 5.2 shows concentration-response curves for [^{3}H]inositol phosphate accumulation (a) and MAP kinase activity (b) in response to LHRH (0-1000 nM) in COS 7 (▲) , CHO (■) transfected with the wild type LHRH receptor and αT3-1 (●) cells. Each value is the mean ± SEM from 4-6 separate determinations.

In (a), all transfections were preincubated with 10 mM LiCl and represents the total [^{3}H]inositol phosphate accumulation in 30 min incubations with the appropriate agonists in the presence of 10 mM LiCl. Basal levels of inositol phosphate accumulations were similar in all transfections and were in the range of 400-700 dpm per assay. In (b), the specific [^{35}S]thiophosphorylation of MAP kinase substrate peptide was measured at various concentrations of agonist. Baseline activity in unstimulated cells was subtracted and the LHRH-induced curves were normalised to the activity obtained with the maximally effective dose. Curve fitting was then conducted as described in Chapter 2. Basal unstimulated levels of MAP kinase activity remained constant between transfections and untransfected cells and were in the range of 1200-1800 dpm per assay. In each transfection a sample was stimulated with PDBu (1μM) as a positive control for the assay and additionally samples were shown to evoke [^{3}H]inositol phosphate responses to the appropriate ligand.

Figure 5.2

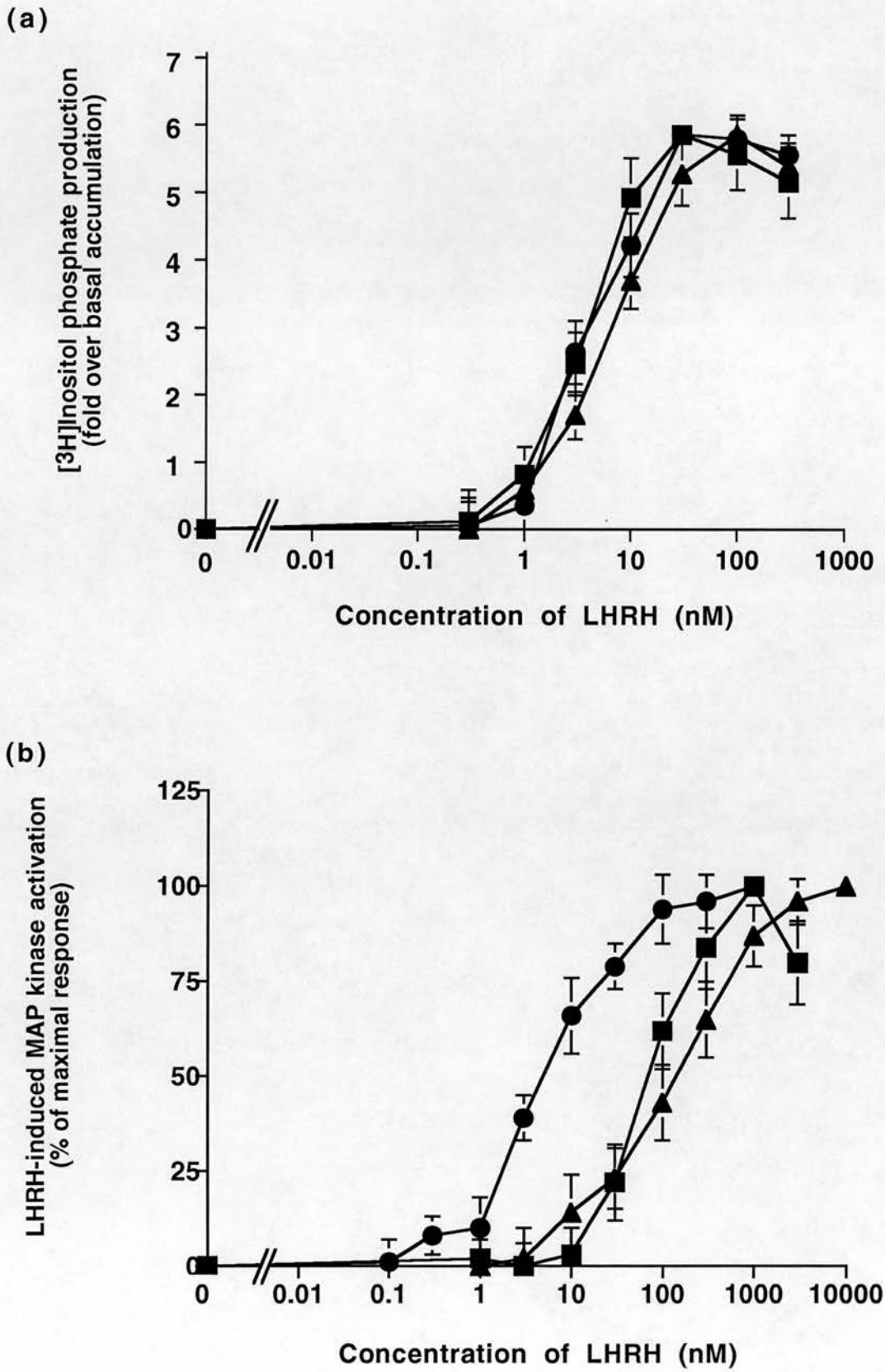

(a)
[3H]Inositol phosphate production (fold over basal accumulation)
7
6
5
4
3
2
1
0
0
0.01
0.1
1
10
100
1000
Concentration of LHRH (nM)
(b)
LHRH-induced MAP kinase activation (% of maximal response)
125
100
75
50
25
0
0
0.01
0.1
1
10
100
1000
10000
Concentration of LHRH (nM)

Figure 5.3

Effects of GF109203X on LHRH- and PDBu-induced MAP kinase activity in COS 7 and CHO cells expressing the mouse LHRH receptor

Figure 5.3 shows the concentration dependence of the effects of a highly selective PKC inhibitor GF109203X on LHRH- (●) and PDBu- (■) induced MAP kinase activity in COS 7 (a) expressing the wild type mouse LHRH receptor.

GF109203X was added 5 min prior to addition of LHRH 100 nM or PDBu 1 μM for a further 10 min (a time previously reported to see maximal stimulation of MAP kinase activity). Each value is the mean ± SEM of between 4-6 experiments and was calculated as before. Basal activity remained constant between transfections. GF109203X had no effect on inositol phosphate formation (data not shown).

Figure 5.3

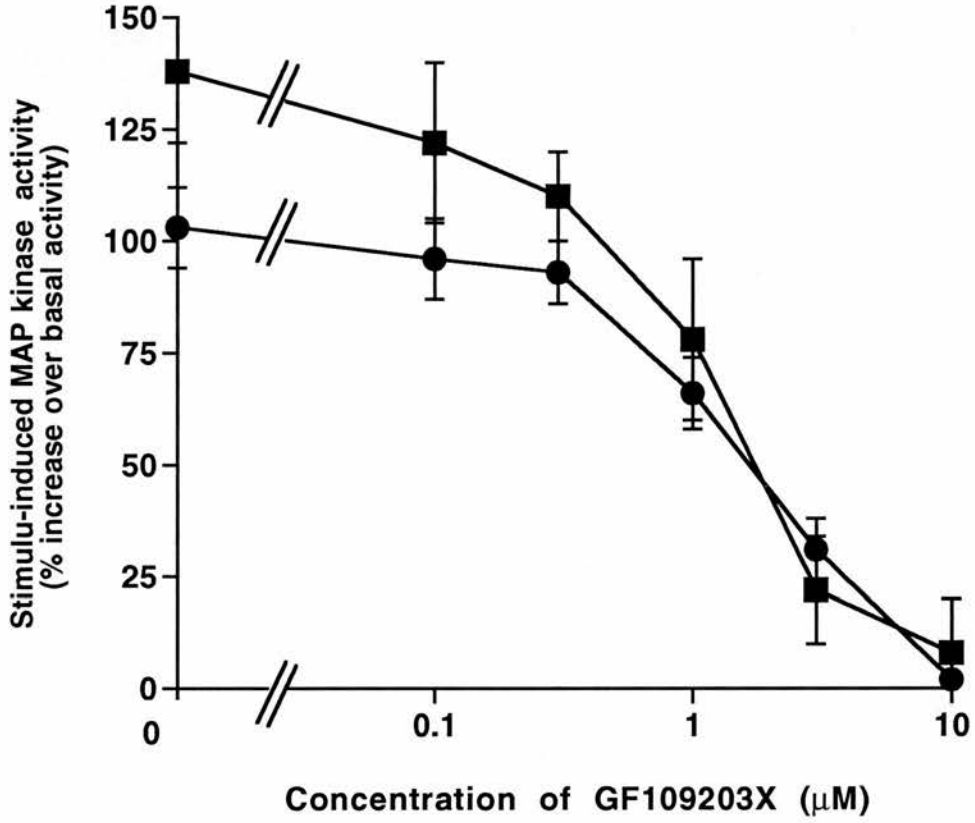

Stimulu-induced MAP kinase activity
(% increase over basal activity)
150
125
100
75
50
25
0
0
0.1
1
10
Concentration of GF109203X (μM)

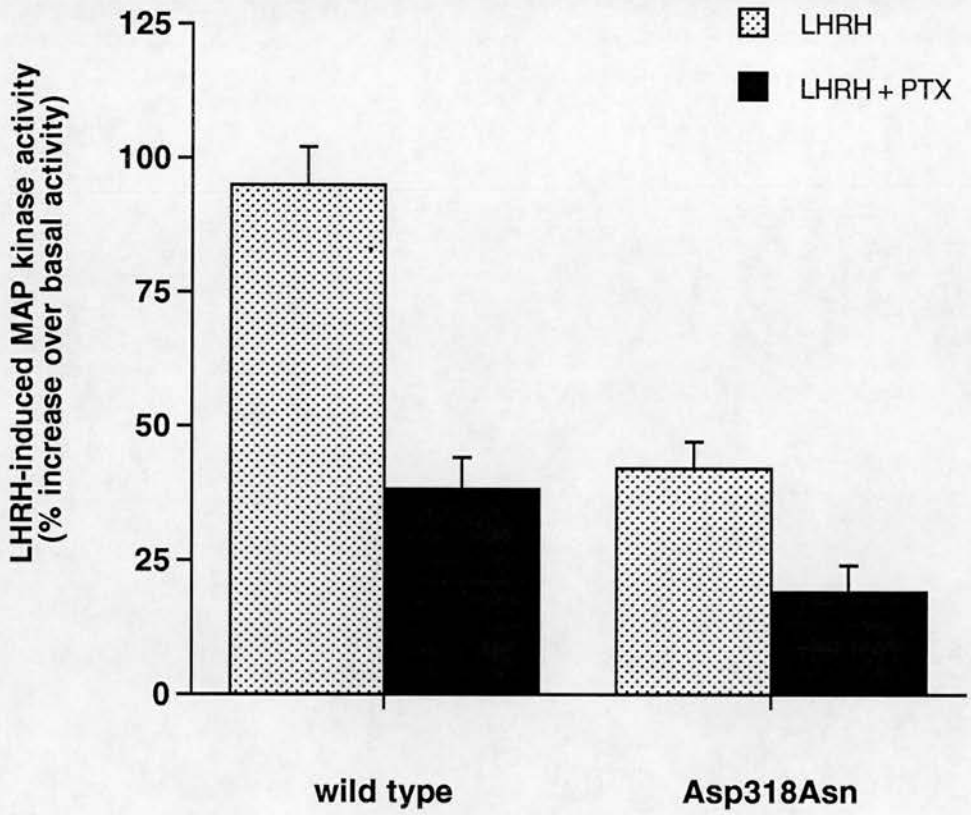
Figure 5.4

Effects of pertussis toxin on LHRH-induced MAP kinase activity in wild type and mutant receptors expressed in COS 7 cells

Figure 5.4 shows the LHRH-induced [^{35}S]thiophosphorylation of MAP kinase substrate peptide by the wild type and Asp318Asn mutant LHRH receptors, and the resulting inhibition when treated with pertussis toxin.

Following an 18h pretreatment ± 100 ng/ml of pertussis toxin, COS 7 cells expressing the wild type and Asp318Asn mutant LHRH receptors were incubated with 100 nM LHRH for 10 min. Inhibition of MAP kinase responses for the wild type and Asp318Asn mutant respectively by 100 ng/ml pertussis toxin were statistically significant ($p < 0.05$, Mann-Whitney U-test). This effect were shown to be a specific effect of an active holotoxin as under the same conditions neither the B-subunit or N-ethyl maleimide (NEM)-inactivated holotoxin had no significant effect on MAP kinase substrate peptide [^{35}S]thiophosphorylation. None of these treatments had any significant effect on basal MAP kinase activity (mean ± SEM, n=6).

Figure 5.4


LHRH
LHRH + PTX
LHRH-induced MAP kinase activity
(% increase over basal activity)
125
100
75
50
25
0
wild type
Asp318Asn

CHAPTER 6

ACTIVATION OF MAP KINASE IN ANTERIOR PITUITARY TISSUE AND THE RELATIONSHIP TO 'LHRH PRIMING' IN FEMALE RAT PITUITARIES

6.1 INTRODUCTION

An apparently unique property of the LHRH receptor is the phenomenon of LHRH self priming (previously described in Chapter 1). This mechanism by which this increase in pituitary responsiveness to LHRH occurs is still fully unresolved however a facilitation of second messenger production and subsequent intracellular events has been observed during LHRH priming [Mitchell et al, 1988] which may point to a plausible mechanism hence clarifying the second messenger pathways involved in priming has been a focus of our laboratory's research interests. The induction of priming is dependent on transcription, translation and the integrity of microfilaments [Pickering & Fink, 1979] and also involves ultrastructural changes in cytoskeletal and secretory elements [Lewis et al, 1985]. Furthermore activation of phospholipase A_2 (PLA_2) is also crucial to the induction of the phenomenon [Thomson et al, 1994].

MAP kinase is involved in many cellular events particularly mitogen-induced transcription , cell cycle control and cellular responses to environmental stress [Davis, 1995; Pelech, 1996; Waskiewicz & Cooper, 1995]. Investigations into downstream targets of MAP kinase have revealed many cytosolic and nuclear proteins which may be phosphorylated by MAP kinase in response to many stimuli, including components of signal transduction cascades, and hence a consensus target phosphorylation sequence for MAP kinase was identified [Clark-Lewis et al, 1991]. Amongst these are transcription factors such as c-Jun and c-Fos, the p90 ribosomal S6 kinase ($p90^{rsk}$), the high molecular weight PLA_2, cytoskeletal proteins including the microtubule associated protein 2 (MAP-2) [Alvarez et al, 1991; Hoshi et al, 1992; Lin et al, 1993; Nguyen et al, 1993; Seth et al, 1992; Sturgill et al, 1988] as well as an increasing list of proteins which contain a potential target MAP kinase phosphorylation motif but which as yet there is no direct evidence for direct MAP kinase regulation [Treisman, 1996]. These observations made MAP kinase of particular interest to our investigations as it appears that there is a remarkable

correlation between MAP kinase targets and proteins involved in the priming effect and therefore the discovery that activation of the LHRH receptor can phosphorylate and activate MAP kinase in the αT3-1 gonadotroph cell line [Sim et al, 1995, Chapter 3+5] (an effect which was maximal within 10 min and remained elevated for 40 min corresponding to the onset of LHRH priming) was of great interest. Additionally, LHRH priming but not initial unprimed LHRH-induced gonadotrophin secretion, is mediated by form of PKC with distinctive properties [Johnson et al, 1992] that appears to be very similar to that of a novel or modified species of PKC which has been characterised in the anterior pituitary [Ison et al, 1993]. Moreover LHRH-induced activation of MAP kinase in αT3-1 cells also is prevented by PKC inhibitors with a low potency by H7 (compared to other PKC inhibitors), similar to the aforementioned PKC.

We investigated whether LHRH can induce MAP kinase activation in anterior pituitaries *in vitro* and whether this may be pertinent to LHRH-priming by examining the potential LHRH-induced MAP kinase activation in anterior pituitary tissue from rats on different days of the oestrous cycle, as the magnitude of LHRH-priming observed changes characteristically throughout the oestrous cycle [Aiyer et al, 1974; Aiyer & Fink, 1974; Fink, 1979; Waring & Turgeon, 1980]. LHRH-priming is also dependent on prior exposure to gonadal steroid hormones (and perhaps other factors) [Fink, 1988c], therefore pituitary tissue from rats which had been ovariectomised was also assessed and the effects of these hormones on LHRH-induced MAP kinase activity in αT3-1 cells was investigated. Finally pilot experiments were carried out to explore the pharmacological characteristics of LHRH-induced MAP kinase activity, but owing to the heterogeneous nature of the tissue and presumed variability in drug access into the tissue a comprehensive study was not possible.

6.2 SPECIFIC METHODOLOGY

αT3-1 cells were grown to ~50% confluency in normal growth medium. The medium was then replaced with DMEM without Phenol red supplemented with 100 mg/ml glucose, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% steroid-stripped foetal calf serum (see Appendix 2) for 72 h and in serum-free medium for a further 24 h. Oestrogen (1 nM), progesterone (200 nM) or both hormones were added 48 h and 3 h respectively prior to the addition of 100 nM LHRH or 1 µM PDBu. Thereafter the αT3-1 cells were treated as usual as described in the methods section.

6.3 RESULTS

Employing the [^{35}S]thiophosphorylation assay described in Chapter 2, the potential activation of MAP kinase in female rat anterior pituitary tissue was investigated over a time course of 0-60 min with 1 nM LHRH (Figure 6.1). In pro-oestrous anterior pituitary tissue but not tissue from oestrous or ovariectomised rats, MAP kinase activity increased rapidly, after a short lag of approximately 5 min, reaching a peak by 15 min that represented an increase of 75 ± 9% over basal activity (mean ± SEM, n=6). This increased activity then gradually declined thereafter leaving a significant residual activation at 60 min. Unstimulated tissue showed no significant change in MAP kinase activity throughout the 60 min incubation (data not shown). LHRH-induced MAP kinase activity was concentration-dependent (Figure 6.2a), being statistically significant at 1-100 nM LHRH (Wilcoxon signed rank test). MAP kinase activity induced by LHRH was nearly maximal by 100 nM and showed an apparent EC_{50} value (the concentration required for 50% of maximal activation) of 0.53 ± 0.19 nM (Figure 6.2). Tissue from ovariectomised rats showed no response to LHRH at concentrations up to 100 nM. The MAP kinase response to LHRH was examined in anterior pituitary tissue taken from each day of the oestrous cycle and from ovariectomised rats. LHRH (100 nM) caused significant activation of MAP kinase

only in tissue from dioestrous and pro-oestrous rats in agreement with the data concerning pro-oestrous and oestrous rats shown in Figure 6.2 and confirming that LHRH could only evoke a MAP kinase response in tissue that was capable of displaying LHRH priming [Mitchell et al, 1994].

It is clear that the majority of the enzymatic activity assayed represented authentic p42/p44 MAP kinase since Table 6.1 shows that 70-80% of either control or LHRH-stimulated activity was immunoprecipitated by the anti-MAP kinase gel conjugate, whereas less than 20% was bound by the control reagent.

Experiments with ionomycin and the phorbol ester, PDBu were performed to assess whether the products of phosphoinositide hydrolysis evoked by the LHRH receptor such as Ca^{2+} mobilisation or PKC activation could mimic the LHRH-induced activity. Table 6.2 shows the effects of ionomycin. At 30 μM, a maximal concentration for this drug, ionomycin failed to increase MAP kinase activity alone or have any significant effect in combination with LHRH. PDBu (1 μM), activated MAP kinase in pro-oestrous tissue with a maximal effect also at around 15 min (Figure 6.4a) indicating an involvement of PKC in this response. Ionomycin also failed to potentiate the PDBu (1 μM)-evoked response (Table 6.2). PDBu-evoked MAP kinase activation was also seen in tissue from ovariectomised rats (Figure 6.4a) acting as a positive control in those tissues where LHRH fails to elicit priming of LHRH release [Mitchell et al, 1994], or activation of MAP kinase. Interestingly both LHRH- and PDBu-induced MAP kinase was apparent in pituitary tissue from male rats (Figure 6.4b).

LHRH-induced MAP kinase activation appears to be mimicked by phorbol ester in anterior pituitary tissue and is sensitive to both PKC and tyrosine kinase inhibitors in αT3-1 cells [Sim et al, 1995; Chapter 3+5]. The effects of these agents were therefore investigated on agonist-mediated MAP kinase activation in pro-oestrous anterior pituitary tissue. The selective PKC inhibitor GF109203X [Toullec et al, 1991] inhibited the MAP kinase response induced by LHRH (100 nM) with an IC_{50} of 2.1 ±

0.9 μM and that evoked by PDBu (1 μM) with an IC_{50} of 1.2 ± 0.4 μM (means ± SEM, n=6). Neither the PKC inhibitor H7 (at concentrations up to 300 μM) [Hidaka et al, 1984] nor the tyrosine kinase inhibitor lavendustin A (at concentrations up to 10 μM) [Hsu et al, 1991] reduced LHRH-induced MAP kinase activation (Figure 6.5). At 10-30 μM, H7 slightly increased the response to LHRH. None of the inhibitors had any effect on basal MAP kinase activity (data not shown).

The effects of pertussis toxin on LHRH-induced MAP kinase activation were examined. Pituitaries from pro-oestrous rats were cut in quarters to aid tissue penetration and incubated in MEM with pertussis toxin (300 ng/ml) for 3-4 h. After this time pertussis toxin had no significant effect on LHRH-induced MAP kinase activation or on basal activity. The LHRH response was slightly reduced compared to normal incubation periods, perhaps indicating that after this time the viability of the tissue was diminished. Longer incubations with pertussis toxin as examined in αT3-1 cells [Sim et al, 1995; Chapter 3+5] were therefore impossible.

Since LHRH priming is prevented by the protein synthesis inhibitor cycloheximide (CHX) [Pickering & Fink, 1979], we investigated whether it affected the LHRH-induced MAP kinase activation. In the presence of 50 μM CHX the LHRH (100 nM)-induced MAP kinase was elevated with respect to the response to LHRH (100 nM) alone and CHX (50 μM) alone also caused a small increase in the basal MAP kinase activity measured (Table 6.4).

As it seems apparent that LHRH is only capable of eliciting a MAP kinase response in anterior pituitary tissue from rats on days of the oestrous cycle during which LHRH priming is evident and furthermore that LHRH priming is additionally dependent on oestrogen [Fink, 1988c], we investigated the effects of the steroid hormones oestrogen and progesterone on LHRH- and PDBu-induced MAP kinase activation in αT3-1 cells (Figure 6.6). In αT3-1 cells grown in steroid-stripped medium without further supplements a minimal (but significant) LHRH- and PDBu-evoked MAP

kinase response was observed (1.6 ± 0.02 and 1.3 ± 0.09 fold over basal control), although the PDBu-evoked response was on the border of statistical significance; Wilcoxon signed rank test. In the presence of oestrogen (1 nM) significant agonist-evoked MAP kinase activation was clearly evident in αT3-1 cells. In these cells the MAP kinase activation evoked by LHRH (100 nM) was an increment of 2.77 ± 0.03 fold over the basal control MAP kinase activity and the corresponding response to PDBu (1 μM) was 1.95 ± 0.06 fold over control. In αT3-1 cells supplemented with progesterone, the MAP kinase responses to LHRH and PDBu were significantly above basal MAP kinase activity (1.8 ± 0.04 and 1.56 ± 0.03) however they were significantly reduced (approximately 42% and 20% respectively) with respect to the responses observed in oestrogen-supplemented cells and were not significantly greater than responses observed in steroid-stripped medium. Finally in steroid-free medium supplemented with both oestrogen and progesterone the LHRH- and PDBu-evoked MAP kinase activity was also significantly greater than basal activity (1.8 ± 0.02 and 1.5 ± 0.06 fold over basal control) but was again significantly reduced compared to those responses in oestrogen-supplemented medium. LHRH- but not PDBu-induced MAP kinase activity was greater in the presence of oestrogen and progesterone than in the presence of progesterone alone.

6.4 DISCUSSION

LHRH has a number of distinct actions on the anterior pituitary gland. In addition to stimulating the release and synthesis of gonadotrophins [Schally et al, 1973], LHRH also has a self-potentiating effect so that intermittent stimulation with repeated identical doses of LHRH causes a progressively increasing secretion of LH in response [Aiyer et al, 1974]. This self potentiation effect, observed in humans, monkeys, sheep and rats is probably involved in the generation of the massive LH surge which precedes ovulation [Fink, 1995]. The precise mechanisms underlying

the priming effect are still unclear, however they are clearly distinct from the usual (unprimed) LH secretion [Fink, 1988a] (see also Table 1.1).

The magnitude of the priming phenomenon varies characteristically on different days of the oestrous cycle being maximal on pro-oestrous. Compared to pro-oestrous, the priming effect of LHRH was less evident at dioestrous, was much less at metoestrous and could not be demonstrated at oestrous [Aiyer et al, 1974]. In contrast initial (unprimed) LH release responses to LHRH varied with a rank order showing the greatest LH release in pro-oestrous and then from oestrous/ovariectomised and male rats with little change in metoestrous and dioestrous rats [Johnson et al 1992; Mitchell et al 1994]. The first hour of incubation with LHRH, is important to the development of LHRH priming. During this period inclusion of various agents such as protein synthesis inhibitors, PLA_2 inhibitors and various protein kinase inhibitors can prevent the subsequent facilitated LH release brought about by priming (but not the standard unprimed LH release) [Curtis et al, 1985; Fink, 1988a; Johnson et al, 1992; Thomson et al, 1993b].

From our data presented here it is clear that activation of the LHRH receptor can lead to MAP kinase activation in the anterior pituitary gland *in vitro* in a concentration- and time-dependent manner. LHRH-induced MAP kinase activity is achieved with an EC_{50} value of 0.53 nM LHRH therefore occurs at physiological relevant concentrations (LHRH priming is typically induced by a physiologically relevant dose of 0.85 nM). Activation of MAP kinase is detectable within 10-60 min of incubation with LHRH and therefore is active during the period which is critical to the induction of the priming phenomenon. Furthermore it seems that the LHRH-induced MAP kinase activation may be selectively involved in the development of the LHRH self priming phenomenon, as significant MAP kinase activation was observed only in rat pituitary tissue from pro-oestrous and dioestrous when the LHRH self priming effect is at its greatest. Furthermore a degree of LHRH priming has been

noted in male rat pituitaries [Nazian, 1986] which is likely to explain the apparent anomaly in our hypothesis that LHRH-induced MAP kinase activation is only important to LHRH priming.

At the time these experiments were performed the recently described MEK inhibitor [Alessi et al, 1995a] was unavailable. Furthermore to date no specific inhibitors of MAP kinases have been described. Therefore we assessed whether any of the common kinase inhibitors had any direct effect on constitutive MAP kinase activity detected *in vitro* from rat hippocampus tissue [see Appendix 1]. However although some compounds displayed weak effects none of the agents tested appeared to potently inhibit MAP kinases. Furthermore approaches such as the use of antisense oligonucleotides to downregulate the ERK proteins or expression of dominant positive or negative mutants of ERKs would be inappropriate as they cannot be employed in intact anterior pituitary tissue and cultured dispersed anterior pituitary cells are known to lose the ability to display LHRH priming [Fink, 1988b]. In view of this it is difficult as yet to directly probe the functional contribution of MAP kinase to LHRH priming. Nevertheless, there is a remarkable correlation between the targets of MAP kinases and the array of cellular events known to be involved in priming. Relevant targets may include c-Jun and c-Fos, RNA polymerase II, $p90^{rsk}$, high molecular weight PLA_2 and MAP-2 and it has been reported that a cdc2-like kinase with a similar substrate motif phosphorylates neurofilaments [Shetty et al, 1993]. The priming phenomenon is dependent upon protein synthesis, activation of PLA_2, the integrity of microfilaments and involves ultrastructural changes in the marginal presentation of secretory granules in gonadotrophs [Fink, 1988a]. Phosphorylation of MAP-2 protein by MAP kinase is reported to disrupt microtubule-microfilament interaction [Hoshi et al, 1992] which may lead to facilitated stimulus-secretion coupling [Malaisse et al, 1975].

MAP kinase activation has also been reported in other secretory responses including thyrotrophin-releasing hormone-induced prolactin secretion in GH_3 and GH_4 pituitary cells [Kanda et al, 1994; Ohmichi et al, 1994] which interestingly involves divergent pathways including a PKC- and tyrosine kinase-dependent mechanism similar to our observations in LHRH-induced MAP kinase activation in αT3-1 cells. Similarly MAP kinase activation has been implicated in secretion of digestive enzymes in response to cholecystokinin and acetylcholine receptor stimulation in pancreatic acinar cells [Williams, 1995], insulin secretion in response to secretagogues [Frodin et al 1995; Persaud et al 1996], catecholamine secretion from bovine adrenal chromaffin cells [Cox et al 1996], responses to secretory stimuli in rat basophillic leukaemia (RBL)-cells and serotonin release in RBL cells [Offermans et al, 1994b]. Additionally MAP kinase is also selectively activated during thrombin-induced platelet activation and aggregation and since platelets are nonproliferative cells MAP kinase activation cannot lead to a mitogenic signal therefore may regulate cytoskeletal or secretory changes during platelet activation [Papkoff et al, 1994]. In *Saccharomyces cerevisiae* bud emergence requires polarisation of the cytoskeleton and secretory vesicles to a specific site on the cell surface. A SLT2 (MPK1) MAP kinase homologue has been identified which functions downstream or in parallel with a cdc28 protein kinase in promoting bud formation and apical growth [Levin & Errede, 1995]. Accordingly activation of MAP kinases may be a common element in a wide spectrum of cellular responses involving cytoskeletal changes and vesicle movement.

During priming LHRH induces the release of LH by two routes. LHRH causes an up-regulation of LHRH-induced second messenger formation as prior exposure to LHRH specifically enhanced LHRH-induced inositol phosphate production and mobilisation of Ca^{2+} from intracellular stores [Mitchell et al, 1988], whilst the ligand binding and Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels remains unaltered. In addition, the priming effect involves a general facilitation of stimulus-secretion

coupling such that other secretagogues including K^+ and ionomycin can elicit enhanced secretory responses [Johnson & Mitchell, 1991; Pickering & Fink, 1979] However K^+ and ionomycin can only increase the amount of hormone available for release [Johnson & Mitchell, 1991] and only LHRH has the capacity to increase cellular responsiveness to itself [Pickering & Fink, 1979]. Although LHRH-induced mobilisation of intracellular and extracellular Ca^{2+} appears to be of importance to LHRH-induced gonadotrophin secretion [Bates & Conn, 1984; Bourne & Baldwin, 1980; Limor et al, 1987; Pickering & Fink, 1979] and there is evidence that the release of intracellular Ca^{2+} may participate in LHRH priming [Curtis et al, 1985], it appears that increased Ca^{2+} is not critical to the induction of priming [Johnson et al, 1991; Johnson et al, 1993b]. Mobilisation of Ca^{2+} appears incidental to LHRH-induced MAP kinase activation in priming as ionomycin was ineffective either alone or in combination with LHRH (Table 6.2). It is unlikely that Ca^{2+} failed to elicit any response due to inability of ionomycin to penetrate the tissue as the studies on the role of Ca^{2+} in LH release have shown a response in hemipituitaries under similar incubation conditions with similar concentrations of this agent [Johnson et al, 1991; Johnson et al, 1993b; Mitchell et al, 1988]. Indeed this result is consistent with evidence that elevation of Ca^{2+} does not elicit the induction of LHRH priming, but rather that it is critically dependent upon a form of PKC [Johnson et al, 1993b].

LHRH-mediated MAP kinase activation also seems to be dependent on PKC as the response is strongly inhibited by the potent PKC inhibitor GF109203X and can be mimicked by phorbol ester in both tissue which can display priming and tissue from males and ovariectomised rats. This data is consistent with LHRH-mediated MAP kinase activation in the αT3-1 gonadotroph cell line and would suggest that activation of MAP kinase in these tissues is under similar regulation. Rat pituitary tissue has been shown to contain the α, β, δ, ε and ζ isoforms of PKC [Maeda & Lloyd, 1993]. The αT3-1 cell line has been shown to express at high levels the α, ε and ζ isoforms [Johnson et al, 1993a; Johnson et al, 1996]. Interestingly LHRH-induced MAP

kinase is not inhibited by the PKC inhibitor H7 at the concentrations used here. This is not uncommon to PKC dependent responses observed in pro-oestrous anterior pituitary tissue [Johnson et al, 1991; Johnson & Mitchell, 1991; Johnson et al, 1992; Johnson et al, 1994; Johnson et al, 1995; MacEwan & Mitchell, 1991; MacEwan et al, 1991; Thomson, 1992b; Thomson et al, 1994; Thomson et al, 1993b; Thomson & Mitchell, 1993]. The induction of the priming phenomenon is dependent upon a species of PKC (which is H7-resistant but sensitive to GF109203X, staurosporine and Ro31-8220) acting subsequently through a protein synthesis-dependent step to activate PLA_2 (Johnson et al 1992; Thomson & Mitchell, 1993; Thomson et al 1993). A PKC-like enzyme considered to correspond to this species has recently been characterised in anterior pituitary and lung but not in the other tissues tested (including mid brain, cerebellum, liver, spleen and COS 7 cells) [Ison et al, 1993]. Its Ca^{2+}-independent activity was notably resistant to H7 but sensitive to staurosporine and Ro 31-8220. The distribution of this activity and its pharmacological characteristics do not obviously correlate with any of the 10 currently described PKC isoforms and thus probably reflects a novel, or modified PKC species. It is possible that MAP kinase is regulated by this PKC species. Such a signalling sequence would be further consistent with the proposed involvement of PKC species as an intermediary in LHRH priming as its activation by LHRH (like priming) is dependent on a PKC which is sensitive to PKC inhibitors including GF109203X but not to H7. PKCs other than those transducing signals from the LHRH receptor can clearly activate MAP kinase in heterogeneous pituitary tissue because PDBu can still activate the enzyme in tissue where LHRH is ineffective (Figure 6.4). Both α, β and ε isoforms of PKC can activate Raf and hence MAP kinase [Dent et al, 1992; Sözeri et al, 1992], although both PKC α and β are H7-sensitive [Ison et al 1993; Thomson et al 1993a]. The evidence here for a modest facilitation of the LHRH response by H7 is consistent with the possibility of reciprocal regulation by H7-resistant and H7-sensitive forms of PKC (or other kinases) (Figure 6.5). There is evidence for such

dual regulation of voltage-sensitive Ca^{2+} channels by different forms of PKC [Johnson et al 1991; Johnson et al 1993b; MacEwan et al 1991]. The regulated step underlying the varying ability of LHRH to activate MAP kinase may well be upstream of MAP kinase itself but downstream of the LHRH receptor since PDBu was still effective in tissue which does not respond to LHRH but has adequate LHRH receptor numbers (Figure 6.4; [Mitchell et al, 1988]). Interestingly it is clear that expression of a variety of PKC isoforms is induced by E_2 treatment of anterior pituitary cells [Thomson et al, 1994a].

In response to LHRH receptor-activation a number of proteins are tyrosine phosphorylated [Wolbers et al 1995; Johnson et al 1995] including the non-receptor tyrosine kinases (nrTK) Src and Fyn [Fennell et al, 1994]. LHRH-, but not PDBu-induced phospholipase D (PLD) activity is inhibited by the tyrosine kinase inhibitor lavendustin A in αT3-1 cells [Fennell et al, 1993], implying a possible role for a nrTK in LHRH signal transduction. From our initial experiments in αT3-1 cells and pro-oestrous pituitary tissue lavendustin A was unable to prevent LHRH-induced MAP kinase activation therefore we surmised that there was no role for tyrosine kinases in this response in anterior pituitary tissue [Mitchell et al, 1994] Since then we have investigated the role of tyrosine kinases more thoroughly in αT3-1 cells and it is now apparent that LHRH-induced MAP kinase activity is sensitive to certain tyrosine kinase inhibitors (although not lavendustin A). It is possible that the LHRH receptor may be activating a different subset of nrTKs in parallel cascades which lead to PLD and MAP kinase in αT3-1 cells. Recently in our laboratory we have demonstrated a role for tyrosine phosphorylation in LHRH-induced gonadotrophin secretion probably in the later stages of the stimulus-secretion pathway although this is not specific to the priming phenomenon as both initial and augmented release were affected to some degree [Johnson et al, 1995]. Furthermore in the αT3-1 cell line, the LHRH receptor can lead to activation of tyrosine kinases downstream of PLC, which appears to play a critical role in the augmentation of LHRH-induced inositol

phosphate production which underlies the LHRH self-priming phenomenon [Mitchell et al, 1994]. In view of these more recent observations it is clear that a more thorough investigation is required in pituitary tissue regarding the role of tyrosine kinases in the MAP kinase response.

The lack of effect of cycloheximide on LHRH-induced MAP kinase activation here is consistent with the possibility that the protein synthesis-dependent step in priming may be downstream of MAP kinase activation or that these steps may occur in parallel rather than in series. The slight increase observed in response to cycloheximide may be due to the loss of synthesis of a negative regulatory protein or some other component of a cascade which crosses with the MAP kinase activation cascade. It would be difficult to determine the protein responsible as the regulation of MAP kinase represents the convergence of many as yet unresolved inputs, but it is unlikely to be specifically relevant to LHRH-induced activation as there is no further potentiation in the presence of LHRH.

In αT3-1 cells LHRH-induced MAP kinase activation is dependent on both PKC and a pertussis toxin-sensitive step [Sim & Mitchell, 1995a; Sim & Mitchell, 1995b] Chapter 3+5]. We were unable to make any reasonable analysis of the involvement of $G_{i/o}$ proteins in LHRH signal transduction in the anterior pituitary gland. Techniques currently used for this analysis involve long periods of incubation either with pertussis toxin, to inactivate the $\alpha_{i/o}$ subunit by an ADP ribosylation catalysed by the toxin, or with antisense oligonucleotides to effectively deplete the protein from cells by blocking *de novo* synthesis of the protein. Pituitary prisms are unsuitable for these treatments as tissue samples may be impenetrable to these agents and do not survive well for long periods *in vitro*. Furthermore as discussed earlier, dispersed anterior pituitary cells could not be used in these experiments as these cells do not display LHRH-priming [Fink, 1988b]. The effect of pertussis toxin on LHRH-induced LH secretion in pituitary pieces was investigated as a pilot experiment to determine

whether long term incubations *in vitro* would be viable for future experiments of this nature. Anterior pituitaries were cut into four and maintained for 24 h in culture dishes under O_2/CO_2 at 37°C in the presence or absence of oestrogen (E_2; 1 nM) and or pertussis toxin (PTx; up to 1 μg/ml). Basal or LHRH-induced LH secretion was measured over 3 h. In the first hour of exposure to LHRH, LH was secreted from the pituitary pieces with a rank order of potency of PTx with E_2 (60 ± 8 ng/ml of LH) > PTx (47 ± 9 ng/ml) > E_2 (38 ± 5 ng/ml) > control (ie LHRH alone; 24 ± 4 ng/ml) with the responses to E_2, PTx, E_2 with PTx being significant greater than control, (LHRH alone)-mediated LH release and E_2 with PTx being significantly greater than E_2 (Students t-test) however the secretion observed in the 2nd hour (when primed release is typically observed) were not significantly different from 1st hour secretion therefore LHRH priming was not maintained under these conditions [Johnson et al unpublished observation]. At present we are therefore unable to investigate aspects of PTx and the LHRH priming phenomenon as this requires long term incubations, however from these observations it appears that PTx may have some anomalous effects on LHRH-mediated LH secretion.

We have shown that LHRH-induced MAP kinase activation in the αT3-1 cell line is dependent on exposure to oestrogen (Figure 6.6). Indeed in a pilot experiment, supplementing the normal growth medium with 0.3 nM oestrogen for 48 h slightly increased the LHRH-induced MAP kinase response in αT3-1 cells (data not shown) to approximately 1.2 fold of the usual agonist-induced response. This appears to mimic the conditions required in the anterior pituitary. LHRH priming is dependent on prior exposure to oestrogen [Fink, 1988b], furthermore oestrogen levels peak on the morning of pro-oestrous therefore coinciding with the onset of LHRH priming [Aiyer & Fink, 1974]. Synthesis of the β-subunit of LH or FSH does not occur in the αT3-1 cell line and as a consequence the gonadotrophins are not secreted [Windle et al, 1990]. This limits the usefulness of this cell line as a model system for studying the physiological aspects of LHRH responses (especially the primed release of LH)

but this is the only gonadotroph cell line available. Despite this, αT3-1 cells appear to be a good model for studying receptor mediated responses [Windle et al, 1990; Eidne et al, 1992; Anderson et al, 1993; Davidson et al, 1994; Shah & Milligan, 1994; Mitchell et al, 1995a, b; Sim et al, 1995]. The result demonstrating a dependence on oestrogen exposure for strong LHRH-induced MAP kinase activation in αT3-1 cells (Figure 6.6) also mirrors the dependence on oestrogen of both the priming effect [Fink, 1988c] and LHRH-induced MAP kinase activation in the anterior pituitary gland (Figure 6.3). A similar result was obtained for LHRH-induced PLD activity in αT3-1 cells grown in steroid-free medium augmented by oestrogen [Fennell, 1995]. Oestrogen is also capable of bringing about an ability of LHRH to prime other LHRH-mediated responses in αT3-1 cells including inositol phosphate formation [Mitchell et al, 1995a] and can restore levels of LHRH-stimulated arachidonic acid release in ovariectomised rats to levels observed in pro-oestrous rat pituitaries [Thomson et al, 1994]. Oestrogen is likely to have many effects on pituitary cells particularly the increased expression of critical proteins which may include many signal transduction components or regulatory proteins. LHRH-induced MAP kinase, PLA_2 (in the pituitary) and PLD activation are all dependent on PKC [Fennell, 1995; Sim & Mitchell, 1995a; Thomson et al, 1994]. Additionally there is an increase in PKC expression and activity following exposure to oestrogen [Thomson et al, 1993a]. Therefore one of the effects of oestrogen which is relevant to our investigations is an increased expression of one or more PKC isoform. Oestrogen treatment has been shown to increase mRNA levels of the α, β, δ, ε and ζ isoforms of PKC in normal and tumorigenic rat pituitaries [Maeda & Lloyd, 1993].

αT3-1 are usually grown in medium containing Phenol red which has a modest oestrogenic activity [Berthois et al, 1986; Welshons et al, 1988]. Moreover Phenol red concentrations typically used in culture media are capable of modulating LHRH responsiveness in superfusion of dispersed anterior pituitary cell cultures (an effect which is more prominent at certain stages of the oestrous cycle) [O'Conner & Kellom,

1990]. Therefore the inclusion of Phenol red in a tissue culture medium should be considered where a putative oestrogen-exposure could modulate the response as it is likely to be critical to the outcome of any measured response. The status of αT3-1 cells with respect to modelling a gonadotroph exposed *in situ* to cyclic hormonal changes is unknown. This is clearly important to αT3-1 cells also, as (shown in Figure 6.6), strong MAP kinase responses were only observed in oestrogen-supplemented hormone-free growth medium. However these were of a similar magnitude to cellular responses typically observed in normal growth medium containing Phenol red.

In summary, from the results presented in this chapter it appears that LHRH-induced MAP kinase activation may have a critical role in the unique LHRH-self priming effect in anterior pituitary tissue, as this was only apparent on appropriate days of the hormonal cycle and furthermore like primed LH secretion this is likely to be dependent on exposure to oestrogen as it was not observed in ovariectomised rats. Additionally we have shown that αT3-1 cells appear to have a requirement for oestrogen and whilst normal growth medium is not supplemented by oestrogen, the oestrogenic effects of Phenol red appear sufficient to account for this requirement. Taken together, αT3-1 cells grown in a Phenol red containing medium may well represent a reasonably faithful model of oestrogen-exposed gonadotrophs *in situ*.

Table 6.1

Immunoprecipitation of MAP kinase activity in pro-oestrous rat anterior pituitary tissue with protein G-Sepharose-4B derivatised reagent

Anterior hemipituitary pieces were incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium only or containing 100 nM LHRH for 15 min. Thereafter the tissue was treated as in protocol (Chapter 2) for anti MAP kinase immunoprecipitation then pellet (P) and supernatant (S) fractions were included in a cytosolic MAP kinase assay. Values are means ± SEM from 3 determinations.

Condition	Antibody reagent	Supernatant	Pellet
		MAP kinase activity recovered ($x10^3$ dpm per assay)	
Control	Nil	1.84 ± 0.26	NA
	Anti-MAP kinase	0.56 ± 0.18	1.39 ± 0.10
	Anti-β-tubulin	1.62 ± 0.15	0.40 ± 0.06
LHRH	Nil	2.93 ± 0.16	NA
	Anti-MAP kinase	0.55 ± 0.13	2.09 ± 0.09
	Anti-β-tubulin	2.58 ± 0.19	0.64 ± 0.15

Table 6.2

Effects of ionomycin on MAP kinase activation in pro-oestrous rat anterior pituitary tissue

Anterior pituitary tissue from pro-oestrous rats was pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium containing either nil, ionomycin (30 μM), 100 nM LHRH, LHRH (100 nM) + ionomycin (30 μM), 12, 13 phorbol dibutyrate (PDBu; 1 μM) or PDBu (1 μM) + ionomycin (30 μM). After 15 min the tissue was removed and immediately homogenised in 2 vol homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Values are means ± SEM from 6 determinations. All values marked (+) are significantly greater than MAP kinase activity in control unstimulated pro-oestrous pituitary tissue matched pair t-test on raw data ($p>0.05$).

Condition	**MAP kinase activity** ($x10^3$ dpm per assay)
control	2.28 ± 0.14
ionomycin	2.45 ± 0.14
LHRH	3.85 ± 0.29 (+)
ionomycin + LHRH	3.93 ± 0.21 (+)
PDBu	3.99 ± 0.31 (+)
PDBu + ionomycin	4.05 ± 0.29 (+)

Table 6.3

Effects of pertussis toxin on MAP kinase activation in pro-oestrous rat anterior pituitary tissue

Pairs of anterior pituitary quarters from pro-oestrous rats were pre-incubated in MEM or MEM containing of pertussis toxin (300 ng/ml) for 3 h. The medium was discarded and replaced with fresh medium containing either nil, pertussis toxin (300 ng/ml), 100 nM LHRH or LHRH (100 nM) + pertussis toxin (300 ng/ml). After 15 min the tissue was removed and immediately homogenised in 2 vol homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Values are means ± SEM from 4 determinations. MAP kinase activity values marked (+) are significantly greater than those in control unstimulated pro-oestrous anterior pituitary tissue by matched pair t-test on raw data ($p>0.05$). PTx + LHRH was not significantly different than LHRH-induced MAP kinase activity ($p<0.05$).

Condition	**MAP kinase activity** ($x10^3$ dpm per assay)
Control	2.08 ± 0.09
pertussis toxin	2.35 ± 0.15
LHRH	2.95 ± 0.25 (+)
pertussis toxin + LHRH	2.93 ± 0.21 (+)

Table 6.4

Effects of cycloheximide on MAP kinase activation in pro-oestrous rat anterior pituitary tissue

Anterior hemipituitaries from pro-oestrous rats were pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium alone or additionally containing cycloheximide (CHX; 50 μM). After 10 min the medium was again replaced with fresh medium containing either nil, CHX (50 μM), 100 nM LHRH or LHRH (100 nM) + CHX (50 μM). After 15 min the tissue was removed and immediately homogenised in 2 vol homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Values are means ± SEM from 6 determinations. MAP kinase activity that was significantly greater than control unstimulated activity is indicated (+) ($p<0.05$). CHX + LHRH was not significantly greater than LHRH-induced MAP kinase activity by matched pair t-test on raw data ($p> 0.05$).

Condition	**MAP kinase activity** ($x10^3$ dpm per assay)
control	3.08 ± 0.43
CHX	4.05 ± 0.86 (+)
LHRH	7.05 ± 0.38 (+)
CHX + LHRH	7.93 ± 0.91 (+)

Figure 6.1

Time course of LHRH-induced MAP kinase activation in female rat anterior pituitary tissue

Anterior hemipituitaries from pro-oestrous, oestrous and ovariectomised rats was pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium alone or containing 1 nM LHRH (●). After the appropriate time the tissue was removed and immediately homogenised in 2 vol of homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Basal MAP kinase activity showed no apparent difference between tissue from all origins falling consistently between 800-1200 dpm per assay. Values are means ± SEM from 8 determinations.

Figure 6.1

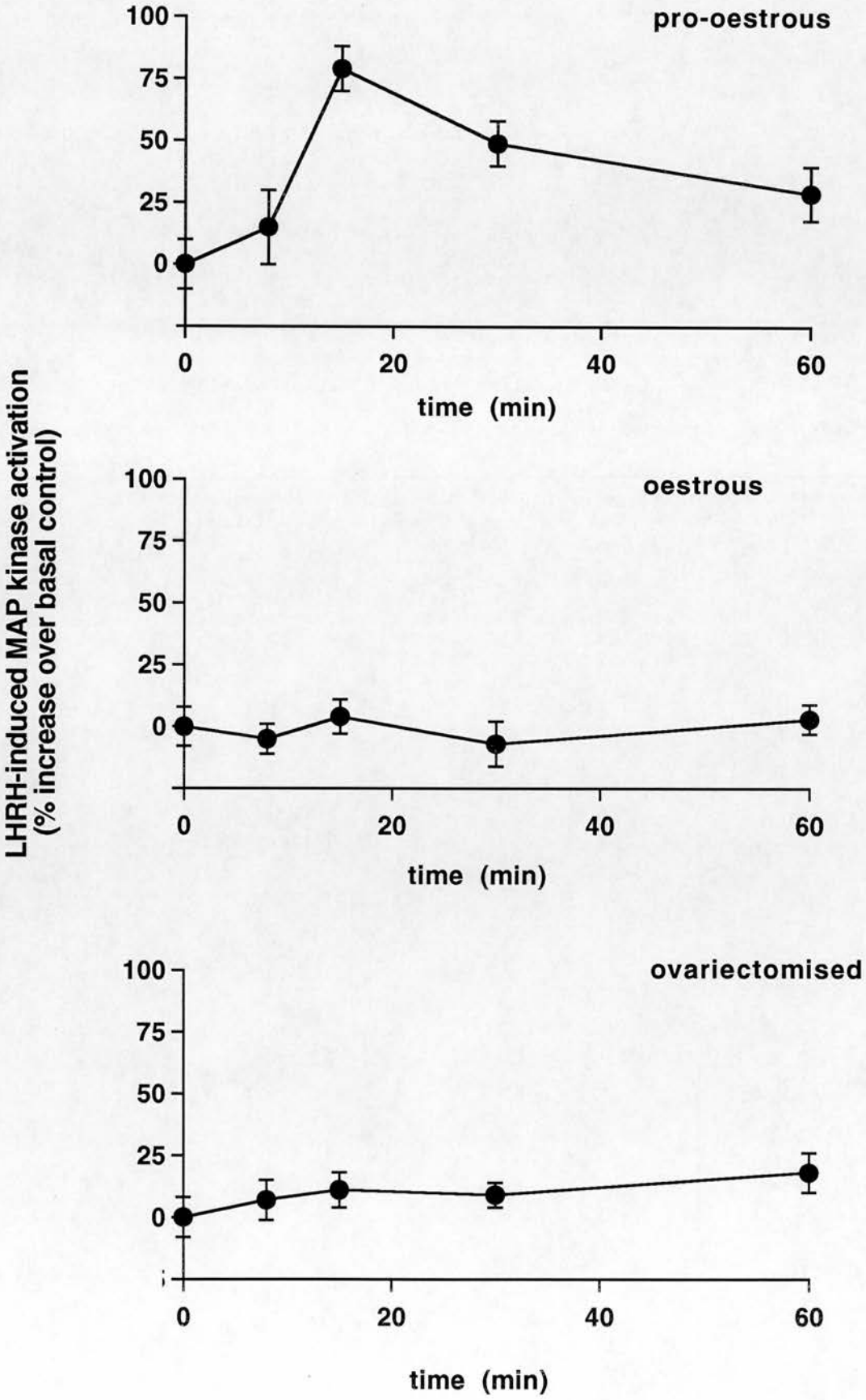

pro-oestrous
oestrous
ovariectomised
LHRH-induced MAP kinase activation
(% increase over basal control)
100
75
50
25
0
0
20
40
60
time (min)

Figure 6.2

Concentration-dependence of LHRH activation of MAP kinase in female rat anterior pituitary tissue

Anterior hemipituitaries from pro-oestrous and ovariectomised rats were pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium alone or containing LHRH (●). After 15 min the tissue was removed and immediately homogenised in 2 vol of homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Basal MAP kinase activity showed no apparent difference between tissue from different origins falling consistently between 800-1200 dpm per assay. Values are means ± SEM from 8 determinations.

Figure 6.2

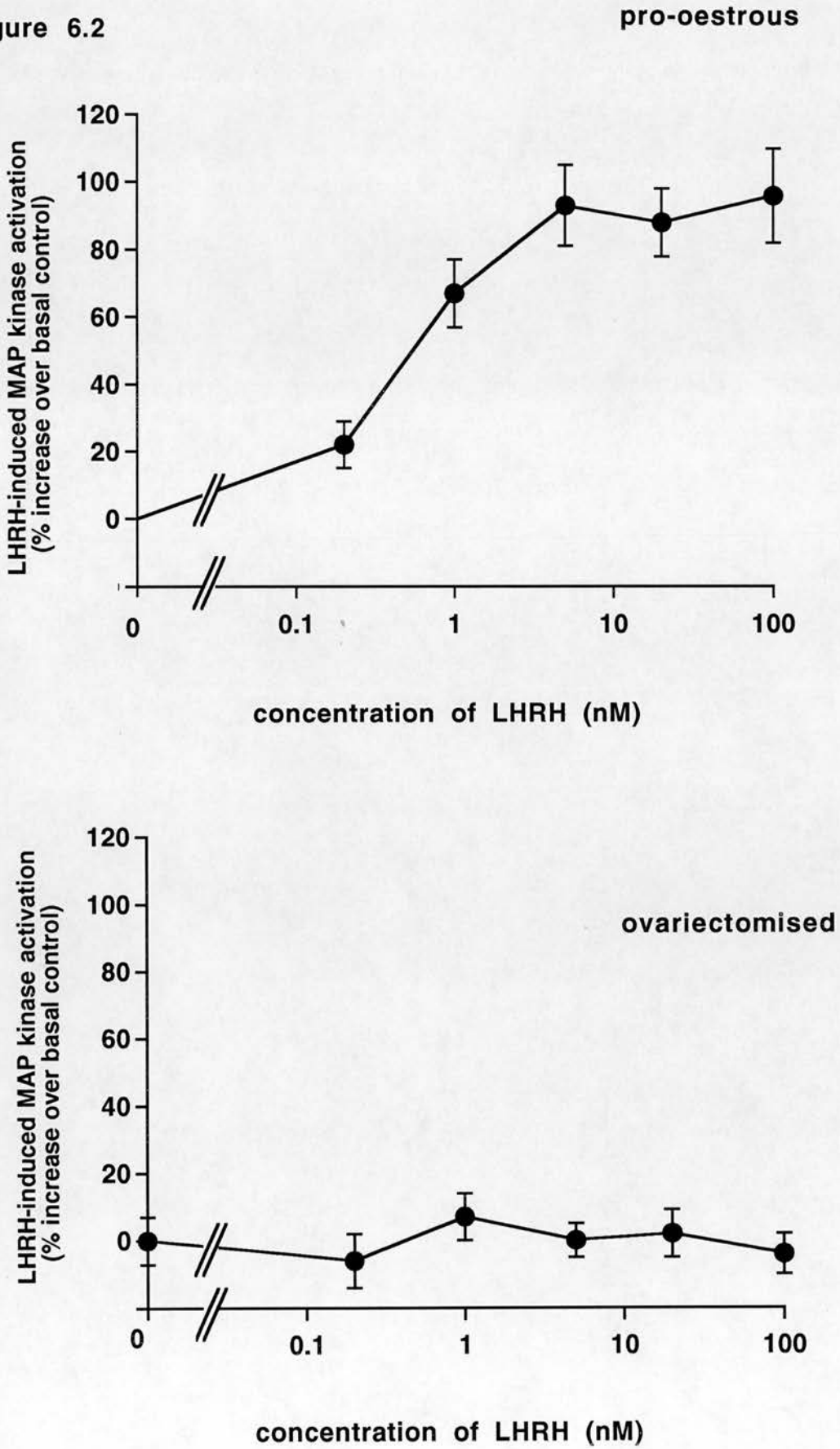

pro-oestrous
LHRH-induced MAP kinase activation
(% increase over basal control)
120
100
80
60
40
20
0
0
0.1
1
10
100
concentration of LHRH (nM)
ovariectomised
LHRH-induced MAP kinase activation
(% increase over basal control)
120
100
80
60
40
20
0
0
0.1
1
10
100
concentration of LHRH (nM)

Figure 6.3

LHRH-induced MAP kinase activation in anterior pituitary tissue taken from different stages of the oestrous cycle in female rats

Anterior hemipituitaries from metoestrous, dioestrous, pro-oestrous, oestrous and ovariectomised rats were pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium alone or containing 100 nM LHRH. After 15 min the tissue was removed and immediately homogenised in 2 vol of homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Basal MAP kinase activity showed no apparent difference between tissue from all origins falling consistently between 800-1200 dpm per assay. Values are means ± SEM from 6 determinations.

Figure 6.3

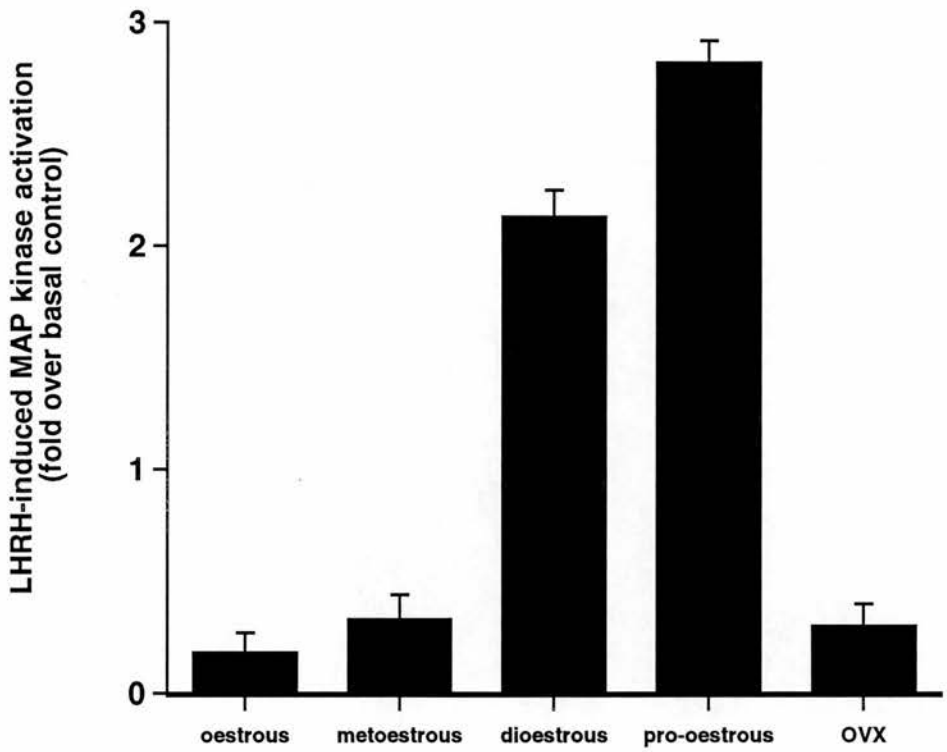

LHRH-induced MAP kinase activation
(fold over basal control)
3
2
1
0
oestrous
metoestrous
dioestrous
pro-oestrous
OVX

Figure 6.4

(a) Time course of phorbol ester-induced activation of MAP kinase in female rat anterior pituitary tissue

Anterior hemipituitaries from pro-oestrous (●) and ovariectomised (■) rats were pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium containing 1 μM phorbol 12, 13-dibutyrate (PDBu) for the appropriate time.

(b) MAP kinase activation in male anterior hemipituitaries

Anterior pituitary tissue was removed from male rats and following a 25 min preincubation with warm MEM the medium was replaced with fresh medium containing or that additionally containing LHRH (100 nM) or PDBu (1 μM) for 15 min.

After the appropriate time the tissue was removed and immediately homogenised in 2 vol of homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Basal MAP kinase activity showed no apparent difference between tissue from all origins falling consistently between 800-1200 dpm per assay and did not alter significantly over 40 min control incubations (data not shown). Values are means ± SEM from 4-6 determinations.

Figure 6.4

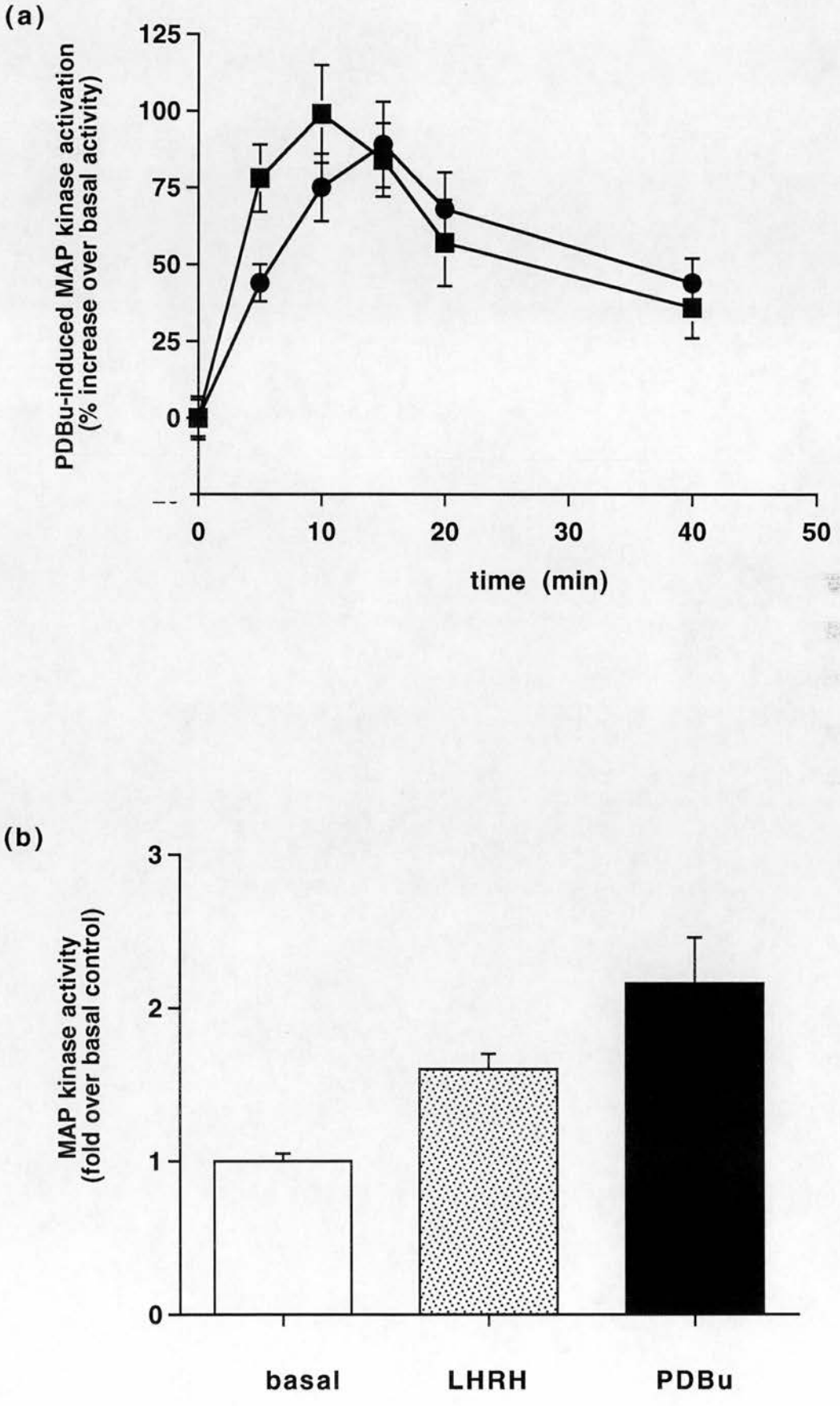

(a)
PDBu-induced MAP kinase activation
(% increase over basal activity)
125
100
75
50
25
0
0
10
20
30
40
50
time (min)
(b)
MAP kinase activity
(fold over basal control)
3
2
1
0
basal
LHRH
PDBu

Figure 6.5

Effects of kinase inhibitors on LHRH- and PDBu-induced activation of MAP kinase activity in rat anterior pituitary tissue

Anterior hemipituitaries from pro-oestrous and male rats were pre-incubated in MEM for 25 min. The medium was discarded and replaced with fresh medium alone or additionally containing GF109203X (●), H7 (▲).or lavendustin A (■). After 10 min the medium was again replaced with fresh medium alone or containing 100 nM LHRH in addition to the appropriate concentration of inhibitor. After 15 min the tissue was removed and immediately homogenised in 2 vol of homogenisation buffer. Thereafter MAP kinase activity was determined as in methods section. Basal MAP kinase activity was unaffected by inclusion of the inhibitors alone (not shown) and as before values were in the region of 800-1200 dpm per assay. Values are means ± SEM from 4-6 determinations.

Figure 6.5

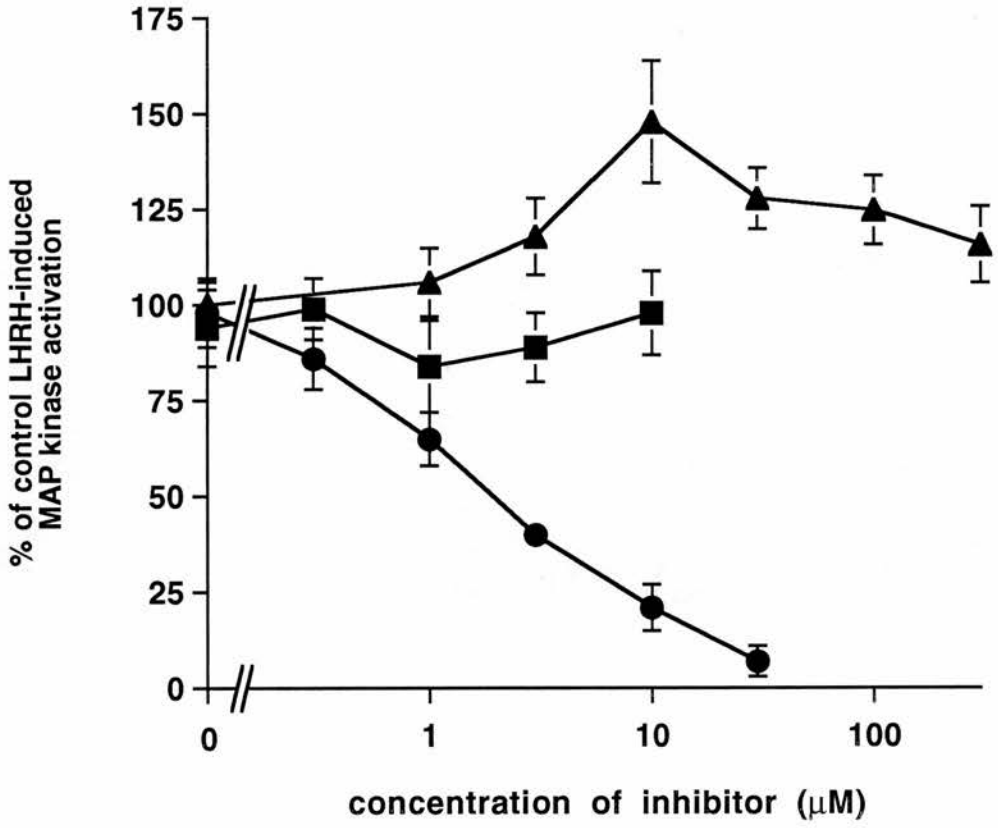
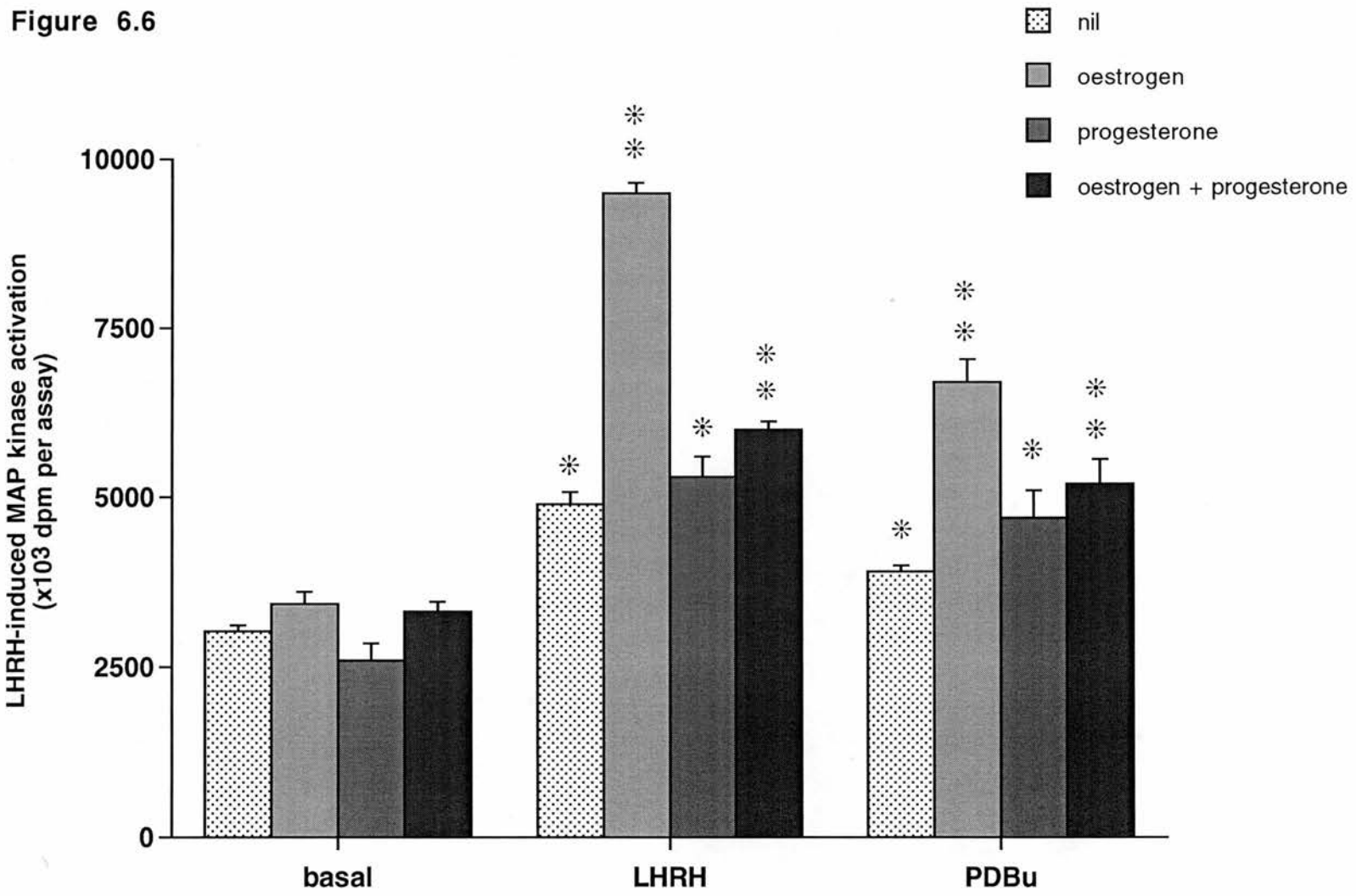

175
150
125
100
75
50
25
0
% of control LHRH-induced MAP kinase activation
0
1
10
100
concentration of inhibitor (μM)

Figure 6.6

Effects of oestrogen and progesterone on LHRH- and PDBu-induced MAP kinase activity in αT3-1 cells

αT3-1 cells were grown in steroid-free medium for 96 h. Oestrogen (1 nM), progesterone (200 nM) or oestrogen (1 nM) and progesterone (200 nM) were added 48 h or 3 h respectively prior to the addition of 100 nM LHRH or 1 μM phorbol 12, 13-dibutyrate (PDBu) for 10 min. Thereafter MAP kinase activity was determined as in methods section. Basal MAP kinase activity was not significantly different between the treatments. Values are means ± SEM from 4-6 determinations.

Figure 6.6


nil
oestrogen
progesterone
oestrogen + progesterone
LHRH-induced MAP kinase activation
(x103 dpm per assay)
10000
7500
5000
2500
0
basal
LHRH
PDBu

CHAPTER 7

ACTIVATION OF PLA_2 BY THE LHRH RECEPTOR DOES NOT APPEAR TO REQUIRE UPSTREAM ACTIVATION OF MAP KINASE

7.1 INTRODUCTION

Phospholipases A_2 (PLA_2) selectively release fatty acids from the sn-2 position of phospholipids and are widely distributed in mammalian tissues. This reaction is particularly important when arachidonic acid (AA) is released as a consequence, as it serves as the rate-limiting step in the formation of the biologically active eicosonoids, prostaglandins and leukotrienes, important mediators in inflammatory responses [Irvine, 1982; Samuelsson et al, 1987]. The other product of PLA_2 is a lysophospholipid which can be further metabolised into platelet activating factor (PAF) which is also important in inflammation [Hanahan, 1986; Irvine, 1982].

PLA_2 can be classified into two distinct forms based on their apparent cellular roles and location. The secreted form of PLA_2 ($sPLA_2$) which function in phospholipid digestion and inflammatory responses and a more recently described cytosolic PLA_2 ($cPLA_2$) which are probably involved in receptor-linked signal transduction mechanisms [Mayer & Marshall, 1993]. Owing to their relative abundance earlier studies had focused on the secreted forms which are classified into two groups [Scott et al, 1990; Thunissen et al, 1990]. Group 1 (pancreatic type) enzymes have been characterised in lung, spleen, pancreas and snake venom whereas Group 2 PLA_2s are found in platelets, spleen and liver and additionally in inflammatory exudes. Although the primary structure of these enzymes differ, they exhibit close similarities in their Ca^{2+} binding regions [Wery et al, 1991] and high Ca^{2+}-dependence for activation [Vadas et al, 1985]. $cPLA_2$ was purified and cloned around 1990 by several groups from a variety of tissues including a macrophage cell line, RAW 264 [Leslie et al, 1988], U937 cells [Clark et al, 1990; Kramer et al, 1991; Sharp et al, 1991] and rat kidney [Gronich et al, 1990]. $cPLA_2$ is expressed in a variety of cells including platelets, macrophages, fibroblasts, kidney mesangial cells and epithelial cells [Gronich et al, 1990; Leslie et al, 1988; Rehfeldt et al, 1993; Takayama et al, 1991].

There are considerable differences between the two forms of PLA_2s. The $sPLA_2$s have an apparent molecular mass of approximately 14 kDa, contain seven disulphide bonds (which render them sensitive to reducing agents such as dithiothreitol) and are present in secretory granules to protect the enzymes from the reducing environment found in the cytosol. They require millimolar concentrations of Ca^{2+} for their action [Dennis, 1983] which is more consistent with the calcium concentrations present in the extracellular environment and display no fatty acid preference at the sn-2 position [Schalkwijk et al, 1990]. These characteristic are more suited to $sPLA_2$s role as a digestive enzyme or in the maintenance of membrane homeostasis or in the extracellular production of inflammatory mediators. $sPLA_2$ is also found to a high degree in arthritic joints [Vadas et al, 1985] therefore it is also a key enzyme in the pathology of certain disease conditions such as arthritis [Murakami et al, 1990].

In contrast the $cPLA_2$ appear to be heterogeneous with an apparent molecular mass ranging in size between 30-110 kDa [Mayer & Marshall, 1993] and are insensitive to reducing agents. They are active at submicromolar Ca^{2+} concentrations which are equivalent to those within the cytosol and are translocated to the plasma membrane following activation in response to hormonal stimulation. This was apparent from the observation that in the presence of a Ca^{2+} chelator the PLA_2 enzymatic activity was present in the cytosol, but on addition of submicromolar Ca^{2+} the activity was found mainly in the membrane fraction [Channon & Leslie, 1990; Clark et al, 1991]. Unlike $sPLA_2$, $cPLA_2$ display a high selectivity towards AA at the sn-2 position of phospholipids especially phosphatidylcholine and phosphatidylethanolamine [Channon & Leslie, 1990; Clark et al, 1990; Rehfeldt et al, 1993], generating AA. This is more consistent with their role in initiation of inflammatory responses by providing the precursor for inflammatory mediators. Additionally it has been demonstrated that when both forms of PLA_2 were overexpressed in CHO cells it was $cPLA_2$ (but not $sPLA_2$) that was responsible for the rapid thrombin- or ATP-stimulated formation of AA, as demonstrated by the increased release of arachidonic

acid in response to the stimulus [Lin et al, 1992]. Molecular cloning and expression of an 85 kDa $cPLA_2$ have revealed no sequence homology between this and the $sPLA_2$s. However the amino-terminal 140 amino acids of $cPLA_2$ includes a 45 amino acid phospholipid binding motif (CaLB) with distinct homology to the C-2 region which is conserved in Ca^{2+}-dependent PKC isoforms, in the synaptic vesicle protein p65, (which has been implicated in vesicle fusion with the plasmalemma), in GTPase-activating protein (GAP) and in phospholipase Cγ (PLCγ) [Clark et al, 1991]. This motif is probably responsible for targeting the enzyme to the plasma membrane (or natural membrane vesicles), in a Ca^{2+}-dependent manner upon activation by agonists, in order to facilitate access to the phospholipid substrate [Channon & Leslie, 1990; Lin et al, 1992].

Arachidonic acid and its metabolites formed in response to ligands that occupy G-protein coupled receptors, have also been implicated as both first and second messengers in a number of cellular processes [Axelrod, 1990; Burch et al, 1986; Gupta et al, 1990; Tsujishita et al, 1994]. Therefore the regulation of production of AA may be an important control point in many biological responses as well as inflammation. $cPLA_2$ is often activated in response to Ca^{2+}-mobilising receptors such as bradykinin, ATP and thrombin which also activate phospholipase C and hence activate PKC. Additionally growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) have also been shown to increase AA release. Serine phosphorylation of $cPLA_2$ by PKC in response to agonist, or direct activation of PKC by phorbol esters has been shown to increase activity several fold in addition to the presence of Ca^{2+} [Hasegawa-Saskai, 1985; Lin et al, 1992; Margolis et al, 1988]. Furthermore staurosporine prevented phosphorylation and activation of $cPLA_2$ and agonist-induced activity was sensitive to phosphatase treatment, all of which imply that phosphorylation may have some regulatory role in $cPLA_2$ activity. More recently a role has also been suggested for tyrosine

phosphorylation [Glaser et al, 1993; Goldman et al, 1992; Kast et al, 1993; Schramek et al, 1994; Tsujishita et al, 1994; Zor et al, 1993].

The activity of $cPLA_2$ can also be increased by phosphorylation by MAP kinase [Lin et al, 1993; Nemenoff et al, 1993]. Lin and co-workers suggested the site of $cPLA_2$ phosphorylation by MAP kinase (Ser-505) is identical to the major site phosphorylated in response to phorbol ester treatment and further that site-directed mutagenesis of this Ser to Ala prevented MAP kinase activation of $cPLA_2$. In a slightly contradictory report on a study investigating phosphorylation of expressed domains of $cPLA_2$ by PKC and MAP kinase, it was suggested that MAP kinase selectively phosphorylates the domain of $cPLA_2$ which contains the MAP kinase consensus sequence, whereas PKC phosphorylates all three expressed domains and the results of peptide mapping indicated these sites were different to those phosphorylated by MAP kinase [Nemenoff et al, 1993]. However both reports indicate at least some regulatory role by this family of enzymes. Recently it has been reported that $cPLA_2$ is a target of the proline-directed kinase p38 (a MAP kinase family enzyme) in response to either thrombin or the thrombin receptor agonist peptide in platelets [Kramer et al, 1995].

The role of PLA_2 in LHRH-mediated responses has also been investigated. AA and its lipoxygenase and epoxygenase metabolites may have a second messenger function in LHRH receptor signalling. In cultured anterior pituitary cells, LHRH can induce an increase in [^{3}H]-AA release from prelabelled cells and AA can induce LH release [Chang et al, 1986; Naor & Catt, 1981]. Addition of a PLA_2 activator, mellitin or PLA_2 exogenously to cultured cells also induced LH release from cultured anterior pituitary cells [Kiesel et al, 1985]. The PLA_2 inhibitor, quinacrine, prevented 40% of the LH/FSH secretory response to LHRH and the remainder was blocked by a DAG lipase inhibitor [Chang et al, 1987a]. These results would suggest that LHRH may induce AA release by a route involving both DAG lipase and PLA_2 action. In our

laboratory the role of PLA_2 in the LHRH self-priming effect was investigated [Thomson et al, 1994]. The induction of LHRH priming but not LHRH-induced LH release was inhibited by a number of inhibitors of PLA_2. Furthermore, LHRH-induced [^{3}H]-AA release was relatively resistant to the PKC inhibitor H7, consistent with earlier findings for H7-inhibition with respect to the PKC responsible for the induction of priming. This would indicate that the form of PKC responsible for induction of LHRH priming can activate PLA_2.

There is clear evidence that both PLA_2 and MAP kinase are involved in the physiological event of LHRH priming [Thomson et al, 1994; Mitchell et al, 1994 and Chapter 6]. It would evidently be of great interest to ascertain if these two LHRH-mediated responses were linked in that PLA_2 represented a downstream target for MAP kinase in the anterior pituitary cells. Given that $cPLA_2$ contains the MAP kinase consensus phosphorylation sequence and further that phosphorylation of PLA_2 may increase the activity of this enzyme this appears to be a plausible hypothesis. The anterior pituitary is of a heterogeneous nature and possible difficulties may have arisen with respect to tissue permeability to various agents and long term viability *in vitro* required for some of the experiments it was desirable to perform. Consequently this study was undertaken in the αT3-1 cell line as a model system.

7.2 RESULTS

In pilot experiments, it has previously been demonstrated that a 15 min incubation with LHRH 100 nM resulted in a significant release of [^{3}H]-AA (3-7 fold of basal) in αT3-1 cells [Thomson, 1992a]. This effect was further investigated using the [^{3}H]-AA release assay described in Chapter 2. In αT3-1 cells LHRH-induced [^{3}H]-AA release was concentration-dependent over a range of 0.3-300 nM after a 15 min incubation with LHRH and displayed an apparent EC_{50} value of 3.56 ± 0.8 nM (Figure 7.1a). Basal and 100 nM LHRH [^{3}H]-AA release was in the region of 0.4 and 1.2-2.8% of the total [^{3}H]-AA incorporated into the αT3-1 cells. In COS 7 cells transiently

transfected with the "wild type" LHRH receptor, LHRH also resulted in the release of [^{3}H]-AA which was 2.97 ± 0.25 fold over basal release with 100 nM LHRH after 15 min. The ATP-induced [^{3}H]-AA release at 15 min was also investigated to compare the magnitude of the release induced by occupation of a transfected receptor (the LHRH receptor) with that of an endogenously expressed receptor (P_2 purinergic). ATP (100 μM), similar to LHRH, resulted in a 3.15 ± 0.4 fold of basal [^{3}H]-AA release. Basal and 100 nM LHRH-mediated [^{3}H]-AA release was in the region of 0.25% and 0.6-0.8% of the total radioactivity incorporated into COS 7 cells. Both basal and stimulated [^{3}H]-AA release were lower in the COS 7 cells compared to αT3-1 cells. However the signal to noise ratio of the LHRH-induced response was approximately equal in both cell lines and it would appear that any differences in [^{3}H]-AA release can be attributed to cellular differences rather than a receptor-mediated defect.

AA can be liberated from arachidonyl phospholipids in a number of ways but is often released primarily as a result of the actions of phospholipase A_2 (PLA_2). The high molecular weight species of PLA_2 (otherwise known as $cPLA_2$) has been implicated in AA release in response to hormone stimulation [Lin et al, 1992]. To investigate whether the [^{3}H]-AA produced in response to LHRH in αT3-1 cells can be attributed to $cPLA_2$ action, a number of PLA_2 inhibitors were tested on LHRH-induced [^{3}H]-AA release. However to date, no effective inhibitors of the high molecular weight species have been identified, although some non selective 14 kDa PLA_2 inhibitors are reported to have a weak activity towards the high molecular weight species [Mayer & Marshall, 1993]. We have tested the following PLA_2 inhibitors on LHRH-induced PLA_2 activity in αT3-1 cells: 4-(4-octadecyl)-4-oxobenzenebutenoic acid [Kohler et al, 1992]; manoalide [Potts et al, 1992] and a benzenesulphonamide compound, N-[1-(2-phenylethyl)-4-piperidinyl]-4-[N-methyl-N-[(E)-3-[4-(methylsulphonyl) phenyl]-2-propenoyl] amino]-benzenesulphonamide hereafter referred to as benzenesulphonamide 4 [Oinuma et al, 1990]. These agents

appeared to have a weak potency, but significantly inhibited the LHRH-induced [^{3}H]-AA release, with 4-(4-octadecyl)-4-oxobenzenebutenoic acid (500 μM) and manoalide (5 μM) inhibiting >80% of the LHRH response and benzenesulphonamide 4 (10 μM) inhibiting ~60% of the LHRH-mediated response at the maximum concentrations tested here (Table 7.1). In contrast aristolochic acid (0-300 μM) and Br-Phe-Ac-Br (0- 300 μM), which are reported to be highly selective towards the low molecular weight species of PLA_2 [Mayer & Marshall, 1993], had no effect. Additionally the DAG lipase inhibitor RHC 80267 (200 μM) did not inhibit LHRH-mediated [^{3}H]-AA release (results not shown). These results indicate that the majority of the [^{3}H]-AA release observed appears to be from the action of a $cPLA_2$, although a residual component of between 20-40% of the [^{3}H]-AA release was not prevented by these agents at the maximum concentration tested. None of these inhibitors had any significant effect on basal [^{3}H]-AA release (results not shown).

Stimulation of the LHRH receptor is known to result in the activation of PKC as a consequence of the formation of DAG through PLC activation [Stojilkovic et al, 1994]. Phosphorylation of $cPLA_2$ by kinases including PKC may have a role in activation of this enzyme in response to hormonal stimulation. The effects of phorbol esters, PKC activators, on [^{3}H]-AA release were assessed. Table 7.2 shows that none of the phorbol esters tested including 4-β phorbol 12,13 dibutyrate (300 nM), mezerein (300 nM) and 1,2-dioctanoyl-*sn*-glycerol (200 μM) was able to elicit any significant change in [^{3}H]-AA release from αT3-1 cells. A role for PKC upstream of PLA_2 was also investigated by determining the effects of a number of PKC inhibitors on LHRH-induced [^{3}H]-AA release. As can be seen in Table 7.3, neither H7 (30 μM), GF109203X (5 μM) nor staurosporine (300 nM) was able to inhibit the LHRH-induced [^{3}H]-AA release in αT3-1 cells. None of these inhibitors had any significant effect on basal [^{3}H]-AA release (results not shown).

$cPLA_2$ has also been proposed to be regulated by tyrosine phosphorylation. The role of tyrosine phosphorylation in LHRH-induced [^{3}H]-AA release in αT3-1 cells was therefore assessed. However as shown in Table 7.4 none of the tyrosine kinase inhibitors or pervanadate, a tyrosine phosphatase inhibitor [Grinstein et al, 1990], displayed any inhibition of the LHRH-mediated response measured. None of these inhibitors had any significant effect on basal [^{3}H]-AA release (results not shown).

The pharmacology of the LHRH-induced [^{3}H]-AA release was further analysed by assessing the effects of agents which act at the level of G-proteins. One of these agents, isotetrandrine, is reported to be a specific inhibitor of PLA_2 activation of receptors, which acts by uncoupling PLA_2 from G-proteins [Hashizume et al, 1991]. Isotetrandrine was able to inhibit >75% of the LHRH-induced [^{3}H]-AA release which suggested the involvement of a G-protein-mediated coupling (Figure 7.2 a). In αT3-1 cells a high concentration of pertussis toxin (300 ng/ml; for 18 h) had no effect on basal or LHRH-induced [^{3}H]-AA release (Figure 7.2 b). To further investigate the role of G-proteins in PLA_2 activity in αT3-1 cells, single stranded phosphorothioate oligonucleotides antisense to non-conserved sequences around the translocation initiation site of $G\alpha_{q/11}$ (5'-CGC CAT (GC)AT GGA CTC CAG AGT-3') and $G\alpha_{12}$ (5'-GGG TCC GCA CCC CGG ACA TGG-3') were prepared. Cells were treated with antisense reagents for 18 h as described in Chapter 2. In Figure 7.3a it can be seen that the relevant antisense reagents successfully downregulated expression of $G\alpha_{12}$ or $G\alpha_{q/11}$ without effecting expression of the other G-protein. The transfection procedure had no significant effect on protein synthesis or cell mortality as determined by Bradford protein concentration assay and Coomassie blue protein staining [Davis, 1988] of the Immobilon-P membranes following electroblotting of the proteins from the SDS gels (on which the proteins in the cell samples were separated by electrophoresis). Transfection of antisense oligonucleotides to $G\alpha_{12}$ but not $G\alpha_{q/11}$ almost entirely inhibited the LHRH-induced [^{3}H]-AA release (Figure

7.3 b) providing evidence to suggest that the LHRH receptor interacts with another G-protein class, G_{12} to activate an isoform of $cPLA_2$.

To determine if PLA_2 was a target of MAP kinase in αT3-1 cells the antisense approach was again used as at present there is no commercially available MAP kinase inhibitor. αT3-1 cells were transfected with single stranded phosphorothioate oligonucleotides antisense to non-conserved sequences around the translocation initiation site of ERKs 2 (5'-CGC CAT GTT GGC TGC ACA GCC GCC-3') for 24 h then returned to normal growth medium for 48 h. This treatment almost entirely removed the p42 and p44 ERK proteins, therefore this oligonucleotide could not be used as a specific reagent towards ERK 2 activation, although this treatment had no significant effect on the LHRH-induced [^{3}H]-AA release (Fig 7.4).

7.3 DISCUSSION

Addition of LHRH to αT3-1 cells clearly results in a concentration-dependent increase in [^{3}H]-AA release, presumably through the action of a phospholipase A_2. The assay employed here has several drawbacks; it measures [^{3}H]-AA release and not directly PLA_2 activity, it measures the total [^{3}H]-AA released into the extracellular medium over a 15 min stimulation and therefore does not give true indication of the kinetics of the response or the [^{3}H]-AA released within the cytosol, there is no information on absolute [^{3}H]-AA concentrations just relative levels of radioactivity and no lysophospholipid measurements are made to confirm that it is a PLA_2 activity that is measured. Regardless of this, the assay has been shown to be an accurate measure of cellular AA production [Powell, 1982]. Given the evidence from this Chapter regarding the attenuation by the PLA_2 inhibitors in addition to evidence from other labs concerning receptor mediated PLA_2 activation and the Ca^{2+} requirements for the PLA_2s, it is most likely that the [^{3}H]-AA release measured largely reflects the activity of a high molecular weight species of PLA_2.

The high molecular weight forms of phospholipase A_2 ($cPLA_2$) have been implicated in hormone-stimulated release [Lin et al, 1992]. This is the most likely candidate for AA release as this isoform and not the low molecular weight forms has a preference for AA at the sn-2 position and are more likely to be active at the physiological levels of Ca^{2+} present in the activated cells. However AA can be mobilised in response to hormone stimulation by other routes such as the actions of DAG lipase on DAG produced from the action of PLC on membrane phospholipids [Bell et al, 1979] or from PLD followed by phosphatidate phosphohydrolase [Billah & Anthes, 1990]. Although the type II $sPLA_2$s display no preference for AA at the sn-2 position and are generally secreted, the actions of this class of enzymes in response to hormonal stimulus cannot be entirely ruled out. In one study addressing this possibility, the functions of type II $sPLA_2$s was investigated by stable transfection into a fibroblast cell line [Pernas et al, 1991]. Although Type II $sPLA_2$s were secreted from these cells by phorbol esters or the G-protein activator, AlF_4^-, and the rate of secretion was unaffected by these agents, a considerable amount of $sPLA_2$ remained associated with the cell and the transfected cells displayed significantly elevated levels of stimulated radiolabelled AA release in response to AlF_4^- or phorbol esters compared to similarly treated untransfected cells. This suggests that type II $sPLA_2$s may in some circumstances be involved in stimulated AA release, however this study is not conclusive and other explanations may exist. By contrast another study reported that CHO cells overexpressing type II $sPLA_2$ did not show an increased AA release in response to thrombin or ATP, whereas stimulated CHO cells transfected with the 85 kDa PLA_2 displayed increased AA release over control untransfected cells [Lin et al, 1992].

The effects of several pharmacological agents was tested on the LHRH-mediated [^{3}H]-AA release. There is a lack of good effective inhibitors for the high molecular weight species of PLA_2 [Marki et al, 1993; Mayer & Marshall, 1993]. In the study by Marki and co-workers where the potencies of several PLA_2 inhibitors was tested on

both $cPLA_2$ and $sPLA_2$, some weak inhibition of $cPLA_2$ was only seen with two of seven reference compounds and some phosphate free analogues or 1,2-amino alcohols [Marki et al, 1993]. As well as the classical inhibitors, aristolochic acid and an active site-directed inhibitor, 4-bromophenol-acylbromide (Br-Phe-Ac-Br; BPB), several very potent type II $sPLA_2$ inhibitors have been identified including the natural marine sponge products, manoalide and scalaradial (IC_{50} ~10-15 nM). However these agents are largely unselective as they act through reversible inhibition of lysine residues [Mobilo & Marshall, 1989; Potts et al, 1992]. We tested manoalide, a new agent from the aroylacrylic acid series of inhibitors, OBAA (4-(4-octadecyl)-4-oxobenzenebutenoic acid) and benzenesulphonamide 4. These agents, at high concentrations were able to inhibit a significant portion of the LHRH-mediated [^{3}H]-AA release whilst other agents such as aristolochic acid, Br-Phe-ac-Br were ineffective at the concentrations tested (Table 7.1). Where assessed previously in the literature IC_{50} values for manoalide against $sPLA_2$ and $cPLA_2$ action have been reported as 3.2 ± 0.5 μM and ineffective at 30 μM (highest concentration tested) [Marki et al, 1993] or 0.017 μM and >30 μM [Mayer & Marshall, 1993], 43 μM and >100 μM for BPB against $sPLA_2$ and $cPLA_2$ action. No literature was found where the potency of the agents aristolochic acid, OBAA or benzenesulphonamide 4 were compared between the two forms of PLA_2, however IC_{50} values of 40 μM [Mayer & Marshall, 1993], 70 nM [Kohler et al, 1992] and 9 nM [Oinuma et al, 1990] respectively have been quoted for these agents on $sPLA_2$ activity. Additionally the DAG lipase inhibitor RHC 80267 did not inhibit LHRH-mediated [^{3}H]-AA release (results not shown). Taken together these results would argue against the possibility that $sPLA_2$ is responsible for the AA release observed in response to LHRH in αT3-1 cells as the potencies of inhibition should be much greater than those observed and further suggest that a $cPLA_2$ species is activated here.

The LHRH-mediated [^{3}H]-AA release was unaffected by incubation with phorbol esters or with PKC or tyrosine kinase inhibitors and would therefore indicate that

these kinases play no direct regulatory role in LHRH-induced activation of PLA_2 in αT3-1 cells. Nevertheless $cPLA_2$ can be activated in response to a variety of agents including thrombin, ATP, bradykinin, vasopressin, phorbol esters and growth factors such as PDGF and EGF [Bonventre et al, 1990; Goldberg et al, 1990; Gronich et al, 1988; Margolis et al, 1988; Rehfeldt et al, 1991; Wijkander & Sundler, 1991] all of which may stimulate kinases in the cytosol. Phosphorylation of $cPLA_2$ has been demonstrated to increase activity in conjunction with raised intracellular Ca^{2+} in a variety of tissues including intact cells and lysates from kidney mesangial cells treated with EGF, macrophage cell lines and a monocyte cell line, THP-1 [Bonventre et al, 1990; Goldberg et al, 1990; Gronich et al, 1988; Rehfeldt et al, 1991]. Lin and co-workers also reported serine phosphorylation of $cPLA_2$ induced by either ATP, thrombin, phorbol esters or the calcium ionophore A23187 and demonstrated that this stable modification correlates with the agonist induced increase in $cPLA_2$ activity [Lin et al, 1992]. It has been suggested that Ca^{2+} is required to promote $cPLA_2$ membrane binding as with the Type II $sPLA_2$s and is not involved in catalysis [Ghomashchi et al, 1992; Wijkander & Sundler, 1992]. One possible function of a phosphorylation event is to decrease the Ca^{2+} concentrations required for membrane association. The properties of a partially purified PLA_2 from phorbol ester-stimulated cells were compared to the $cPLA_2$ from unstimulated cells [Rehfeldt et al, 1993]. Both enzymes displayed an identical Ca^{2+}-dependence for enzyme activation but significant differences were found in the Ca^{2+}-dependent membrane association of the enzymes obtained from the two sources. The PLA_2 from the phorbol ester-treated cells showed membrane association at much lower Ca^{2+} concentrations due to a stable modification of the enzyme for example phosphorylation. Phosphorylation of $cPLA_2$ however does not necessarily lead to increased activity. In the study by Wijkander and Sundler, PKC dependent phosphorylation of PLA_2 was observed without a corresponding increase in enzyme activity [Wijkander & Sundler, 1991] and additionally two other studies reported that PLA_2 was not activated in response to

short term phorbol ester treatment despite the translocation of PLA_2 to the membrane within 5-10 min [Rehfeldt et al, 1991; Shibata et al, 1992]. Thus factors additional to PKC-dependent phosphorylation may be required in some instances.

Tyrosine phosphorylation may additionally have a role in $cPLA_2$ responses. In murine resident peritoneal macrophages eicosanoid formation in response to various stimuli including zymosan, A23187 and PMA was prevented by the tyrosine kinase inhibitors genistein and tyrphostin-25. These inhibitors had no effect on human synovial fluid PLA_2 activity *in vitro* or in intact cells. Additionally monoclonal anti-phosphotyrosine blots of agonist treated peritoneal cells demonstrated an increase in protein tyrosine phosphorylation [Glaser et al, 1993]. These results suggest a protein tyrosine kinase-mediated phosphorylation event in PLA_2 signal transduction. Pervanadate enhanced by 4-10 fold the PLA_2 activity induced by reactive oxygen species, phorbol ester and a Ca^{2+}-ionophore in macrophages [Goldman et al, 1992]. Further investigation of this pathway demonstrated that a combination of pervanadate and TPA led to massive tyrosine phosphorylation, the sustained activation of tyrosine kinase activity and inhibition of tyrosine phosphatase activity as well as activation of PKC and a myelin basic protein kinase activity [Zor et al, 1993]. In a mouse keratinocyte cell line HEL-30 transforming growth factor-alpha (TGF-α) stimulated the rapid release of AA in a time- and dose-dependent manner due to the action of a $cPLA_2$ activity which was found to be membrane associated [Kast et al, 1993]. This activity was shown to be attributable to a $cPLA_2$ activity, as the activity was found in the cytoplasmic fraction and was Ca^{2+} dependent, furthermore it co immunoprecipitated with an anti-PLA_2 antibody and had an apparent molecular weight of 100-110 kDa following SDS-PAGE electrophoresis. This activation of $cPLA_2$ was found to be independent of the PLC products DAG and IP_3 or PKC. Moreover $cPLA_2$ was found to be tyrosine phosphorylated by this treatment. In rat mesangial cells, chronic exposure to endothelin-1 (ET-1) stimulates prostaglandin release via the increased activity and gene expression of $cPLA_2$ [Schramek et al,

1994], acute exposure to ET-1 also stimulates $cPLA_2$ activity in these cells. Herbimycin A, a cellular-TK inhibitor, abrogated the ET-1-induced $cPLA_2$ expression and hence activity whilst PKC inhibitors or heparin (which can inhibit intracellular Ca^{2+} increase as well as MAP kinase activation and cell proliferation) were ineffective on this response [Schramek et al, 1994]. Suspension of permeabilised HL-60 or U937 cells in acidic or alkaline conditions causes the release of various fatty acids, particularly AA [Tsujishita et al, 1994]. The effects of pervanadate and genistein on this reaction were assessed. The concomitant production of lysophospholipids, particularly lysophosphatidylcholine, suggested that PLA_2 played a major role in this action. In alkaline conditions the release of fatty acids, stimulated by permeabilisation, and lysophospholipids was enhanced by pervanadate and this enhancement was reversed by the inclusion of genistein, suggesting a role for tyrosine phosphorylation in this release reaction. Additionally this effect was increased further in the presence of a G-protein activator GTP[γS] or AlF_4^-. However this last result should be interpreted with caution as some of the activity measured may reflect an arachidonic acid-non selective PLA_2 isoform.

Since it was first reported that $cPLA_2$ contains a MAP kinase consensus phosphorylation sequence and that phosphorylation of $cPLA_2$ by the family of MAP kinases can increase activity [Lin et al, 1993; Nemenoff et al, 1993], considerable evidence has accumulated regarding the inter-relationship of these enzymes. Nevertheless whilst MAP kinase is capable of activating $cPLA_2$ and these two enzymes are often both stimulated in response to one agonist in many cells, PLA_2 is not always a downstream target for MAP kinase. In one study macrophage cells were exposed to a range of stimuli including zymosan, phorbol ester, okadaic acid and A23187, all of which had previously shown to induce AA release through $cPLA_2$ activity [Qui & Leslie, 1994]. The results demonstrated a complex pattern of $cPLA_2$ regulation. PMA and zymosan trigger PKC activation that leads to activation of MAP kinase and PLA_2, whereas in okadaic acid treated cells, MAP kinase and PLA_2

activation are PKC independent. Alternatively in A23187-treated cells PLA_2 activation is both PKC and MAP kinase independent [Qui & Leslie, 1994]. In fibroblast cells both PLA_2 and MAP kinase are stimulated in response to thrombin and ATP. Both of these responses are inhibited by PTx pre treatment or down-regulation of PKC. Expression of a mutant $G\alpha_i$ subunit having Gly 203 mutated to Thr (α_{i2}G203T) inhibited AA release independent of adenylyl cyclase inhibition, Ca^{2+}-mobilisation and MAP kinase activation [Winitz et al, 1994]. This result defines a role of $G\alpha_i$ in the control of PLA_2 activity in addition to regulation by PKC and MAP kinase. Arachidonic acid may also serve to regulate the activity of MAP kinase. In vascular smooth muscle cells (VSMC), AA activated MAP kinase in a time- and dose-dependent manner. It appears likely that this is, at least in part, due to the conversion of AA to 15-hydroxyeicosatetraenoic (15-HETE) and is additionally dependent on PKC, as AA activation of MAP kinase was blocked by PKC downregulation or nordihydroguaiaretic acid (NDGA), a potent inhibitor of the lipoxygenase system [Rao et al, 1994]. Furthermore in rat liver epithelial WB cells, it is reported that p42 and p44 MAP kinase is activated in response to polyunsaturated fatty acids, especially AA. This mechanism is highly dependent on PKC as it is prevented by phorbol ester sensitive PKC downregulation and translocation of the α, δ and ε isoforms has been demonstrated in response to AA [Hii et al, 1995]. Although receptor mediated AA release was not investigated in this study, it is evident that a downstream consequence of PLA_2 action in this cell type may be MAP kinase activation

Our original intention was to ascertain whether PLA_2 represented a downstream target for MAP kinase activated in response to LHRH in the αT3-1 cell line, as activation of both these enzymes appear critical to the LHRH self-priming effect observed in rat pro-oestrous tissue [Mitchell et al, 1994; Thomson et al, 1994] Chapter 6]. Significant responses to LHRH were observed for both these enzymes, however depletion of ERK by transfection of the antisense oligonucleotide

corresponding to ERK2 had no effect on LHRH-induced [^{3}H]-AA release. This oligonucleotide appeared to be non specific to ERK 2, as the corresponding band to ERK 1 was also diminished by this treatment. Thus it would appear that in this instance phosphorylation of a PLA_2 by ERK is unnecessary and that this activity is otherwise regulated. It would obviously be of interest in the future to investigate a putative role of p38 sub-family of MAP kinase members in LHRH-induced PLA_2 activation in the light of the recent observations by Kramer and co workers who have shown that p38 like $cPLA_2$ is activated in response to thrombin in platelets and have further suggested that $cPLA_2$ may be a target for p38 kinase in platelet activation [Kramer et al, 1995].

PLA_2 regulation by G-proteins became evident with the observation that some PLA_2 responses in intact cells were pertussis toxin-sensitive indicating that the receptor was coupled to PLA_2 by a $G_{i/o}$ protein [Burch et al, 1986]. Furthermore GTP analogues and AlF_4^- are able to stimulate arachidonate release [reviewed in Axelrod, 1990; Cockcroft et al, 1991; Cockroft, 1992]. Many receptors that couple to PLA_2 also couple to PLC, which can in principle activate PLA_2. However it has been demonstrated that G_i proteins mediate this effect based on a number of observations; pertussis toxin selectively inhibits PLA_2 and not PLC; that neomycin (which selectively inhibits PLC activation by substrate sequestration) selectively inhibits PLC and not PLA_2 and that PMA can inhibit PLC without affecting PLA_2 [Burch & Axelrod, 1987; Burch et al, 1986; Conklin et al, 1988]. However (excluding the neomycin data) this evidence does not rule out the possibility that PLC activation is required in parallel. Indeed it has been suggested in some instances that a requirement for PLC activation prior to PLA_2 activation is necessary for Ca^{2+} mobilisation [Cockcroft et al, 1991]. Collagen- and thrombin-induced AA release and platelet aggregation has been extensively investigated by a number of groups [Akiba et al, 1989; Fuse & Tai, 1987; Nakashima et al, 1987; Silk et al, 1989]. Both AA release and platelet aggregation has been suggested to be caused mainly by $cPLA_2$,

and furthermore that this PLA_2 activation is also regulated by G-proteins. One group has investigated the effects of bisclaurine alkaloids, including isotetrandine, on PLA_2 and PLC activity in this system [Akiba et al, 1992; Hashizume et al, 1991]. Their findings suggest that these agents but especially isotetrandine, has a specific potency against PLA_2 activity [Hashizume et al, 1991] and that bisclaurine alkaloids appear to exert their effects by uncoupling of the G-protein from the enzyme (probably through alkaloid-induced changes in the membrane properties) rather than by affecting the enzyme directly or the ligand-receptor interaction [Akiba et al, 1992].

The pertussis toxin-sensitive G-proteins $G\alpha_{i/o}$ appear to be most commonly associated with $cPLA_2$ activation [Axelrod et al, 1988; Cockcroft et al, 1991]. Recently expression of a mutant $G\alpha_i$ subunit (α_iG203T) has been reported to inhibit AA release by at least two groups [Murray-Whelan et al, 1995; Winitz et al, 1994]. This mutant α_i-subunit was further shown to specifically inhibit Ca^{2+} activation (but not phorbol ester activation) of AA release [Murray-Whelan et al, 1995]. The high affinity cholecystokinin (CCK) receptor is coupled to PLA_2 by a G-protein in pancreatic acini [Tsunoda & Owyang, 1993; Tsunoda & Owyang, 1995]. A non-hydrolysable guanosine nucleotide analogue, GTP[γS] stimulated amylase secretion which was inhibited by anti PLA_2 antibodies in permeabilised pancreatic acini. Furthermore the actions of the high affinity CCK receptor agonist (CCK-OPE) were inhibited by isotetrandrine and G_q antagonist-2A which inhibits G_q activation, however the actions of CCK-OPE were not inhibited by $G_{q\alpha}$ antibodies but were inhibited by a G_β subunit antibody [Tsunoda & Owyang, 1995]. From these observations it appears that the $\beta\gamma$ subunit of G_q may be critical in mediating this response.

Another G-protein proposed to be involved in AA release responses is the recently cloned $G\alpha_{12}$ [Chan et al, 1993; Xu et al, 1993]. To date little is known about the function of this family of α-subunits. It is known that the thrombin receptor and the

thromboxin A_2 receptor couple to G_{12} and G_{13} during platelet activation [Offermanns et al 1994] and platelet activation has also been implicated with PLA_2 activation in platelets [Kramer et al 1995]. In one study the oncogenic effects of the G_{12} were investigated [Xu et al, 1993]. Overexpression of the wild type α_{12} subunit was weakly transforming in contrast to the activated α_{12} subunit which acted as a potent oncogene. This observation is confirmed in another study, activated α_{12} and α_{13} induced neoplastic transformation in Rat-1 and NIH 3T3 fibroblasts [Voynoyasenetskaya et al, 1994]. Transformation by α_{12} correlates with alterations in the eicosanoid pathway but not with alterations in PI-specific PLC or other G-protein linked second messengers [Xu et al, 1993]. Interestingly G_{12} deficient mice have been proposed as a model for inflammatory bowel disease perhaps also suggesting a role for α_{12} in AA mobilisation as part of an inflammatory response [Rudolph et al, 1995].

In summary, LHRH-induced [^{3}H]-AA release which may involve a $cPLA_2$ seems to be unaffected by protein kinase inhibition, ERK 2 depletion or phorbol esters, but is potently inhibited by isotetrandine in αT3-1 cells. Despite the ability of this receptor to activate the $G_{i/o}$ family of G-proteins [Sim et al, 1995; Chapter 3+4], LHRH-mediated [^{3}H]-AA release was unaffected by pertussis toxin pretreatment thereby eliminating a role for $G\alpha_{i/o}$ in this response. Interestingly using the antisense oligonucleotide approach, depletion of the α_{12} subunit and not $\alpha_{q/11}$ subunits abolished the LHRH-mediated [^{3}H]-AA release. Taken together these data would indicate that this response may be substantially regulated by a signalling pathway reliant on G-proteins probably of the G_{12} family.

Table 7.1

Effects of various PLA_2 inhibitors on LHRH-induced [^{3}H]arachidonic acid release from αT3-1 cells

αT3-1 cells pre-labelled with 0.5 μCi/ well of [^{3}H]-AA were incubated in medium containing no drug or a PLA_2 inhibitor for 15 min. The medium was discarded and replaced with fresh medium containing LHRH and inhibitor where appropriate for a further 15 min. Thereafter the [^{3}H]-AA release measurements were determined. All values are the mean ± SEM of between 3-8 determinations (actual values are shown in brackets). LHRH-induced [^{3}H]-AA release was significantly greater than baseline and this was significantly inhibited by all the PLA_2 inhibitors shown here.

Table 7.1

Treatment (Concentration)	[^{3}H]AA released as % of total label incorporated	mean % of LHRH-stimulated [^{3}H]AA release
basal	0.4 ± 0.03 (10)	
LHRH (100 nM)	1.8 ± 0.59 (10)	100% (+)
LHRH + aristolochic acid		
300 μM	1.76 ± 0.42 (8)	98%
LHRH + br-phe-ac-br		
300 μM	1.75 ± 0.32 (8)	97%
LHRH + 4-(4-octadecyl)-4-oxobenzenebutenoic acid		
1.5 μM	1.65 ± 0.89 (8)	92%
5.0 μM	1.45 ± 0.30 (8)	81%
50 μM	1.08 ± 0.12 (8)	60% (*)
500 μM	0.33 ± 0.05 (8)	18% (*)
LHRH + manoalide		
0.5 μM	1.73 ± 0.10 (6)	96%
1.0 μM	1.48 ± 0.13 (6)	82%
2.5 μM	1.01 ± 0.02 (6)	56% (*)
5.0 μM	0.26 ± 0.05 (6)	14% (*)
LHRH + benzenesulphonamide 4		
0.3 μM	1.67 ± 0.12 (6)	93%
1.0 μM	1.35 ± 0.09 (6)	75%
3.0 μM	1.24 ± 0.04 (6)	69% (*)
10 μM	0.68 ± 0.05 (6)	38% (*)

Table 7.2

The effects of phorbol esters on [^{3}H]arachidonic acid release from αT3-1 cells

αT3-1 cells pre-labelled with 0.5 μCi/ well of [^{3}H]-AA were incubated in medium containing either vehicle or a phorbol ester for 15 min. Thereafter the [^{3}H]-AA release measurements were determined. All values are the mean ± SEM of between 4-8 determinations (actual values are shown in brackets). As a control response, LHRH-induced [^{3}H]-AA release was significantly greater than baseline. None of the phorbol esters tested significantly increased the [^{3}H]-AA release.

Table 7.2

The effects of a phorbol ester and related PKC-activating agents on [^{3}H]arachidonic acid release from αT3-1 cells

αT3-1 cells pre-labelled with 0.5 μCi/ well of [^{3}H]-AA were incubated in medium containing either vehicle or drugs for 15 min. Thereafter the [^{3}H]-AA release measurements were determined. All values are the mean ± SEM of between 4-8 determinations (actual values are shown in parentheses). As a positive control response in the same experiment, LHRH-induced [^{3}H]-AA release was shown to be significantly greater than baseline (+). None of the PKC-activators tested significantly increased the [^{3}H]-AA release matched pair t-test on raw data.

Treatment	Concentration	[^{3}H]-AA (% of total label incorporated)
basal		0.59 ± 0.03 (8)
LHRH	100 nM	1.61 ± 0.18 (8) (+)
12, 13-phorbol dibutyrate	300 nM	0.60 ± 0.04 (6)
mezerein	300 nM	0.62 ± 0.05 (6)
1, 2-sn-dioctanoyl glycerol	200 μM	0.58 ± 0.04 (6)

Table 7.3

Effects of PKC inhibitors on LHRH-induced [^{3}H]arachidonic acid release in αT3-1 cells

αT3-1 cells pre-labelled with 0.5 μCi/ well of [^{3}H]-AA were incubated in medium containing no drug or a PKC inhibitor for 15 min. The medium was discarded and replaced with fresh medium containing LHRH and inhibitor where appropriate for a further 15 min. Thereafter the [^{3}H]-AA release measurements were determined. All values are the mean ± SEM of between 4-8 determinations (actual values are shown in brackets). LHRH-induced [^{3}H]-AA release was significantly greater than baseline. None of the PKC inhibitors tested significantly inhibited the LHRH-induced [^{3}H]-AA release

Table 7.3

Effects of PKC inhibitors on LHRH-induced [^{3}H]arachidonic acid release in αT3-1 cells

αT3-1 cells pre-labelled with 0.5 μCi/ well of [^{3}H]-AA were incubated in medium containing no drug or a PKC inhibitor for 15 min. The medium was discarded and replaced with fresh medium containing LHRH and inhibitor where appropriate for a further 15 min. Thereafter the [^{3}H]-AA release measurements were made. All values are the mean ± SEM of between 4-8 determinations (actual values are shown in parentheses). LHRH-induced [^{3}H]-AA release was significantly greater than baseline (+). None of the PKC inhibitors tested significantly inhibited the LHRH-induced [^{3}H]-AA release by matched pair t-test on raw data.

Treatment	Highest concentration tested	[^{3}H]-AA (% of total label incorporated)
basal		0.59 ± 0.03 (8)
LHRH	100 nM	1.61 ± 0.18 (8) (+)
LHRH +H7	30 μM	1.54 ± 0.10 (4) (+)
LHRH + GF109203X	5 μM	1.45 ± 0.03 (4) (+)
LHRH + staurosporine	300 nM	1.58 ± 0.06 (4) (+)

Table 7.4

Effects of various tyrosine kinase inhibitors on LHRH-induced [^{3}H]arachidonic acid release in αT3-1 cells

αT3-1 cells pre-labelled with 0.5 μCi/ well of [^{3}H]-AA were incubated in medium containing no drug or a tyrosine kinase inhibitor for 15 min. The medium was discarded and replaced with fresh medium containing LHRH and inhibitor where appropriate for a further 15 min. Thereafter the [^{3}H]-AA release measurements were determined. All values are the mean ± SEM of between 4-8 determinations (actual values are shown in brackets). LHRH-induced [^{3}H]-AA release was significantly greater than baseline. None of the tyrosine kinase inhibitors tested significantly inhibited the LHRH-induced [^{3}H]-AA release

Table 7.4

Treatment	Highest concentration tested	[^{3}H]-AA (% of total label incorporated)
basal		0.32 ± 0.03 (8)
LHRH	100 nM	1.11 ± 0.18 (8)
LHRH + geldanamycin	30 μM	1.12 ± 0.18 (6)
LHRH + genistein	100 μM	1.01 ± 0.24 (6)
LHRH + lavendustin A	3 μM	1.08 ± 0.06 (6)
LHRH + N-methyl 2,5-dihydroxycinnamate	300 μM	1.20 ± 0.02 (6)
LHRH + piceatannol	30 μM	0.92 ± 0.04 (6)
LHRH + ST 271	100 μM	1.28 ± 0.5 (6)
LHRH + pervanadate	1 mM	0.95 ± 0.06 (6)

Figure 7.1

LHRH-stimulated [^{3}H]arachidonic acid release

(a) Dose-dependent [^{3}H]arachidonic acid release in αT3-1 cells

The specific [^{3}H]-AA release from αT3-1 cells was measured at various concentrations of LHRH for 15 min. Each value is the mean ± SEM of 6 determinations. Baseline [^{3}H]-AA release in unstimulated cells was typically in the region of 0.4% of the total radioactivity incorporated by the cells, this was subtracted and the agonist induced curves were normalised to the activity obtained with the maximally effective dose. Curve fitting was then conducted as described in Chapter 2. The EC_{50} value for the response was 3.4 ± 0.4 nM.

(b) Agonist-induced [^{3}H]arachidonic acid release in COS 7 cells transfected with the wild type LHRH receptor.

COS 7 cells were transfected with cDNA for the wild type LHRH receptor and subsequently challenged with LHRH (100 nM) or ATP (100 μM) for 15 min. Each value represents the mean ± SEM of 6 determinations.

Figure 7.1

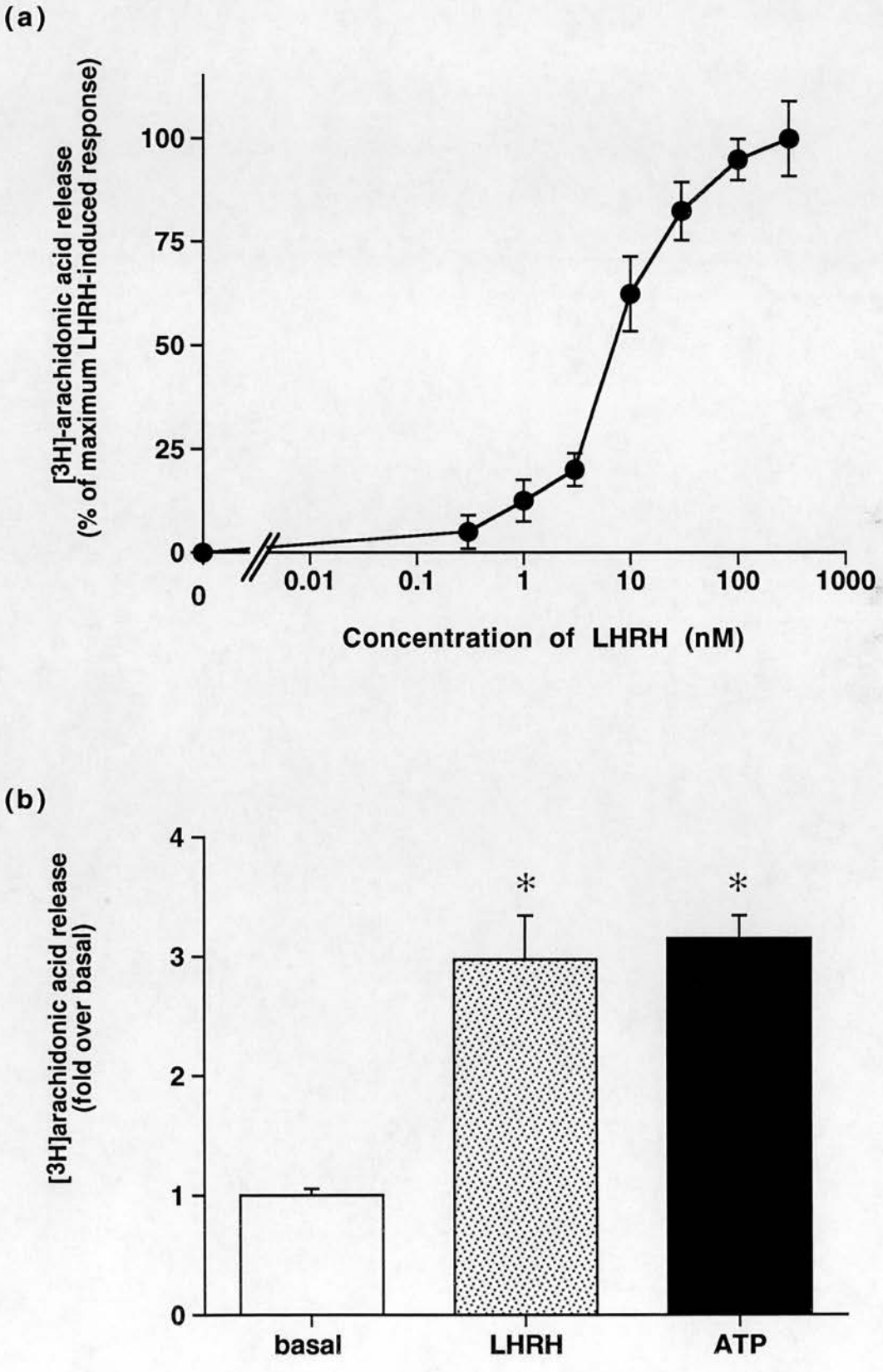

(a)
[3H]-arachidonic acid release
(% of maximum LHRH-induced response)
100
75
50
25
0
0
0.01
0.1
1
10
100
1000
Concentration of LHRH (nM)
(b)
[3H]arachidonic acid release
(fold over basal)
4
3
2
1
0
*
*
basal
LHRH
ATP

Figure 7.2

Effects on LHRH-induced [^{3}H]arachidonic acid release in αT3-1 cells of agents which inhibit at the level of G-proteins.

Figure 7.2 (a) shows the effect of various concentrations of a G-protein/PLA2 inhibitor, isotetrandine on LHRH-induced [^{3}H]-AA release. Basal [^{3}H]-AA release in unstimulated cells was subtracted and the values expressed as a % of the maximum LHRH-induced response. Each value represents the mean ± SEM of at least 4 determinations.

The effects of pertussis toxin, the B-oligomer of pertussis toxin and a chemically-inactivated holotoxin on LHRH-induced [^{3}H]-AA release are shown in figure 7.2 (b). None of the treatments had any significant effect on either basal or LHRH-induced release. Means ± SEM, n=6.

Figure 7.2

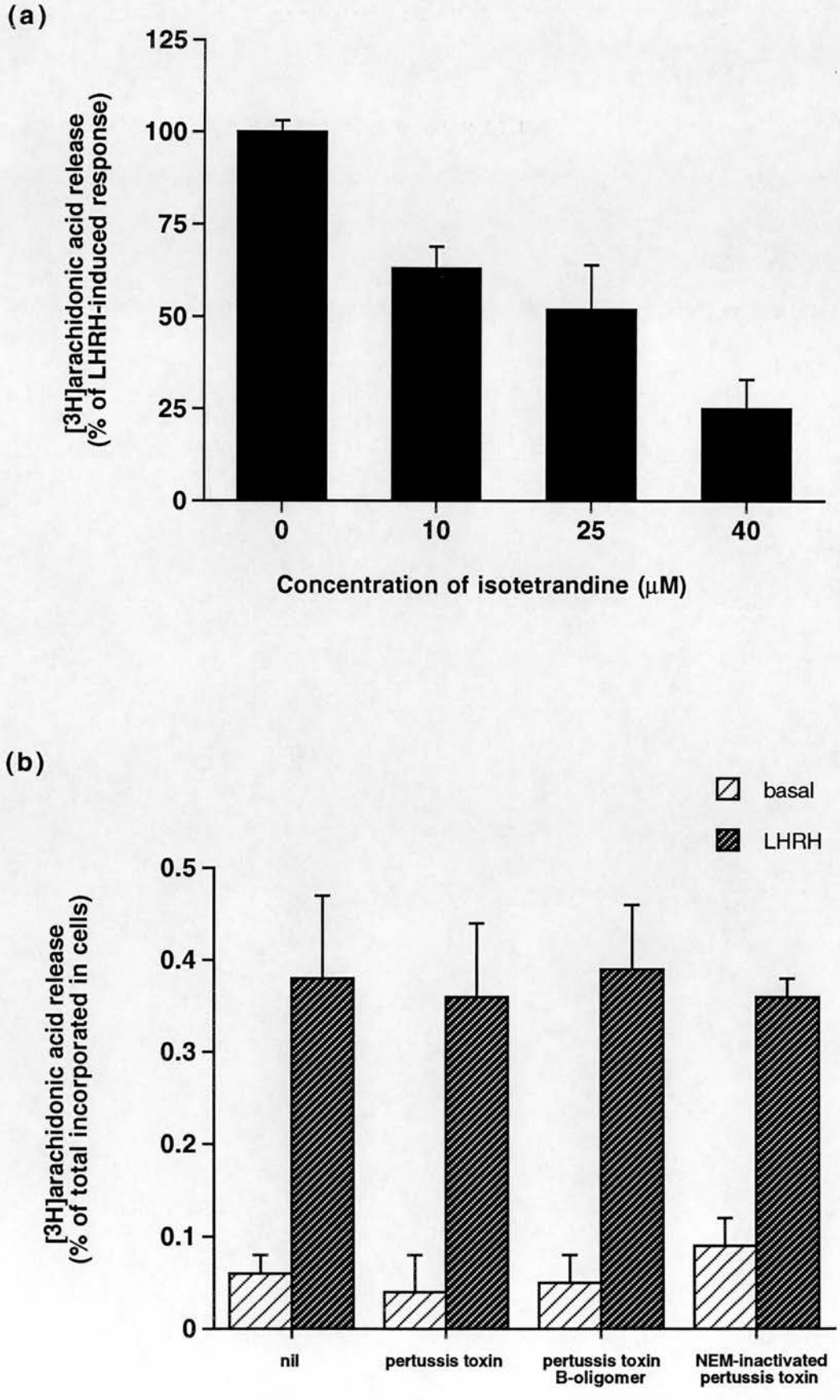

(a)
[3H]arachidonic acid release
(% of LHRH-induced response)
125
100
75
50
25
0
0
10
25
40
Concentration of isotetrandine (μM)
(b)
basal
LHRH
[3H]arachidonic acid release
(% of total incorporated in cells)
0.5
0.4
0.3
0.2
0.1
0
nil
pertussis toxin
pertussis toxin
B-oligomer
NEM-inactivated
pertussis toxin

Figure 7.3

Effects of expression of antisense oligonucleotides to the G-proteins G_{12} or $G_{q/11}$.

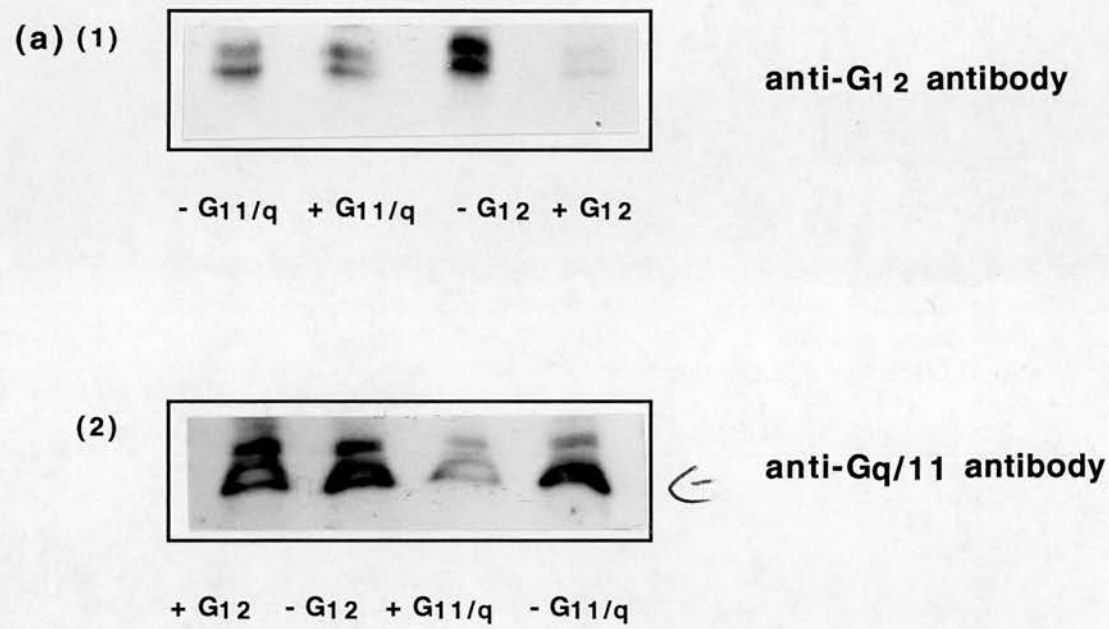
αT3-1 cells were transfected for 18 h with oligonucleotides of the antisense sequence to the G-proteins G_{12} or $G_{q/11}$. A control set of cells underwent the transfection procedure but with no antisense oligonucleotides. The cells were allowed to grow for a further 36-48 h in normal growth medium before on [^{3}H]-AA release was assayed.

(a 1) shows anti-G_{12} immunoblots which were carried out after protein separation by electrophoresis. Lanes 1 and 2 show ± $G_{q/11}$ oligonucleotides in lipofectamine vesicles and lanes 3 and 4 show ± G_{12} antisense oligonucleotide in lipofectamine vesicles.

(a 2) shows anti $G_{q/11}$ immunoblots which were carried out after protein separation by electrophoresis. Lanes 1 and 2 show ± G_{12} antisense oligonucleotides in lipofectamine vesicles and lanes 3 and 4 show ± $G_{q/11}$ oligonucleotide in lipofectamine vesicles.

(b) shows the corresponding [^{3}H]-AA release data for αT3-1 cells from the same transfection as panel (a) incubated with nil or 100 nM LHRH for 15 min. Each value is the mean ± SEM where n=4. Basal levels of [^{3}H]-AA release were not seen to be significantly different between sham-transfected and untreated αT3-1 cells.

Figure 7.3


(a) (1)
anti-G12 antibody
- G11/q + G11/q - G12 + G12
(2)
anti-Gq/11 antibody
+ G12 - G12 + G11/q - G11/q

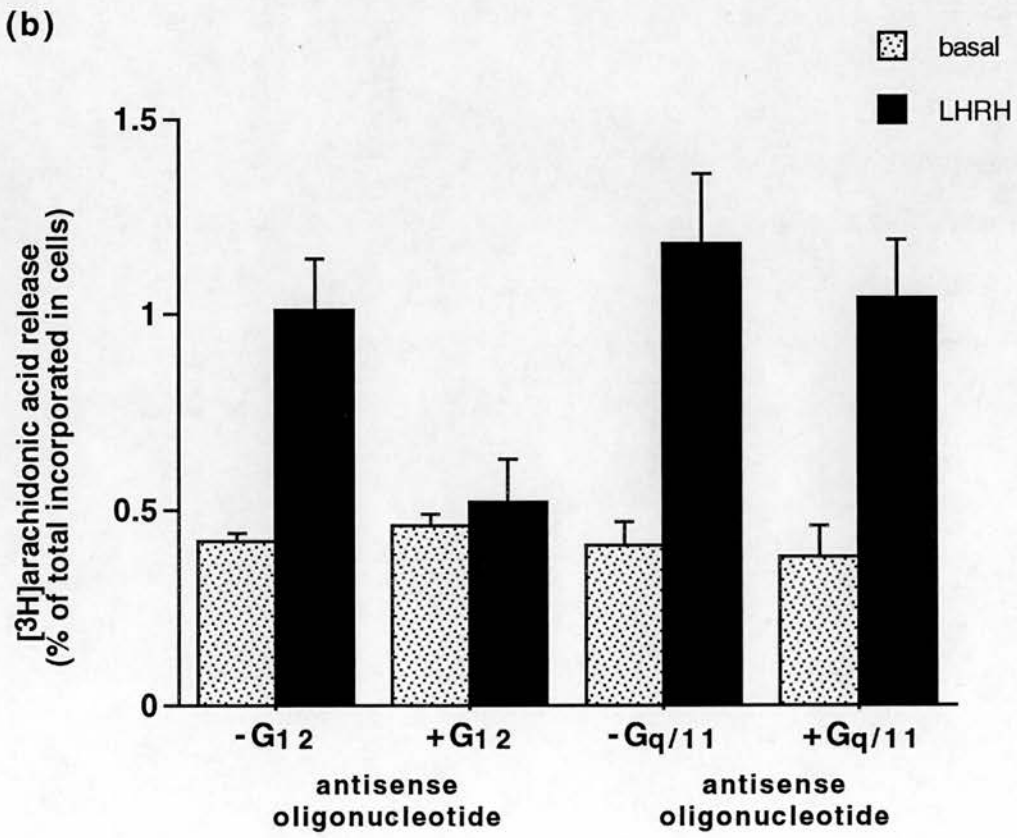

(b)
basal
LHRH
[3H]arachidonic acid release
(% of total incorporated in cells)
1.5
1
0.5
0
-G12
+G12
-Gq/11
+Gq/11
antisense oligonucleotide
antisense oligonucleotide

Figure 7.4

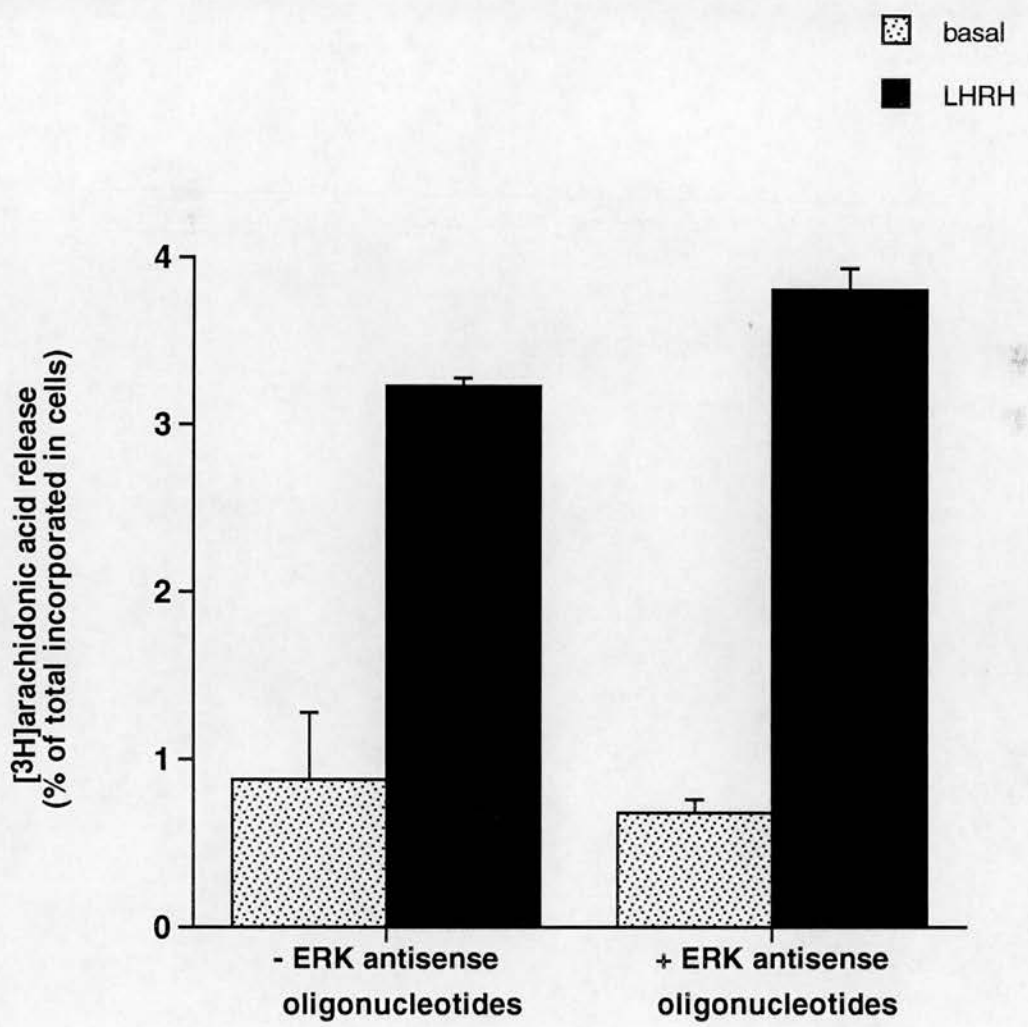
Effects of expression of antisense oligonucleotides to ERK 1+2

αT3-1 cells were transfected for 18 h with oligonucleotides of the antisense sequence to ERKs 1+2. A control set of cells underwent the transfection procedure but with no antisense oligonucleotides. The cells were allowed to grow for a further 36-48 h in normal growth medium before the effect on [^{3}H]-AA release was assayed.

Panel (a) shows anti-ERK 1+2 immunoblots which were carried out after protein separation by electrophoresis. Lanes 1 shows untreated αT3-1 cells, lanes 2 and 3 show ± ERK 1+2 antisense oligonucleotide in lipofectamine vesicles.

Figure 7.2 b shows the corresponding [^{3}H]-AA release data for αT3-1 cells from the same transfection as panel (a) incubated with nil or 100 nM LHRH for 15 min. Each value is the mean ± SEM where n=4. Basal levels of [^{3}H]-AA release were not seen to be significantly different between sham transfected and untreated αT3-1 cells.

Figure 7.4


basal
LHRH
[3H]arachidonic acid release
(% of total incorporated in cells)
4
3
2
1
0
- ERK antisense
oligonucleotides
+ ERK antisense
oligonucleotides

Chapter 8

Overview

The luteinising hormone-releasing hormone (LHRH) receptor is a member of the rhodopsin family of seven transmembrane-spanning domain, G-protein coupled receptors. To date the predominant signalling pathways that have been described in response to LHRH receptor activation, are phosphoinositide hydrolysis and Ca^{2+} mobilisation following activation of phospholipase β (PLCβ) via a heterotrimeric G-proteins of the $G_{q/11}$ class [Anderson et al, 1993; Davidson et al, 1994a; Davidson et al, 1994b; Stojilkovic et al, 1994]. In this study we have investigated the potential activation by the LHRH receptor of a key signalling enzyme, the mitogen-activated protein (MAP) kinase. At the onset of this study, there was little evidence implicating MAP kinase in G-protein-coupled receptor signalling cascades and most literature centred on the role of MAP kinases in growth factor tyrosine kinase-mediated signalling [Cobb et al, 1991; Sturgill & Wu, 1992]. Consequently we devised an *in vitro* kinase activity assay [Sim et al, 1994], to investigate whether a G protein-coupled receptor, such as the LHRH receptor, could lead to MAP kinase activation in the αT3-1 mouse clonal gonadotroph cell line (which endogenously expresses the LHRH receptor), in anterior pituitary tissue or in COS 7 and CHO host cells, transfected with wild type and mutated LHRH receptors (tCOS 7 and tCHO).

In the αT3-1 cell line, the LHRH receptor was shown to bring about a marked and sustained activation of MAP kinase (Chapter 3). These data from the *in vitro* kinase assay were confirmed by anti-ERK 1 and 2 immunoblots which showed an LHRH-induced, phosphorylation-dependent decrease in electrophoretic mobility on SDS-PAGE gels of p42 and to a lesser extent of p44 MAP kinase. LHRH-induced MAP kinase activation could be partially mimicked by the phorbol ester, 12,13-phorbol dibutyrate (PDBu) and was abolished in cells where phorbol-ester sensitive PKC isoforms had been downregulated by chronic exposure to PDBu. Further investigations revealed LHRH- and PDBu-induced MAP kinase activation was sensitive to a number of selective PKC inhibitors including GF109203X, Ro-31 8220 and H7. These data suggested an obligatory role for PKC in LHRH-induced MAP kinase activation, although LHRH-evoked responses may rely on a different isoform or subset of isoforms of PKC than those activated by phorbol ester, as evident from the varying IC_{50}s observed on inhibition of MAP kinase responses evoked by these two agonists. This pharmacological profile is consistent with a novel or modified

PKC isoform [Sim et al, 1994] which has been associated with LHRH-induced PLD activation in αT3-1 cells and with PLA_2 activity associated with LHRH priming in pro-oestrous pituitary tissue [Fennell et al, 1994; Thomson et al, 1994]. Tyrosine phosphorylation may also be an important mediator of this response as LHRH-induced MAP kinase activity was additionally sensitive to the tyrosine kinase inhibitors genistein, piceatannol and MDC and could be partially mimicked by the tyrosine phosphatase inhibitor pervanadate. Furthermore LHRH-induced MAP kinase phosphorylation (and in pilot experiments using the activity assay; not shown) was prevented by PLC inhibitors. These findings concur with the pathways recently proposed for MAP kinase activation downstream of a number of G_q-coupled receptors (for example M_1 muscarinic) where phosphoinositide turnover and PKC activation also appear to be an upstream requirement in this response [Buhl et al, 1995; Gupta et al, 1992; Hawes et al, 1995]. Interestingly LHRH-evoked MAP kinase activity is additionally sensitive to pertussis toxin and could be partially mimicked (in a pertussis toxin-sensitive manner) by a reported G_i activating peptide, mastoparan. We therefore propose a hypothesis where the LHRH receptor is capable of activating MAP kinase by a $G\alpha_{q/11}$- and PKC-dependent mechanism or a $G\alpha_{i/o}$-dependent pathway, but to see the full extent of this response a dual signalling input through $G\alpha_{q/11}$ and $G\alpha_{i/o}$ is required. This dual mechanism is relatively unusual in G-protein-mediated MAP kinase activation at present. However a small number of examples such as the PAF receptor [Honda et al, 1994; Takano et al, 1994], the thrombin receptor [Kahan et al, 1992b; Kramer et al, 1995; Seuwen et al, 1990] and the $C5_a$ receptor [Buhl et al, 1995] would appear to require input from both pertussis toxin-sensitive and insensitive G-proteins. This type of dual input requirement to bring about a maximal response, may become more widely apparent in the future.

The ability of the LHRH receptor to phosphorylate and activate MAP kinase has further been demonstrated by expression of the wild type mouse LHRH receptor in COS 7 and CHO host cells (Chapter 5). This response was also sensitive to PKC inhibition, although the unusual sensitivity to the PKC inhibitor H7 was not observed here suggesting that the LHRH receptor can utilise other PKC isoforms to mediate MAP kinase activation. This response was also sensitive to pertussis toxin,

highlighting the importance of the dual input mechanism in LHRH signalling and confirming that this was not a cell-specific artefact. We have also shown that the ability to bring about a strong PLC response and cause translocation of PKC is not always sufficient to bring about MAP kinase activation (Chapter 5). Following expression of the wild type mouse LHRH receptor or the 5-HT_{2C} (another rhodopsin family member) or the mGlu 1a receptor (a more distantly related receptor) in COS 7 cells, only the LHRH receptor was able to induce a strong MAP kinase response, whilst sustained inositol phosphate formation and translocation of PDBu binding sites to the membrane fraction was observed for all these receptors. The ability to cause MAP kinase activation could not be directly attributed to one of the unusual structural motifs within this receptor, the natural reciprocal mutation between Asn and Asp in TMD II and TMD VII [Zhou et al, 1994]. Mutation of the mouse LHRH receptor to restore the more typically observed residues in these domains, and transient transfection of these receptors in COS 7 host cells, resulted in attenuated MAP kinase responses. However significant activation was observed and the magnitude of the response was proportionate to the reduced IP responses observed in mutant compared to wild type receptors (Chapter 5). From these studies alone it is impossible to explain what property of the LHRH receptor (but not all PLC-coupled/ PKC activating receptors), confers the ability to particularly strongly activate MAP kinase, although it is likely to be reliant upon the ability to interact with multiple G-proteins or possibly other atypical regions within the receptor.

The LHRH receptor is unusual in that as well as the atypical structural motifs found in the LHRH receptor [Zhou et al, 1994; Davidson et al, 1994], it displays a unique 'self priming' phenomenon, whereby LHRH causes an augmentation of secretory responsiveness following prior exposure to LHRH [Fink et al, 1988]. There is a notable correlation between MAP kinase targets and the changes expressed in LHRH priming [Mitchell et al, 1994]. Accordingly, having clearly established an LHRH-induced activation of MAP kinase in a clonal pituitary cell line, it was of great interest to see that this response was apparent in intact anterior pituitary tissue. In anterior pituitary tissue from pro-oestrous rats, LHRH caused concentration-dependent activation of MAP kinase, furthermore the magnitude of this response correlated with that of LHRH priming on various days of the oestrous cycle. This

response could also be mimicked with phorbol esters and was inhibited (in a similar manner to LHRH-induced activity in αT3-1 cells) by PKC inhibitors (Chapter 6). Taken together this demonstrates an association of MAP kinase with the priming effect of LHRH [Fink, 1988] and suggests that it may have a significant role in the induction and development of this phenomenon. Definitive test awaits specific inhibitors of MAP kinase or its activation pathways or its deletion or overexpression by antisense or expression of mutant constructs. The regulated step determining the onset of the priming phenomenon probably lies upstream of MAP kinase but downstream of the LHRH receptor as phorbol ester was still effective in tissue where priming was not observed (but which had adequate LHRH receptor numbers) [Mitchell et al, 1994]. At present we have found no evidence to support the hypothesis that in response to LHRH, PLA_2 represents an important cellular target of MAP kinase (Chapter 7). PLA_2 activation may also have been associated with the priming phenomenon as both of these responses are implicated in this effect [Mitchell et al, 1994; Thomson et al, 1994] and are observed in αT3-1 cells (Chapter 3 and 7). Both PLA_2 and ERK responses have been associated with platelet activation in response to thrombin; however these responses were also unconnected in platelet activation [Kramer, personal communication]. Further investigation revealed that p38 (not ERK) was both activated in response to thrombin and responsible for PLA_2 activation, therefore it would obviously be interesting to investigate the effects on MAP kinase and PLA_2 activation of expression of antisense oligonucleotides against p38 in αT3-1 cells, especially since recent literature includes several examples of G-protein activation (including notably activated G_{12} and G_{13} subunits [Vara Presad et al 1995]) of p38 and the JNK/SAPK family of MAP kinases [Kramer et al, 1995]. Additionally the importance of ovarian steroids to the ability of the LHRH receptor to cause activation of MAP kinase has been demonstrated. In αT3-1 cells grown in steroid-free medium, a predominant LHRH-induction of MAP kinase activity was only observed in the additional presence of oestrogen (Chapter 6). Taken together with the observations that in female rat anterior pituitaries MAP kinase activity was co-ordinately regulated with the oestrous cycle (and thus cyclic changes in oestrogen exposure), it suggests that the ability of this receptor to interact with MAP kinase may not necessarily be an intrinsic property of this receptor.

Alternatively whilst the LHRH receptor can cause a strong activation of MAP kinase at higher concentrations in cultured cells (i.e. αT3-1, tCOS 7 and tCHO) under physiological conditions *in vivo*, this response is only significant when a number of criteria are fulfilled (including prior exposure to steroid hormones), which further suggest that the MAP kinase response may be critical to the 'self priming phenomenon'. This observation could obviously have significance in a number of clinical applications such as the development of novel contraceptives or treatments for infertility. Furthermore any agent which could selectively modify LHRH receptor activation of a particular signalling pathway such as the MAP kinase cascade, could offer therapeutic advantages in tumours associated with the presence of low affinity LHRH receptors [Emons et al, 1993].

It has become apparent in as a result of these studies that the LHRH receptor joins an increasing number of receptors which are promiscuous in their interactions with G-proteins [Milligan, 1993; Offermans & Schultz, 1994; Chapters 3, 4, 5 and 7]. In addition to the well documented functional coupling to $G_{q/11}$ proteins [Anderson et al, 1993; Shah & Milligan, 1994; Stojilkovic et al, 1994], the data concerning the inhibition of the MAP kinase response by pertussis toxin (Chapter 3 and 5) and partial mimicry by mastoparan, in a pertussis toxin-sensitive manner (Chapter 3), provided the first evidence for $G_{i/o}$-mediated signal transduction by the LHRH receptor. Additionally we have clearly demonstrated a pertussis toxin-sensitive inhibition of forskolin- and PACAP-induced cAMP formation in αT3-1 cells (Chapter 4). This inhibition may additionally be dependent on other factors such as intracellular Ca^{2+} concentrations or the phosphorylation state of the cells, all of which are potentially altered following LHRH activation of the receptor. However we found no evidence to suggest that this effect was directly due to LHRH-induced Ca^{2+} mobilisation or PKC activation. Furthermore in experiments examining LHRH activation of PLA_2 we have provided the first evidence suggesting that the LHRH receptor can also interact with the G_{12} class of G-proteins in αT3-1 cells (Chapter 7).

Thus it appears (as is the case for many receptors) that it is no longer correct to regard the LHRH receptor, as a receptor coupled to one G-protein which activates one signalling cascade. It is evident from the results presented here, that the LHRH

receptor can activate a variety of different G-proteins to generate multiple intracellular signals. Over the course of this study evidence has accumulated to support this concept in many other receptors [Offermans & Shultz, 1994; Milligan, 1993; Gudermann et al 1996]. It is also an intriguing possibility that G-protein-coupled receptors can intercept intracellular signalling cascades classically associated with growth factor- and cytokine-stimulated responses, an avenue of LHRH receptor signalling which may be of interest to investigate in the future.

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PUBLICATIONS

ACTIVATION OF MAP KINASE ASSOCIATED WITH THE PRIMING EFFECT OF LHRH

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ABSTRACT

A MAP kinase activity assay was developed to determine whether the LHRH receptor could activate this enzyme (particularly during LHRH priming). In anterior pituitary tissue from pro-oestrous rats LHRH caused concentration-dependent activation of MAP kinase after 5-10 min and continued for up to 60 min of incubation. The magnitude of this response correlated with that of LHRH priming on various days of the oestrous cycle but not with the magnitude of 1st hour (unprimed) LHRH-induced LH release. The response to LHRH was mimicked by a phorbol ester but not by ionomycin and was blocked with high potency by GF 109203X but not by H7 (in a similar manner to the PKC species that mediates LHRH priming). Neither the tyrosine kinase inhibitor lavendustin A nor the protein synthesis inhibitor cycloheximide blocked LHRH-induced MAP kinase activation. The possible functional significance of MAP kinase activation in gonadotrophs is considered with respect to LHRH priming.

INTRODUCTION

The priming effect of LHRH is an augmentation of the secretory responsiveness of gonadotrophs which develops fully within about 30-45 min of exposure to LHRH and is dependent on prior exposure to gonadal steroids (and perhaps other factors) (Fink, 1988). The induction of priming is mediated by a form of protein kinase C (PKC) (Johnson *et al.* 1992) with distinctive properties similar to those of an apparently novel species recently characterized in anterior pituitary tissue (Ison *et al.* 1993). Downstream of this PKC, activation of phospholipase A_2 (PLA_2) is essential for induction of the phenomenon (Thomson *et al.* 1993a). Inhibitors of transcription, translation and microfilament integrity disrupt LHRH priming (Pickering & Fink, 1976; 1979) which also involves ultrastructural changes in cytoskeletal/secretory elements (Lewis *et al.* 1979).

The mitogen-activated protein kinase (MAP kinase) family is a group of serine/threonine protein kinases which are believed to participate in mitogen-induced transcription and translation (Cobb *et al.* 1991). The consensus target motif for MAP kinase (Clark-Lewis *et al.* 1991) has been recognised in a number of cellular proteins including jun and myc, p90 ribosomal S6 protein kinase ($p90^{rsk}$), high molecular weight PLA_2 and microtubule-associated protein 2 (MAP-2) all of which appear to be targets *in vivo* (Sturgill *et al.* 1988; Alverez *et al.* 1991; Hoshi *et al.* 1992; Seth *et al.* 1992; Lin *et al.* 1993; Nguyen *et al.* 1993). In view of the concordance between the targets of MAP kinase and the changes brought about in LHRH priming, we investigated the possibility that this enzyme may be activated and play a functional role during LHRH priming.

MATERIALS AND METHODS

Cob Wistar rats were maintained and vaginal smears examined as described previously (Johnson *et al.* 1992; Thomson *et al.* 1993b). Bilateral ovariectomy was performed under halothane anaesthesia and the rats were left for a period of 4 weeks before experiments. All chemicals were obtained from LC Laboratories/Calbiochem, Novabiochem, Nottingham, Notts, U.K., or Sigma Chemical Co., Poole, Dorset, U.K. The MAP kinase substrate peptide was prepared by solid phase synthesis. ATP-$\gamma[^{35}S]$ (specific activity 1198 Ci/mmol) was from NEN, Dreieich, Germany. Statistical analyses were carried out using the Mann-Whitney U-test and curve-fitting was performed by the program P.fit (Biosoft, Cambridge, Cambs, U.K.), as described previously (Thomson *et al.* 1993b).

Hormone release experiments: Anterior pituitary glands were removed between 11.00 and 12.00 h on specified days of the oestrous cycle and experiments carried out as described previously (Johnson *et al.* 1992; Thomson *et al.* 1993b), with consecutive hourly incubations in the presence of LHRH (1 nmol/l). Medium was radioimmunoassayed for LH using NIADDK rat LH-RP2 as standard.

MAP kinase assay: Hemipituitaries (3 per 2 ml) were pre-incubated for a recovery period of 30 min in minimal essential medium (37°C, under 95% O_2/5% CO_2) and then any inhibitory drugs were added with new medium. Five min later LHRH (0.2 - 100 nmol/l) 4β-phorbol 12,13-dibutyrate (PDBu, 1 μmol/l), or ionomycin (30 μmol/l) were added for 15 min unless otherwise stated. Where required, dimethylformamide was present at 0.03% (v/v). This concentration of vehicle was shown to have no effect on MAP kinase activity. Tissue was homogenized in 100 μl of ice-cold homogenization medium: 20 mmol Tris/l; 12 mmol EDTA/l, 50 mmol 2-mercaptoethanol (EtSH)/l, 1 mmol phenylmethyl-sulphonylfluoride/l, pH 7.4 with HCl, containing 0.01% (w/v) leupeptin, 20 μmol trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane/l, 2 mg aprotinin/l, 1 μmol pepstatin/l, 2.5 mmol Na_3VO_4/l, 62.5 mmol β-glycerophosphate/l and 200 nmol okadaic acid/l. The supernatant fraction was collected after centrifugation. The 25 μl assay finally contained 20 mmol Tris/l, 0.5 mmol EGTA/l, 2.4 mmol EDTA/l, 20 mmol $MgCl_2$/l, pH 7.5 with HCl, with 50 μmol NaF/l, 50 mmol EtSH/l and 200 nmol okadaic acid/l as well as the other peptidase and phosphatase inhibitors diluted 1 in 5 from their concentrations in the 5 μl aliquot of cytosolic extract added to the assay. The selective MAP kinase substrate peptide (APRTPGGRR; Clark-Lewis *et al.* 1991) was used at 2 mmol/l. Substrate-free blanks were determined in all cases. Assays were started with 100 μmol ATP-γS/l (containing 0.58 μCi ATP-$\gamma[^{35}S]$/tube) and brief centrifugation. Tubes were incubated for 40 min at 30°C (within the linear range of the assay). Incubations were stopped by addition of 25 μl ice-cold trichloroacetic acid (120 g/l) and 10 μl bovine serum albumin (20 g/l). After 15 min on ice, samples were centrifuged (12000 g for 5 min at 4°C), then 30 μl aliquots were spotted onto 4 cm^2 pieces of Whatman P81 paper. Papers were washed for 3x2 min in 10 ml of H_3PO_4 (75 mmol/l) and dried before scintillation counting. Specific MAP kinase activity was defined by subtracting the substrate-free blanks which were generally less than 25% of values with substrate and were unaltered by any of the present treatments.

Immunoprecipitation: Protein G-Sepharose-4B (100 μl packed gel) was washed and resuspended in 140 μl 25 mmol Tris/l, 150 mmol NaCl/l, 0.04% (v/v) Nonidet-P40, 0.25% (w/v) gelatin, pH 7.4 with HCl. MAP kinase antibody (Zymed Z033, specific for p44 and p42 species; 50 μg), or a control anti-β tubulin antibody (Amersham N357) was added to the gel and incubated for 2 h at room temperature with rolling. The derivatized gel was washed twice and resuspended in homogenization buffer to a final volume of 140 μl. Fifty μl aliquots, together with 50 μl cytosolic extract were incubated for 18 h at 4°C with rolling. After brief centrifugation, the supernatant was removed, the gel washed once

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and resuspended to 100 µl. Aliquots (10 µl) of supernatant, of resuspended gel and of cytosolic extract (diluted 1:1) were assayed.

RESULTS

It is clear that majority of the enzymic activity assayed represented authentic p44/p42 MAP kinase since Table 1 shows that 70-80% of either control or LHRH-stimulated activity was immunoprecipitated by the anti-MAP kinase gel, whereas less than 20% was bound by the control reagent.

TABLE 1: Immunoprecipitation of MAP kinase activity with protein G-Sepharose-4B derivatized reagents.

Condition	Antibody reagent	MAP kinase activity recovered ($\times 10^3$ dpm per assay) Supernatant	Pellet
a. Control	Nil	1.84 ± 0.26	—
	Anti-MAP kinase	0.56 ± 0.08	1.39 ± 0.18
	Anti-β-tubulin	1.62 ± 0.32	0.40 ± 0.08
b. LHRH (100 nmol/l)	Nil	2.93 ± 0.32	—
	Anti-MAP kinase	0.55 ± 0.18	2.09 ± 0.34
	Anti-β-tubulin	2.58 ± 0.36	0.64 ± 0.20

Values are the means ± S.E.M. from 3 determinations.

In pro-oestrous anterior pituitary tissue but not that from oestrous or ovariectomized rats, LHRH (1 nmol/l) induced a marked increase in MAP kinase activity after a brief delay (in the order of 5 min), peaking at around 15 min and then declining gradually to 60 min (Fig. 1). Unstimulated tissue showed no significant change in MAP kinase activity through 60 min incubation (data not shown). The response of pro-oestrous tissue to LHRH (15 min incubation) was statistically significant at 1-100 nmol LHRH/l and showed an EC_{50} value of 0.53 ± 0.19 nmol/l (Fig. 2). Tissue from ovariectomized rats showed no response to LHRH even up to a concentration of 100 nmol/l. The responses of anterior pituitary tissue from rats on different days of the oestrous cycle and from ovariectomized rats were compared in terms of acute LHRH-induced LH release, LHRH priming and LHRH-induced MAP kinase activation (Table 2). The 1st hour (unprimed) release of LH induced by LHRH (1 nmol/l) was greatest in tissue from pro-oestrous followed by ovariectomized and oestrous rats. In contrast, LHRH priming (the increment in LH release in a 2nd hour with LHRH) was greatest in tissue from pro-oestrous and dioestrous rats. LHRH (100 nmol/l) caused significant activation of MAP kinase only in tissue from pro-oestrous and dioestrous rats.

Experiments were carried out to assess whether either of the obvious consequences of phosphoinositide hydrolysis evoked by the LHRH receptor, ie Ca^{2+} mobilization and PKC activation, could mimic the LHRH-induced activation of MAP kinase. Stimuli were deliberately presented at concentrations giving near-maximal responses. Despite causing marked release of LH from pro-oestrous anterior pituitary tissue (Johnson *et al.* 1992), ionomycin (30 µmol/l) failed to increase MAP kinase activity (84 ± 12% of control at 15 min; mean ± S.E.M. of 4 determinations). Although facilitation of LHRH-induced mobilisation of intracellular Ca^{2+} is one of the downstream consequences of

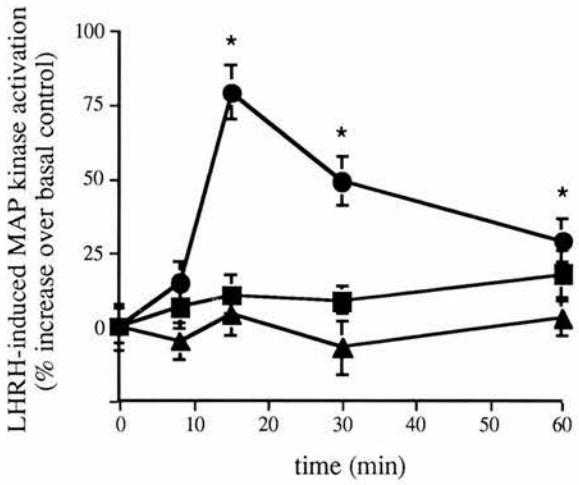

FIGURE 1. Time course of LHRH (1 nmol/l) activation of MAP kinase in anterior pituitary tissue from pro-oestrous (●), oestrous (▲) and ovariectomized (■) rats. All basal activity at zero time was within 850-1000 dpm per assay. Values are means ± S.E.M. from 5-6 determinations. *p < 0.05 compared with activity at zero time.

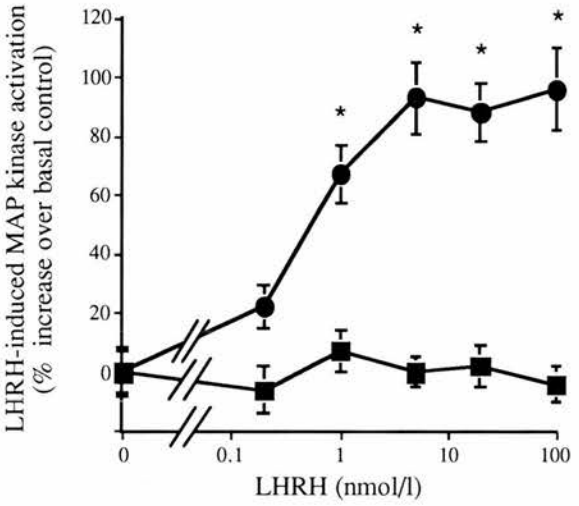

FIGURE 2. Concentration-dependence of LHRH activation of MAP kinase in anterior pituitary tissue from pro-oestrous (●) and ovariectomized (■) rats are shown. Values are means ± S.E.M. from 5 determinations. *p < 0.05 compared with basal activity.

LHRH priming (Mitchell *et al.* 1988), this result is consistent with evidence (Johnson *et al.* 1992) that elevation of Ca^{2+} does not elicit induction of the phenomenon, but rather that it is critically dependent upon a form of PKC. Indeed, PDBu (1 µmol/l) activated MAP kinase in pro-oestrous tissue with a maximal effect after approximately 15 min incubation (Table 3). A similar effect of PDBu was seen in tissue from ovariectomized rats (Table 3); acting as a positive control in this tissue where LHRH failed to elicit either priming of LH release or activation of MAP kinase (Table 2, Fig. 2) and indicating that PKCs other than those transducing signals from the LHRH receptor can activate

TABLE 2: LHRH-induced LH release, priming and MAP kinase activation in anterior pituitary tissue taken from different stages of the oestrous cycle.

Tissue	LHRH-induced LH release (μg/l) 1st hour	2nd hour	LHRH-induced MAP kinase activation ($x10^3$ dpm/assay
Oestrous	13.6 ± 1.4*	28.0 ± 2.5*	0.06 ± 0.09
Metoestrous	4.3 ± 1.0*	25.5 ± 3.0*	0.11 ± 0.14
Dioestrous	6.1 ± 0.9*	57.9 ± 4.8*	0.71 ± 0.12*
Pro-oestrous	29.2 ± 1.8*	142.3 ± 16.7*	0.94 ± 0.10*
Ovariectomized	14.8 ± 1.6*	15.4 ± 2.0*	0.10 ± 0.12

LHRH was used at a concentration of 1 nmol/l for the LH release experiments and 100 nmol/l for the MAP kinase activation experiments. Basal release of LH from tissue from all origins fell within the range 3-6 μg/l with the one exception of tissue from ovariectomized rats (14-20 μg/l). Basal MAP kinase activity showed no apparent differences in tissue from all origins, falling consistently within the range 800-950 dpm per assay. Values are means ± S.E.M. from 6-12 determinations. *$p < 0.05$ compared with corresponding basal incubations.

MAP kinase in heterogeneous pituitary tissue. Preliminary experiments carried out with the αT3-1 gonadotroph cell line have also demonstrated LHRH-induced activation of MAP kinase through a PKC-dependent route which is dependent on previous exposure to steroids (Sim *et al.* 1993).

The selective PKC inhibitor GF 109203X (Toullec *et al.* 1991) inhibited the MAP kinase activation evoked by LHRH (100 nmol/l) with an IC_{50} of 2.1 ± 0.9 μmol/l and that evoked by PDBu (1 μmol/l with an IC_{50} of 1.2 ± 0.4 μmol/l (means ± S.E.M., n = 4-6). LHRH priming elicited by 1 nmol LHRH/l was inhibited by GF 109203X with an IC_{50} of 3.9 ± 1.6 μmol/l (mean ± S.E.M., n = 5), whereas 1st hour (unprimed) responses were unaffected at 10 μmol GF 109203X/l. Neither the PKC inhibitor H7 (Hidaka *et al.* 1984) nor the tyrosine kinase inhibitor lavendustin A (Hsu *et al.* 1991) reduced LHRH-induced MAP kinase activation (Fig. 3). At 10-30 μmol/l, H7 slightly increased the response to LHRH. None of the inhibitors affected basal MAP kinase activity. Since LHRH priming is prevented by the protein synthesis inhibitor cycloheximide (Pickering & Fink, 1979), we investigated whether it affected LHRH-induced MAP kinase activation. In the presence of 50 μmol cycloheximide/l, the activation of MAP kinase induced by 100 nmol LHRH/l in pro-oestrous tissue was unaltered (92 ± 14% of that in cycloheximide-free controls; mean ± S.E.M., n = 4), although cycloheximide alone caused a small increase in the basal MAP kinase activity measured.

DISCUSSION

Activation of MAP kinase in pro-oestrous rat anterior pituitary tissue occurs at physiologically-relevant concentrations of LHRH and is detectable from 10-60 min of incubation (Figs. 1, 2). The magnitude of LHRH-induced MAP kinase activation correlates closely with that of priming phenomenon on different days of the oestrous cycle; showing significant activation only on pro-oestrus and dioestrus when the priming phenomenon is at its greatest (Table 2). The magnitude of 1st hour (unprimed) responses to LHRH varied with a different rank order showing the greatest responses in tissue from pro-oestrous and then from oestrous/ovariectomized rats. This demonstrates an association of MAP kinase activation with the priming effect of LHRH and may suggest that this enzyme plays a significant role in development of the phenomenon. Because specific inhibitors are unavailable,

TABLE 3: Time course of phorbol ester-induced MAP kinase activation in anterior pituitary tissue.

Incubation time (min)	Phorbol 12,13-dibutyrate-induced MAP kinase activation (% increase over basal activity) Pro-oestrous	Ovariectomized
0	0 ± 7	0 ± 6
5	44 ± 4*	78 ± 11*
10	75 ± 11*	99 ± 16*
15	89 ± 14*	84 ± 12*
20	68 ± 12*	57 ± 14*
40	44 ± 5*	36 ± 10*

Values are the means ± S.E.M. from 4-6 determinations. *$p < 0.05$ compared with zero time controls.

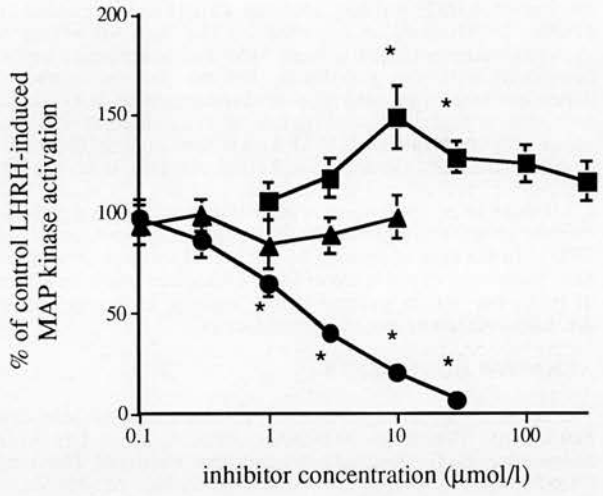

FIGURE 3: Effects of GF 109203X (●), H7 (■) and lavendustin A (▲) are shown on the MAP kinase activity induced by incubation of pro-oestrous anterior pituitary tissue with 100 nmol LHRH/l. Values are the means ± S.E.M. from 4-8 determinations. *$p < 0.05$ compared with drug-free controls.

it is difficult as yet to directly probe the functional contribution of MAP kinase to LHRH priming. Nevertheless, there is a remarkable correlation between the targets of MAP kinases and the array of cellular events known to be involved in priming. Relevant targets may include jun and myc, RNA polymerase II, $p90^{rsk}$, high molecular weight PLA_2 and MAP-2 and it has been reported that a cdc2-like kinase with a similar substrate motif phosphorylates neurofilaments (Shetty *et al.* 1993). The priming phenomenon is dependent upon protein synthesis, activation of PLA_2, the integrity of microfilaments and involves ultrastructural changes in the marginal presentation of secretory granules in gonadotrophs. Phosphorylation of MAP-2 protein by MAP kinase is reported to disrupt microtubule-microfilament interaction (Hoshi *et al.* 1992) which may lead to facilitated stimulus-secretion coupling (Malaisse *et al.* 1975).

The induction of the priming phenomenon is dependent upon an apparently novel H7-resistant species of PKC which acts through a protein synthesis-dependent step to activate PLA_2 (Johnson *et al.* 1992; Thomson & Mitchell, 1993; Thomson *et al.* 1993a). It is possible that MAP kinase is an intermediary in this sequence because its activation by LHRH (like priming) is dependent on a PKC which is sensitive to PKC inhibitors including GF 109203X but not to H7 (Fig. 3; Johnson *et al.* 1992; 1993; Thomson *et al.* 1993b; Ison *et al.* 1993). The evidence here for a modest facilitation of the LHRH response by H7 is consistent with the possibility of reciprocal regulation by H7-resistant and H7-sensitive forms of PKC (or other kinases) (Fig. 3). There is evidence for such dual regulation of voltage-sensitive Ca^{2+} channels by different forms of PKC (Johnson *et al.* 1989; MacEwan *et al.* 1991; Johnson *et al.* 1993). Other forms of PKC can clearly activate MAP kinase in anterior pituitary tissue because PDBu can still activate the enzyme in tissue where LHRH is ineffective (Table 2). Both α and β isoforms of PKC can activate raf and thence MAP kinase (Sozeri *et al.* 1992; Dent *et al.* 1992), although both PKC α and β are H7-sensitive (Ison *et al.* 1993; Thomson *et al.* 1993b). The regulated step underlying the varying ability of LHRH to activate MAP kinase may well be upstream of MAP kinase itself but downstream of the LHRH receptor since PDBu was still effective in tissue which does not respond to LHRH but has adequate LHRH receptor numbers (Table 3; Mitchell *et al.* 1988). The lack of effect of cycloheximide on LHRH-induced MAP kinase activation here is consistent with the possibility that the protein synthesis-dependent step in priming may be downstream of MAP kinase activation or that these steps may occur in parallel rather than in series. The mechanism of MAP kinase activation by G protein-coupled receptors (such as the LHRH receptor here, the M_1 muscarinic receptor and the thrombin receptor; Ely *et al.* 1990; L'Allemain *et al.* 1992; Qian *et al.* 1993) may involve a pathway distinct from that for growth factor receptors (Lange-Carter *et al.* 1993). In the case of priming by the LHRH receptor at least, we have shown the dependence of MAP kinase activation on a form of PKC, but not on tyrosine kinase activity, as is required for activation of the enzyme by growth factors.

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Rapid paper

Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein kinase C and a pertussis toxin-sensitive G protein

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Abstract

The LHRH receptor in αT3-1 gonadotrope cells was shown to bring about a marked and sustained activation of MAP kinase. This response was prevented by protein kinase C inhibition or down-regulation and could be partially mimicked by phorbol ester. Additional evidence for inhibition of this response by pertussis toxin and partial mimicry by mastoparan (in a pertussis toxin-sensitive manner) provides the first evidence for G_i/G_o-mediated signal transduction by the LHRH receptor. This dual mechanism of MAP kinase activation appears to be exceptional amongst the G protein-linked receptors that have been investigated.

Keywords: MAP kinase; Protein kinase C; G protein; LHRH receptor

1. Introduction

Mitogen-activated protein kinases (MAP kinases) are activated in response to various external stimuli, and regulate a diverse array of cellular events which are important not only in cell cycle regulation but also in the transduction of non-mitogenic signals, sometimes in highly-differentiated cell types (Davis, 1993). MAP kinases are activated by phosphorylation on both tyrosine and threonine residues by a family of dual specificity kinases which in turn are activated by several distinct kinases including *raf* isoforms, MEK kinase, and *mos*. Whilst tyrosine kinase growth factor receptors have been shown to activate MAP kinase in a multistep process that involves *raf*, *ras*, adaptor proteins and GTP exchange factors, the pathways of activation utilised by G protein-linked receptors are less well understood.

Luteinising hormone-releasing hormone (LHRH) controls gonadotropin release from the anterior pituitary gland and regulates gonadal function. The LHRH receptor is a seven transmembrane domain, G protein-linked receptor (Tsutsumi et al., 1992), activation of which (by means of $G_{q/11}$ (Hsieh and Martin, 1992; Shah and Milligan, 1994)) results in phosphoinositide hydrolysis, Ca^{2+} mobilisation and activation of PKC (Stojilkovic et al., 1994). The present experiments utilise the αT3-1 gonadotrope cell line (Horn et al., 1991) to investigate the potential activation of MAP kinase by the LHRH receptor and the mechanisms responsible.

2. Materials and methods

2.1. Chemicals and data analysis

All chemicals were obtained from Calbiochem, Nottingham, Notts, UK, or Sigma Chemical Co., Poole, Dorset, UK. The MAP kinase substrate peptide was prepared by solid phase synthesis. [γ-^{35}S]ATP and [^{3}H]inositol (specific activity 1198 and 17 Ci/mmol respectively) were from NEN, Dreieich,

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Germany. Statistical analyses were carried out using the Mann-Whitney *U*-test and curve-fitting was performed by the program P.fit (Biosoft, Cambridge, Cambs, UK).

2.2. Cytosolic MAP kinase assay

αT3-1 cells were grown (in DMEM with 0.11 g/l Na pyruvate, 100 U/ml each of penicillin/streptomycin and 10% foetal calf serum) to confluency in sets of 25-cm^2 flasks and quiesced with serum-free medium for 24 h prior to assay. Agonists were present for 10 min (unless otherwise stated), with antagonists additionally for 1 min beforehand. Vehicle (dimethylformamide 0.03% (v/v)) was present in controls and had no effect on MAP kinase activity. After stimulation, the medium was aspirated, and cells were homogenized in 200 μl of ice-cold buffer containing EDTA, 2-mercaptoethanol (EtSH), and inhibitors of phosphatases and peptidases before assaying as described previously (Mitchell et al., 1994b). Briefly, the supernatant fraction was collected after centrifugation at 12000 $\times$ *g* for 30 min at 4°C, and 5-μl aliquots were assayed in buffer containing EGTA, EDTA, $MgCl_2$, EtSH and inhibitors of phosphatases and peptidases, in the presence of the selective MAP kinase substrate peptide (APRTPGGRR) (Mitchell et al., 1994b) and 100 μM [γ-^{35}S]ATP (containing 0.58 μCi [γ-^{35}S]ATP/tube). After 40 min at 30°C (within the linear range of the assay), incubations were stopped by addition of ice-cold trichloroacetic acid and bovine serum albumin. Following 15 min on ice, samples were centrifuged (12000 $\times$ *g* for 5 min at 4°C), then supernatant aliquots were spotted onto phosphocellulose paper squares (Whatman P81) which were washed extensively in 75 mM H_3PO_4 before scintillation counting. Specific MAP kinase activity was defined by subtracting the substrate-free blanks which were generally less than 25% of control values with substrate and were unaltered by any stimuli. The specificity of this assay for MAP kinases was monitored by immunoprecipitation with a monoclonal anti-p42/p44 antibody (Zymed Z033; Zymed Laboratories Inc., San Francisco, USA) conjugated to protein G-Sepharose 4B beads, as described previously (Mitchell et al., 1994b). In cytosolic extracts of αT3-1 cells treated with either nothing, LHRH (100 nM, 10 min) or phorbol 12,13-dibutyrate (PDBu, 300 nM, 8 min), 71 $\pm$ 12, 74 $\pm$ 10 and 67 $\pm$ 9% of the recovered activity was associated with the beads rather than the supernatant. Corresponding values using a control anti-β-tubulin monoclonal antibody of the same IgG subclass (N 357, Amersham) were 12 $\pm$ 8, 4 $\pm$ 8 and 23 $\pm$ 13%, respectively (means $\pm$ SEM, n = 3).

2.3. MAP kinase immunoblots

Phosphorylation of p42 and p44 MAP kinase was determined by the electrophoretic mobility shift assay (Alblas et al., 1993). αT3-1 cells were grown in 12-well plates and quiesced for 24 h before experiments. Following incubation with agonists/antagonists, the medium was aspirated, the cells washed in 1 ml ice-cold Hanks balanced salt solution and scraped into 500 μl SDS buffer (50 mM Tris base, 5% EtSH, 2% sodium dodecyl sulphate, pH 7.2), before heating to 100°C for 5 min and storage at −20°C. Four μl of each sample was separated by electrophoresis on 20% homogenous gels, and proteins were electroblotted to polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a Phast-system apparatus (Pharmacia Biotech). Non-specific binding was reduced by incubating the blots in 5% BSA/PBS overnight. MAP kinases were then identified by incubating with mouse monoclonal anti-MAP kinase antibody (Z033) (diluted 1:1200), and then horseradish peroxidase conjugated-anti-mouse IgG (Scottish Antibody Production Unit, Strathclyde), in 0.25% BSA/PBS/Tween 20 before visualisation using the Enhanced Chemi-Luminescence (ECL) system (Amersham).

2.4. Inositol phosphate formation

αT3-1 cells, cultured in 12-well plates, were incubated for 16 h under 95/5% O_2/CO_2 in 0.5 ml/well Earle's Balanced Salt solution (EBSS) containing 10 mM glucose and 0.2% w/v BSA with *myo*-[^{3}H]inositol 2 μCi/well. After washing, 10 mM LiCl was added for 15 min before stimulation with LHRH for 10 min. Incubations were stopped by aspiration and the addition of 700μl cold 1.34 M trichloroacetic acid before assaying as described previously (Mitchell et al., 1994a). In brief, following centrifugation of cell homogenates at 12000 $\times$ *g* for 5 min (4°C) aliquots of supernatant were transferred to tubes containing EDTA (pH 7.0) and 1:1 1,1,2-trichloro-trifluoro-ethane:tri-*N*-octylamine for solvent extraction of labelled phospholipids. After centrifugation at 12000 $\times$ *g* for 5 min at 4°C aliquots of the upper aqueous phase were adjusted to pH 8 and hydrophilic inositol-containing compounds were separated on 1 ml columns of Dowex 1 $\times$ 8 (formate form, mesh size 200–400). Inositol phosphates were eluted in 1 M ammonium formate 0.1 M formic acid (Mitchell et al., 1994a).

3. Results

3.1. LHRH-induced MAP kinase activation and inositol phosphate formation

The potential activation of MAP kinase was investigated over a time course of 0–120 min with 100 nM LHRH (Fig. 1a). MAP kinase activity increased rapidly, reaching a peak by 10 min which represented an increase to 245 $\pm$ 9% of basal (mean $\pm$ SEM, n = 6). This elevated activity was sustained for approxi-

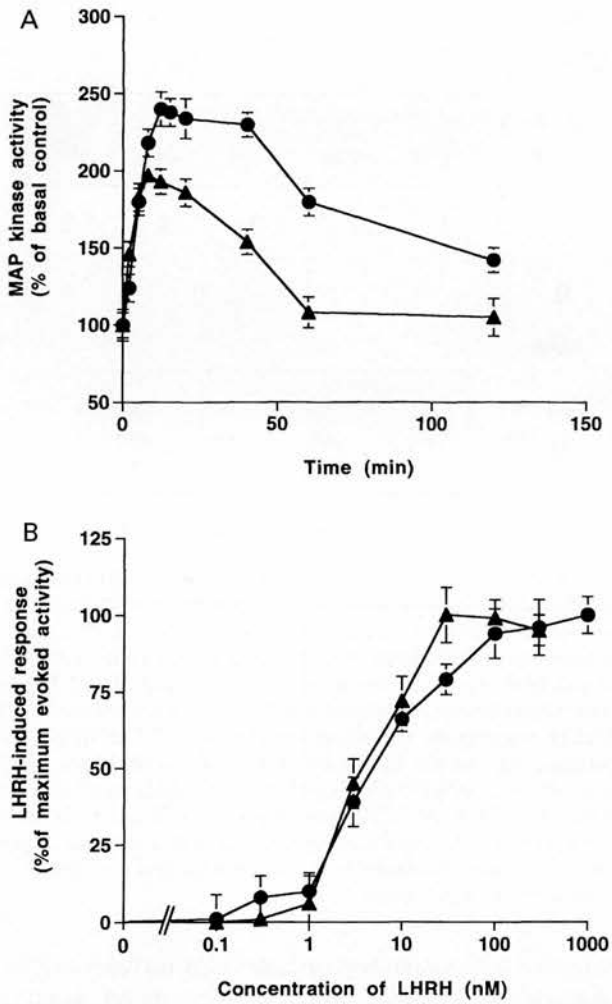

Fig. 1. Characteristics of LHRH-induced MAP kinase activation. (a) Shows the specific [^{35}S]thiophosphorylation of MAP kinase substrate peptide after different times of incubation of αT3-1 cells with 100 nM LHRH (●) or 300 nM phorbol 12,13-dibutyrate; PDBu (■). Each value is the mean ± SEM from four to six separate determinations. (b) Shows the concentration-dependence of LHRH-induced MAP kinase activation (●) and [^{3}H]inositol phosphate formation (▲) after 10 min incubation. Each value is the mean ± SEM from four to six determinations.

mately 40 min, and then gradually declined leaving a significant residual activation at 120 min. The basal (unstimulated) activity showed no detectable change through an equivalent time course. The concentration-dependence of LHRH-induced MAP kinase activity and of [^{3}H]inositol phosphate formation were closely concurrent, with both responses being nearly maximal by 100 nM and showing comparable EC_{50} values (the concentration required for 50% of maximal activation) of 3.4 ± 0.4 and 2.8 ± 0.3 nM respectively (Fig. 1b).

3.2. Involvement of protein kinase C and a pertussis toxin-sensitive component in MAP kinase activation

LHRH-induced MAP kinase activation appears to depend on PKC since the response was inhibited by GF109203X, a highly selective inhibitor of PKC (Toullec et al., 1991) with an IC_{50} value of 1.8 ± 0.2 μM (Fig. 2a), similar to its potency on a number of well-characterized PKC-mediated cellular responses (Toullec et al., 1991). At a concentration of 3 μM, GF109203X had no detectable effect on basal MAP kinase activity. In contrast to the MAP kinase response, LHRH-induced [^{3}H]inositol phosphate production was unaffected by GF109203X at concentrations up to 5 μM. Furthermore, downregulation of phorbol ester-sensitive PKC isoforms for 20 h with 300 nM PDBu caused 92 ± 10% inhibition of LHRH-induced MAP kinase activation (means ± SEM, n = 4), with no change in basal activity. Treatment with pertussis toxin (3–300 ng/ml for 18 h) showed no effect on [^{3}H]inositol phosphate formation in response to LHRH (Fig. 2b). In contrast, pertussis toxin strongly inhibited LHRH-induced MAP kinase activity (IC_{50} value, 32 ± 4 ng/ml; 75% inhibition by 300 ng/ml; Fig. 2b). Under similar conditions, treatment of αT3-1 cells with either the inactive B-subunit of pertussis toxin (O'Neill et al., 1992) or *N*-ethylmaleimide-inactivated holotoxin (Banga et al., 1987) at a concentration of 100 ng/ml, had no effect on LHRH-induced MAP kinase activity (104 ± 10 and 85 ± 13% of control LHRH responses, respectively (mean ± SEM, n = 3) compared with an inhibition to 38 ± 8% of controls caused by 100 ng/ml holotoxin (mean ± SEM, n = 6)). In order to confirm these results with an independent technique, the phosphorylation-induced gel mobility shift in immunoreactive p42 and p44 MAP kinases was monitored. Both p42 and (to a lesser extent) p44 species displayed a component of reduced electrophoretic mobility after LHRH stimulation for 10 min (Fig. 3a). This effect was concentration-dependent (Fig. 3a) and, in the case of p42 at least, was clearly inhibited by either 3 μM GF 109203X or by preincubation with 100 ng/ml pertussis toxin (Fig. 3b).

3.3. Mastoparan- and phorbol ester-induced MAP kinase activity

Mastoparan is an activator of G proteins with reported selectivity for G_i/G_o over other G proteins (Gil et al., 1991). Exposure of αT3-1 cells to mastoparan (5–15 μM) for 10 min resulted in a marked increase in MAP kinase activity (Fig. 4a). The effect was maximal at 10 μM where it represented an increase to 190 ± 9% of basal activity (mean ± SEM, n = 6). No significant effect on [^{3}H]inositol phosphate production was noted at any concentration up to 12.5 μM. Mastoparan-induced activation of MAP kinase was substantially inhibited by pertussis toxin, but not by GF 109203X (Fig. 4b), suggesting that the PKC activation required for LHRH action was not downstream of G_i/G_o but probably a result of $G_{q/11}$-mediated stimulation of phospholipase C. The PKC

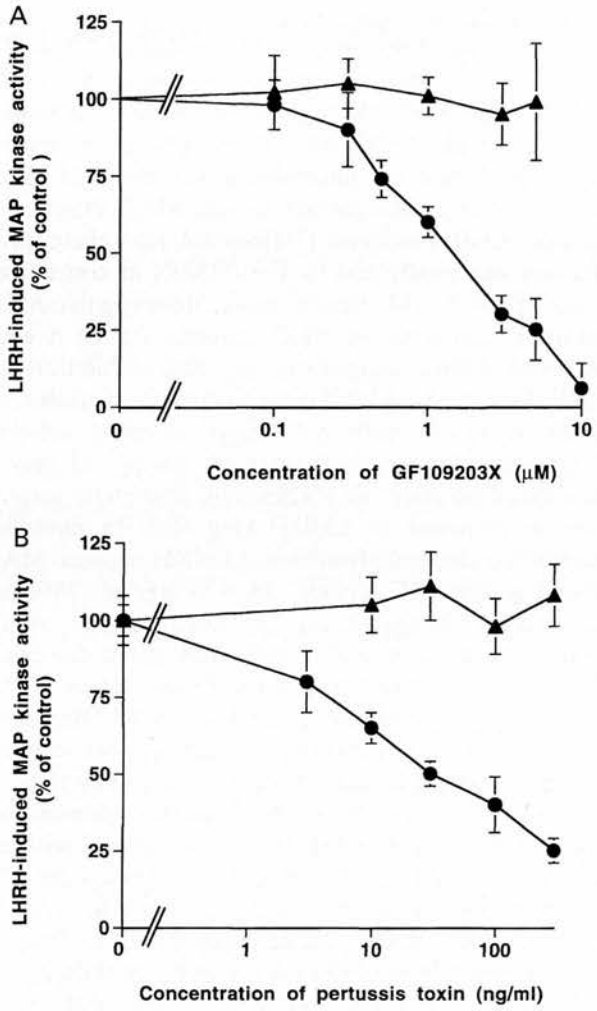

Fig. 2. Effects of the PKC inhibitor GF 109203X and pertussis toxin on LHRH-induced MAP kinase activity and [^{3}H]inositol phosphate formation. The concentration-dependence of effects on LHRH (100 nM)-induced MAP kinase substrate peptide [^{35}S]thiophosphorylation (●) and [^{3}H]inositol phosphate formation (▲) is shown in (a) for GF 109203X and in (b) for pertussis toxin. Values are the means ± SEM from four to six separate determinations. Inhibition of MAP kinase responses by ≥ 0.5 μM GF 109203X and by ≥ 10 ng/ml pertussis toxin was statistically significant ($P < 0.05$, Mann-Whitney U-test).

activator 4β-PDBu (300 nM) caused marked stimulation of MAP kinase activity (197 ± 13% of basal at 8 min; mean ± SEM, $n = 6$; Fig. 1a) which was not mimicked by the inactive isomer 4α-PDBu (300 nM, 8 min; 110 ± 9% of basal, $n = 4$). The effect of PDBu peaked slightly earlier than that of LHRH (at 8 min) but had returned to the basal level by 1 h, at which point there was still a significant residual activation by LHRH (Fig. 1a). Ionomycin (10 μM for 10 min) caused no detectable activation of MAP kinase (106 ± 7% of control, mean ± SEM, $n = 4$). PDBu-induced MAP kinase activation was prevented by GF 109203X but was unaffected by pertussis toxin (Fig. 4b), excluding the possibility that the pertussis toxin-

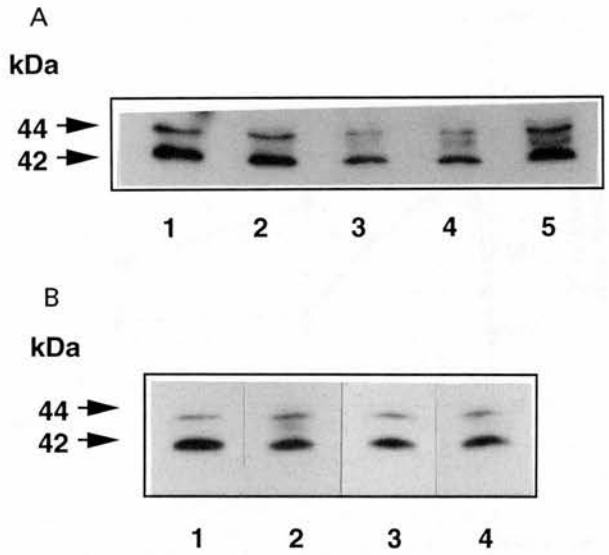

Fig. 3. Anti-MAP kinase immunoblots showing LHRH-induced mobility shifts. Anti-p42/p44 MAP kinase immunoblots were carried out after protein separation by electrophoresis. (a) Shows the concentration-dependence of LHRH-induced reduction in mobility of p42 (and to a lesser extent p44). Lanes 1, 2, 3, 4 and 5 show results from 10-min incubations with 0, 1, 10 and 100 nM and 1 μM LHRH, respectively. (b) Shows the effects of GF 109203X and pertussis toxin on the LHRH-induced mobility shift. Lanes 1, 2, 3 and 4 show control, 100 nM LHRH, 100 nM LHRH with 3 μM GF 109203X, and 100 nM LHRH after 100 ng/ml pertussis toxin for 18 h, respectively. No other staining was detected in the lanes. Each observation was replicated in the experiment and was typical of three separate experiments.

sensitive component was operated downstream of PKC (Katada et al., 1985) rather than by direct receptor interaction. The combination of mastoparan and PDBu caused greater MAP kinase activation than either alone (Fig. 4b), but from the present experiments it is not possible to make any conclusions about additivity or synergy.

4. Discussion

It has recently become clear that MAP kinases can be activated not only by growth factor and cytokine receptors but also by a variety of G protein-coupled receptors. These include receptors that interact predominantly with $G_{q/11}$ such as M_1 and M_3 muscarinic, endothelin, angiotensin II, bombesin and TRH receptors (Ely et al., 1990; Duff et al., 1992; Wang et al., 1992; Offermans et al., 1993; Pang et al., 1993; Crespo et al., 1994; Faure et al., 1994; Ohmichi et al., 1994), those that interact predominantly with $G_{i/o}$ such as α_2-C10 adrenergic, M_2 muscarinic, D_2 dopaminergic and A_1 adenosine receptors (Alblas et al., 1993; Crespo et al., 1994; Faure et al., 1994) and those that readily interact with both families of G proteins, such as thrombin and lysophosphatidic acid (LPA) receptors (Kahan et al., 1992; Hordijk et al., 1994).

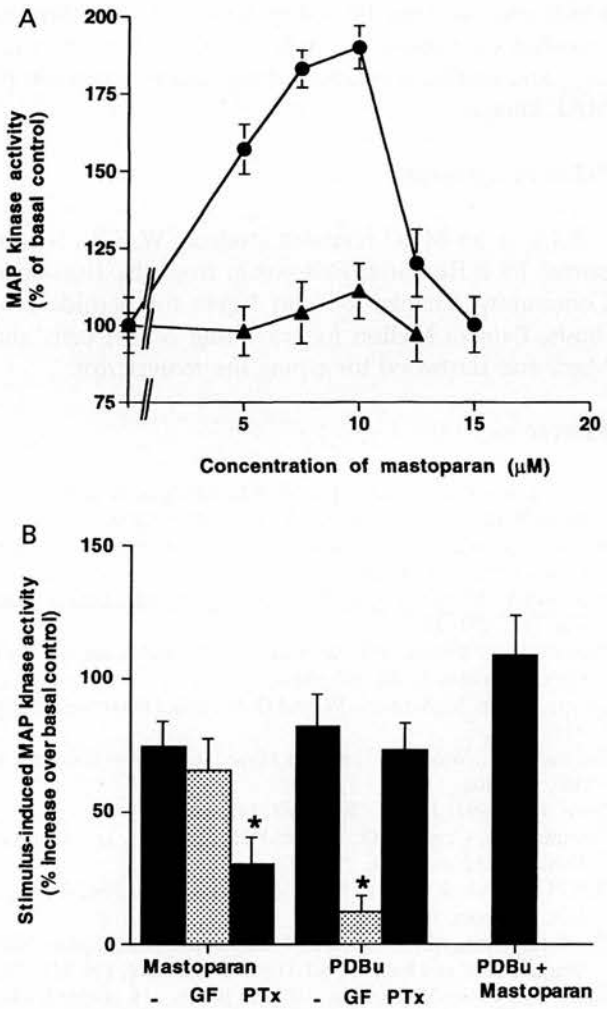

Fig. 4. Characteristics of mastoparan- and phorbol ester-induced MAP kinase activation (a) Shows the concentration-dependence of the effects of mastoparan (10 min incubation) on MAP kinase substrate peptide [^{35}S]thiophosphorylation (●) and [^{3}H]inositol phosphate formation (▲). Values are the means ± SEM from six and four separate determinations, respectively. (b) Shows the activation of MAP kinase induced by 10 min incubations with mastoparan (10 μM), phorbol 12,14-dibutyrate (PDBu; 300 nM) and the combination, as well as the effects thereon of GF 109203X (GF; 3 μM) and pertussis toxin (Ptx; 100 ng/ml for 18 h). Values are the means ± SEM from six to nine separate determinations. (*) Indicates statistically significant inhibition of responses ($P < 0.05$, Mann-Whitney U-test).

A partial PKC-dependence of M_1 muscarinic receptor- and TRH receptor-mediated MAP kinase activation has been observed in both CCL 39 and COS 1 host cells and in GH_3 cells, respectively (Kahan et al., 1992; Qian et al., 1993; Ohmichi et al., 1994). In contrast, the M_3 receptor response in SH-SY5Y cells and the bombesin receptor response in Swiss 3T3 cells were essentially prevented by PKC down-regulation or by PKC inhibitors (Offermans et al., 1993; Pang et al., 1993). It is not yet clear whether the different degrees of PKC dependence displayed by these G_q-linked receptors reflect transducer preferences of the receptors or cell-specific differences in pathway components. However, activation of MAP kinase by endothelin-1 was substantially inhibited by PKC down-regulation and was unaffected by pertussis toxin in a range of different cell types (Wang et al., 1992; Bogoyevitch et al., 1993; Cazaubon et al., 1993).

$G_{i/o}$ receptor-mediated MAP kinase activation appears not to be dependent on PKC, since the pertussis toxin-sensitive MAP kinase response to α_2-C10 receptors in Rat-1 fibroblasts was unaccompanied by any phosphoinositide hydrolysis and could not be reliably mimicked by phorbol esters (Alblas et al., 1993; Hordijk et al., 1994). Furthermore, the response to M_2 but not to M_1 muscarinic receptors expressed in COS 7 cells was inhibited by pertussis toxin, whereas the converse effect was seen with PKC down-regulation (Crespo et al., 1994). Correspondingly, the activation of MAP kinase by overexpression of a GTPase-deficient mutant G_{i2} α subunit (in Rat-1 but not COS 7 cells) was unaltered by PKC down-regulation (Gupta et al., 1992; Qian et al., 1993). However, recent evidence has suggested that $\beta\gamma$ rather than α subunits of G proteins may play the predominant role in subsequent activation of MAP kinase by a variety of G protein-linked receptors (Crespo et al., 1994; Faure et al., 1994).

In the case of thrombin and LPA receptors, MAP kinase activation is pertussis toxin-sensitive whilst other responses such as phosphoinositide hydrolysis and tyrosine phosphorylation are unaffected (Hung et al., 1992; Kahan et al., 1992; Hordijk et al., 1994). Interestingly, the MAP kinase activation caused by LPA in Rat-1 cells cannot be robustly mimicked by endothelin, despite the ability of endothelin to elicit phosphoinositide hydrolysis and tyrosine phosphorylation in these cells (Hordijk et al., 1994). As found with the receptors signalling singly through $G_{i/o}$, MAP kinase responses to LPA and thrombin do not display a consistent dependence on PKC. In Rat-1 cells, the MAP kinase response to LPA was not mimicked by phorbol esters (although it was attenuated by a general kinase inhibitor staurosporine) (Hordijk et al., 1994) and the response to thrombin was unaffected by PKC down-regulation (Gupta et al., 1992). In umbilical vein endothelial cells or CCL 39 cells, however, thrombin-evoked MAP kinase activation was attenuated by a selective PKC inhibitor, Ro 31-8220, or by PKC down-regulation (Wheeler-Jones and Pearson, 1995; Kahan et al., 1992).

Where tested, both the $G_{q/11}$- and the $G_{i/o}$-linked MAP kinase responses appear to be accompanied by increased GTP loading of p21*ras* (Ohmichi et al., 1994; Hordijk et al., 1994; Alblas et al., 1993) and are severely attenuated by the overexpression of domi-

nant negative mutant *ras* (Crespo et al., 1994). This suggests that the later stages of these responses are predominantly mediated by a conventional *ras-raf* cascade. In the case of the $G_{q/11}$-linked receptors, the site at which PKC acts is not clear. PKC may inactivate the p21*ras* GTPase-activating protein, GAP (Downward et al., 1990) or phosphorylate the *ras* target, *raf* (Kolch et al., 1993). Although TRH promotes both GTP loading of p21*ras* and phosphorylation of *raf-1*, these effects are reported to be PKC-independent but accompanied by tyrosine phosphorylation of a potential regulator of *ras* activation, *shc* (Ohmichi et al., 1994). The precise role of PKC in $G_{q/11}$-linked receptor activation of MAP kinase therefore remains unclear.

The remarkable aspect of MAP kinase activation by the LHRH receptor is that it depends strongly on pathways characteristic of both $G_{q/11}$- and $G_{i/o}$-linked receptors, in being both highly dependent on PKC and sensitive to pertussis toxin (Fig. 2). From the data with mastoparan and PDBu, it appears that either $G_{i/o}$ or PKC can elicit MAP kinase activation. Although the precise way these signals integrate in compiling the LHRH response is unclear, it is possible that PKC may exert a facilitatory action on the $G_{i/o}$-mediated signal either at the level of p21*ras* GTP loading or at the level of *raf*.

Interestingly, until the present study, there has been no clear evidence that the LHRH receptor can interact with any G protein other than $G_{q/11}$ (Fig. 2; Hsieh and Martin, 1992; Shah and Milligan, 1994; Stojilkovic et al., 1994). Only one isolated report has described partial sensitivity to pertussis toxin of LHRH-induced [^{3}H]inositol phosphate production, but that was in the absence of any corresponding change in gonadotropin secretion (Hawes et al., 1993). However, pertussis toxin can demonstrate anomalous effects, independent of the attenuation of $G_{i/o}$ activation. Both the blockade of LHRH-induced MAP kinase activation here and authentic ADP-ribosylation of $G_{i/o}$ are seen only with pertussis holotoxin and not with *N*-ethylmaleimide-treated toxin or the toxin B oligomer (Banga et al., 1987; O'Neill et al., 1992). In accordance with this new evidence for $G_{i/o}$ activation by the LHRH receptor, we have recently observed pertussis toxin-sensitive LHRH-induced suppression of forskolin-stimulated cAMP formation in αT3-1 cells (MacKenzie, C., Johnson, M.S., Sim, P.J. and Mitchell, R., unpublished observations).

The LHRH receptor is a member of the rhodopsin family of G protein-linked receptors but contains a number of non-canonical motifs and lacks a carboxyl terminal tail (Tsutsumi et al., 1992). This presumably accounts for its characteristic resistance to rapid homologous densensitisation (Davidson et al., 1994) which may in turn be a key factor in its ability to maintain interaction not only with $G_{q/11}$ but also with $G_{i/o}$ and sustain a relatively long-lasting activation of MAP kinase.

Acknowledgements

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UNCONVENTIONAL SIGNALLING BY THE LHRH RECEPTOR

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The LHRH receptor is a member of the family of G protein-linked receptors coupled to hydrolysis of phosphoinositides [1]. The dependence of LHRH-induced gonadotrophin secretion upon phospholipase C activity has however been questioned [2]. In view of the unique ability of the LHRH receptor to elicit the phenomenon of self-priming [3], we have sought to define unconventional signals emanating from this receptor that may not be generated by other members of the receptor family.

The phenomenon of LHRH self-priming is dependent upon an apparently novel species of PKC with a pituitary-selective distribution [4] that we have partially purified and characterised [5]. Amongst the cellular targets of a PKC with these properties are PLA_2 and PLD.

Signalling by means of tyrosine kinase and MAP kinase cascades is classically associated with growth factor receptors. Nevertheless we have shown that each of these is strongly activated by LHRH both in normal pituitary tissue and in the αT3-1 gonadotroph cell line. LHRH-induced production of phosphotyrosine-immunoreactive proteins is mimicked in part by phorbol esters but not by ionomycin and is essential for LHRH-induced PLD activation and LH secretion [6]. LHRH-induced MAP kinase activation correlates strongly with the ability of gonadotrophs to demonstrate self-priming and occurs through a PKC-dependent rather than Ca^{2+}-dependent or tyrosine kinase-dependent mechanism [7]. Since MAP kinase is involved in the regulation of transcriptional, translational and cytoskeletal organisation, it is a strong candidate for a central role in the self-priming phenomenon which is reliant on the rapid induction of protein synthesis and cytoskeletal changes.

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P14 THE EFFECTS OF PROTEIN KINASE C INHIBITORS ON CONSTITUTIVELY-ACTIVE MAP KINASE FROM RAT HIPPOCAMPUS

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The MAP kinases (mitogen-activated protein kinases), otherwise known as ERKs (extracellular signal-regulated kinases) are a family of serine/threonine kinases that are rapidly activated by mitogens (Cobb *et al.* 1991). A consensus target motif for phosphorylation by MAP kinases has been defined and recognised within the amino acid sequences of cytoskeletal proteins, other kinases and transcription factors (Clark-Lewis *et al.* 1991). In hippocampal neurons, phosphorylation and increased activity of MAP kinase is brought about by NMDA receptor activation (Bading & Greenberg, 1991). MAP kinase is also activated by phorbol esters, presumably by means of their ability to promote protein kinase C (PKC) activity (Cobb *et al.* 1991).

In order to assess some of the pharmacology of MAP kinases, we have examined the effects of a number of widely-used protein kinase inhibitors on MAP kinase activity from hippocampus cytosol. Tissue was homogenised in ice-cold 20 mM Tris HCl pH 7.4 with 12 mM EDTA, 50 mM β-mercaptoethanol, protease inhibitors and 2.5 mM sodium orthovanadate, 62.5 mM β-glycerophosphate and 200 nM okadaic acid. Five µl samples of a crude cytosolic fraction were assayed in 25 µl assays also containing 16 mM Tris HCl pH 7.4, 40 mM β-mercaptoethanol, 160 nM okadaic acid and 50 µM ATP [γ-^{35}S] (specific activity 1285 Ci/mmol; 0.35 µCi/tube). After 40 min at 30°C, with or without the selective peptide substrate APRTPGGRR (2 mM; Clark-Lewis *et al.* 1991), the reaction was stopped with ice-cold trichloroacetic acid and the thiophosphorylated peptide collected by spotting onto phosphocellulose paper and extensive washing in 75 mM H_3PO_4. The constitutive kinase activity could be immunoprecipitated by a protein G-sepharose coupled monoclonal antibody (Zymed Z033) to the p44 and p42 species of MAP kinase and was inhibited by pretreatment of the cytosolic extract with sweet potato acid phosphatase (0.3 - 10U) in the absence but not in the presence of 10 mM pyrophosphate.

A number of protein kinase inhibitors showed the following IC_{50} values (mean ± s.e.mean, n = 6 - 9 separate determinations): staurosporine, 1.5 ± 1.2 µM; K252a, 5.8 ± 1.4 µM; Ro 31-8220 (Davis *et al.* 1989), 3.8 ± 0.9 µM; H7, 66 ± 1 µM; HA 1004, 7.0 ± 2.5 µM; chelerythrine, 69 ± 5 µM. It is important to consider that higher concentrations of these compounds used in some experiments on cellular responses may well influence MAP kinase as well as PKC. Interestingly, the 10-fold greater potency of HA 1004 than H7 seen here has not been observed for any of the other kinases investigated (Hidaka *et al.* 1984).

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QZ 113 EVIDENCE FOR A NOVEL H7-RESISTANT SPECIES OF PROTEIN KINASE C IN PITUITARY GLAND THAT LEADS TO ACTIVATION OF MAP KINASE. Rory Mitchell, Angela Ison, Pauline Sim, James Simpson, Melanie Johnson, Roger Clegg* and Kevin Connor*, MRC Brain Metabolism Unit, 1 George Square, Edinburgh and *Hannah Research Institute, Ayr, U.K.

Models of the role of PKC in stimulus-secretion coupling, in the activation of phospholipases A_2 and D and in the regulation of Ca^{2+} channels in the anterior pituitary gland have pointed to the presence of a form of PKC which is resistant to H7 but sensitive to GF 109203X and Ro 31-8220. Cytosolic PKCs from anterior pituitary tissue were partially purified on DEAE cellulose followed by fractionation on hydroxyiapatite, revealing an unusual late-eluting component of activity absent from a range of other tissues tested. This fraction contained a phorbol-activated, PS-dependent histone IIIs- or GS peptide-kinase activity which was Ca^{2+}-independent and resistant to H7 but not to Ro 31-8220. All other eluting activity was H7-sensitive. Immunoreactivity for PKC α, β_1, (no γ), δ, ε, (no η) and 81 kDa ζ eluted at different positions from the H7-resistant species. Only 68 kDa ζ immunoreactivity was present in the same fraction. Although all bands were blocked in the presence of the relevant peptide antigens, the identity of the high MWt ζ product is uncertain. [^{32}P]auto-phosphorylation experiments revealed an unusual high MWt product (>130 kDa) predominantly in the H7-resistant fraction from pituitary but not midbrain control, whereas other fractions all produced autophosphorylation signals clustered around 90 - 95 kDa. The H7-resistant PKC may be a novel species, one of the newly-described species such as θ, λ or μ or a modified form of a known species such as ζ. In the α T3-1 gonadotroph cell line, stimulation of the phosphoinositide-hydrolysing LHRH receptor evokes activation of MAP kinase as measured by phosphorylation of a selective peptide substrate in an assay incorporating immunoprecipitation with an anti-ERK1/2 reagent. This activation is unaffected by tyrosine kinase inhibitors such as lavendustin A yet is sensitive to PKC inhibitors such as GF 109203X but not H7. This suggests that one of the cellular roles of this H7-resistant PKC is in the G protein receptor-induced cascade of MAP kinase activation involving either MEKK- or perhaps raf-mediated pathways.

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ASSESSMENT OF THE POTENTIAL ROLE OF THE NON-CANONICAL TM7 RESIDUE Asp_{318} IN THE PROMINENT ACTIVATION OF MAP KINASE BY THE GnRH RECEPTOR (BUT NOT OTHER G_q-LINKED RECEPTORS) EXPRESSED IN COS 7 CELLS. R. Mitchell, P. Sim, E. Lutz, M. Jonsson, G. Flink, W. Zhou* and S. Seaton*. MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ UK and *Fishberg Centre for Neurobiology, Mount Sinai School of Medicine, New York, NY 10029 USA

We have previously shown that the GnRH receptor elicits a prominent activation of MAP kinase in gonadotropes through a similar concentration range to that for (pertussis toxin-insensitive) [^{3}H]inositol phosphate generation. GnRH-induced MAP kinase activation is relatively long lasting and is dependent on both protein kinase C and a pertussis toxin-sensitive component; an unusual profile, otherwise described only for the thrombin receptor (another member of the small group of receptors with a TM7 Asp for Asn substitution). In COS 7 cells transfected with GnRH, 5-HT_{1C} or $mGluR_1$ receptors, appropriate agonists caused concentration-dependent [^{3}H]inositol phosphate formation and PKC translocation to a similar extent, but only the GnRH receptor caused detectable activation of MAP kinase (a PKC-dependent and pertussis toxin-sensitive response). Mutation of Asp_{318} to Asn produced a GnRH receptor displaying similar potency to the wild type in ligand binding, [^{3}H]inositol phosphate and MAP kinase assays, but which was expressed at rather lower levels. Activation of MAP kinase by the mutant was still pertussis toxin-sensitive, indicating that the special ability of the GnRH receptor to activate an auxiliary pertussis toxin-sensitive G protein and thereby lead to prominent MAP kinase activation is not simply due to its Asp_{318} motif.

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ACTIVATION OF MAP KINASE BY THE GnRH RECEPTOR THROUGH A PKC-DEPENDENT PERTUSSIS TOXIN-SENSITIVE MECHANISM. P. Sim and R. Mitchell. MRC Brain Metabolism Unit 1 George Square, Edinburgh EH8 9JZ, UK

Mitogen activated protein (MAP) kinases are known to have a central role in a diverse array of intracellular signalling pathways. These include cell cycle regulation induced by growth factors whose receptors have intrinsic tyrosine kinase activity and also, as has become increasingly apparent, in the transduction of signals through G protein-linked receptors, suggesting a role for MAP kinases in non-proliferative signalling cascades. We have developed an *in vitro* assay based on [^{35}S]thiophosphorylation of a selective peptide substrate to investigate potential activation of MAP kinase by gonadotropin releasing-hormone (GnRH) and the mechanisms responsible. In the αT3-1 gonadotrope cell line, stimulation of the GnRH receptor (a phosphoinositide-hydrolysing receptor, known to couple to G_q) results in a marked and sustained increase in MAP kinase activity. This response was clearly protein kinase C (PKC)-dependent as the increased MAP kinase activity was potently inhibited by the selective PKC inhibitors Ro 31-8220 and GF109203X and less strongly by another, H7. Phorbol ester down regulation of PKCs completely prevented GnRH-induced MAP kinase activity. Interestingly this response was also highly sensitive to pertussis toxin and could be partially mimicked by mastoparan (a G-protein activator with reported selectivity for G_i/G_o) in a pertussis toxin-sensitive manner. These results suggest that the GnRH receptor may interact with 2 or more G-proteins and that activation of MAP kinase by this receptor may require involvement of more than one concurrent signalling pathway.

Phospholipase C-dependent activation of tyrosine kinases by LHRH in αT3-1 cells, and its role in LHRH priming of inositol phosphate production

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The LHRH receptor is a member of the superfamily of 7-transmembrane domain receptors coupled by G proteins to the activation of phospholipase C (PLC) [1]. Because this receptor has the unusual ability to bring about an upregulation of its own signal transduction in terms of inositol phosphate production [2] we have been exploring its ability to activate unconventional cellular signalling pathways [3] which might participate in modifying signal transduction by the receptor. We previously provided the first evidence that the LHRH receptor in αT3-1 cells can rapidly elicit increases in the cellular content of anti-phosphotyrosine immunoreactive proteins and that this pathway is of functional importance in leading to the activation of phospholipase D [4].

In order to distinguish the alternative hypotheses of a regulation of tyrosine kinases or tyrosine phosphatases being responsible, we carried out the following experiments to explore directly any alterations in enzymic activity of αT3-1 cell tyrosine kinases.

After quiescing for 20 h and stimulation in serum-free medium, cells were homogenised in 350 µl of 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 2 mM $MgCl_2$, 0.2 mg/ml benzamidine, 0.3 mM 4-(2-aminoethyl)benzene sulphonyl-fluoride) HCl, 0.1 mg/ml soybean trypsin inhibitor and centrifuged at 25000 g for 20 min (4°C) twice, with resuspension of the pellet in fresh buffer. The pellet was solubilised in 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 3% Nonidet-P40, 10% glycerol, 50 mM 2-mercaptoethanol with peptidase inhibitors, by stirring on ice for 1 h. After centrifugation at 55000 g for 45 min (4°C), the supernatant was loaded onto DEAE cellulose columns and washed with 6 column volumes of column equilibration buffer: 25 mM HEPES (pH 7.0), 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Nonidet-P40 and peptidase inhibitors, before eluting in 3 column volumes of buffer containing 100 mM NaCl. Samples were assayed by a method modified considerably from that of Litwin et al 1991 [5]. Assays (100 µl) contained 50 mM Tris HCl (pH 7.4), 12.5 mM $MgCl_2$, 50 µM sodium orthovanadate, 7 mg/ml p-nitrophenylphosphate, 0.6 µCi ATP [γ-^{33}P], 20 µl sample, with or without 1.6 mg/ml poly(Glu-Tyr; 4:1). After 45 min at 30°C (linear rate), assays were quenched with 50 µl of 0.1 M EDTA, 0.1 M ATP and aliquots were spotted onto 3MM paper squares and washed extensively in 12% trichloroacetic acid, 70 mM phosphoric acid.

Substrate-free blanks showed no time-dependent change in [^{33}P] incorporation (~7000 dpm per assay) whereas incubation of cells with 100 nM LHRH for 20 min caused an approximately 2 fold increase in rate of [^{33}P] incorporation into the substrate (~11000 dpm per 45 min in controls and (~23000 dpm per 45 min with LHRH). The greatest increase in tyrosine kinase activity was seen at 5 min incubation with LHRH, declining gradually thereafter. This response was inhibited in a concentration-dependent manner by the PLC inhibitor U 73122 (83 ± 11% inhibition at 25 µM) and only slightly reduced by the less active congener U 73433 (36 ± 8% inhibition at 25 µM).

In order to assess adaptive changes in LHRH receptor-induced [^{3}H]inositol phosphate formation, a method based on that of Stephens et al 1988 [6] was employed. Cells were labelled for 16 h with 1 µCi/ml [^{3}H]inositol in Earles's Balanced Salt Solution with 10 mM glucose and 0.2% bovine serum albumin. After washing, cells to be primed were incubated with 2 nM LHRH for 15 min; all cells extensively washed, then after a further 30 min, 10 mM LiCl was added for 15 min before stimulation with 100 nM LHRH for 30 min. Assays were stopped by aspiration and the addition of 700 µl cold 1.34 M trichloroacetic acid. Cell scrapings were centrifuged at 12000 g for 5 min (4°C) and 500 µl of supernatant was transferred to tubes with 50 µl 0.1M EDTA (pH 7.0) and 500 µl 1:1 freon:tri-N-octylamine. After extensive vortexing and centrifugation at 12000 g for 5 min (4°C), 300 µl of the upper aqueous phase was added to 200 µl 1M $NaHCO_3$ with Universal Indicator (overall pH of approximately 8). Labelled inositol phosphates were separated on 1 ml columns of Dowex 1x8 (formate form, mesh size 200-400). The columns were washed extensively with water before sample application and then 20 column volumes of water, 5 column volumes of 60 mM ammonium formate and 10 column volumes of 1M ammonium formate. 0.1M formic acid were applied to elute unmodified inositol, glycerophosphoinositols and inositol phosphates respectively.

As in pituitary [3], in αT3-1 cells pretreated for 16 h with 1 nM oestrogen, a brief exposure to a low dose of LHRH (2 nM for 15 min) greatly facilitated the production of [^{3}H]inositol phosphates elicited by a subsequent challenge with 100 nM LHRH for 30 min (206 ± 12% of unprimed responses) with no change in unstimulated basal [^{3}H]inositol phosphate production as a result of this conditioning preincubation. This augmentation was prevented by the tyrosine kinase inhibitors genistein (10-100 µM) and methyl 2,5-dihydroxycinnamate (30 µM) and reduced by lavendustin A (10 µM).

These results demonstrate that the LHRH receptor can lead to activation of tyrosine kinases downstream of PLC and that they play a critical role in the augmentation of LHRH-induced inositol phosphate production which underlies the LHRH-self priming phenomenon.

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Activation of MAP kinase by the LHRH receptor through a PKC-dependent pertussis toxin-sensitive mechanism

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Mitogen-activated protein (MAP) kinases, a family of Ser/Thr protein kinases, are known to be involved in a wide variety of intracellular signal transduction pathways initiated by binding of a variety of agonists to their respective cell surface receptors [1]. MAP kinases have most commonly been associated with cell cycle regulation induced by growth factors whose receptors have intrinsic tyrosine kinase activity. However, it is becoming increasingly evident that receptors belonging to the superfamily of seven transmembrane domain, G protein-coupled receptors may also activate MAP kinase and consequently MAP kinase may also be involved in non-proliferative signalling cascades [2].

To enable us to investigate stimulus-induced MAP kinase activation we developed an *in vitro* assay measuring [^{35}S]thiophosphorylation of a selective peptide substrate containing the consensus target motif characteristic of MAP kinases [3]. The identity of the enzymic activity as authentic MAP kinase was confirmed using a monoclonal anti-MAP kinase antibody (Z033, Zymed, anti-p44^{ERK1}/p42^{ERK2}). In the αT3-1 gonadotropin cell line, stimulation of the luteinising hormone-releasing hormone (LHRH) receptor (a phosphoinositide-hydrolysing receptor, known to couple to Gq [4,5]) results in increased MAP kinase activity. This increased activity peaked by 10 min at a near maximal concentration of 100 nM LHRH and was partially mimicked by phorbol 12,13-dibutyrate (PDBu) [6]. We have used a number of pharmacological agents to further investigate the upstream components of this signalling cascade.

The LHRH-induced increase in MAP kinase activity was potently inhibited by two PKC inhibitors, GF109203X and Ro 31-8220 (showing IC_{50} values of 1.84 ± 0.14 μM and 0.64± 0.08 μM respectively) but with extremely low potency by another PKC inhibitor of a different structural class, H7 (IC_{50} 168±12 μM). This pharmacology is consistent with an apparently novel PKC isoform found selectively in anterior pituitary [7]. Pre-incubation for 18 h with PDBu (300 nM) to down-regulate the phorbol ester-sensitive PKC isoforms, completely abolished LHRH-induced MAP kinase activity. The tyrosine kinase inhibitor lavendustin A had no effect on LHRH-induced MAP kinase activity at concentrations up to 10 μM. The role of G proteins was also investigated. Incubation for 18 h with pertussis toxin in the range of 30-200 ng/ml revealed that this LHRH-induced MAP kinase activity was highly sensitive to this toxin suggesting that the G protein involved may be Gi or Go.

The present results clearly demonstrate an involvement of PKC or a "PKC-like" kinase upstream of MAP kinase in this signalling cascade. Also for the first time we have shown that the LHRH receptor can couple to multiple G protein species, ie Gi/Go in addition to Gq. Taken together these results suggest that activation of MAP kinase by this receptor may require concurrent involvement of more than one signalling pathway. Activation of MAP kinases through a Gi- and ras-dependent route has been reported for the lysophosphatidic acid (LPA) receptor and α_2-C10 adrenergic receptor [8,9]. However, the exact mechanism remains unclear. In the case of the LPA receptor, it has been hypothesised that a genistein- and staurosporine-sensitive kinase may be involved downstream of Gi, perhaps at the level of membrane-associated proteins regulating ras. Certain isoforms of PKC have also been reported to phosphorylate raf [10] so may potentially exert a co-activation role further downstream in the MAP kinase activation cascade. It seems possible that the ability of certain G protein-coupled receptors to activate MAP kinase in a non-proliferative role may be determined by their ability to co-activate convergent signalling pathways.

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