THE DOPAMINE RECEPTORS OF THE COCKROACH SALIVARY GLAND

by

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CONTENTS

ABSTRACT

A study has been made of the secretory response and the electrical response (a hyperpolarization followed by a depolarization) mediated by dopamine receptors of the cockroach (*Nauphoeta cinerea* Olivier) salivary gland *in-vitro*.

Receptor classification

The selective D_1 agonists fenoldopam and SK38393, and the selective D_2 agonists LY163502 and quinpirole induced, in descending order of potency, secretion and a concomitant electrical response from the acinar cells. The secretory and electrical responses to dopamine were found to be antagonised by, in descending order of potency, chlorpromazine (a non-selective dopamine antagonist), SCH23390 (a selective D_1 antagonist), haloperidol and metaclopramide (selective D_2 antagonists). Another D_2 antagonist (þ)sulpiride and the highly selective D_2 antagonist domperidone did not inhibit either the secretory or electrical responses. These results taken together suggest that the receptor mediating the secretory and electrical response are the same, and analogous to the mammalian D_1 sub-type. This suggestion is strengthened by the fact that SCH23390 inhibited the secretory and electrical response to all the selective agonists, while (þ)sulpiride did not.

Although domperidone did not inhibit the electrical response to dopamine, three other actions were observed: one, post-synaptic, led to the potentiation of the hyperpolarization; this action was shared by (þ)sulpiride. A separate post-synaptic action resulted in the inhibition of the depolarizing phase of the response. Finally a presynaptic action led to the abolition of the response to nerve stimulation.

Effects of the calmodulin antagonists W7 and calmidazolium

In an attempt to investigate the role of calmodulin in stimulus-secretion coupling within the salivary gland, the actions of two calmodulin antagonists, W7 and calmidazolium, were studied. In high concentrations, but within the range in which they are known to inhibit calmodulin, W7 and calmidazolium were found to inhibit dopamine-induced secretion and hyperpolarize the acinar cells. The hyperpolarization was not inhibited by SCH23390, and resulted from an increase in cytosolic free calcium, released from the same source as that accessed by dopamine. Lower concentrations of these two antagonists caused submaximal secretion and potentiated dopamine-induced hyperpolarizations. An interpretation of these results is that calmodulin promotes secretion, and exerts a negative control on cytosolic free calcium by an independent process which can be selectively inhibited.

SUMMARY

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I A study has been made into the pharmacology of the dopamine receptors of the cockroach salivary gland.

II An historical account is given in Chapter I of the secretory and electrical responses of the salivary gland to dopamine. The electrical response consists of a hyperpolarization which is occasionally followed by a depolarization. It has been suggested previously that secretion, the hyperpolarization and the depolarization might be mediated by three separate receptors.

III The subclassification of dopamine receptors is discussed in Chapter II.

IV In Chapters III to V an account is given of the effects of antagonists on the three responses to nerve stimulation and dopamine, and the ability of agonists to mimic dopamine. The antagonists used were: chlorpromazine (non-selective), SCH23390 (selective for D_1 receptors), haloperidol, metoclopramide, domperidone and (±) sulpiride (all four selective for D_2 receptors). The agonists used were: fenoldopam and SKF38393 (selective for D_1 receptors), and LY163502 and quinpirole (selective for D_2 receptors).

V Chapter III describes a study of fluid secretion. Secretion was stimulated by addition of agonists to the superfusate. Secretory rates were measured before and after a standard

30 min exposure to the antagonists. K_Ds were estimated by "three point assay". Because the antagonists did not always equilibrate the K_Ds obtained should be regarded as apparent values.

VI Chlorpromazine, SCH23390, haloperidol and metoclopramide inhibited dopamine-induced secretion with K_Ds of 0.12μM, 2.2μM, 17.4μM and 1.2mM, respectively. Domperidone and (±)sulpiride failed to inhibit secretion.

VII Fenoldopam, SKF38393 and quinpirole induced secretion. Within a factor of three the minimum effective concentrations were 4.8μM, 41μM and 132μM, respectively.

VIII The responses to all three agonists were inhibited by SCH23390, but not by (±)sulpiride.

IX The rank order of potency of agonists and antagonists at the secretory receptor supports the idea that this receptor is similar to the mammalian D_1 sub-type, but distinct from the D_2 sub-type.

X Chapter IV is devoted to the pharmacology of the receptor mediating the hyperpolarization to dopamine and nerve stimulation. Membrane potentials of individual acinar cells were measured with intracellular electrodes. Responses to agonists were usually induced by local application via pressure ejection from a microelectrode with a broken tip. Occasionally agonists were added to the superfusate.

Responses to nerve stimulation were induced by applying trains of stimuli at 20Hz. The protocol for studying antagonists was generally as described for the study of the secretory receptors. The effects of antagonists on the responses to dopamine and nerve stimulation were studied in parallel. Responses to dopamine, D, and nerve stimulation, N, were evoked at intervals of 90s with the sequence D_1, D_2, N. D_1 and D_2 represent different effective concentrations. The use of two concentrations allowed estimates of the K_D of antagonists by "three point assay". No quantitative analysis of the effects of antagonists on nerve stimulation was attempted.

XI Responses to nerve stimulation were inhibited by chlorpromazine, SCH23390, haloperidol and metoclopramide. These four antagonists also inhibited the hyperpolarization to dopamine with K_Ds of: chlorpromazine 0.2μM, SCH23390 4.1μM, haloperidol 3.3μM and metoclopramide 265μM. In contrast (±)sulpiride and domperidone potentiated the hyperpolarization to dopamine (see XVII and XVIII).

XII Fenoldopam, SKF38393, LY163502 and quinpirole induced a hyperpolarization with a minimum effective concentration, within a factor of 2, of 3.3μM, 5.3μM, 44μM and 100μM respectively.

XIII The responses to all four agonists were inhibited by SCH23390, but not by (±)sulpiride.

XIV The rank order of potency of agonists and antagonists suggests that the same receptor subserves secretion and the hyperpolarization.

XV Chapter V describes an investigation into the depolarizing component of the electrical response. The methods used were as described for Chapter IV, but detailed quantitative results were not obtained. Fenoldopam, SKF38393, LY163502 and quinpirole induced a depolarization. Chlorpromazine, SCH23390, haloperidol and metoclopramide were effective antagonists of the depolarization to dopamine and nerve stimulation. The range of concentrations over which the antagonists inhibited the depolarization were the same as for the hyperpolarization. The results do not support the idea that separate receptors mediate the hyperpolarizing and depolarizing components of the response.

XVI In contrast to its effect on the hyperpolarization, domperidone inhibited the depolarization to dopamine. This finding might be taken to support the idea that different receptors mediate the hyperpolarization and depolarization, but the absence of an effect on the depolarization of (±)sulpiride and the inhibition of the depolarization by SCH23390 argue against the idea that the receptor subserving the depolarization is a D_2 receptor. The possibilities therefore arise that this receptor is a hitherto unknown subtype, or that domperidone acts at a level beyond the dopamine receptor.

XVII Chapter VI describes a further investigation into the pharmacology of domperidone. As mentioned above domperidone potentiated the hyperpolarization to dopamine. The hyperpolarization induced by local application of 5-HT was also potentiated by domperidone. It is known that 5-HT acts on different receptors (Bowser-Riley *et al*, 1978). Domperidone did not however potentiate the secretory response to dopamine. (±)Sulpiride was also tested against the dopamine-induced hyperpolarization and secretion, and acted like domperidone. These results are consistent with the idea that the potentiation of the hyperpolarization to dopamine by domperidone and (±)sulpiride occurs beyond the point of receptor activation.

XVIII In Chapter VII a description is given of a study of the role of calmodulin in the secretory and electrical responses of the salivary gland to dopamine. The effects of two calmodulin antagonists, W7 and calmidazolium, were investigated.

XIX Low concentrations of W7 and calmidazolium induced submaximal secretion. At higher concentrations the rate of secretion fell and was not increased by the addition of dopamine.

XX Low concentrations of W7 and calmidazolium potentiated the hyperpolarization to dopamine. Higher concentrations of W7 and calmidazolium induced a hyperpolarization which did not decline during prolonged exposures to these substances. The hyperpolarization to W7 was not inhibited by SCH23390, in a

concentration which blocked matching responses to dopamine. This strongly suggests that the hyperpolarization to W7 and calmidazolium occurs independently of activation of the dopamine receptor.

XXI After the preparation had been exposed to calcium free solution for 10 min, the hyperpolarization to W7 declined during prolonged exposure. No response was obtained to W7, in the absence of added calcium, after prior exposure of the preparation to dopamine in calcium free solution. Prior exposure of the preparation to W7, in the absence of added calcium, inhibited the response to dopamine. The results indicate that the hyperpolarization is calcium-dependent and that W7 and calmidazolium appear to release calcium from the same source as does dopamine.

XXII A model is presented which accounts for simultaneous inhibition of secretion and activation of the hyperpolarization. In a qualitative way, the model predicts oscillations in the concentration of intracellular calcium in situations in which they have been observed.

CHAPTER I

THE COCKROACH SALIVARY GLAND

Structure of the salivary gland

The cockroach salivary gland is a bilateral structure lying on the ventral surface of the crop and consists of groups of acini and two reservoirs. Two pairs of ducts, one from the acini and one from the salivary reservoirs, join to form the main duct which opens in to the hypopharynx. The acini are composed of three layers: an external envelope, a membrane contiguous with the secretory ducts and, sitting between these, the acinar cells (for review see Bowser-Riley, 1978a). Kupffer (cited by Bowser-Riley, 1978a) distinguished two cell types, peripheral and central. The peripheral cells lie beneath the basement membrane, they are oval or pyramidal in shape and have an intracellular ductule which is continuous with the lumen of the gland. These cells contain a single nucleus, mitochondria and ribosomes, but no endoplasmic reticulum or secretory vesicles (Bland and House, 1971; Kessel and Beams, 1963). The central cells form the bulk of the acinus, contain endoplasmic reticulum, large secretory vesicles and a mucoprotein (Bland and House, 1971). The central cells are joined by septate desmosomes and have microvilli near the junction of the cell with the beginning of the duct. Although the majority of the ducts are composed of non-secretory cells, duct cells near the acini have large vesicles of dense granular material containing sialoglycans and a high tryptophan concentration.

Innervation

The salivary gland of *Nauphoeta cinerea* receives two sources of innervation, the principle source being from the suboesophageal ganglion. In addition a limited innervation, particularly of the acini lying adjacent to the reservoir ducts, arises from the somatogastric nerve (Bowser-Riley, 1978a). Lateral groups of acini are innervated by the ipsilateral duct nerve, while central cells receive their innervation from both duct nerves. Three types of axons have been distinguished in the salivary gland (Maxwell, 1978); one, clear, containing no vesicles, while the other types, A and B, do contain vesicles. Type A axons are more numerous and innervate cells throughout the acinus, while type B axons innervate only the peripheral cells underneath the basement membrane.

The neurotransmitter

It was initially proposed (House, 1973) that 5-HT might be the transmitter at this synapse. However microspectrofluorimetry indicated that a catecholamine, not 5-HT, was present in the nerve terminals (Bland *et al*, 1973). Radiochemical assay and HPLC identified the catecholamine as dopamine (Fry *et al*, 1974; Kapoor *et al*, 1983). It should however be noted that dopamine, adrenaline, noradrenaline and 5-HT all evoke secretion and an electrical response from the acinar cells (Bowser-Riley and House, 1976; Bowser-Riley *et al*, 1978; Breward *et al*, 1980; Ginsborg *et al*, 1976b; House, 1973).

Secretion

It has been suggested that the peripheral cells may be involved in the production of dilute watery secretion (Cholodkowsky, 1881; Hofer, 1887; Lebedeff, 1899; all three cited by Bowser-Riley, 1978a; Bland and House, 1971), while the central cells are known to secrete mucin and amylase (Bland and House, 1971). In addition maltase, invertase and protease have been found in the secretion, although the cells of origin have not been identified (Bland and House, 1971). The rate of secretion is dependent on the concentration of sodium, potassium, calcium and chloride ions. The sodium ions are thought to be the driving force for water movement, while the potassium ions have been presumed to be necessary for the normal functioning of the sodium/potassium pump. However, though the rate of secretion is dependent on the external chloride concentration, chloride ions are thought to be distributed passively across the membrane, and thus unlikely to have an active role in the secretory response. In contrast, calcium appears to play an essential role in the induction of the secretory response, as substitution of the external calcium by magnesium reduces consecutive responses to dopamine (Smith and House, 1979; Gray and House, 1982).

The electrical response

Nerve stimulation and dopamine evoke a hyperpolarization of the salivary gland which is occasionally followed by a depolarization (House, 1973; Ginsborg *et al*, 1974; Ginsborg *et al*, 1976a; Bowser-Riley *et al*, 1978). The cells in the acinus are

electrically coupled, and dopamine induces a hyperpolarization of both central and peripheral cells (House, 1975). The hyperpolarization results from an increase in potassium conductance (House, 1973; Ginsborg *et al*, 1974) which, like secretion, has been shown to be calcium-dependent, consecutive responses being reduced and eventually abolished under conditions where the external calcium concentration is near zero (Ginsborg *et al*, 1980). Support for this conclusion arises from the fact that the calcium ionophore A23187 alone induces a hyperpolarization (Mitchell and Martin, 1980). The conductance underlying the depolarization is not yet known, although it has been suggested that different receptor sub-types mediate the depolarizing and hyperpolarizing components of the response respectively (Ginsborg and House, 1976).

Signal transduction

Until the early 1980's, with the exception of the role of calcium, little was known about signal transduction in the cockroach salivary gland. However more recent studies have shown that dopamine induces an increase in cytoplasmic c-AMP levels, and it is clear that c-AMP is an intermediate in the secretory, but not the electrical response to dopamine (Gray *et al*, 1984; Grewe and Kebabian, 1982). This fact led to the suggestion that the secretory and electrical responses were mediated by different receptors (Gray *et al*, 1984).

CHAPTER II

DOPAMINE RECEPTOR SUB-CLASSIFICATION

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Mammalian systems

The last twenty years have seen many advances in the knowledge of the function and pharmacology of dopaminergic systems. It is now apparent that there are at least two sub-types of dopamine receptor, namely D_1 and D_2.

The early 1970's saw the first steps towards sub-division of the dopamine receptors following the discovery of a dopamine-sensitive adenylyl cyclase in neuronal tissue from the CNS (Brown and Mackman, 1972; Kebabian *et al*, 1972). Subsequently the actions of a variety of dopamine agonists and antagonists on this system were found to correlate well with actions on physiological preparations (Clement-Cormier *et al*, 1974; Miller *et al*, 1974). The possibility of dopamine receptor sub-classification was first broached when the butyrophenone dopamine antagonists, in particular haloperidol, were found to be weak inhibitors of the dopamine-induced increase in c-AMP, relative to their effects in other functional models (Iversen, 1975; Snyder *et al*, 1975). In addition, a number of ergot derivatives were found to mimic the action of dopamine in the pituitary gland, whilst they inhibited the dopamine-induced adenylyl cyclase activity of the striatum without activating adenylyl cyclase in their own right (Kebabian *et al*, 1977; Pieri *et al*, 1978). Thus dopamine receptors were sub-divided: on one hand those receptors positively coupled to adenylyl cyclase, which were named D_1 receptors and those on the pituitary, originally thought to be without any influence on adenylyl cyclase,

which were named D_2 receptors (Kebabian, 1978; Kebabian and Calne, 1979; Spano *et al*, 1978). Later investigations showed that the D_2 receptors of the pituitary (Cote *et al*, 1982; Swennen and Denef, 1982; De Camilli *et al*, 1979; Enjalbert and Bockaert, 1983) and striatum (Cooper *et al*, 1986; Onali *et al*, 1984; Stoof and Kebabian, 1981; Weiss *et al*, 1985; Saller and Sallama, 1986a) were in fact negatively coupled to adenylyl cyclase. However this may not be universally true. Initially central and peripheral dopamine receptors were presumed to be different and named D_1 and D_2 and DA_1 and DA_2, respectively. However, as yet, no pharmacological distinction has been accepted. Thus the nomenclature has now been streamlined, with both central and peripheral receptors being classified as either D_1 or D_2.

Dopamine receptors were also sub-divided with respect to their anatomical location. It was noted that dopamine receptors located pre-synaptically on nerve terminals (controlling dopamine synthesis and release), cell somata and dendrites (regulating impulse traffic along dopaminergic nerves) were more sensitive to dopamine agonists than post-synaptic dopamine receptors (Carlsson, 1975; Roth, 1979; Skirboll *et al*, 1979). In common with pre-synaptic receptors for other neurotransmitters, these have been termed autoreceptors (Carlsson, 1975). Subsequent pharmacological characterization of these receptors has shown that they are of the D_2 sub-type. Although putative D_2 autoreceptor selective agonists have been reported (Johansen and White, 1987), subsequent studies have shown no evidence of agonist selectivity for either the pre- or post-synaptic D_2 receptors.

Pharmacological profile of D1 and D2 receptors

Since the selective action of the butyrophenone dopamine antagonists for D_2 receptors was first observed, a number of selective agonists and antagonists have come of age. For the sake of clarity the pharmacology of the two receptors will be discussed in relation to the action of the compounds used in the present investigation; namely SKF38393, fenoldopam (selective D_1 agonists), quinpirole, LY163502 (selective D_2 agonists), SCH23390 (a selective D_1 antagonist), haloperidol, metoclopramide, (±)sulpiride (antagonists selective for D_2 receptors) and domperidone (highly selective for D_2 receptors).

D1 Receptors

Selective agonists SKF38393 was first synthesised in 1978 by Pendleton and co-workers and was found to dilate the renal artery. Further studies established that this compound had an atypical agonist profile at dopamine receptors. Thus SKF38393 was found to stimulate dopamine-sensitive adenylyl cyclase in the caudate nucleus but failed to induce emesis and did not inhibit dopamine turnover, or prolactin release (Setler *et al*, 1978). Following the sub-classification of the dopamine receptors by Kebabian and Calne (1979), it was proposed that SKF38393 was a selective D_1 agonist (Stoof and Kebabian, 1981). Subsequent ligand binding studies revealed that SKF38393 has a much higher affinity for D_1 as opposed to D_2 receptors in the CNS (Anderson *et al*, 1985; Hyttel, 1983 & 1984; Sibley *et al*, 1982b; O'Boyle and Waddington, 1987). In addition this compound has been shown to inhibit noradrenaline-

induced contractions of the rabbit splenic artery (Ohlstein *et al*, 1984) and relax the dog mesenteric vascular bed (Hilditch and Drew, 1985). It also stimulates adenylyl cyclase, and secretion from the parathyroid gland by activation of D_1 receptors (Brown *et al*, 1980). In all D_1 receptor models SKF38393 has been shown to be less potent than dopamine and it has been proposed as a partial agonist (Hilditch and Drew, 1985). In most D_2 receptor models SKF38393 was found to be inactive. Thus it fails to inhibit tachycardia induced by sympathetic nerve stimulation; nor does it relax the sympathetically-induced contraction of the rat anococcygeus muscle (Hilditch and Drew, 1985). However Enjalbert and Bockaert (1983) showed SKF38393 to bind to D_2 receptors of the rat pituitary but with a low affinity (K_D = 1mM).

Fenoldopam (SKF82526) is a close analogue of SKF38393 and, like SKF38393, it has been shown to be a selective D_1 agonist (Weinstock *et al*, 1980). Thus it binds selectively to D_1 receptors in the CNS and periphery (O'Boyle and Waddington, 1987; Niznik *et al*, 1988), activates dopamine-sensitive adenylyl cyclase in the CNS and periphery (Weinstock *et al*, 1980; Sigala, 1989), and relaxes the rabbit splenic artery (Ohlstein *et al*, 1984) and dog mesenteric vascular bed (Hilditch and Drew, 1985). Fenoldopam is more potent an agonist than SKF38393, but is still consistently less potent than dopamine. As regards an action at D_2 receptors, little information is available. However it is clear that fenoldopam shows only low affinity for D_2 receptors of human caudate putamen membranes (O'Boyle and Waddington, 1987), and as an agonist in functional models it fails to inhibit prolactin release in the dog (Hahn *et al*,

1982), sympathetically-induced tachycardia in the cat and sympathetically-induced contractions of the rabbit anococcygeus muscle (Hilditch and Drew, 1985).

Selective antagonists SCH23390 was proposed as the first selective D_1 antagonist (Hyttel, 1983; Iorio *et al*, 1983). This compound was shown to inhibit dopamine-stimulated adenylyl cyclase activity with 2,000 times greater potency than it inhibited ^{3}H-spiperone binding to D_2 receptors. It has since been shown that SCH23390 blocks D_1 receptors of the parathyroid gland (Niznik *et al*, 1986), and dopamine-induced relaxation of the rabbit splenic artery and dog mesenteric vascular bed (Hilditch and Drew, 1985). It does not raise prolactin levels in rats (Iorio *et al*, 1983; Saller and Salama, 1986b) and does not block the inhibition of sympathetic nerve-induced tachycardia in the cat, or sympathetically mediated contractions of the rabbit anococcygeus muscle (Hilditch and Drew, 1985). This suggests that SCH23390 does not posess significant affinity for D_2 receptors. Consistent with these findings is the fact that SCH23390 displaces ^{3}H-piflutixol from D_1 receptors with a K_D of 0.3μM whereas it displaces ^{3}H-spiperone from D_2 receptors in the striatum with a K_D of 1.3μM (Boyce *et al*, 1985; Cross *et al*, 1983; see also O'Boyle and Waddington, 1987). However SCH23390, in relatively low concentrations, has been shown to block the inhibition by dopamine of acetylcholine release from striatal slices, an event which is thought to be mediated by D_2 receptors (Plantje *et al*, 1984b).

D2 receptors

Selective agonists The first of the selective D_2 receptor agonists was LY141865. Tsuruta *et al* (1981) established that this compound was a full agonist of D_2 receptors in the intermediate lobe of the rat pituitary, which upon activation inhibit adenylyl cyclase and thus secretion of α-MSH. In contrast LY141865 did not activate the dopamine-sensitive adenylyl cyclase in the carp retina (Tsuruta *et al*, 1981). Subsequent investigations have shown that LY141865 is an antagonist of ^{3}H-spiperone binding to bovine pituitary membranes (Seeman *et al*, 1987), and, by activating pre-synaptic D_2 receptors, inhibits the release of nor-adrenaline from sympathetic nerve terminals in the cardiovascular system (Hahn *et al*, 1982). In the central nervous sytem LY141865 has been shown to be an agonist of post-synaptic D_2 receptors in the nucleus accumbens (White and Wang, 1986).

LY141865 has now been superseded by its active enantiomer quinpirole (LY171555), which has a four fold greater affinity for D_2 receptors than its parent compound (Seeman *et al*, 1987). Quinpirole has now become the classical D_2 receptor agonist, and more recent studies have shown that it inhibits transmission at the stellate ganglion (Sabouni *et al*, 1986), inhibits adenylyl cyclase in the rat anterior pituitary (Enjalbert and Bockaert, 1983), inhibits Ach release from the caudate putamen and inhibits dopamine turnover in the CNS (Foreman *et al*, 1989; Brown *et al*, 1985). It also slows the heart and relaxes vascular smooth muscle by inhibiting nor-adrenaline release through the activation of pre-synaptic D_2 receptors on sympathetic nerve terminals (Hilditch and Drew, 1985). Although

quinpirole has a low affinity for D_1 receptors in the CNS and periphery (Niznik *et al*, 1985), it has been reported to be a weak agonist of D_1 receptors in the dog pancreas (Horiuchi *et al*, 1989).

LY163502 is the latest addition to the line of selective D_2 agonists. Murray and Waddington (1989) have shown that this compound has a far greater affinity for D_2 as opposed to D_1 receptors and it has subsequently been found to inhibit prolactin secretion, inhibit dopamine turnover in the CNS, induce emesis in the dog and inhibit ACh release from the caudate putamen (Foreman *et al*, 1989).

Selective antagonists As mentioned above, butyrophenone dopamine antagonists, in particular haloperidol, were the first compounds identified as being selective for D_2 receptors (Iversen, 1975; Snyder *et al*,1975). However haloperidol is not a highly selective antagonist having a K_D at D_1 receptors within an order of magnitude of that obtained at D_2 receptors (Anderson *et al*, 1987; Hilditch and Drew, 1985; Seeman *et al*, 1987). Since these observations were made a number of antagonists showing a high degree of selectivity for D_2 receptors have been introduced.

(±)Sulpiride was first proposed as a selective D_2 antagonist by Jenner and Marsden (1981). Thus (±)sulpiride inhibits ^{3}H-spiperone binding to pituitary membranes (Seeman *et al*, 1987; Enjalbert and Bockaert, 1983) and blocks dopamine-induced inhibition of adenylyl cyclase in the intermediate lobe of the rat pituitary gland (Munemura *et al*, 1980; Tsuruta *et al*, 1981). In addition (±)sulpiride has been shown to block the dopamine-induced inhibition

of both tachycardia of the cat heart and contractions of the rabbit anococcygeus muscle evoked by stimulation of sympathetic nerves (Hilditch and Drew, 1985). It also inhibits binding to D_2 receptors and D_2 mediated events in the CNS, including dopamine-induced inhibition of adenylyl cyclase (Hytell, 1983; Lacey *et al*, 1987; Alkadhi *et al*, 1986; Stoof and Kebabian, 1981). It is clear that of the two enantiomers (-)sulpiride is more potent at D_2 receptors than is (+)sulpiride (Sabouni *et al*, 1986; Tsuruta *et al*, 1981; White and Wang, 1984; Seeman *et al*, 1987; Enjalbert and Bockaert, 1983; Alkadhi *et al*, 1986). The action of (±)sulpiride at D_1 receptors is not so clear cut. Goldberg and Kohli (1983) suggested that (+)sulpiride blocks D_1 receptor mediated events in the cardiovascular system, while (-)sulpiride does not (see also Ohlstein *et al*, 1984). Subsequent investigations have established that this is also the case at other D_1 receptors in the CNS and periphery (Niznik *et al*, 1988; Basse and Robie, 1984; Brodde, 1982). However studies of other D_1 receptors in the CNS and periphery have found little or no activity with either isomer (Anderson *et al*, 1985; Fleminger *et al*, 1983; Hilditch and Drew, 1985).

In 1979 Laduron and Leyson suggested that domperidone was a selective antagonist of D_2 dopamine receptors in the rat CNS, as it displaced ^{3}H-haloperidol binding in the striatum with far greater potency than it inhibited dopamine-stimulated adenylyl cyclase activity. This conclusion was supported by the fact that domperidone was a poor antagonist of dopamine-stimulated adenylyl cyclase activity in the retina of the guinea-pig and carp (Watling and Dowling, 1981). Subsequently domperidone has been shown to

have a high affinity for D2 receptors of the anterior pituitary (Sibley *et al*, 1982; Seeman *et al*, 1987), striatum (Kazmi *et al*, 1986; Anderson *et al*, 1985) and to block dopamine-induced inhibition of nor-adrenaline release from sympathetic nerve terminals (Hilditch and Drew, 1985). In D_1 receptor models domperidone, as mentioned above, has been found to be a poor antagonist. Thus it does not inhibit dopamine-stimulated adenylyl cyclase in retinal tissue (Watling and Dowling, 1981) or rat striatal homogenates (Flaim *et al*, 1985; Anderson *et al*, 1987), and does not inhibit dopamine-induced relaxation of the rabbit splenic artery, or vasodilation of the dog mesenteric vascular bed (Hilditch and Drew, 1985). In ligand binding studies it has been shown that domperidone has a very low affinity for D_1 receptor binding sites (Anderson *et al*, 1987; O'Boyle and Waddington, 1984; Flaim *et al*, 1985)

Metoclopramide is another selective D_2 antagonist, but like haloperidol it does not lack activity at the D_1 receptor. In ligand binding studies, metoclopramide has been shown to inhibit ^{3}H-spiperone binding to the anterior pituitary (Seeman *et al*, 1987) and to rat striatal membranes (O'Boyle and Waddington, 1984; Fleminger *et al*, 1983). In functional models metoclopramide has been found to block dopamine mediated inhibition of catecholamine release from sympathetic nerve terminals (Hope *et al*, 1978) and dopamine-induced inhibition of adenylyl cyclase in the rat pituitary (Munemura *et al*, 1980). In studies of D_1 receptors metoclopramide failed to inhibit binding of selective ligands and, in concentrations up to 100μM, was without activity against dopamine-stimulated adenylyl cyclase activity in the rat striatum (Flaim *et al*, 1985; Fleminger *et al*, 1983; O'Boyle and Waddington, 1984).

In the periphery metoclopramide was found to inhibit fenoldopam-induced relaxation of the rabbit splenic artery (Ohlstein *et al*, 1984), dopamine-evoked relaxation of the rabbit mesenteric artery (Brodde *et al*, 1981) and D_1-mediated reduction in renal vascular pressure (Hahn *et al*, 1982) with K_D's of approximately 5μM. However metoclopramide failed to antagonise dopamine-induced relaxation of the guinea-pig aorta (Cox and Ennis, 1979).

Prototypical D1 and D2 receptor systems

From the information presented above, one can define prototypical D_1 and D_2 receptor systems on a functional and pharmacological basis. D_1 receptors stimulate adenylyl cyclase; the consequential responses would be activated by the selective D_1 agonists fenoldopam and SKF38393, but not the selective D_2 agonist quinpirole. In turn, the responses induced by dopamine, fenoldopam and SKF38393 would be blocked by the selective D_1 antagonist SCH23390, but not by the selective D_2 antagonists (±)sulpiride or domperidone. D_2 receptors inhibit adenylyl cyclase; consequential responses would be activated by quinpirole, but not by fenoldopam or SKF38393. In turn; the responses induced by dopamine and quinpirole would be blocked by the selective D_2 antagonists (±)sulpiride and domperidone, but not by SCH23390. Haloperidol and metaclopramide would be expected to antagonise both responses to dopamine, but with greater potency with respect to the D_2 receptor mediated event. The classical non-selective dopamine antagonists such as α-flupenthixol and chlorpromazine, would be expected to block both responses with equal potency.

Interactions between D1 and D2 receptors

The analysis of the pharmacology of dopamine in some systems is not as clear cut as would seem from the information provided above, as it appears that in many cases both D_1 and D_2 receptors are present on the post-synaptic membrane. Stoof and Kebabian (1981) showed that dopamine-induced efflux of c-AMP from the rat striatum was inhibited by LY141865 through the activation of a post-synaptic D2 receptor. More recently the stimulation of adenylyl cyclase activity in striatal homogenates by dopamine was found to be switched to an inhibitory effect in the presence of the D_1 antagonist SCH23390 and this effect was antagonised by (±)sulpiride (O'Boyle and Waddington, 1985). Such a relationship has also been proposed in the regulation of prolactin release from the anterior pituitary by dopamine (Treiman and Greengard, 1985). Thus dopamine inhibits adenylyl cyclase and secretion as does the selective D_2 agonist quinpirole , while the selective D_1 agonist SKF38393 activates adenylyl ayclase and induces secretion; these effects are blocked by selective D_2 and D_1 antagonists respectively (Treiman and Greengard, 1985; Enjalbert and Bockaert, 1983; Saller and Salama, 1986a & b).

Dopamine receptors in invertebrates

Studies of the action of dopamine in invertebrates in the 70's and early 80's identified this substance as an important neurotransmitter both in the CNS and the periphery. In addition a number of dopamine receptor-mediated functions were shown to be dependent on an increase in cellular c-AMP levels, and at least four

dopamine receptor sub-types have been proposed in invertebrates (for review see Walker *et al*, 1980; Gospe Jr., 1983). However the majority of these studies preceded the introduction of selective agonists and antagonists, while others lacked sufficient pharmacological data for classification of the receptors. It is now however clear that receptors similar to the mammalian D_1 and D_2 receptor exist together with three other sub-types. In 1985 Stoof *et al* characterized two dopamine receptors on the growth hormone producing cells of the snail (*Lymnae stagnalis*), one of which was similar to the mammalian D_1 receptor and the other to the D_2 receptor. The D_2 receptor subserved a hyperpolarization of the cells, which was the dominant event, a response which could be induced by the D_2 agonist LY141865 and inhibited by the D_2 antagonists (±)sulpiride and YM09151-2. The D_1 receptor subserved a c-AMP dependent increase in the excitability of these cells, this effect being mimicked by the D_1 agonist SKF38393 and antagonised by the D_1 antagonist SCH23390. Thus it appears that these receptors interact to control growth hormone secretion in much the same way as D_1 and D_2 receptors regulate secretion from the anterior pituitary. A more recent study has now suggested that the D_2 receptor on these growth hormone secreting cells may be different from D_2 receptors in the mammal as domperidone does not act as an antagonist here and the selective D_2 receptor agonist N-0437 is ineffective (Werkman *et al*, 1987). The use of selective agonists and antagonists has also identified a D_1-like receptor on the locust (*Locusta migratoria* L.) salivary gland (Lafon-Cazal and Bockaert, 1983), the effects of dopamine being mimicked by SKF38393 and inhibited by a number of non-selective dopamine antagonists, but not by the selective D_2 antagonist domperidone.

Dopamine receptors distinct from either the mammalian D_1 or D_2 receptor have been found in a number of preparations. Studies of the buccal-2 neurone and pedal giant neurone of *Lymnae stagnalis* (Audesirk, 1989) revealed a dopamine receptor which subserves a hyperpolarization. This response was not mimicked by selective D_1 or D_2 agonists and was not inhbited by either selective or non-selective dopamine antagonists. In the cerebral and buccal ganglia of *Limax maximus* dopamine induces a feeding motor program, a response which was also induced by the D_1 agonist SKF38393, but not inhibited by a range of neuroleptics (Wieland and Gelprin, 1983). In addition Orr *et al* (1987) characterized a dopamine-sensitive adenylyl cyclase system in the cockroach (*Periplaneta americana*) CNS whose pharmacology showed little if any similarity to that of either the D_1 or D_2 dopamine receptor. Thus the D_2 agonist quinpirole induced c-AMP accumulation, whereas the D_1 agonists fenoldopam and SKF38393 inhibited the response to dopamine. Furthemore the D_1 antagonists SCH23390 and SKF83566 had a greater affinity for the dopamine receptor than did the D_2 antagonist (±)sulpiride, while haloperidol failed to inhibit the response to dopamine.

CHAPTER III

CHARACTERIZATION OF THE SECRETORY RECEPTORS

INTRODUCTION

It is now well established that secretion from the cockroach (*Periplaneta americana* and *Nauphoeta cinerea*) salivary gland is controlled by the suboesophageal nerve (Whitehead, 1970; Smith and House, 1977), the neurotransmitter at this synapse being dopamine (Bland *et al*, 1973; Fry *et al*, 1974; Kapoor *et al*, 1983). Little is known however about the target receptors, and as yet there have been no pharmacological investigations to determine whether or not they are similar to one or other of the mammalian sub-types of dopamine receptor, namely the D_1 and D_2 receptors. Moreover, and perhaps more importantly, comparison of the pharmacology of the receptors mediating secretion from and the hyperpolarization of the acinar cells, respectively, is required to address the conclusions of Gray *et al* (1984), who suggested that a different type of receptor was responsible for each event.

In the present investigation the rank order of potency of the following antagonists was obtained against the secretory response to dopamine: SCH23390 (a selective D_1 antagonist), domperidone, (±)sulpiride, haloperidol and metoclopramide (selective D_2 antagonists) and chlorpromazine (a non-selective dopamine antagonist). Furthermore, the ability of two D_1 receptor agonists, SKF38393 and fenoldopam, and two D_2 receptor agonists, quinpirole and LY163502, to induce secretion from the gland was studied, together with the effects on the responses obtained of the D_1 receptor antagonist SCH23390, and the D_2 receptor antagonist (±)sulpiride.

Cockroaches (*Nauphoeta cinerea*, Olivier) were reared in glass aquaria and provided with rat cake, fruit and water *ad libitum*.

Bathing solution

Cockroaches, during dissection, and the salivary apparatus, after removal from the animal, were bathed in a solution of the following composition (mM): NaCl, 160; KCl, 1; $CaCl_2$, 5; glucose, 20; tris-(hydroxymethyl)-methylamine; pH adjusted to between 7.7 and 7.8 with HCl. For drugs and chemicals see Appendix 1.

General dissection

A cockroach (male or female) was anaesthetised by cooling, and the legs and wings removed. The animal was then pinned, ventral side down, through the head and tip of the abdomen to the base of a shallow perspex dissecting chamber lined with silicone resin (Sylgard, Dow Corning). The body of the cockroach was then covered in bathing solution, one of the abdominal segments was raised and the exoskeleton ventral and lateral to this was cut. The dorsal segments were dissected away at their lateral edges and the tracheal tubing and other tissues were removed to expose the internal organs. The gut was pinned out to the right to reveal the salivary apparatus (Figure 1). The glands were held away from the gut by gripping the salivary reservoirs by forceps, and the connective tissue holding the glands to the crop and oesophagus was cut, along with some tracheal tubing supplying the glands. The glands were then held

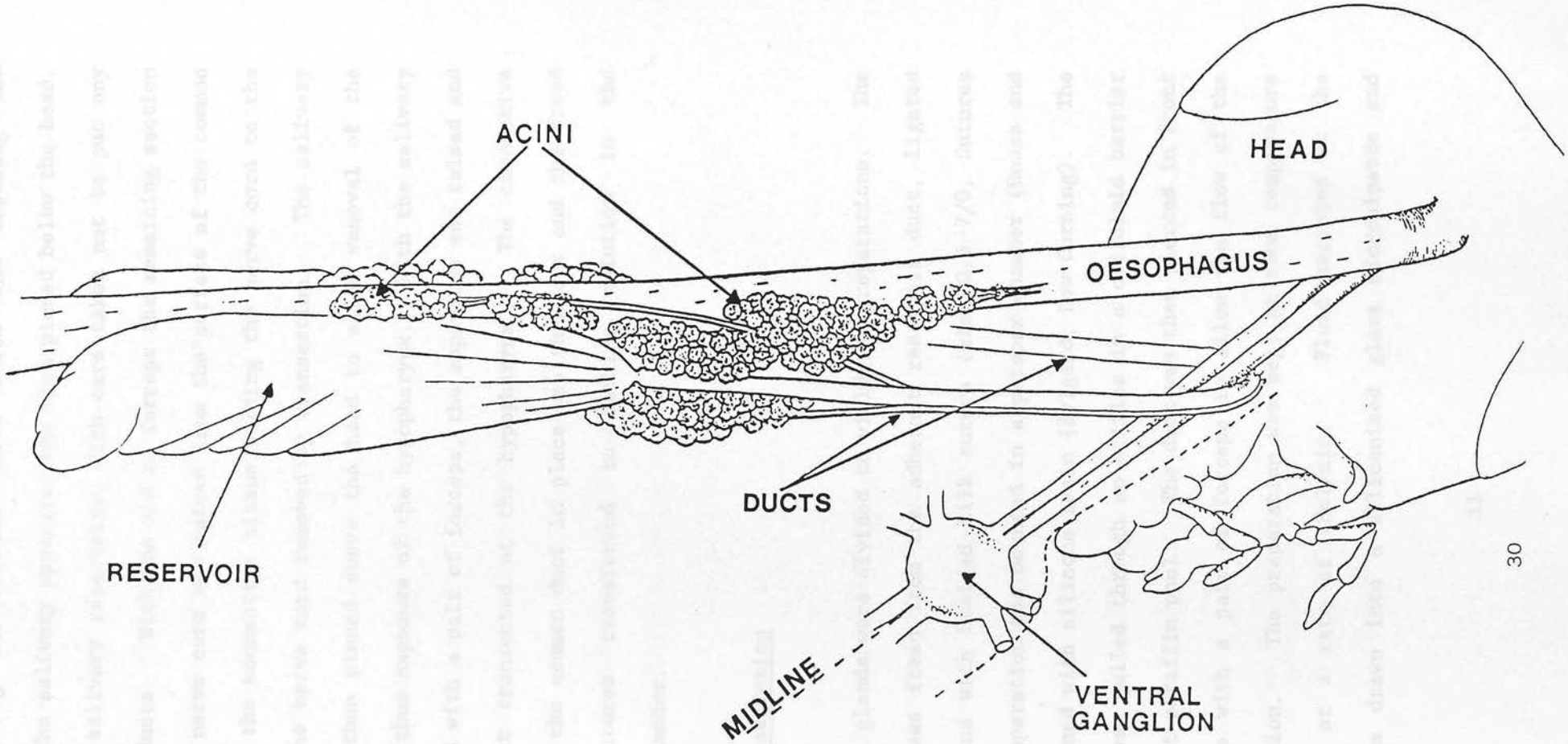

FIGURE 1 The cockroach salivary gland

forward while the uppermost section of the thorax was cut away from the head, freeing the ventral nerve cords and exposing the mouthparts. The salivary apparatus was then pinned below the head, by way of the salivary reservoirs, with care taken not to put any strain on the ducts. With the aid of forceps the remaining section of the ventral nerve cord was raised from the surface of the common salivary duct, the connective tissue holding the nerve cord to the duct cut and the nerve cord removed by transection. The salivary apparatus was then pinned above the head to allow removal of the mouthparts and thus exposure of the hypopharynx. With the salivary reservoirs held with a pair of forceps, the apparatus was raised and the common duct transected at the hypopharynx. The connective tissue holding the common duct in place was then cut and the freed salivary structures transferred to bathing solution in the experimental chamber.

Measurement of secretion

Paired salivary glands were divided to yield two preparations. The salivary duct was freed from the adherent reservoir duct, ligated near its cut end with braided silk suture (size 0.5 7/0, Sutures Ltd). The preparation was mounted in a perspex chamber (House and Smith, 1978) lined with silicone resin (Sylgard, Dow Corning). The salivary duct was pulled through an orifice in a celluloid barrier into an adjacent paraffin pool. The duct was then pierced in front of the ligature with a pair of forceps to allow the flow of the salivary secretion. The preparation was kept at room temperature and superfused at a rate of 18ml/min. Fluid secreted at the ligated end was drawn into a siliconized glass micropipette and

transferred to a second paraffin pool where it assumed a spherical shape. The volume was calculated from the diameter of the sphere which was measured using a microscope with a micrometer eyepiece (House and Smith, 1978). Agonists were added to the superfusate cumulatively, with the fluid secreted being sampled at 5 min intervals. Full equilibration with the agonist was assumed after two equivalent responses had been obtained, and the mean rate of secretion over this 10 min period was calculated.

The accuracy of measurement

The diameter of each droplet could be measured to an accuracy of 0.25 divisions on the graticule of the eyepiece. Since the smallest droplets were about 10 divisions in diameter, the maximum error made in estimating the volume of the droplets was 15%.

Schild analysis

The linear relationship between log (dose ratio - 1) and log (antagonist concentration) was determined by linear regression in the conventional way. If log (dose ratio - 1) = y and log (antagonist concentration) = x, then the slope of the line is given by C/A where:

$$C = n \Sigma xy - (\Sigma x)(\Sigma y)$$

and

$$A = n \Sigma x^2 - (\Sigma x)^2$$

The standard error of the mean for the slope is given by

$$\sqrt{(B - C^2/A) / \{(n - 2) A\}}$$

where

$$B = n \Sigma y^2 - (\Sigma y)^2$$

RESULTS

An investigation into the effects of dopamine antagonists

When added to the superfusate dopamine induced secretion in a concentration-related manner. In the presence of the dopamine receptor antagonists chlorpromazine (0.1 - 5μM), SCH23390 (10 - 5μM), haloperidol (10 - 100μM) and metoclopramide (2mM) the secretory response to dopamine was inhibited. Provided the concentration of chlorpromazine was below 5 μM, SCH23390 below 100μM and haloperidol below or equal to 100μM the inhibition was surmountable and was reversed by washing with antagonist free solution for 30 min.

Log-concentration-response curves were obtained in antagonist free solution, and after a 30 min incubation with an antagonist to a range of dopamine concentrations namely, 0.003, 0.001, 0.03, 0.10, 0.30, 1, 3 and 10μM. At least two experiments were completed with the highest concentration of each antagonist used in the present study. The responses, r, in the absence and presence of the antagonist were fitted to the relationship (Waud and Parker, 1971; Barlow and Blake, 1989):

$$r = \frac{M A^P}{A^P + X^P}$$

where r is the response in nl/min and A the concentration of dopamine. Values for M and p, common to both sets of data, and of separate values of X (X_1 in the absence and X_2 in the presence of the antagonist) were estimated by non-linear regression (see Appendix II). The values of X_1 and X_2 correspond to the values for the EC_{50}s and the dose ratio was taken as X_2/X_1. Figure 2 shows the log-concentration-response curve for dopamine in antagonist free solution, and in the presence of 5μM chlorpromazine after a 30 min exposure. The corresponding data for 50μM SCH23390 and 100μM haloperidol are shown in figures 3 and 4 respectively. It is evident that for each antagonist the experimental results are reasonably well fitted by parallel log-concentration-response curves with the same slope and maximum response. This strongly suggests that the antagonists behaved in a competitive manner.

In general, for each antagonist studied, dose ratios were estimated using a "three point assay" (Edinburgh Staff, 1968). Three concentrations of each antagonist were tested on each preparation. The antagonists were added to the superfusate cumulatively, dose ratios being estimated after a 30 min incubation with each concentration. At least four experiments were completed for each antagonist. Table I shows the data from a single experiment in which dose ratios were obtained for 10, 20 and 50μM SCH23390. In table II, III, IV and V the data obtained in individual experiments with chlorpromazine (0.1, 0.5 and 5μM), SCH23390 (10, 20 and 50μM) haloperidol (20, 50 and 100μM) and metoclopramide (2mM), respectively, are summarized.

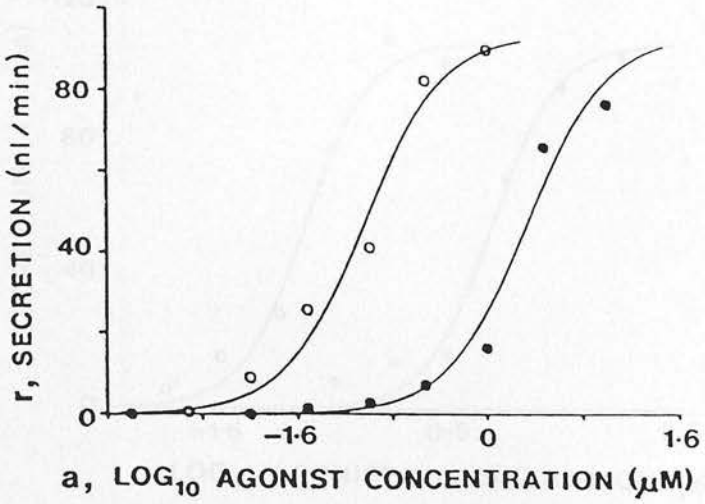

FIGURE 2 Inhibitory effect of chlorpromazine on secretion. Abcissa : log (dopamine concentration; a). Ordinate : rate of secretion in nl/min (r). Open circles show secretory rates in antagonist free solution; closed circles show secretory rates in the presence of 5μM chlorpromazine after a 30 min exposure. Fitted curves are:

$$r = \frac{M \quad A^P}{A^P + X^P}$$

where M is in nl/min, A is 10^a, and represents the agonist concentration in μM and p determines the slope of the curve; X = 0.2μM (X_1) in the absence of chlorpromazine and 7.4μM (X_2) in the presence of chlorpromazine; these values represent the concentration of agonist which produces 50% of the maximal response. The fitting procedure estimated values for M and p (common to the data represented by the open and closed circles), and X_1 and X_2 such that the grand sum of the squared vertical deviations of the data from the curves was minimized. The value of K_D given by these data is given by:

$$\frac{5\mu M}{(X_2/X_1)-1} = 0.12\mu M$$

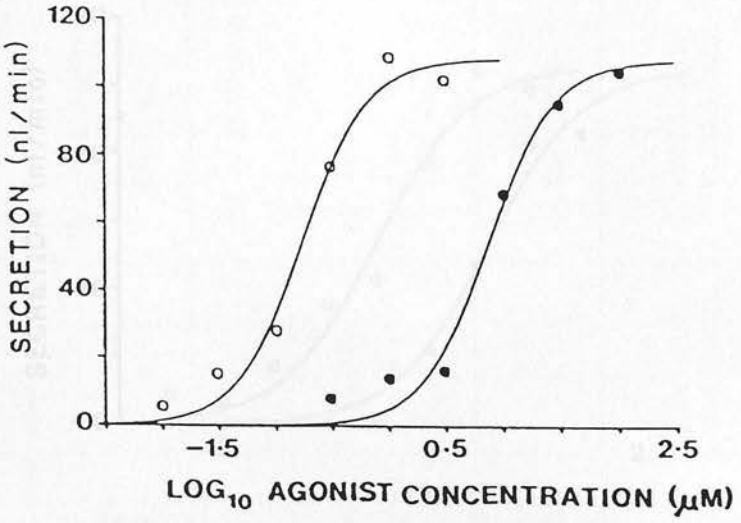

FIGURE 3 Inhibitory effect of SCH23390 on secretion. Abcissa : log (dopamine concentration). Ordinate : rate of secretion in nl/min. Open circles show secretory rates in antagonist free solution; closed circles show secretory rates in the presence of 50μM SCH23390 after a 30 min exposure. Fitted curves are as in figure 1; X = 0.09μM (X_1) in the absence of SCH23390 and 2.2μM (X_2) in the presence of SCH23390. The value of K_D given by these data was 2.2μM.

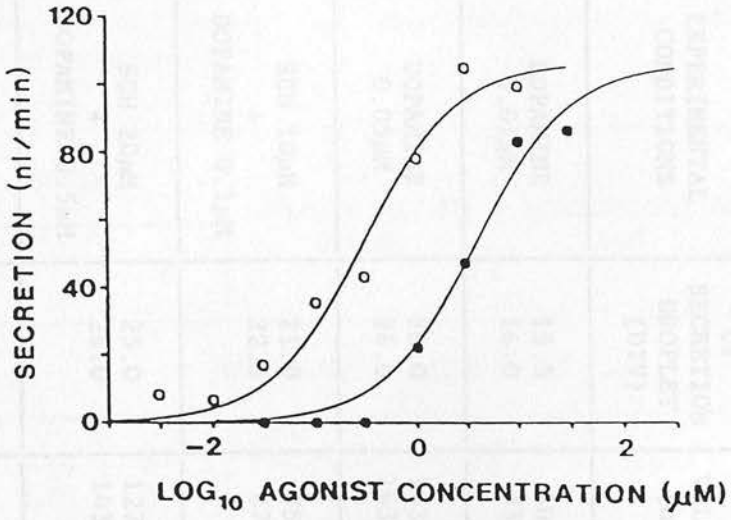

FIGURE 4 Inhibitory effect of haloperidol on secretion. Abcissa: log (dopamine concentration). Ordinate : rate of secretion in nl/min. Open circles show secretory rates in antagonist free solution; closed circles show secretory rates in the presence of 5μM haloperidol after a 30 min exposure. Fitted curves are as in figure 1; X = 0.1μM (X_1) in the absence of haloperidol and 1.6μM (X_2) in the presence of haloperidol. The value of K_D given by these data was 10.2μM.

Table I

Data from a single "three point assay" of the inhibition by SCH23390 of dopamine-induced secretion

EXPERIMENTAL CONDITIONS	DIAMETER OF SECRETION DROPLET (DIV)	VOLUME (nl)	MAXIMUM ERROR %	TIME (min)	SECRETION RATE (nl/min)	MEAN RATE (ml/min)	DOSE RATIO
DOPAMINE 0.01μM	15.5 16.0	30.47 33.51	4.8 4.7	5 5	6.09 6.70	6.40	-
DOPAMINE 0.05μM	26.0 26.0	143.79 143.79	2.9 2.9	5 5	28.76 28.76	28.76	-
SCH 10μM + DOPAMINE 0.1μM	21.0 22.5	75.76 87.11	3.6 3.3	5 5	15.15 17.42	16.28	4.91
SCH 20μM + DOPAMINE 0.5μM	25.0 26.0	127.83 143.79	3.0 2.9	5 5	25.57 28.76	27.16	11.22
SCH 50μM DOPAMINE 1μM	21.0 21.0	75.76 75.76	3.6 3.6	5 5	15.15 15.15	15.15	53.25

Table II

Data from "three point assays" of the inhibition by chlorpromazine of the secretory response to dopamine.

EXPERIMENT NUMBER	CONTROL		CHLORPROMAZINE 0.5μM	CHLORPROMAZINE 1μM	CHLORPROMAZINE 5μM
	A	B			
	DOPAMINE CONCENTRATION (μM)				
1	0.01	0.10	0.10	0.40	1.00
2	0.01	0.05	0.05	0.10	0.50
3	0.03	0.10	0.30	0.30	1.00
	0.10	0.30	0.30	1.00	10.00
	SECRETION RATE (nl/min)				
1	3.36	24.54	13.09	23.40	23.50
2	6.78	27.16	6.70	10.00	18.03
3	11.22	31.39	21.49	10.56	17.53
	35.92	71.76	46.46	52.38	61.56
	DOSE RATIO				
1			3.47	4.53	11.19
2			5.03	7.76	20.57
3			5.42	10.40	22.88
			2.17	6.04	45.58

Table III

Data from "three point assays" of the inhibition by SCH23390 of the secretory response to dopamine.

EXPERIMENT NUMBER	CONTROL		SCH23390 10μM	SCH23390 20μM	SCH23390 50μM
	A	B			
DOPAMINE CONCENTRATION (μM)					
1	0.01	0.10	0.2	0.4	0.80
2	0.01	0.05	0.05	0.10	0.50
3	0.01	0.05	0.05	0.10	0.00
4	0.01	0.05	0.10	0.50	1.00
SECRETION RATE (nl/min)					
1	2.18	28.28	26.07	20.95	13.27
2	10.44	36.31	20.73	14.50	13.09
3	7.55	44.70	13.74	8.75	12.57
4	6.40	28.76	16.29	27.16	9.20
DOSE RATIO					
1			2.43	7.63	30.08
2			2.64	7.77	42.41
3			3.82	9.49	40.22
4			4.91	11.22	53.25

Additional values for dose ratios, estimated from fitted dose response curves were used in the Schild plot (figure 6) and in the calculation of the mean K_D for SCH23390 (see table VI), namely: for 10μM SCH23390, 5.31, 25.17, 5.45 and 13.24; for 20μM SCH23390, 8.92; for 50μM SCH23390, 23.93, 7.67, 23.56.

Table IV

Data from "three point assays" of the inhibition by haloperidol of the secretory response to dopamine.

EXPERIMENT NUMBER	CONTROL		HALOPERIDOL 10μM	HALOPERIDOL 20μM	HALOPERIDOL 50μM
	A	B			
	DOPAMINE CONCENTRATION (μM)				
1	0.10	1.00	0.30	1.00	1.00
2	0.10	0.30	0.30	0.30	1.00
3	0.10	0.30	0.30	1.00	1.00
4	0.01	0.10	0.10	0.20	0.30
	SECRETION RATE (nl/min)				
1	16.9	53.77	21.26	35.95	27.16
2	22.62	42.04	34.06	21.93	35.38
3	26.41	43.09	28.76	44.18	29.67
4	11.32	42.04	27.94	24.81	26.35
	DOSE RATIO				
1			2.28	3.04	5.27
2			1.57	3.12	4.86
3			2.57	3.10	8.07
4			2.88	7.27	9.72

Table V

Data obtained from "three point assays" of the inhibition by metoclopramide of the secretory response dopamine.

EXPERIMENT NUMBER	CONTROL		METOCLOPRAMIDE 1mM
	A	B	
DOPAMINE CONCENTRATION (μM)			
1	0.01	0.50	0.50
2	0.01	0.05	0.10
3	0.01	0.10	0.10
4			
SECRETORY RATE (nl/min)			
1	10.79	43.16	17.85
2	5.06	24.09	5.01
3	4.29	23.34	6.44
4	16.53		15.68
DOSE RATIO			
1			1.27
2			3.52
3			2.25
4			10.04

To test for competitiveness the mean dose ratios for each antagonist were subjected to Schild analysis (Arunlakshana and Schild, 1959; see below). The slopes obtained from the plot of log (mean dose ratio - 1) against log (antagonist concentration) were as follows: chlorpromazine, 0.9 ± 0.3; haloperidol, 1.0 ± 0.4; SCH23390 1.1 ± 0.2. These values appear sufficiently close to unity for the antagonists to be said to act in a competitive manner over the concentration range studied. Figures 5 - 7 show the corresponding plots with the slopes set to unity.

Determination of KDs from dose ratios

The equilibrium dissociation constant, K_D, was estimated from individual dose ratios, DR, from the relationship:

$$K_D = B / (DR - 1)$$

where B is the antagonist concentration. The geometric mean of the K_D for each antagonist was calculated according to the relationship (Sokal and Rohlf, 1969):

$$Y_{G.M.} = \text{antilog } 1/n \ (\Sigma \log y)$$

where n is the number of values for the K_D, Y, and $Y_{G.M.}$ is the geometric mean. The 95% confidence limits were calculated according to the relationship:

$$\text{Lower limit} = L_1 = Y_{G.M.} - (t_{0.05} \cdot S_Y)$$

$$\text{Upper limit} = L_2 = Y_{G.M.} + (t_{0.05} \cdot S_Y)$$

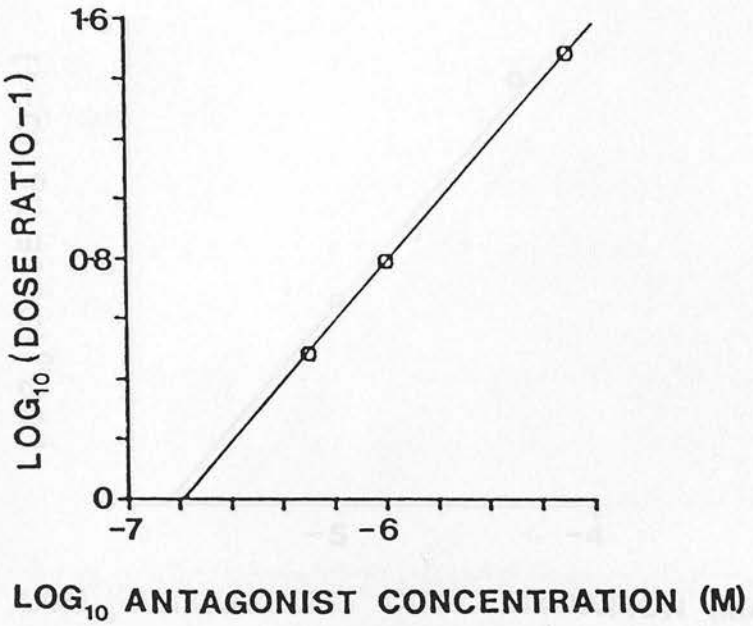

FIGURE 5 "Schild plot" obtained for inhibition by chlorpromazine of the secretory response to dopamine. Abcissa: log (antagonist concentration). Ordinate: log(dose ratio-1). The line was fitted by linear regression with the slope set to 1. Each point represents the mean value from at least four experiments. The intercept on the abcissa indicates a value of 0.2μM for the K_D of chlorpromazine.

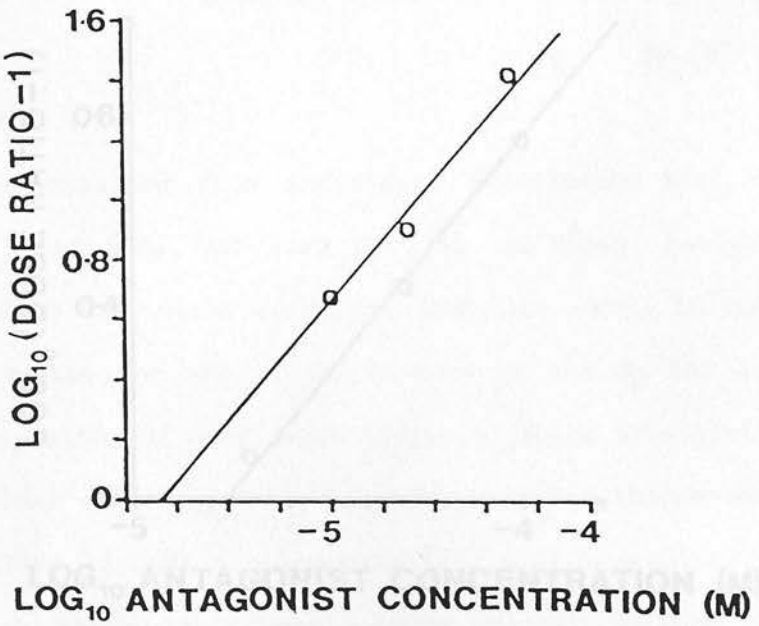

FIGURE 6 "Schild plot" obtained for inhibition by SCH23390 of the secretory response to dopamine. Abcissa: log (antagonist concentration). Ordinate: log (dose ratio-1). The line was fitted by linear regression with the slope set to 1. Each point represents the mean value from at least four experiments. The intercept on the abcissa indicates a value of 2.2μM for the K_D of SCH23390.

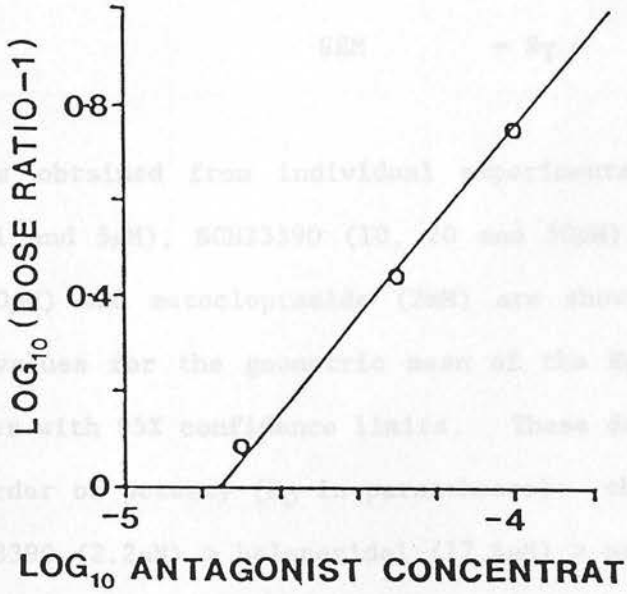

FIGURE 7 "Schild plot" obtained for inhibition by haloperidol of the secretory response to dopamine. Abcissa: log (antagonist concentration). Ordinate: log (dose ratio-1). The line was fitted by linear regression with the slope set to 1. Each point represents the mean value from at least four experiments. The intercept on the abcissa indicates a value of 17.5μM for the K_D of haloperidol.

where $t_{0.05}$ is the significance when $P < 0.05$. S_Y is the standard error of the mean:

$$\text{Standard deviation} = S = \sqrt{\Sigma(y - y)^2/(n-1)}$$

$$\text{SEM} = S_Y = S/\sqrt{n}$$

The K_Ds obtained from individual experiments with chlorpromazine (0.5, 1 and 5μM), SCH23390 (10, 20 and 50μM), haloperidol (20, 50 and 100μM) and metoclopramide (2mM) are shown in table VI, which gives values for the geometric mean of the K_D for each antagonist together with 95% confidence limits. These data give the following rank order of potency (K_D in parentheses): chlorpromazine (0.12μM) > SCH23390 (2.2μM) > haloperidol (17.4μM) > metoclopramide (1.2mM). Although there is a considerable scatter between the individual values (see figure 8), a clear separation between chlorpromazine, SCH23390, haloperidol and metoclopramide is evident.

In contrast to the antagonists discussed above the selective D_2 receptor antagonists domperidone and (±)sulpiride failed to inhibit the secretory response to dopamine.

An investigation into the effects of dopamine receptor agonists

The dopamine receptor agonists fenoldopam, SKF38393, quinpirole and LY163502 (not studied further) all induced secretion from the salivary gland. Figure 9 shows the effect of increasing the concentration of fenoldopam on the secretory response. One can see that secretion was induced in a concentration-related manner. Figure 10 compares the log-concentration-response curve obtained to

Table V1 Dose ratios estimated by 'three point assay for each of the active dopamine antagonists together with the geometric mean K_{Dapp} and upper (L_2) and lower (L_1) confidence limits (95%)

ANTAGONIST	CONCENTRATION (μM)	DOSE RATIO	$K_{D}app$(μM) GEOMETRIC MEAN	95% CONFIDENCE LIMITS (μM)	
				L_1	L_2
CHLORPROMAZINE	0.5	3.5			
	0.5	5.0			
	0.5	5.4			
	0.5	2.2			
	1.0	4.5			
	1.0	7.8			
	1.0	10.4	0.2	0.1	0.3
	1.0	6.0			
	5.0	11.2			
	5.0	20.6			
	5.0	22.9			
	5.0	45.6			
SCH23390	10	2.4			
	10	2.6			
	10	3.8			
	10	4.9			
	10	5.3			
	10	25.2			
	10	5.5			
	10	13.2			
	20	7.6	2.2	1.5	3.0
	20	7.8			
	20	9.5			
	20	11.2			
	20	8.9			
	50	30.1			
	50	42.4			
	50	40.2			
	50	53.3			
	50	23.9			
	50	7.7			
	50	23.6			
HALOPERIDOL	20	2.3			
	20	1.6			
	20	2.6			
	20	2.9			
	50	3.0			
	50	3.1	17.5	13.1	23.2
	50	3.1			
	50	7.3			
	100	5.3			
	100	4.9			
	100	8.1			
	100	9.7			
METOCLOPRAMIDE	2000	3.5			
	2000	1.3			
	2000	10.0	1201.2	117	12356.1
	2000	2.3			

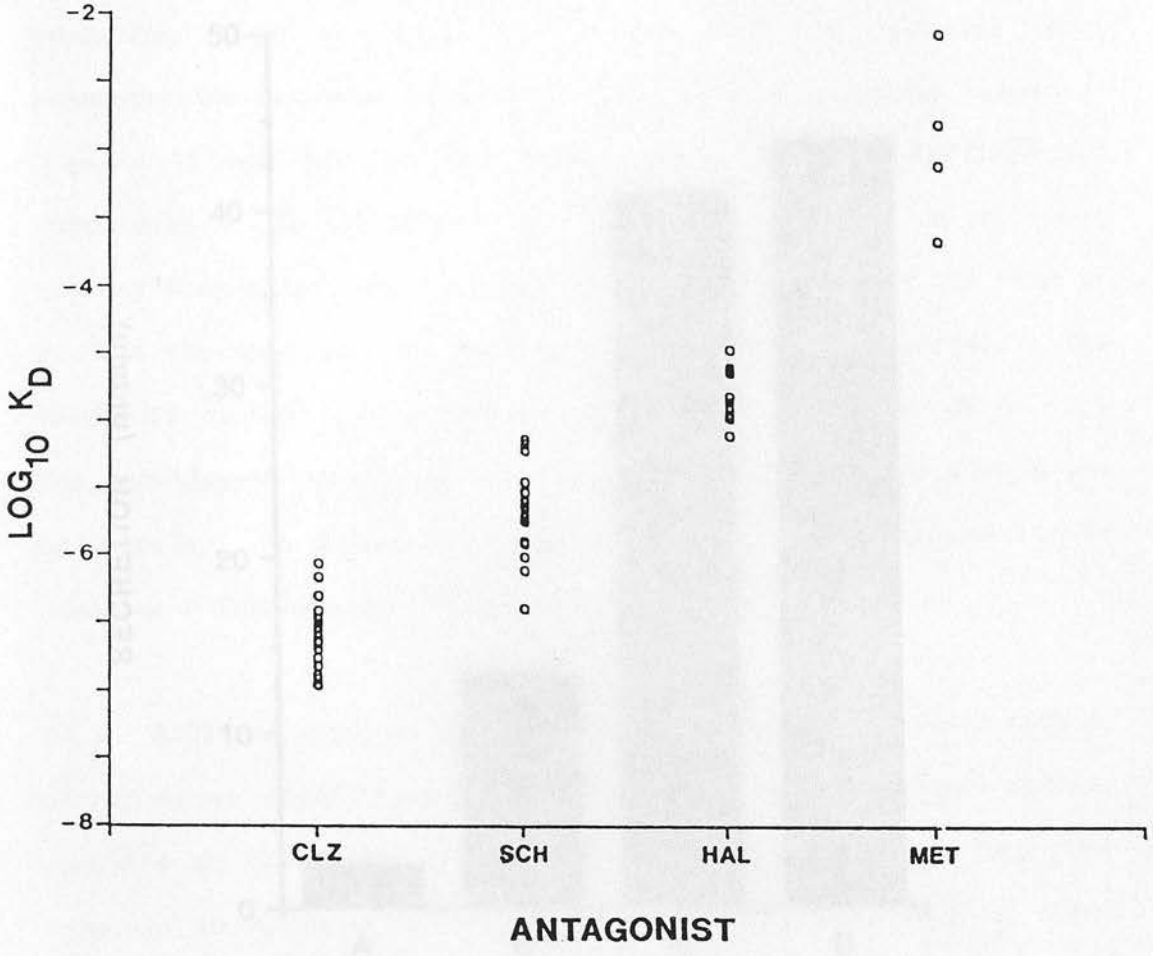

FIGURE 8 Diagram showing the range of values obtained in different experiments for the log K_D of each antagonist tested against the secretory response to dopamine. Ordinate shows the log K_D (M). CLZ is chlorpromazine; SCH is SCH23390; HAL is haloperidol; MET is metoclopramide.

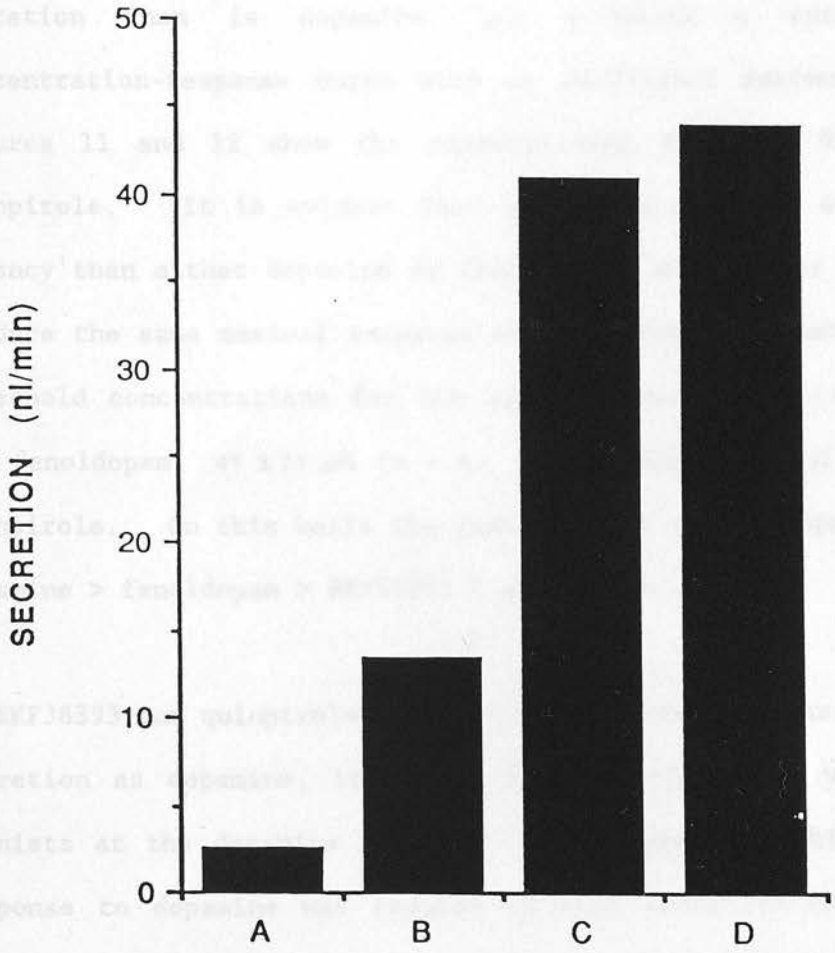

FIGURE 9 Block diagram showing the concentration-related induction of secretion by fenoldopam. Ordinate: rate of secretion in nl/min. The height of the columns indicates the secretory response: A to 1μM, B to 3μM, C to 10μM, and D 100μM fenoldopam.

dopamine (0.003 - 1μM) and to fenoldopam (1 - 100μM) in a single experiment. Fenoldopam is clearly less potent as an agonist of secretion than is dopamine, but produces a parallel log-concentration-response curve with an equivalent maximum response. Figures 11 and 12 show the corresponding data for SKF38393 and quinpirole. It is evident that these two agonists are of lower potency than either dopamine or fenoldopam, and neither was able to produce the same maximal response as the natural transmitter. The threshold concentrations for the agonists was 4.8 $\pm$ 1.8μM (n = 4) for fenoldopam, 41 $\pm$ 21 μM (n = 4) for SKF38393 and 132 $\pm$ 58 μM for quinpirole. On this basis the rank order of potency appeared to be dopamine > fenoldopam > SKF38393 > quinpirole.

As SKF38393 and quinpirole failed to induce the same maximal rate of secretion as dopamine, it seemed possible that they were partial agonists at the dopamine receptor. In support of this idea, the response to dopamine was reduced by high concentrations of these agonists. In one of three experiments in which the interaction of SKF38393 and dopamine was studied, 100nM dopamine produced a secretory response of 62nl/min, 1mM SKF38393 produced a response of 40nl/min and the two together, a response of 40nl/min. The maximum response, produced by 10μM dopamine, was 126nl/min. In a similar experiment, 30nM dopamine produced a response of 42nl/min, 3mM quinpirole, 14nl/min and the two together, 25nl/min. The maximum rate in this preparation, produced by 10μM dopamine, was 66nl/min.

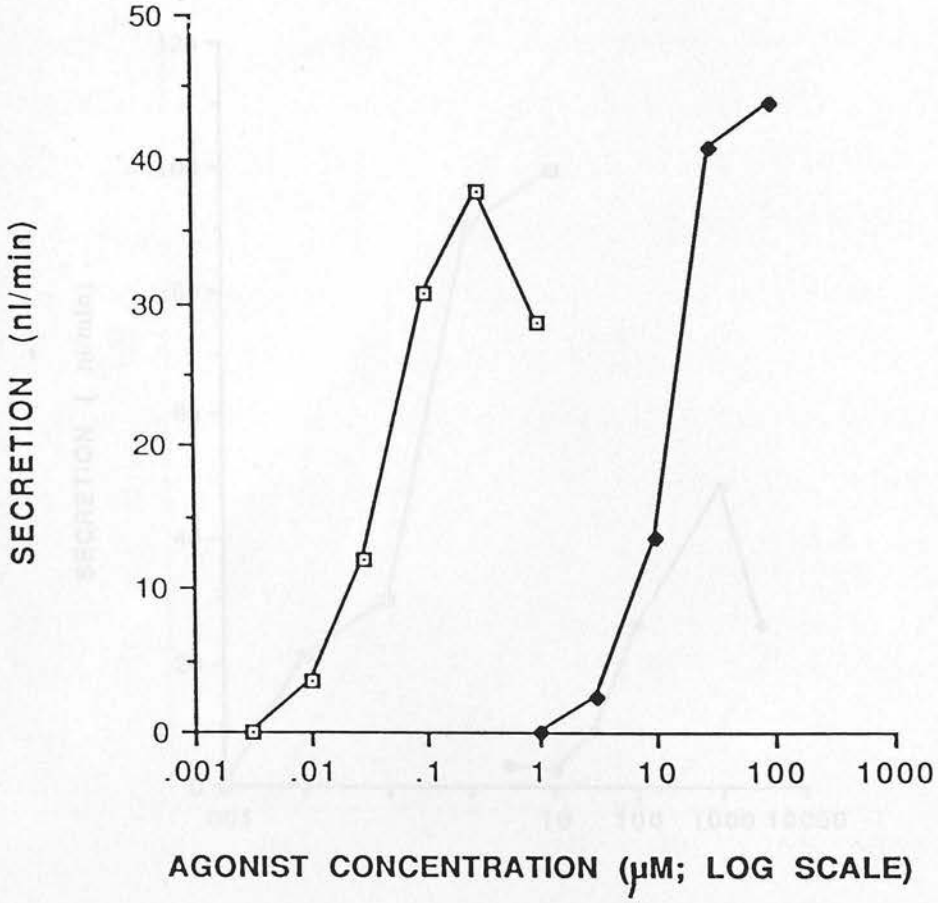

FIGURE 10 Comparison of the log-concentration-response curves for dopamine and fenoldopam. Abcissa: agonist concentration in μM (logarithmic scale). Ordinate: rate of secretion in nl/min. Open squares represent the response to dopamine. Closed squares represent the response to fenoldopam. These data were obtained from a single experiment.

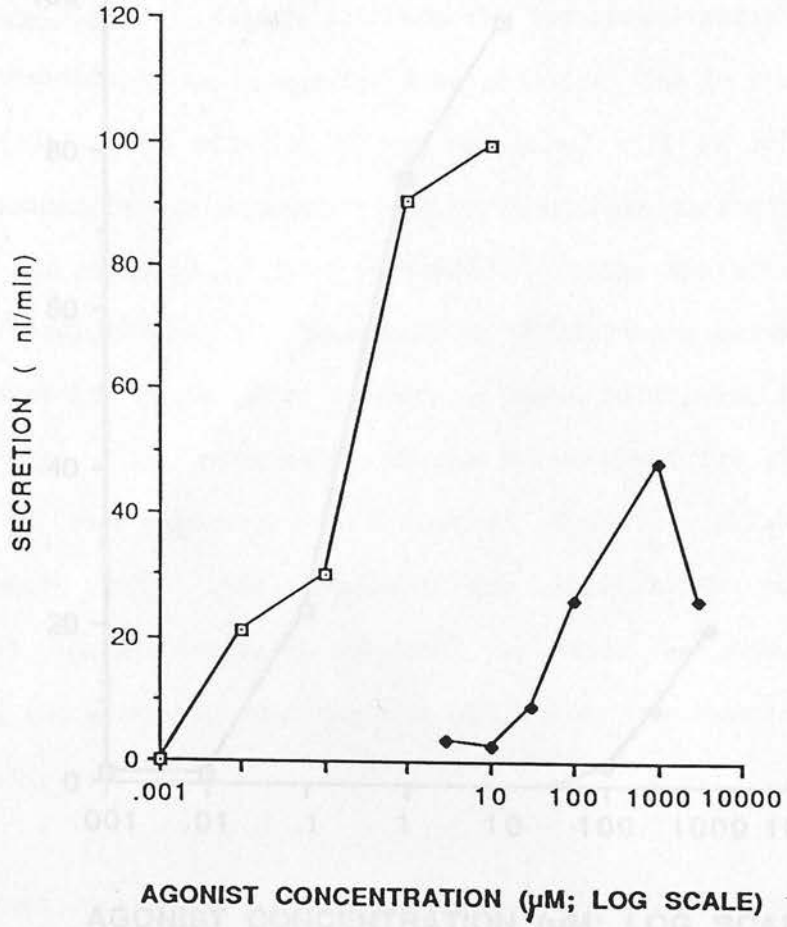

FIGURE 11 Comparison of the log-concentration-response curves for dopamine and SKF38393. Abcissa: agonist concentration (μM; logarithmic scale). Ordinate: rate of secretion in nl/min. Open squares represent the response to dopamine. Closed squares represent the response to SKF38393. These data were obtained from a single experiment.

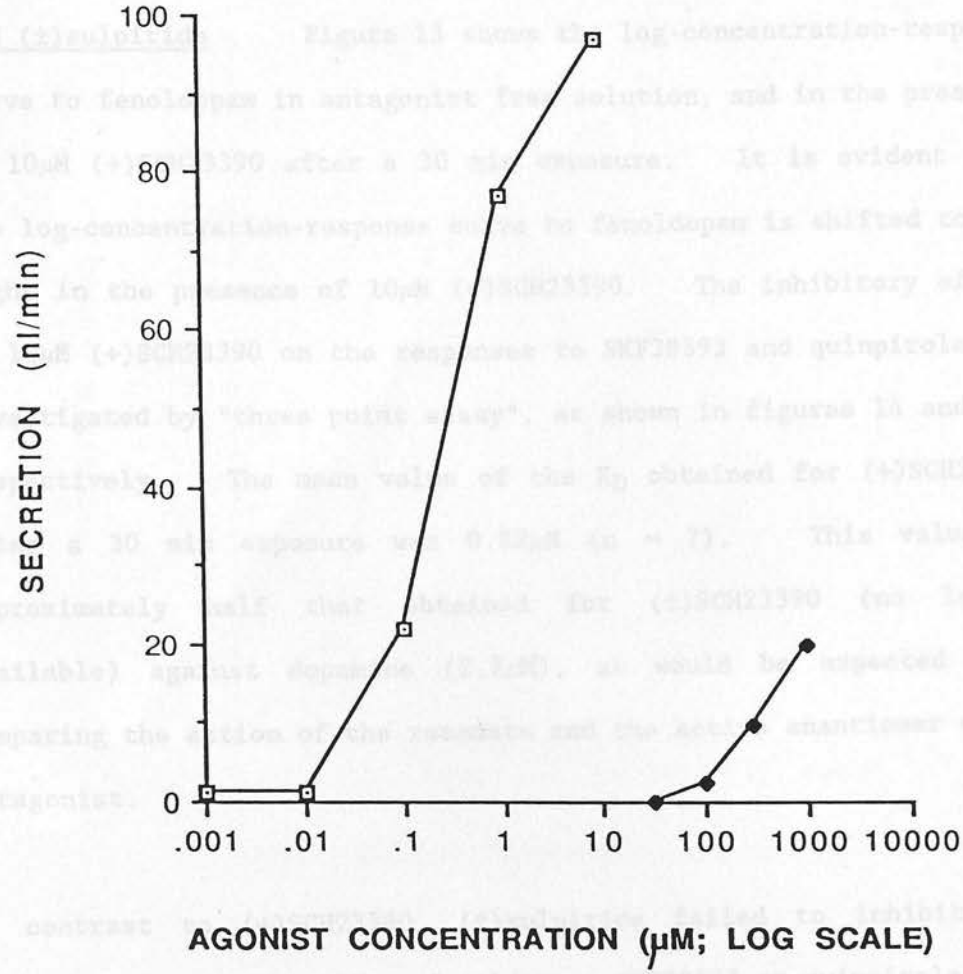

FIGURE 12 Comparison of the log-concentration-response curves for dopamine and quinpirole. Abcissa: agonist concentration (μM; logarithmic scale). Ordinate: rate of secretion in nl/min. Open squares represent the response to dopamine. Closed squares represent the response to quinpirole. These data were obtained from a single experiment.

Antagonism of the effects of the dopamine agonists by (+)SCH23390 and (±)sulpiride Figure 13 shows the log-concentration-response curve to fenoldopam in antagonist free solution, and in the presence of 10μM (+)SCH23390 after a 30 min exposure. It is evident that the log-concentration-response curve to fenoldopam is shifted to the right in the presence of 10μM (+)SCH23390. The inhibitory effect of 10μM (+)SCH23390 on the responses to SKF38393 and quinpirole was investigated by "three point assay", as shown in figures 14 and 15, respectively. The mean value of the K_D obtained for (+)SCH23390 after a 30 min exposure was 0.82μM (n = 7). This value is approximately half that obtained for (±)SCH23390 (no longer available) against dopamine (2.2μM), as would be expected when comparing the action of the racemate and the active enantiomer of an antagonist.

In contrast to (+)SCH23390, (±)sulpiride failed to inhibit the secretory response to either fenoldopam, SKF38893 or quinpirole.

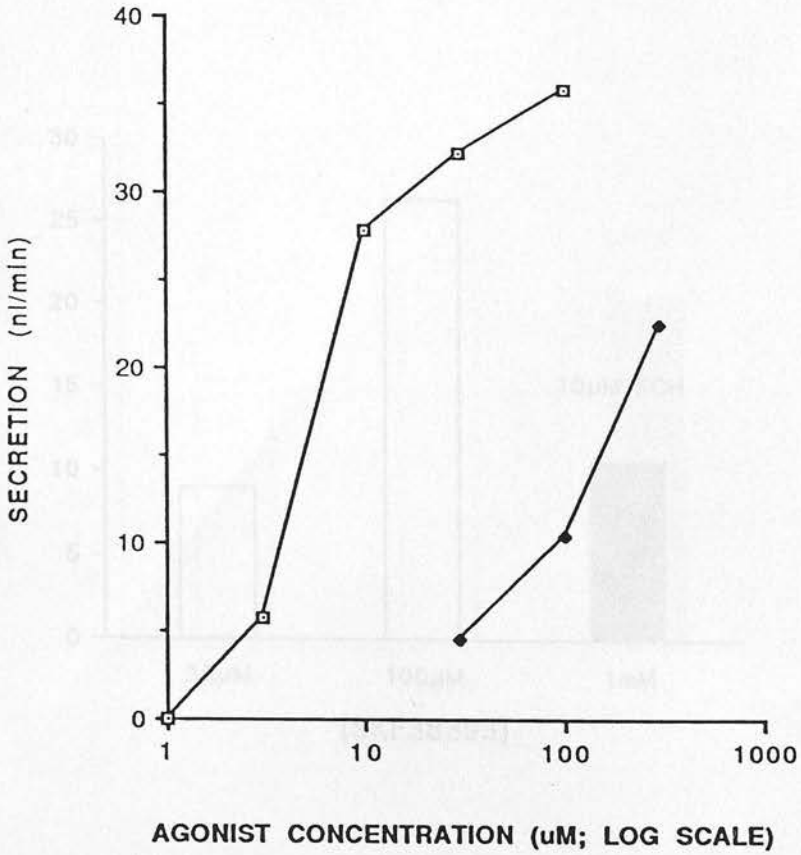

FIGURE 13 Inhibition by SCH23390 of the secretory response to fenoldopam. Abcissa: agonist concentration (μM; logarithmic scale). Ordinate: secretory rate in nl/min. Open squares: secretion in the absence of antagonist. Closed squares: secretion in the presence of 10μM (+)SCH23390 after a 30min exposure.

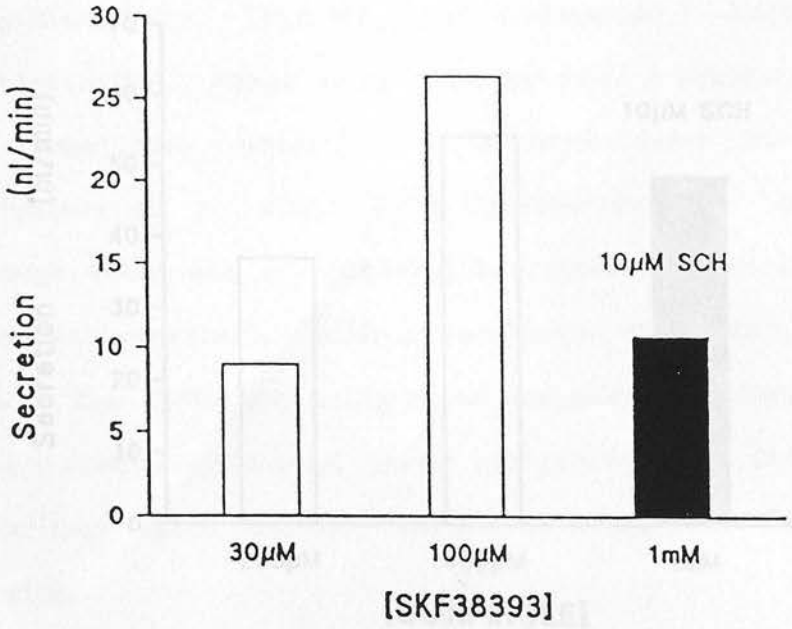

FIGURE 14 Inhibition by SCH23390 of the secretory response to SKF38393. Open columns: rate of secretion in nl/min induced by 10 and 100μM SKF38393 in the absence of the antagonist. Filled column: the response to 1mM SKF38393 in the presence of 10μM (+)SCH23390 after a 30min exposure.

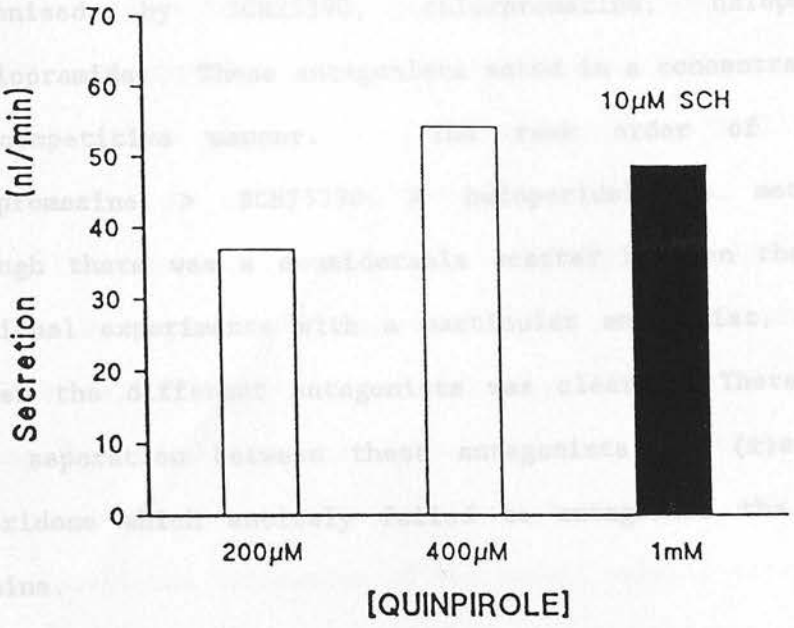

FIGURE 15 Inhibition by SCH23390 of the secretory response to quinpirole. Open columns: rate of secretion in nl/min induced by 200 and 400μM quinpirole in the absence of the antagonist. Filled column the response to 1mM quinpirole in the presence of 10μM (+)SCH23390 after a 30min exposure.

DISCUSSION

Dopamine-induced secretion from the cockroach salivary gland was antagonised by SCH23390, chlorpromazine, haloperidol and metoclopramide. These antagonists acted in a concentration-related and competitive manner. The rank order of potency was chlorpromazine > SCH23390 > haloperidol >> metoclopramide. Although there was a considerable scatter between the results of individual experiments with a particular antagonist, a separation between the different antagonists was clear. There was also a clear separation between these antagonists and (±)sulpiride and domperidone which entirely failed to antagonise the response to dopamine.

Comparison with mammalian D1 receptors

SCH23390 is a selective antagonist of mammalian D_1 receptors, while (±)sulpiride, domperidone, metoclopramide and to a lesser extent haloperidol show selectivity for the D_2 receptor (see Introduction). The fact that SCH23390 antagonised the secretory response to dopamine, while (±)sulpiride and domperidone did not, suggests that the receptor mediating secretion is pharmacologically similar to the mammalian D_1 receptor. At first sight the inhibition of secretion by haloperidol and metoclopramide might be thought to be inconsistent with such a classification. It should be noted however that the concentrations of these compounds required to inhibit the secretory response in the present study are also inhibitory at the mammalian D_1 receptor (Flaim *et al*, 1985; Hilditch and Drew, 1985).

The suggestion that the dopamine receptors on the cockroach salivary gland are similar to the D_1 receptor is supported by the studies with selective agonists. Thus both D_1 and D_2 agonists induced secretion, the rank order of potency being dopamine > fenoldopam > SKF38393 > quinpirole. Furthermore, the response to all agonists was inhibited by the selective D_1 antagonist (+)SCH23390, but not the selective D_2 antagonist (±)sulpiride. The rank order of potency, dopamine > fenoldopam > SKF38393 is the same as for mammalian D_1 receptors (Flaim *et al*, 1985; Hilditch and Drew, 1986; Niznik *et al*, 1988; Ohlstein *et al*, 1984). In addition the finding that SKF38393 has actions consistent with those of a partial agonist mirrors the results of Hilditch and Drew (1985) on the D_1 receptor-mediated relaxation of the rabbit splenic artery. Equally a weak stimulant action of the selective D_2 agonist quinpirole has also been observed on mammalian D_1 receptors subserving pancreatic secretion in dogs (Horiuchi *et al*, 1989). As in the cockroach salivary gland, the induction of pancreatic secretion by quinpirole was antagonised by the D_1 antagonist, SCH23390, but not the selective D_2 antagonist, (±)sulpiride.

Additional support of the D_1 classification for the dopamine receptor in the cockroach salivary gland, arises from the fact that activation of this receptor leads to an increase in cytosolic c-AMP levels (Grewe and Kebabion, 1983), and that c-AMP is an intermediate in the secretory response (Gray *et al*, 1984).

However, a clearly anomalous finding is the low potency of SCH23390 at the receptor on the cockroach salivary gland, the K_{Dapp} being perhaps between 2 and 5 orders of magnitude higher than that found

in other functional studies on mammalian D_1 receptors, where SCH23390 has been found to be the most potent antagonist (Hilditch and Drew, 1985; Anderson *et al*, 1985). Not all D_1 receptors however have an identical pharmacology and there are striking differences in the potency of (±)sulpiride at D_1 receptors in a variety of preparations. The selective D_1 antagonist SCH23390 inhibits the effects of dopamine on the endothelium and smooth muscle of the rabbit splenic artery (Hilditch and Drew, 1985; Ohlstein *et al*, 1984), rabbit mesenteric vascular bed (Missalle *et al*, 1985; Hilditch and Drew, 1985), rat striatum (Anderson *et al*, 1985) and renal cortex (Baldi *et al*, 1988; Felder *et al*, 1989). In contrast (±)sulpiride is only a weak antagonist at D_1 receptors in the mesenteric vascular bed and fails to antagonise the action of dopamine at D_1 receptors on the smooth muscle of the rabbit splenic artery (Hilditch and Drew, 1985; Ohlstein *et al*, 1984; Missalle *et al*, 1985). However, (+)sulpiride is an antagonist at dopamine D_1 receptors of the rabbit splenic artery (Ohlstein *et al*, 1984) and (±)sulpiride inhibits the dopamine-sensitive adenylyl cyclase of the rat striatum (Anderson *et al*, 1985) and renal cortex (Baldi *et al*, 1988; Felder *et al*, 1989). Moreover, the affinity of (±)sulpiride for the receptor in the striatum is much lower than that for the receptors on the renal cortex (Anderson *et al*, 1985; Baldi *et al*, 1988; Felder *et al*, 1989). The potency of dopamine receptor agonists may also prove to be important in the subclassification of D_1 receptors, as, in contrast to its activity in most D_1 receptor models fenoldopam was found to be a more potent agonist than dopamine of D_1 receptors in the rabbit and dog mesenteric vascular bed (Hildith and Drew, 1985; Missalle *et al*, 1985). It is also interesting to note that investigations of D_1

receptors in the amygdala have failed to identify a dopamine-sensitive adenylyl cyclase (Mailman *et al*, 1986; Dawson *et al*, 1986; Walaas and Greengard, 1984). The D_1 receptors described above and the receptor on the cockroach salivary gland, at which SCH23390 is a relatively weak antagonist, may represent hitherto unrecognised subtypes of the D_1 dopamine receptor.

Comparison with other invertebrate dopamine receptors

Of great importance to the understanding of invertebrate physiology and receptor evolution is the comparison of the pharmacology of receptors for individual neurotransmitters within a particular species and also between different species. The most interesting comparison is to be made with the dopamine-sensitive adenylyl cyclase system of the locust (*L. migratoria*) salivary gland, where the antagonist rank order of potency and the K_D of the individual antagonists were almost identical to those obtained for the cockroach salivary gland (Lafon-Cazal and Bockaert, 1984). The most striking similarity was the low potency of SKF38393 which, as in the present investigation, inhibited the response to dopamine when the two compounds were applied in combination. Similarities also exist between the receptor on the cockroach salivary gland and the D_1-like receptor on growth hormone producing cells of the snail (*Lymnaea stagnalis*). Stoof *et al* (1984) established that SKF38393 mimicked one of two actions of dopamine on this preparation, while SCH23390 inhibited this response to both agonists. In contrast, the studies of Orr *et al* (1987) have identified in the cockroach (*Periplaneta americana*) brain, a dopamine receptor which has a pharmacological profile distinct from either the mammalian D_1 or D_2

receptor. This is also true of the dopamine receptor on the buccal-2 neurone and pedal giant neurone of *Lymnaea stagnalis* (Audesirk, 1989).

CHAPTER IV

CHARACTERIZATION OF THE RECEPTOR SUBSERVING THE HYPERPOLARIZATION

INTRODUCTION

The data presented in the previous chapter suggests that the dopamine receptor mediating the secretory response of the cockroach salivary is similar to the mammalian D_1 sub-type. House (1973) found that, in addition to secretion, dopamine induces a hyperpolarization of the salivary gland acinar cells. Subsequent studies established that a calcium-dependent potassium conductance underlies the hyperpolarization (Ginsborg *et al*, 1974 and 1980) which, unlike secretion, is independent of c-AMP (Gray *et al*, 1984). Due to the fact that c-AMP acts an intermediate in the secretory response to dopamine, but not the hyperpolarizing response, it was suggested that a different dopamine receptor subserved each event. It is therefore of some interest to study the pharmacology of the receptor mediating the hyperpolarization. To enable as close a comparison of the pharmacology of the two events, the protocol used in the present investigation follows closely that of the previous study. Thus (i) the rank order of potency was obtained for the selective D_1 antagonist SCH23390, the selective D_2 antagonists domperidone, (±)sulpiride, haloperidol and metoclopramide and the non-selective dopamine antagonist chlorpromazine, (ii) a study was made of the ability of two selective D_1 agonists, fenoldopam and SKF38393, and two selective, D_2 agonists LY163502 and quinpirole, to induce a hyperpolarization and (iii) the effects of SCH23390 and (±)sulpiride was studied on the responses obtained to the agonists used in (ii).

The dissection has been described in Chapter III. Paired salivary glands and associated ducts were pinned to the base of a perspex chamber by way of four troughs filled with silicone resin (Sylgard, Dow Coming). The main ducts of the gland were drawn into a suction electrode to allow stimulation of the encapsulated nerve. The preparation was kept at room temperature (18-25°C) and superfused at a rate of 5ml/min.

Experimental design

Experimental chamber Figure 16 illustrates the chamber used in electrophysiological investigations. It was constructed from a circular piece of perspex with a central channel cut in an H shape, covering approximately 3/4 of the diameter. At each end of the channel corresponding to the 'horizontal bar' of the H at the point of the junction with the vertical line, net baffles were placed to remove air bubbles from and smooth the flow of the superfusate. In the centre of the channel four troughs were positioned in the shape of a square whose area approximated that of the preparation. The troughs were filled with silicone resin (Sylgard, Dow Corning), which allowed the salivary gland to be pinned firmly to the base of the dish. At one side of the channel and in line with these troughs a holder for the suction electrode (see below) was built into the chamber. This was angled at approximately 70° to the horizontal in order to direct the suction electrode to the base of the channel at the front of the trough over which the common duct of the gland would lie. The holder consisted of a groove cut into the

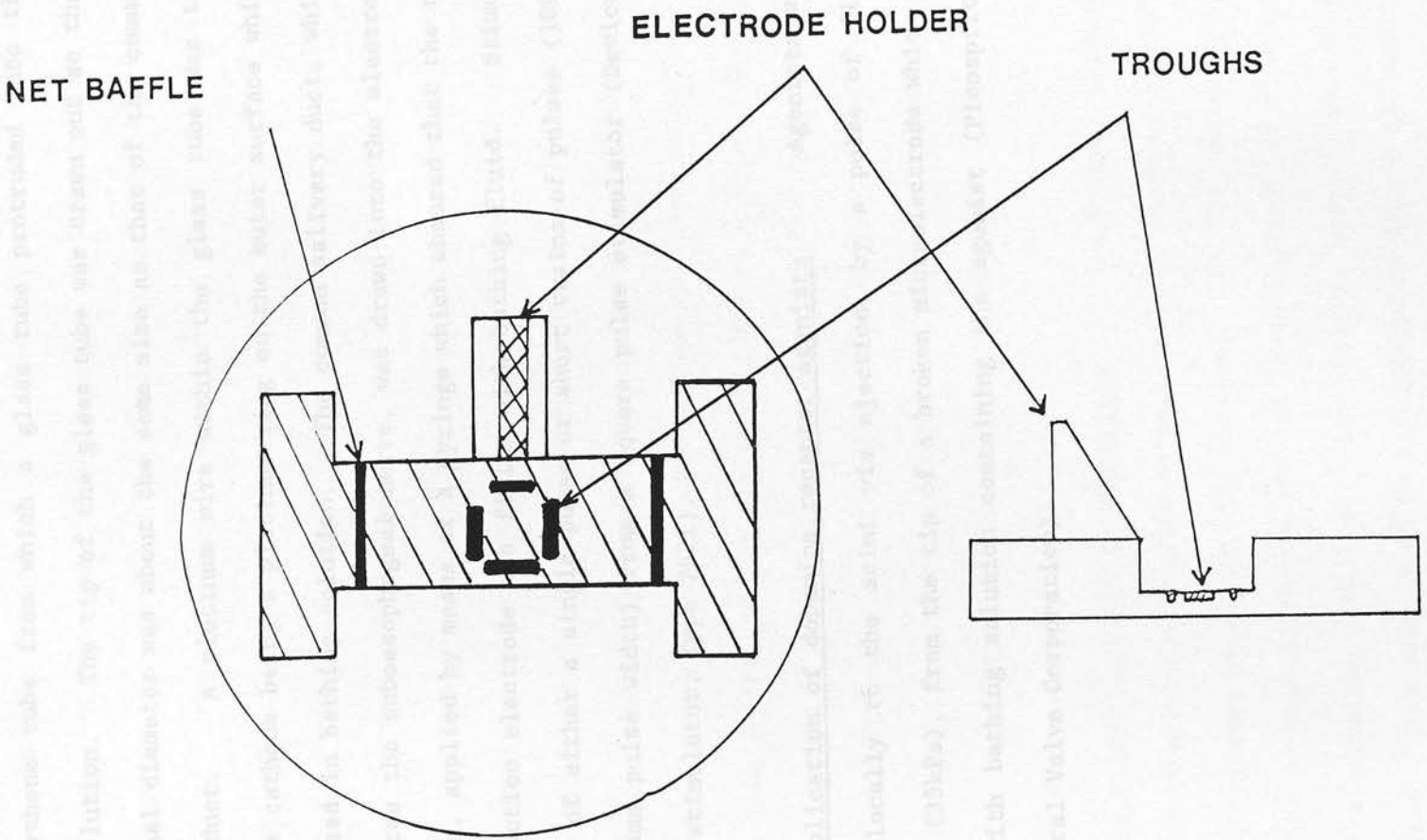

FIGURE 16 Diagram of the experimental chamber. See text for

upper side of a perspex triangular prism, of about the same size as the electrode. When pressed into this channel the suction electrode was held firmly in place.

The suction electrode The suction electrode was made from a rigid polythene tube from which a glass tube protruded into the bathing solution. The tip of the glass tube was drawn out so that its internal diameter was about the same size as that of the common salivary duct. A platinum wire within the glass tube was the anode, the cathode being a platinum ring on the outer surface which was immersed in bathing solution. The common salivary duct, which encapsulates the suboesophageal nerve, was drawn into the electrode by suction, applied by means of a syringe which ensured that the tip of the suction electrode was filled with bathing fluid. Stimuli consisted of either a single pulse or short trains of pulses (100V, 20Hz, 0.5ms pulse width) from a square pulse stimulator (Devices, Isolated stimulator, Type 2521).

Local application of dopamine receptor agonists Agonists were applied locally to the acini via ejection, by a pulse of fixed pressure (35kPa), from the tip of a broken microelectrode which was filled with bathing solution containing the agonist (Picospritzer II, General Valve Corporation).

Recording of electrical activity of the acinar cells Membrane potentials were recorded with glass microelectrodes made on a horizontal electrode puller (P-77 Brown-Flaming Micropipette Puller, Sutter Instrument Company) from borosilicate glass cappillaries, containing an inner filament to aid filling (Clark Electromedical Instruments, Cat.No. GC120F-15). These electrodes (20-40MΩ resistance) were filled with 3M KCl using a syringe, pulled to a fine point under heat to allow its insertion into the tip of the microelectrode. The electrodes were connected, via a pre-amplifier (Dagan 8900 Patch Clamp, current clamp mode), to a digital audio processor (Sony PCM-701ES) and a video recorder (Sony SL-F30) (see Figure 17). From the output of the digital audio processor connections were also made to a dual beam oscilloscope (Tektronix Inc., Type 565) and a pen recorder to allow the membrane potential to be monitored during the experiments. The microelectrodes were lowered into position by means of a Leitz micromanipulator and placed close to the acinus. The acinar cell was penetrated by using the fine control of the manipulator and/or a light tap on the air table on which the apparatus rested. The bath electrode consisted of AgCl-coated Ag wire which was mounted on the microscope stage holding the experimental chamber. The bath electrode was connected to earth via a calibrator (see Figure 17).

Control of stimuli applied to the acinus The stimuli applied to the preparation were controlled automatically by means of a Neurolog system (Digitimer Research Instruments). The setup allowed stimulation of the salivary nerve and local application of agonists to occur alternately with a set time interval between each form of stimulation. The setup used is illustrated in figure 18, and

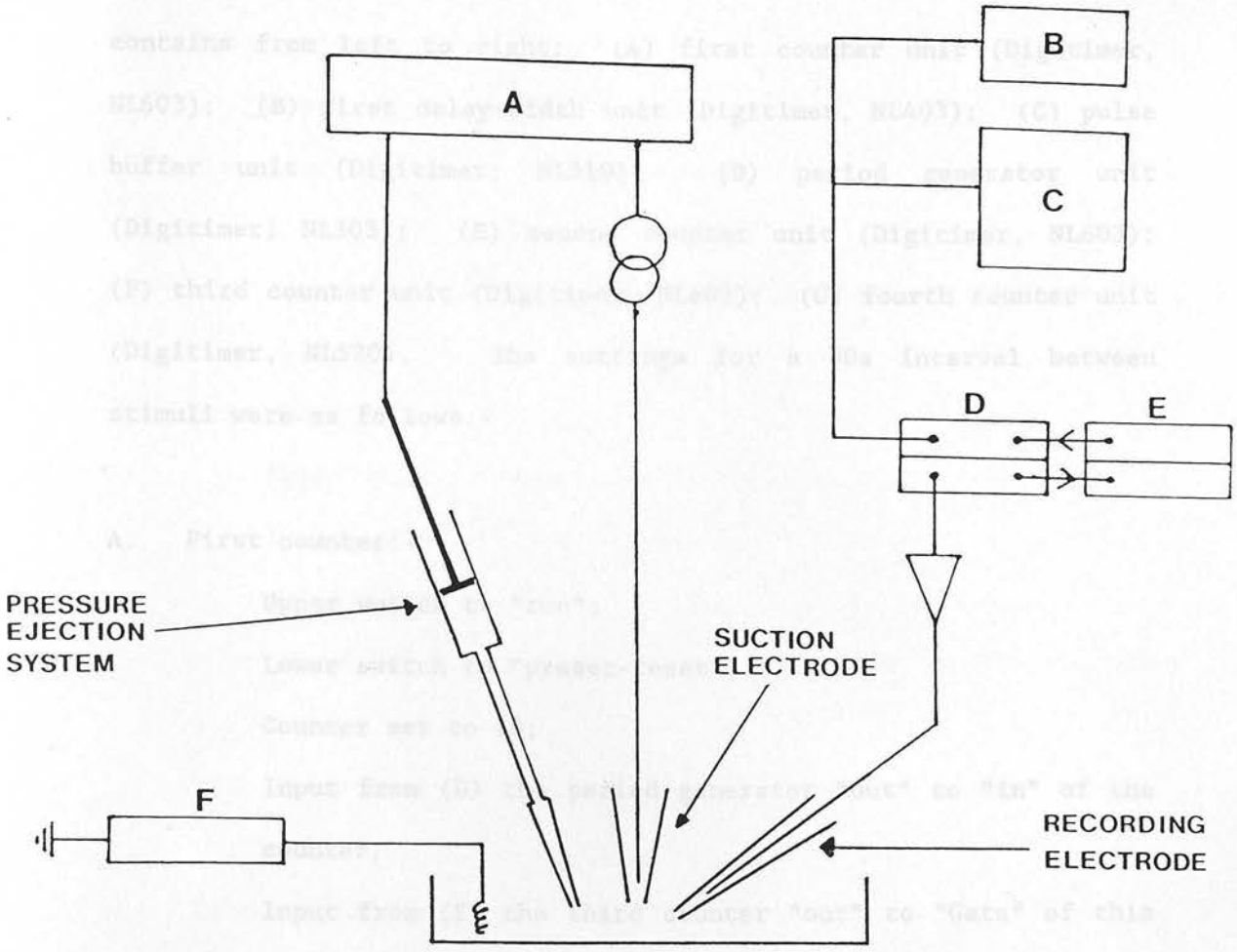

FIGURE 17 Diagram of the apparatus used for stimulation and recording: A = Neurolog System; B = pen recorder; C = oscilloscope; D = digital audio processor; E = video recorder; F = calibrator.

contains from left to right: (A) first counter unit (Digitimer, NL603); (B) first delay-width unit (Digitimer, NL403); (C) pulse buffer unit (Digitimer, NL510); (D) period generator unit (Digitimer; NL303); (E) second counter unit (Digitimer, NL603); (F) third counter unit (Digitimer, NL603); (G) fourth counter unit (Digitimer, NL520). The settings for a 90s interval between stimuli were as follows:-

A. First counter:-

Upper switch to "run";

Lower switch to "preset-reset";

Counter set to 18;

Input from (D) the period generator "out" to "in" of the counter;

Input from (F) the third counter "out" to "Gate" of this counter.

B. First delay-width unit:-

Switch to "Trig + slope";

Delay set to 100μs and 0;

Width set to 500ms

C. Pulse buffer unit:-

Amplitude set to 10V;

Switch to 0-10V;

Input from (B) the delay width unit "out" to "in" on this unit

D. Period generator:-

Switch to "on";

Period set to 1s;

Multiplier set to 5

E. Second counter:-

Upper switch to "run"

Lower switch to "preset-reset"

Counter set to 36

Input from (D) the period generator "out" to "in" on this unit

F. Third counter:-

Upper switch to"run"

Lower switch to "preset-reset";

Counter set to 18;

Input from (D) the period generator "out" to "in" on this unit;

Input from (E) the second counter "out" to "reset" on this unit

G. Fourth counter:-

Upper switch to "run";

Lower switch to "preset-stop";

Counter set to 09;

Input from (H) the convertor "TTL out No.1" to "in" on this unit;

Input from (E) the second counter"out" to "reset" on this unit

H. Convertor:-

Input from "pulses out" of the gated pulse generator (external unit controlling the square pulse stimulator used to stimulate the nerve) to "-Logic in (B)" of this unit

Input from (G) the fourth counter "out" to "TTL in (B)" of this unit

The (D) fourth counter "counter" controls the number of impulses applied to the nerve, in this case 09, which is effectively 10 impulses.

Two further connections were made. Both of these were to external units and allowed the neurolog to trigger the gated pulse generator (Devices, Type 2521) of the square pulse stimulator (Devices, Isolated stimulator, Type 2533) and the pressure ejection system (Picospritzer II, General Valve Corporation):

1. From (C) pulse buffer "out" to the "External Stimulus" input of the Picospritzer unit.
2. From (H) the convertor "-Logic out (B)" to the "gate pulse" input of the Gated Pulse Generator.

Investigation of the effects of dopamine receptor antagonists on responses to nerve stimulation and dopamine

Nerve stimulation Submaximal responses were obtained using a train of 1-10 impulses, 0.5ms pulse width; 1-100V, 20Hz. Antagonists were added to the superfusate after four consistent control responses had been obtained. No quantitative analysis was attempted for the effects of the antagonists on nerve stimulation.

Dopamine Dissociation constants, K_Ds, for each of the antagonists were determined against responses to dopamine applied locally by pressure ejection (5×10^{-7}M, 35kPa, 0.003-5s). When applied in this way the absolute concentration of the agonist at the acinus is, of course, unknown and a function of time. There is

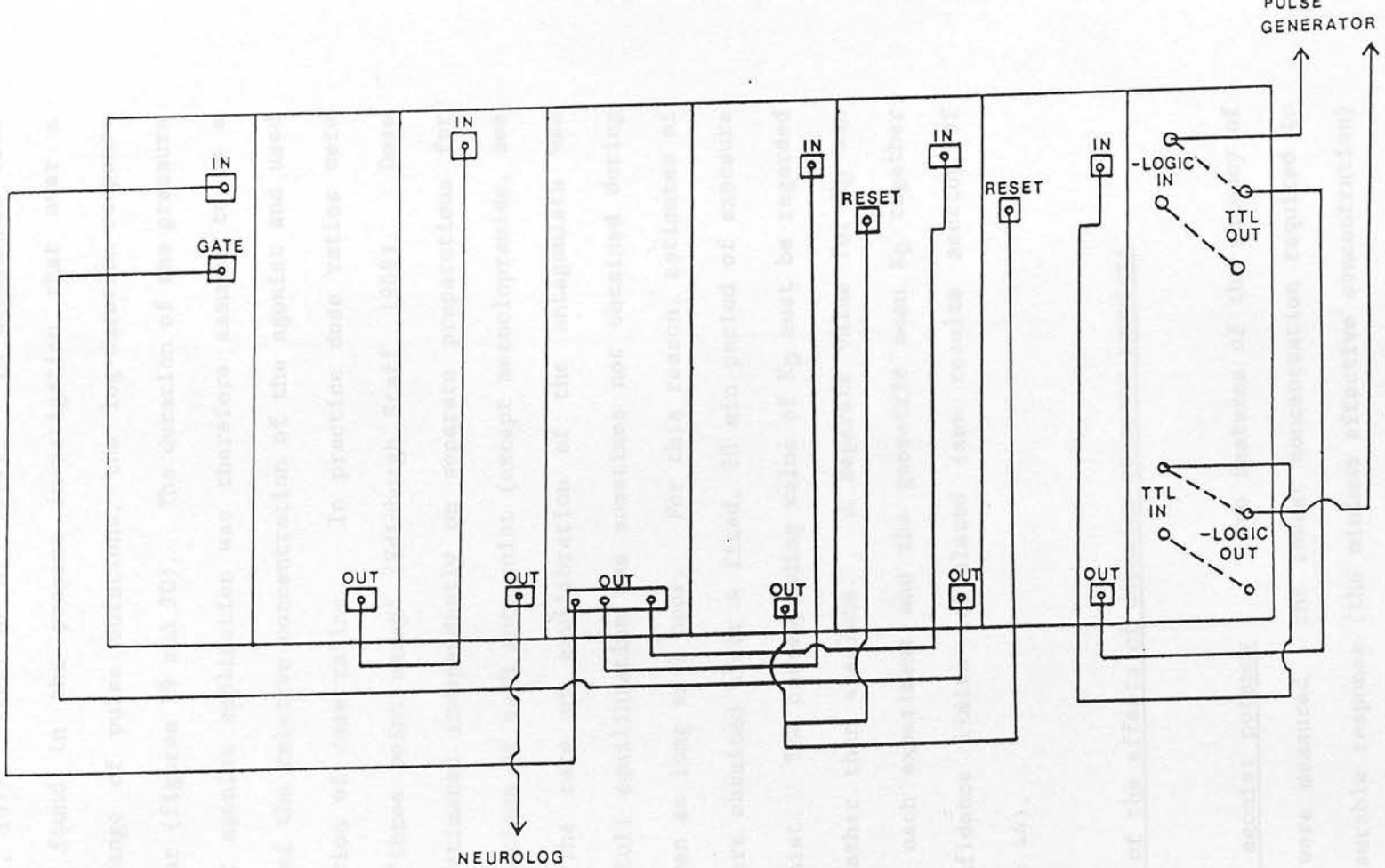

FIGURE 18 Diagram showing connections between individual units of the Neurolog System. See text for details. A - first counter; B - first delay-width unit; C - pulse buffer unit; D - period generator; E - second counter; F - third counter; G - fourth counter; H - convertor.

evidence however, that the effective concentration of agonist at the acinus is proportional to the duration of a pulse of fixed pressure (McCaman *et al*, 1977; Sakai *et al*, 1979). In accordance with this, it was found in the present investigation that over a considerable range of pulse durations, the log-duration-response curve was linear (figures 19 and 20). The duration of the pressure pulse used for agonist application was therefore assumed to be a valid measure of the relative concentration of the agonist and used in the estimation of dose ratios. In practice dose ratios were estimated by "three point assay" (Edinburgh Staff, 1968). Dose ratios were estimated independently on separate preparations for three concentrations of each antagonist (except metoclopramide, see results). The rate of equilibration of the antagonists was variable and full equilibration was sometimes not obtained during incubations even as long as 1 hour. For this reason, estimates of dose ratios were obtained after a fixed, 30 min period of exposure to the antagonist. The corresponding value of K_D must be regarded as apparent rather than absolute. A separate value for K_D was obtained from each experiment and the geometric mean K_D together with 95% confidence limits calculated (see results section of Chapter III, p.44).

Investigation of the effects of dopamine receptor agonists

Estimation of agonist potency Two features of the potency of the agonists were measured : the lowest concentration required to produce a measurable response (the minimum effective concentration) and the equipotent molar ratio of the agonist and dopamine. To obtain an estimate of the minimum effective concentration of an

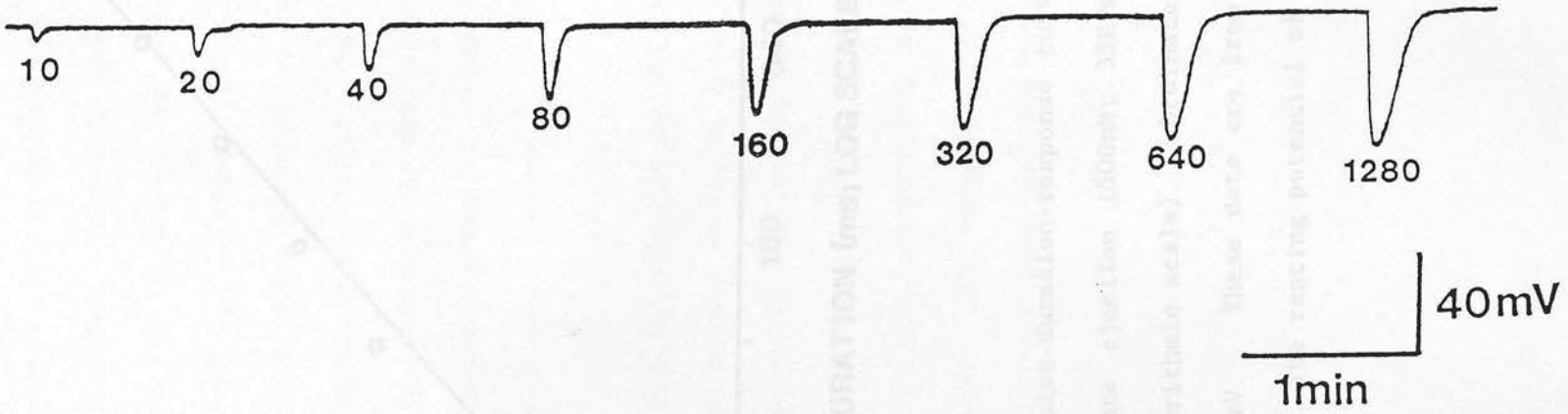

FIGURE 19 The effect of increasing pulse duration on the response to dopamine applied locally by pressure ejection (1μM; 35KPa). The record is continuous. Pulse duration in ms is given below each response. The hyperpolarization in this and all subsequent figures is downwards. The resting potential was -55mV.

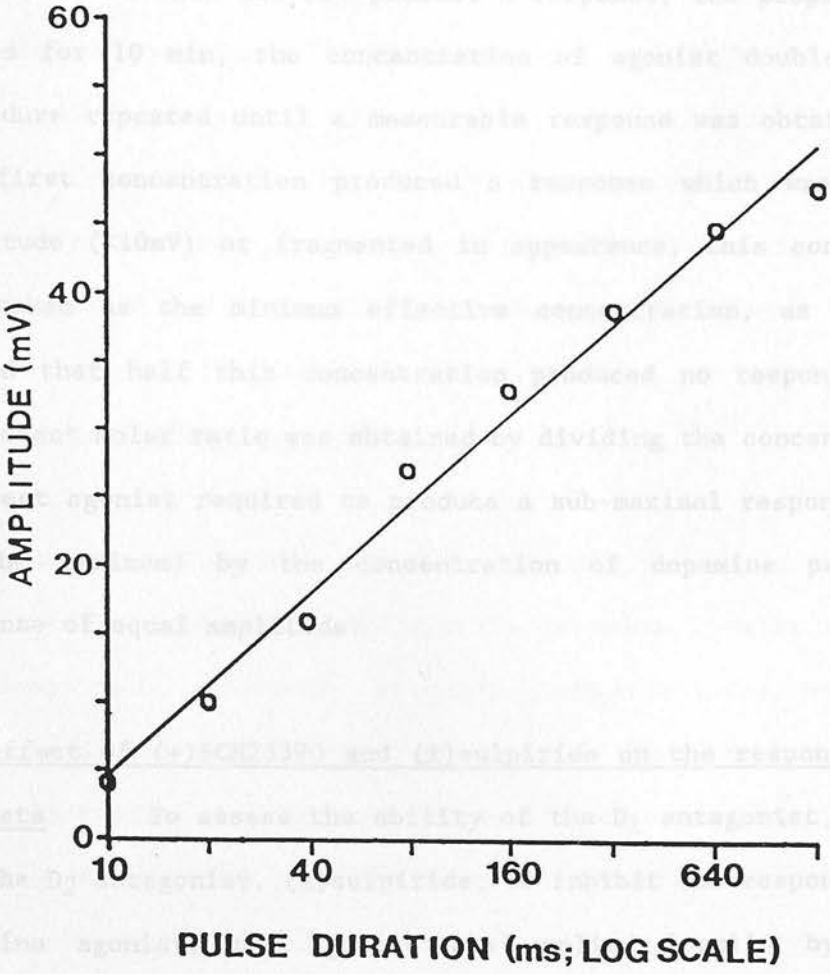

FIGURE 20 A "log-pulse-duration-response curve" to dopamine applied locally by pressure ejection (500nM; 35KPa). Abcissa: pulse duration in ms (logarithmic scale). Ordinate: amplitude of the hyperpolarization in mV. These data are from the experiment illustrated in figure 19. The resting potential of this cell was -55mV.

agonist, tissues were exposed to a low concentration of the agonist for 60s. If this did not produce a response, the preparation was washed for 10 min, the concentration of agonist doubled and the procedure repeated until a measurable response was obtained. If the first concentration produced a response which was small in amplitude (<10mV) or fragmented in appearance, this concentration was taken as the minimum effective concentration, as experience showed that half this concentration produced no response. The equipotent molar ratio was obtained by dividing the concentration of the test agonist required to produce a sub-maximal response (50-90% of the maximum) by the concentration of dopamine producing a response of equal amplitude.

The effect of (+)SCH23390 and (±)sulpiride on the responses to the agonists To assess the ability of the D_1 antagonist, SCH23390, and the D_2 antagonist, (±)sulpiride, to inhibit the responses to the dopamine agonist, the agonist was applied locally by pressure ejection (35kPa, 0.003-5s). The concentration of the agonist in the ejection pipette was 1mM. The antagonists were added to the superfusate after four consistent responses had been obtained, and superfused for 30 min.

RESULTS

As is well known (House, 1973; Ginsborg *et al*, 1974) stimulation of the suboesophageal nerve evokes a stimulus-dependent hyperpolarization of the salivary gland acinar cells. When applied locally by pressure ejection, dopamine mimicked the effect of nerve stimulation in a duration-dependent manner (see Methods).

The effects of dopamine antagonists

Chlorpromazine (0.1-5μM), SCH23390 (10-50μM), haloperidol (10-100μM) and metoclopramide (1mM) inhibited the response to nerve stimulation and dopamine. However, at higher concentrations some of the antagonists had non-specific effects. The inhibition of the hyperpolarization by chlorpromazine at concentrations > 5μM was insurmountable, while SCH23390 destabilised the acinar cell membrane at concentrations ≥100μM. The effect of these compounds could therefore be studied only over a limited range.

Nerve stimulation Figure 21 illustrates the effect of chlorpromazine on the response to nerve stimulation. A shows the response to 2 impulses applied to the nerve in antagonist free solution. B shows the response to an identical stimulus in the presence of 0.5μM chlorpromazine. C shows the effect of increasing the number of impulses to 9 still in the presence of chlorpromazine after a 30 min exposure. One can see that chlorpromazine inhibited the response to nerve stimulation and that increasing the stimulus overcame the blockade. Although Bowser-Riley *et al* (1978) established that useful estimates of an antagonist's K_D can be

obtained against nerve stimulation, no attempt was made to carry out a quantitative analysis in the present study. The effects of SCH23390, haloperidol and metoclopramide are shown in a corresponding way in figures 22, 23 and 24. Chlorpromazine (0.1, 0.5 and 5μM), SCH23390 (10, 20 and 50μM), haloperidol (10, 20 and 100μM) and metoclopramide inhibited the hyperpolarization to nerve stimulation. The inhibition of the response by chlorpromazine (0.1-5μM) and SCH23390 (10-50μM) could be surmounted and was reversed on washing with antagonist free solution. However, the blockade of the hyperpolarization by haloperidol (100μM only) and metoclopramide (1mM) was insurmountable, and the inhibitory action of haloperidol (10-100μM) was not reversed after a 30min wash with antagonist free solution. In contrast, the inhibition by these compounds of the response to dopamine was found to be surmountable and was reversed on washing (see below). Thus haloperidol and metoclopramide may have an additional pre-synaptic action.

Of the more selective D_2 receptor antagonists, (±)sulpiride failed to inhibit the response to nerve stimulation, while domperidone (50 & 100μM) inhibited the response through a pre-synaptic action (see Chapter VI).

Dopamine A more quantitative analysis of the effects of the antagonists was undertaken with respect to the hyperpolarization produced by dopamine applied locally by pressure ejection (see methods). Figure 25 illustrates a typical "three point assay" for chlorpromazine. A shows the response to a 150ms pulse of dopamine and B the response to a 300ms pulse in antagonist free solution. C shows the response to a 300ms pulse of dopamine after addition of

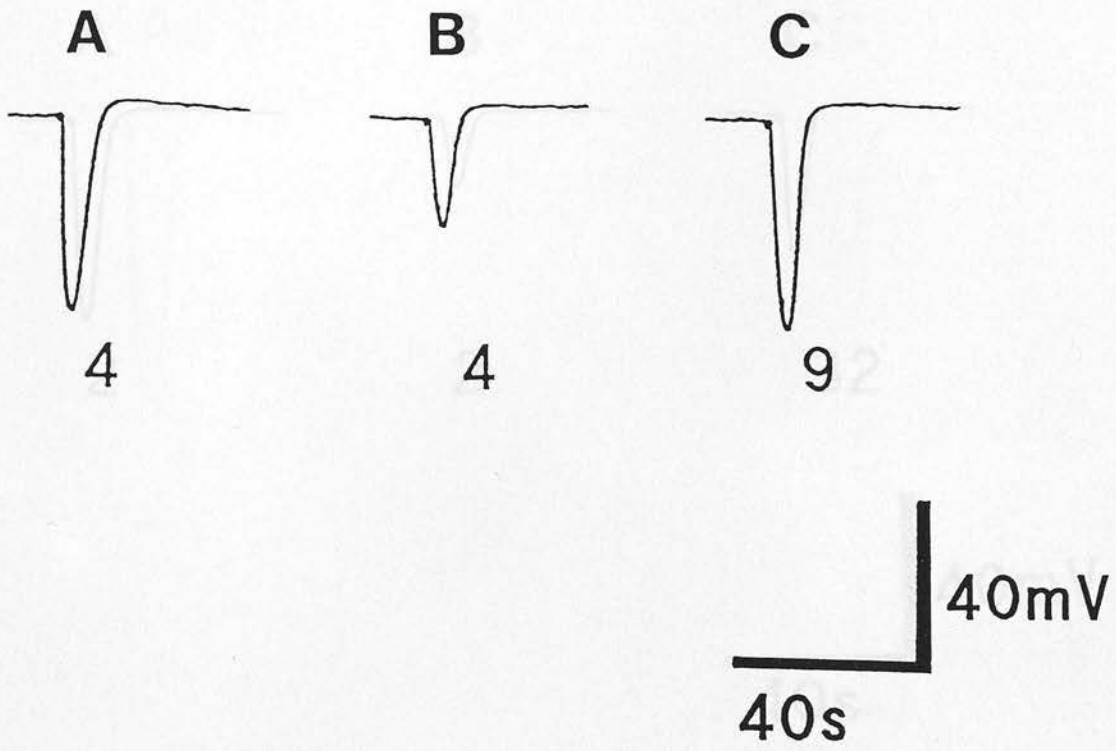

FIGURE 21 Inhibition by chlorpromazine of the electrical response to nerve stimulation (100V; 20Hz). A: the response to 4 impulses in the absence of the antagonist. B: the response to an identical stimulus in the presence of 0.5μM chlorpromazine. C: the effect of increasing the number of impulses to 9; chlorpromazine had been in contact with the preparation for 30min. The resting potential was -40mV.

FIGURE 22 Inhibition by SCH23390 of the electrical response to nerve stimulation (100V; 20Hz). A: the response to 2 impulses in the absence of the antagonist. B: the response to an identical stimulus in the presence of 20μM SCH23390. C: the effect of increasing the number of impulses to 32; SCH23390 had been in contact with the preparation for 30min. The resting potential was -40mV.

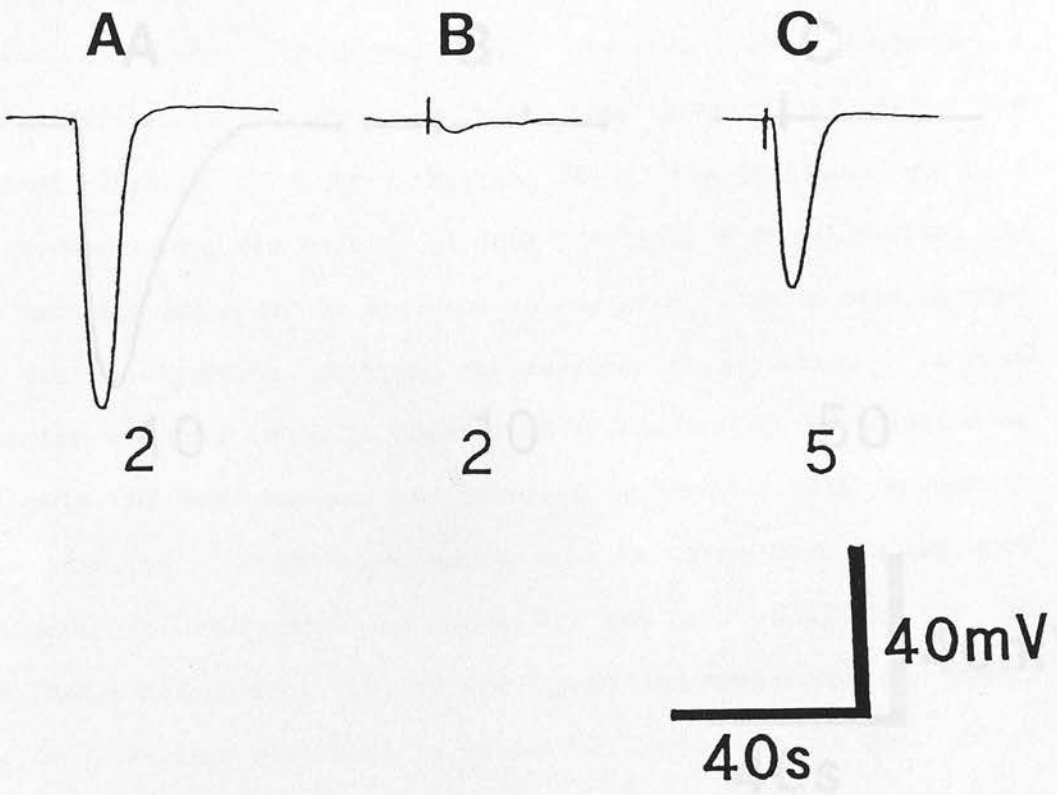

FIGURE 23 Inhibition by haloperidol of the electrical response to nerve stimulation (100V; 20Hz). A: the response to 2 impulses in the absence of the antagonist. B: the response to an identical stimulus in the presence of 20μM haloperidol. C: the effect of increasing the number of impulses to 5; haloperidol had been in contact with the preparation for 30min. The resting potential was -42mV.

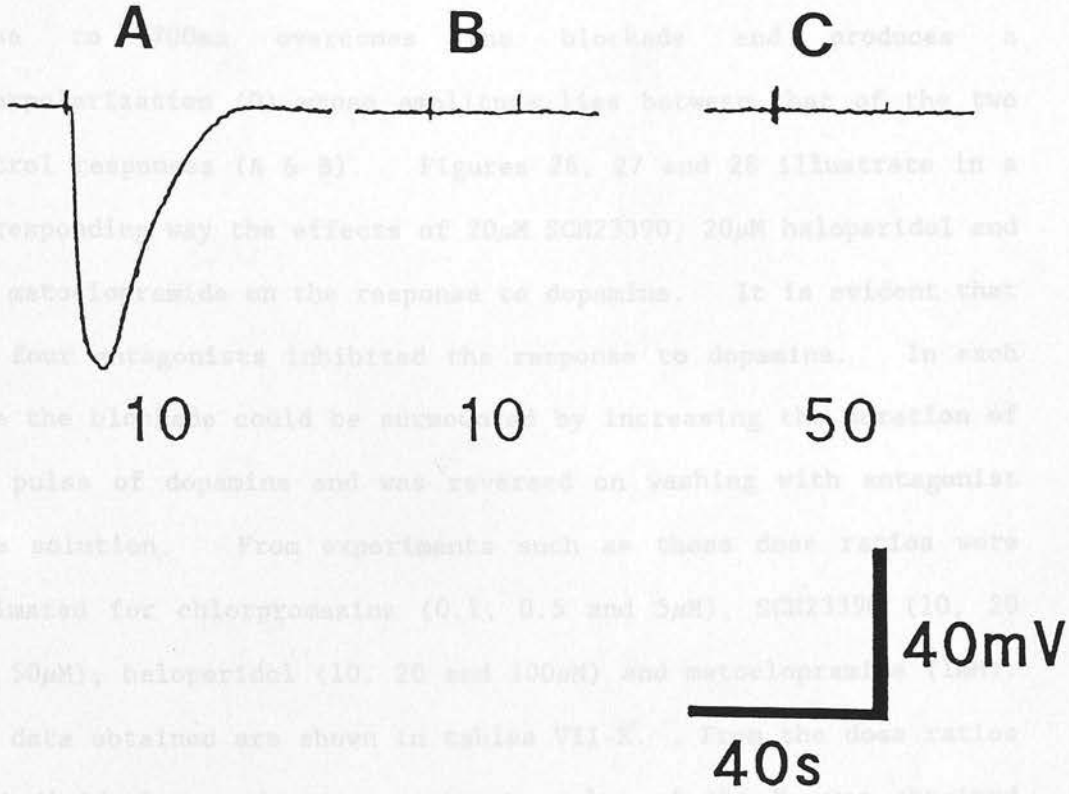

FIGURE 24 Inhibition by metoclopramide of the electrical response to nerve stimulation (100V; 20Hz). A: the response to 10 impulses in the absence of the antagonist. B: the response to an identical stimulus in the presence of 1mM metoclopramide. C: the effect of increasing the number of impulses to 50; metoclopramide had been in contact with the preparation for 30min. The resting potential was -42mV.

0.5μM chlorpromazine to the superfusate. D shows the effect of increasing the duration of the dopamine pulse to 700ms in the presence of chlorpromazine after a 30min exposure. From B and C one can see that the response to dopamine is inhibited by 0.5μM chlorpromazine, and that increasing the duration of the dopamine pulse to 700ms overcomes the blockade and produces a hyperpolarization (D) whose amplitude lies between that of the two control responses (A & B). Figures 26, 27 and 28 illustrate in a corresponding way the effects of 20μM SCH23390, 20μM haloperidol and 1mM metoclopramide on the response to dopamine. It is evident that all four antagonists inhibited the response to dopamine. In each case the blockade could be surmounted by increasing the duration of the pulse of dopamine and was reversed on washing with antagonist free solution. From experiments such as these dose ratios were estimated for chlorpromazine (0.1, 0.5 and 5μM), SCH23390 (10, 20 and 50μM), haloperidol (10, 20 and 100μM) and metoclopramide (1mM). The data obtained are shown in tables VII-X. From the dose ratios of individual experiments a separate value of the K_D was obtained and the geometric mean of the K_D with 95% confidence limits calculated (as described in the results section of Chapter III). These data are summarised in Table XI. The rank order of potency for the antagonists was as follows (K_D in parentheses): chlorpromazine (0.2μM) > SCH23390 (4.1μM) = haloperidol (3.3μM) > metoclopramide (265μM). Although there is a considerable scatter between the K_Ds from individual experiments a clear separation was evident between chlorpromazine, SCH23390 and haloperidol, and metoclopramide (figure 29).

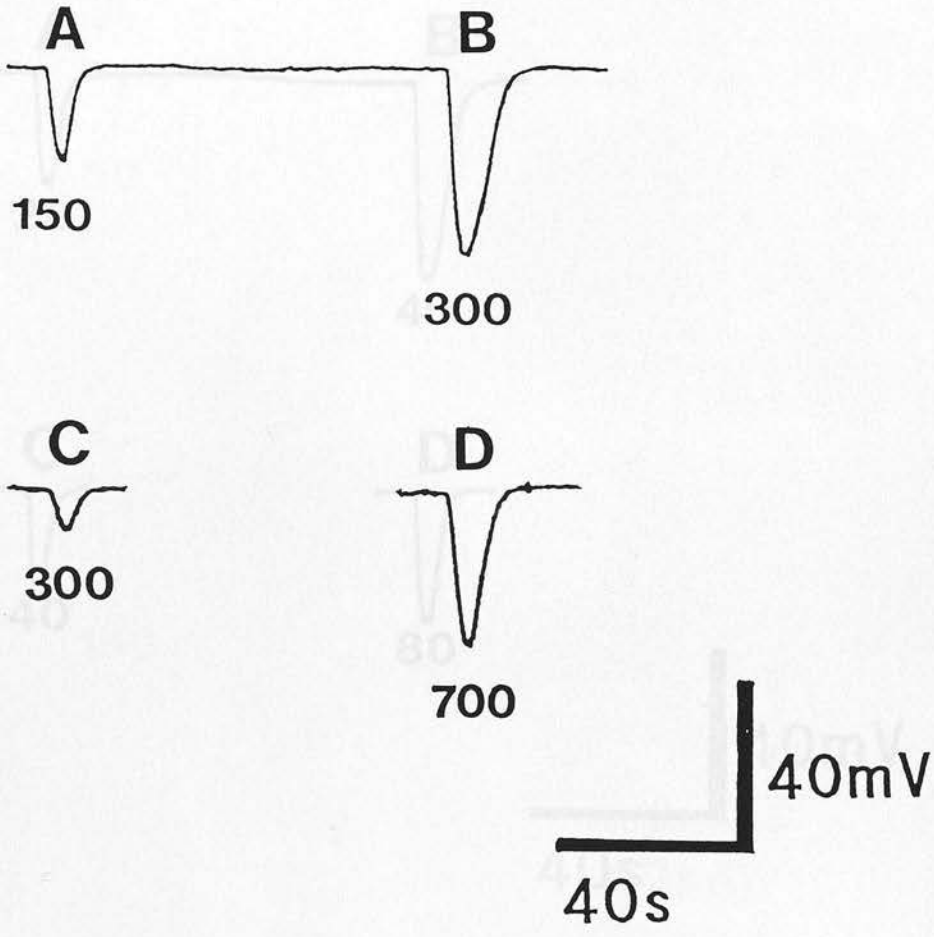

FIGURE 25 Antagonism by chlorpromazine of the hyperpolarization induced by dopamine applied locally by pressure ejection (500nM; 35KPa). A and B show the hyperpolarization to a 150ms and 300ms pulse of dopamine, respectively, in the absence of chlorpromazine; C shows the inhibition of the response to the larger of these two stimuli upon addition of 0.5μM chlorpromazine to the perfusate. D illustrates the effect of increasing the duration of the dopamine pulse to 700ms. At this point the period of exposure to chlorpromazine was 30min. The initial resting potential was -40mV.

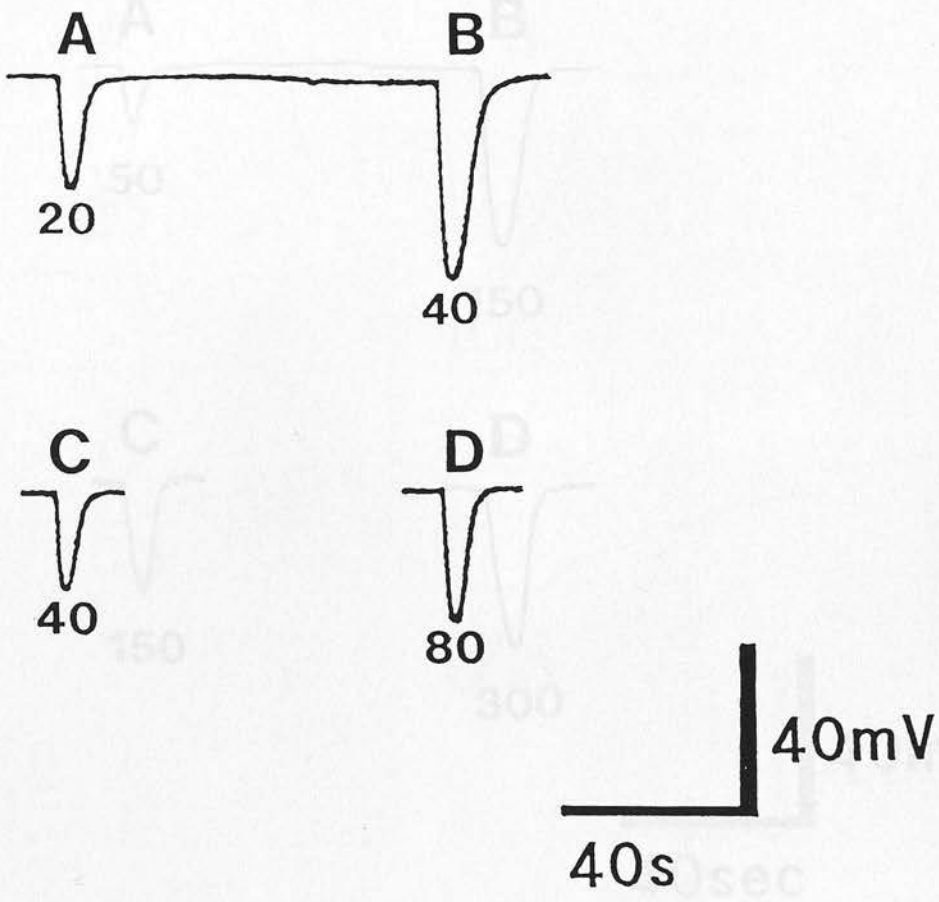

FIGURE 26 Antagonism by SCH23390 of the hyperpolarization induced by dopamine applied locally by pressure ejection (500nM; 35KPa). A and B show the hyperpolarization to a 20ms and 40ms pulse of dopamine, respectively, in the absence of SCH23390; C shows the inhibition of the response to the larger of these two stimuli upon addition of 20μM SCH23390 to the perfusate. D illustrates the effect of increasing the duration of the dopamine pulse to 80ms. At this point the period of exposure to SCH23390 was 30min. The initial resting potential was -42mV.

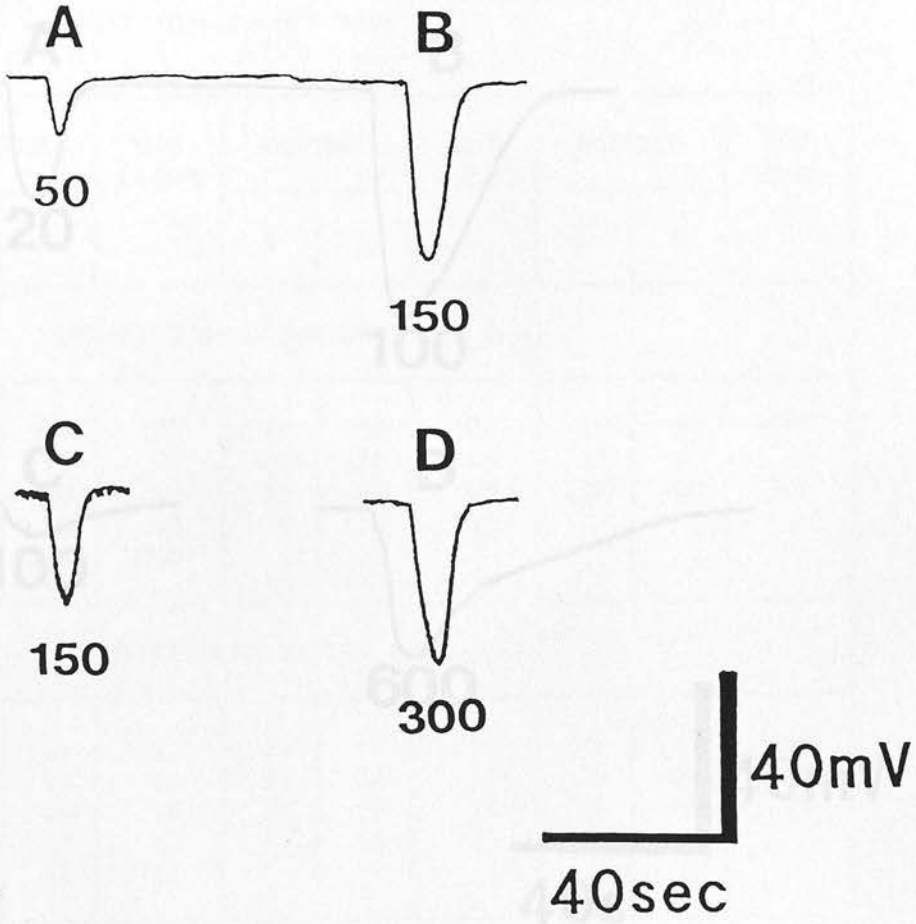

FIGURE 27 Antagonism by haloperidol of the hyperpolarization induced by dopamine applied locally by pressure ejection (500nM; 35KPa). A and B show the hyperpolarization to a 50ms and 150ms pulse of dopamine, respectively, in the absence of haloperidol; C shows the inhibition of the response to the larger of these two stimuli upon addition of 20μM haloperidol to the perfusate. D illustrates the effect of increasing the duration of the dopamine pulse to 300ms. At this point the period of exposure to haloperidol was 30min. The initial resting potential was -36mV.

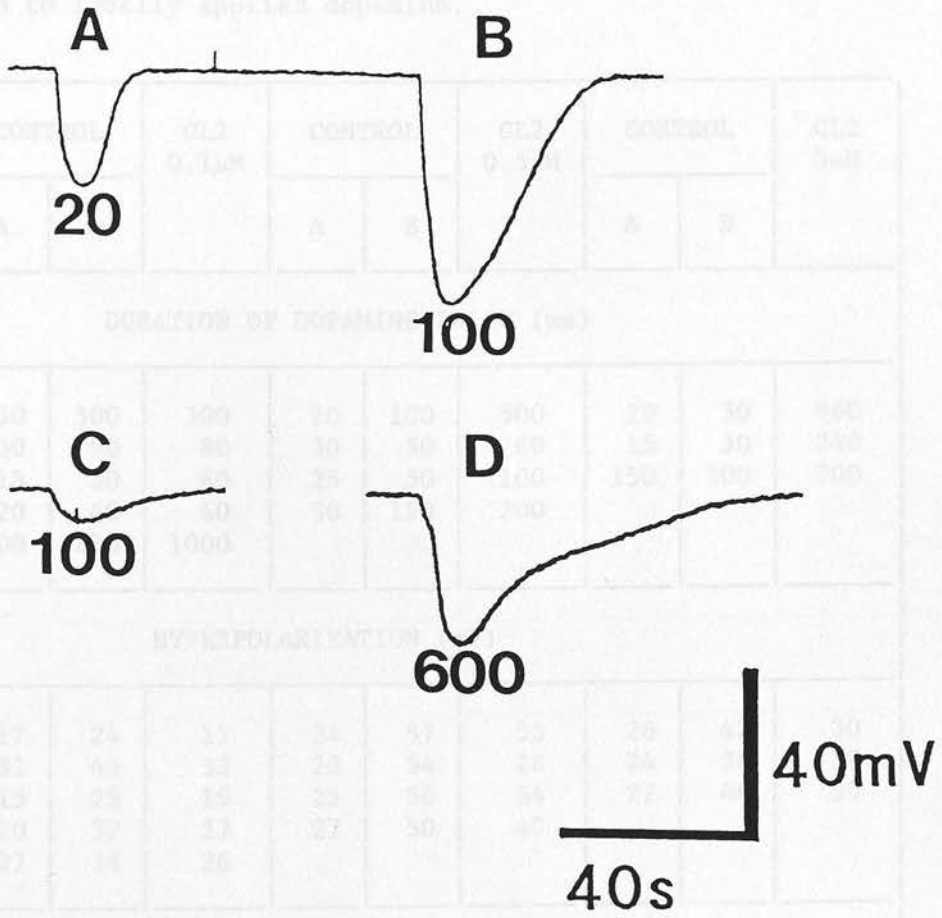

FIGURE 28 Antagonism by metoclopramide of the hyperpolarization induced by dopamine applied locally by pressure ejection (500nM; 35KPa). A and B show the hyperpolarization to a 20ms and 100ms pulse of dopamine, respectively, in the absence of metoclopramide; C shows the inhibition of the response to the larger of these two stimuli upon addition of 20μM metoclopramide to the perfusate. D illustrates the effect of increasing the duration of the dopamine pulse to 600ms. At this point the period of exposure to metoclopramide was 30min. The initial resting potential was -55mV.

Table VII

Data from "three point assays" of the inhibition by chlorpromazine of the hyperpolarization to locally applied dopamine.

EXPERIMENT NUMBER	CONTROL		CL2 0.1μM	CONTROL		CL2 0.5μM	CONTROL		CL2 5μM
	A	B		A	B		A	B	
DURATION OF DOPAMINE PULSE (ms)									
1	150	300	300	20	100	500	20	30	960
2	30	90	90	30	50	60	15	30	240
3	15	30	60	25	50	100	150	300	700
4	20	40	40	50	150	200			
5	400	800	1000						
HYPERPOLARIZATION (mV)									
1	17	24	17	24	57	55	26	42	30
2	31	43	33	22	54	28	24	38	39
3	15	25	19	25	50	54	27	46	35
4	20	37	27	27	50	40			
5	27	34	26						
DOSE RATIO									
1	2.00			5.51			43.37		
2	2.50			1.82			7.61		
3	3.03			2.08			3.49		
4	1.50			2.15					
5	2.76								

CLZ = chlorpromazine

Table VIII

Data from "three point assays" of the inhibition by SCH23390 of the hyperpolarization to locally applied dopamine.

EXPERIMENT NUMBER	CONTROL		SCH 10μM	CONTROL		SCH 20μM	CONTROL		SCH 50μM
	A	B		A	B		A	B	
DURATION OF DOPAMINE PULSE (ms)									
1	40	80	120	20	100	2000	10	20	2400
2	20	40	80	100	200	400	20	80	4800
3	200	300	400	20	40	100	20	60	1200
4	20	40	50	80	110	150	15	40	180
5	100	200	400	5	20	60			
6				25	50	150			
HYPERPOLARIZATION (mV)									
1	14	36	36	22	41	41	42	52	40
2	26	46	30	36	46	46	27	45	28
3	43	63	51	22	34	34	21	54	50
4	22	45	40	26	46	46	24	32	31
5	33	45	32	42	58	41			
6				11	22	10.5			
DOSE RATIO									
1	1.45			20.00			275.69		
2	3.48			2.35			222.21		
3	1.70			3.30			22.85		
4	1.45			1.59			7.08		
5	4.80			14.27					
6				6.19					

SCH = SCH23390

Table IX

Data from "three point assays" of the inhibition by haloperidol of the hyperpolarization to locally applied dopamine.

EXPERIMENT NUMBER	CONTROL		HAL 10μM	CONTROL		HAL 20μM	CONTROL		HAL 100μM
	A	B		A	B		A	B	
	DURATION OF DOPAMINE PULSE (ms)								
1	20	50	250	100	200	2000	10	80	80
2	50	200	200	50	150	300	20	80	3400
3	80	160	400	10	30	700	30	60	2000
4	30	60	100	10	30	80	20	60	720
	HYPERPOLARIZATION (mV)								
1	12	44	40	14	31	24	45	59	52
2	14	22	13	16	40	38	28	46	27
3	31	36	37	20	30	23	34	60	41
4	24	34	31	11	30	19	17	30	16.5
	DOSE RATIO								
1	5.61			10.48			5.66		
2	4.76			2.08			39.24		
3	2.20			50.35			55.32		
4	2.10			5.04			46.21		

HAL - haloperidol

Table X

Data from "three point assays" of the inhibition by metoclopramide of the hyperpolarization to locally applied dopamine.

EXPERIMENT NUMBER	CONTROL		METOCLOPRAMIDE 1mM
	A	B	
DURATION OF DOPAMINE PULSE (ms)			
1	4	5	20
2	120	200	600
3	20	40	40
4	20	40	640
HYPERPOLARIZATION (mV)			
1	24	40	41
2	28	54	33
3	40	56	39
4	23	41	40
DOSE RATIO			
1	3.90		
2	3.50		
3	2.30		
4	16.60		

Table X1 Dose ratios estimated by "three point assay" for each of the active dopamine antagonists together with the geometric mean K_{Dapp} and upper (L_2) and lower (L_1) confidence limits (95%)

ANTAGONIST	CONCENTRATION (μM)	DOSE RATIO	$K_{D}app$ (μM) GEOMETRIC MEAN	95% CONFIDENCE LIMITS (μM)	
				L_1	L_2
CHLORPROMAZINE	0.1	2.0			
	0.1	2.8			
	0.1	2.5			
	0.1	3.0			
	0.1	1.5			
	0.5	5.5			
	0.5	1.8	0.2	0.1	0.5
	0.5	2.1			
	0.5	2.2			
	5.0	43.4			
	5.0	7.6			
	5.0	3.5			
HALOPERIDOL	10	5.6			
	10	4.8			
	10	2.2			
	10	2.1			
	20	10.5			
	20	2.1			
	20	50.4	3.8	0.5	28.1
	20	5.0			
	100	5.7			
	100	46.2			
	100	39.2			
	100	55.3			
SCH23390	10	1.5			
	10	3.5			
	10	1.7			
	10	4.8			
	10	1.5			
	20	6.2			
	20	14.3			
	20	2.4	4.1	0.5	33.7
	20	20.0			
	20	3.3			
	20	1.6			
	50	275.7			
	50	222.2			
	50	22.9			
	50	7.1			
METACLOPRAMIDE	1000	3.9			
	1000	3.5			
	1000	2.3	264.7	30	2180
	1000	16.6			

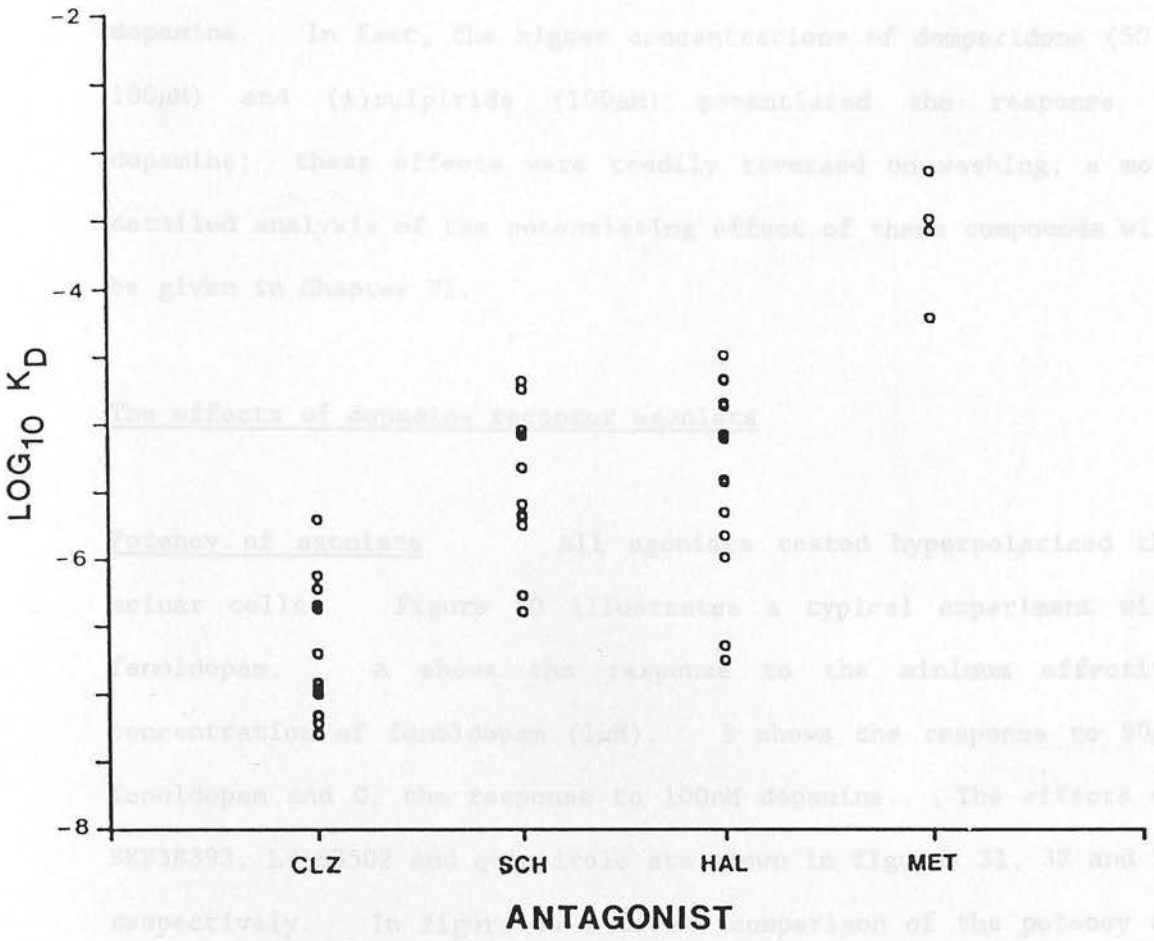

FIGURE 29 Diagram showing the range of values obtained in different experiments for the log K_D of each antagonist tested against the hyperpolarizing response to dopamine. Ordinate: the log K_D (M). CLZ is chlorpromazine; SCH is SCH23390; HAL is haloperidol; MET is metoclopramide.

Unlike the antagonists discussed above, domperidone (1-100μM) and (±)sulpiride (1-100μM) failed to inhibit the hyperpolarization to dopamine. In fact, the higher concentrations of domperidone (50 & 100μM) and (±)sulpiride (100μM) potentiated the response to dopamine; these effects were readily reversed on washing; a more detailed analysis of the potentiating effect of these compounds will be given in Chapter VI.

The effects of dopamine receptor agonists

Potency of agonists All agonists tested hyperpolarized the acinar cells. Figure 30 illustrates a typical experiment with fenoldopam. A shows the response to the minimum effective concentration of fenoldopam (1μM). B shows the response to 50μM fenoldopam and C, the response to 100nM dopamine. The effects of SKF38393, LY163502 and quinpirole are shown in figures 31, 32 and 33 respectively. In figure 34 a direct comparison of the potency of SKF38393, LY163502 and quinpirole on a single preparation. A shows the response to 50μM SKF38393. B shows the hyperpolarization to 500μM LY163502. C shows the hyperpolarization induced by 1mM quinpirole. The minimum effective concentrations and equipotent molar ratios obtained from individual experiments for each agonist, together with the means ± s.e.m. are shown in table XII. The sensitivity of each preparation to agonists varied, as can be seen from the range of minimum effective concentrations for each agonist: fenoldopam 1-10μM (n = 4); SKF38393 1-10μM (n = 4); LY163502 5-100μM (n = 4); quinpirole 100μM (n = 4). Thus the relative equipotent molar ratios (which allow for variations in sensitivity) were used to establish the agonist rank order of potency, which was

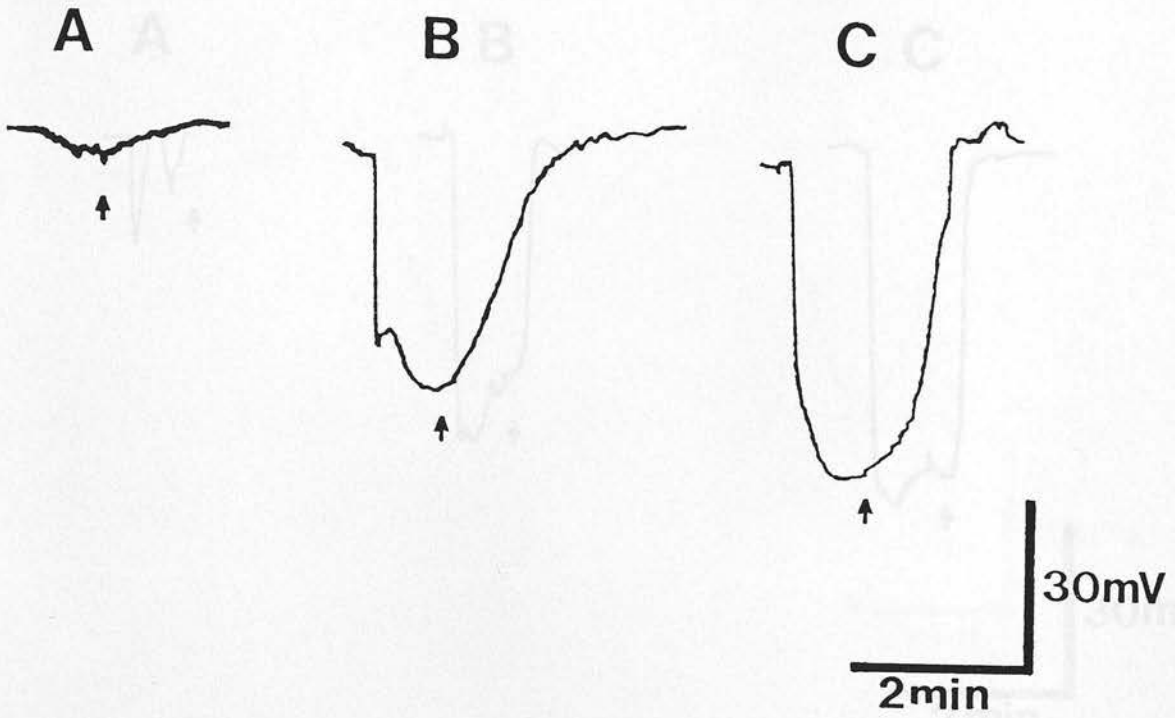

FIGURE 30 Response to the minimum effective concentration of fenoldopam, together with equivalent submaximal responses to fenoldopam and dopamine. A shows the hyperpolarization induced by 1μM fenoldopam (minimum effective concentration). Resting potential was -38mV. The hyperpolarizing responses to 50μM fenoldopam and 100nM dopamine are illustrated in B and C respectively. These two responses were obtained from the same cell. The resting potential was -43mV. In each case the agonist was added to the superfusate and was in contact with the preparation for 1min. In A, B and C the arrow marks the begining of the wash.

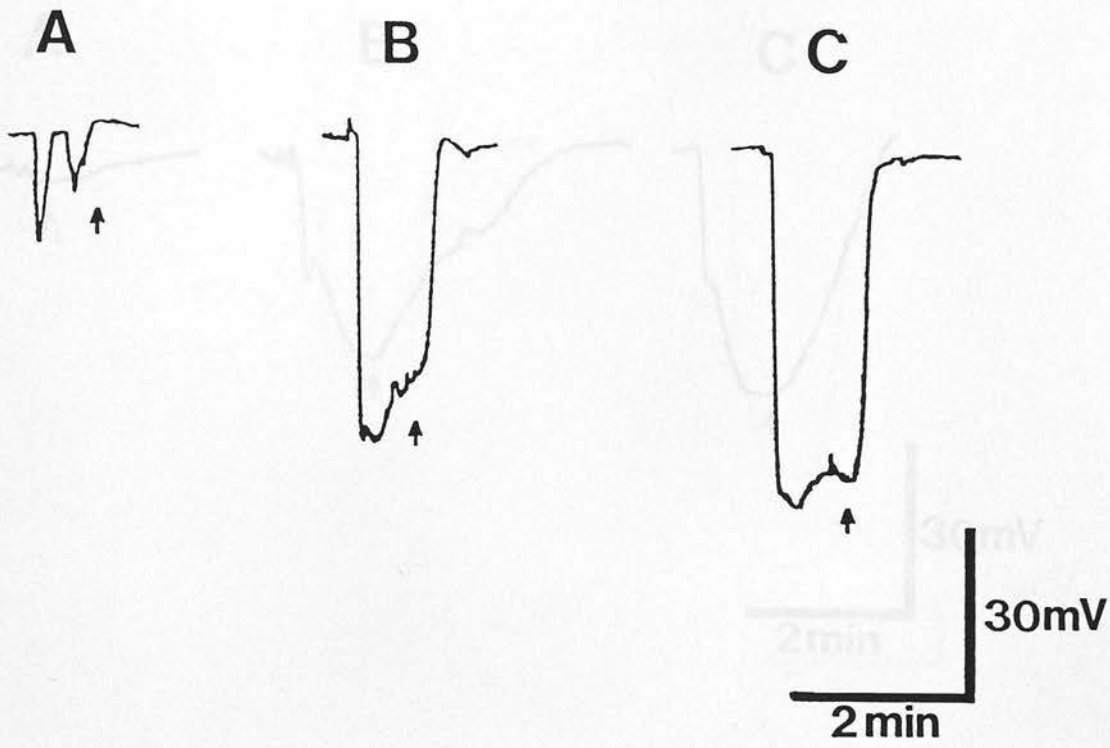

FIGURE 31 Response to the minimum effective concentration of SKF38393, together with equivalent submaximal responses to SKF38393 and dopamine. A shows the hyperpolarization induced by 5μM SKF38393 (minimum effective concentration). The hyperpolarizing responses to 50μM SKF38393 and 100nM dopamine are illustrated in B and C respectively. All three responses were obtained from the same cell. In each case the agonist was added to the superfusate and was in contact with the preparation for 1min. The resting potential was -43mV. In A, B and C the arrow marks the beginning of the wash.

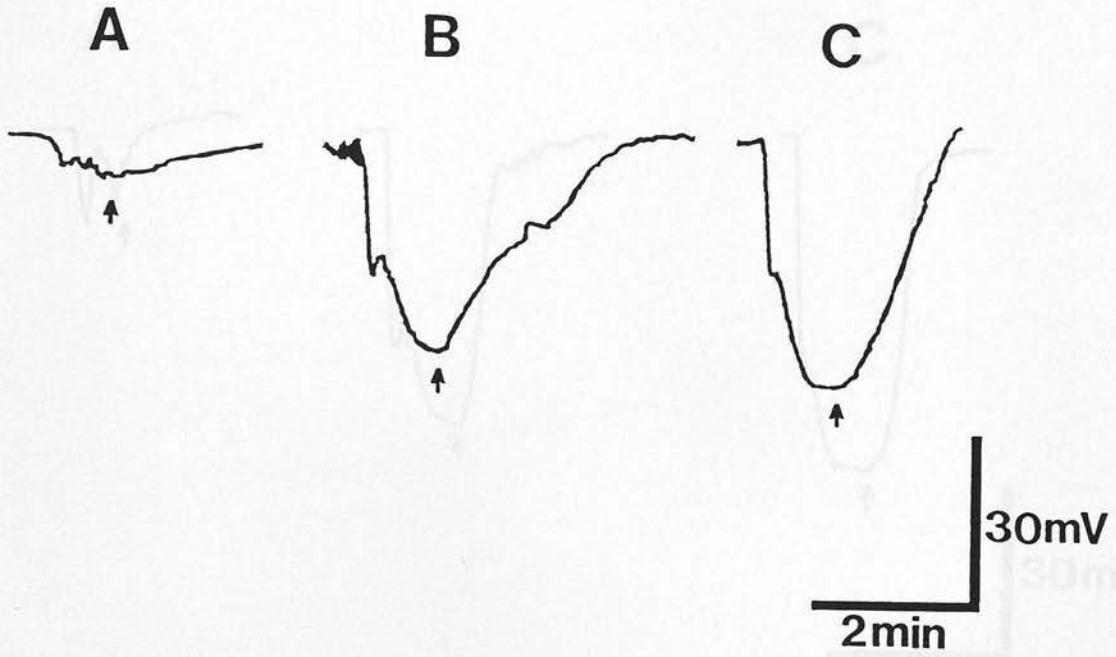

FIGURE 32 Response to the minimum effective concentration of quinpirole, together with equivalent submaximal responses to quinpirole and dopamine. A shows the hyperpolarization induced by 100μM quinpirole (minimum effective concentration). The hyperpolarizing responses to 1mM quinpirole and 50nM dopamine are illustrated in B and C respectively. All three responses were obtained from the same cell. In each case the agonist was added to the superfusate and was in contact with the preparation for 1min. The resting potential was -40mV. In A, B and C the arrow marks the beginning of the wash.

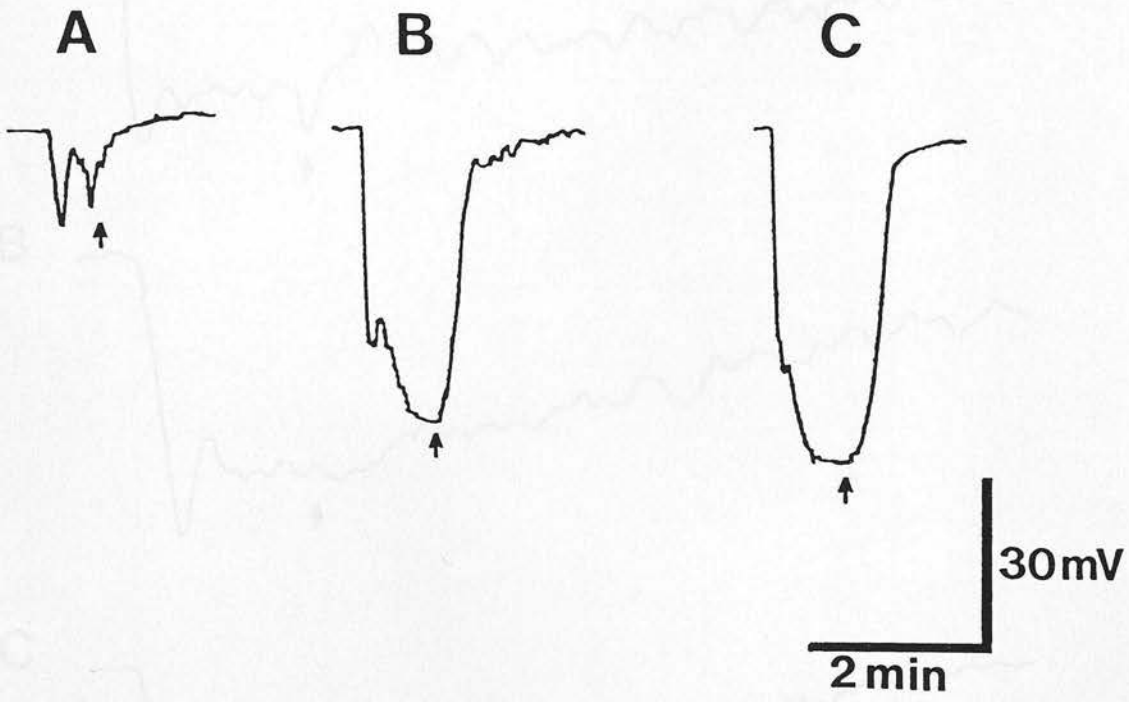

FIGURE 33 Response to the minimum effective concentration of LY163502, together with equivalent submaximal responses to LY163502 and dopamine. A shows the hyperpolarization induced by 50μM LY163502 (minimum effective concentration). The hyperpolarizing responses to 500μM LY163502 and 50nM dopamine are illustrated in B and C respectively. All three responses were obtained from the same cell. In each case the agonist was added to the superfusate and was in contact with the preparation for 1min. The resting potential was -40mV. In A, B and C the arrow marks the beginning of the wash.

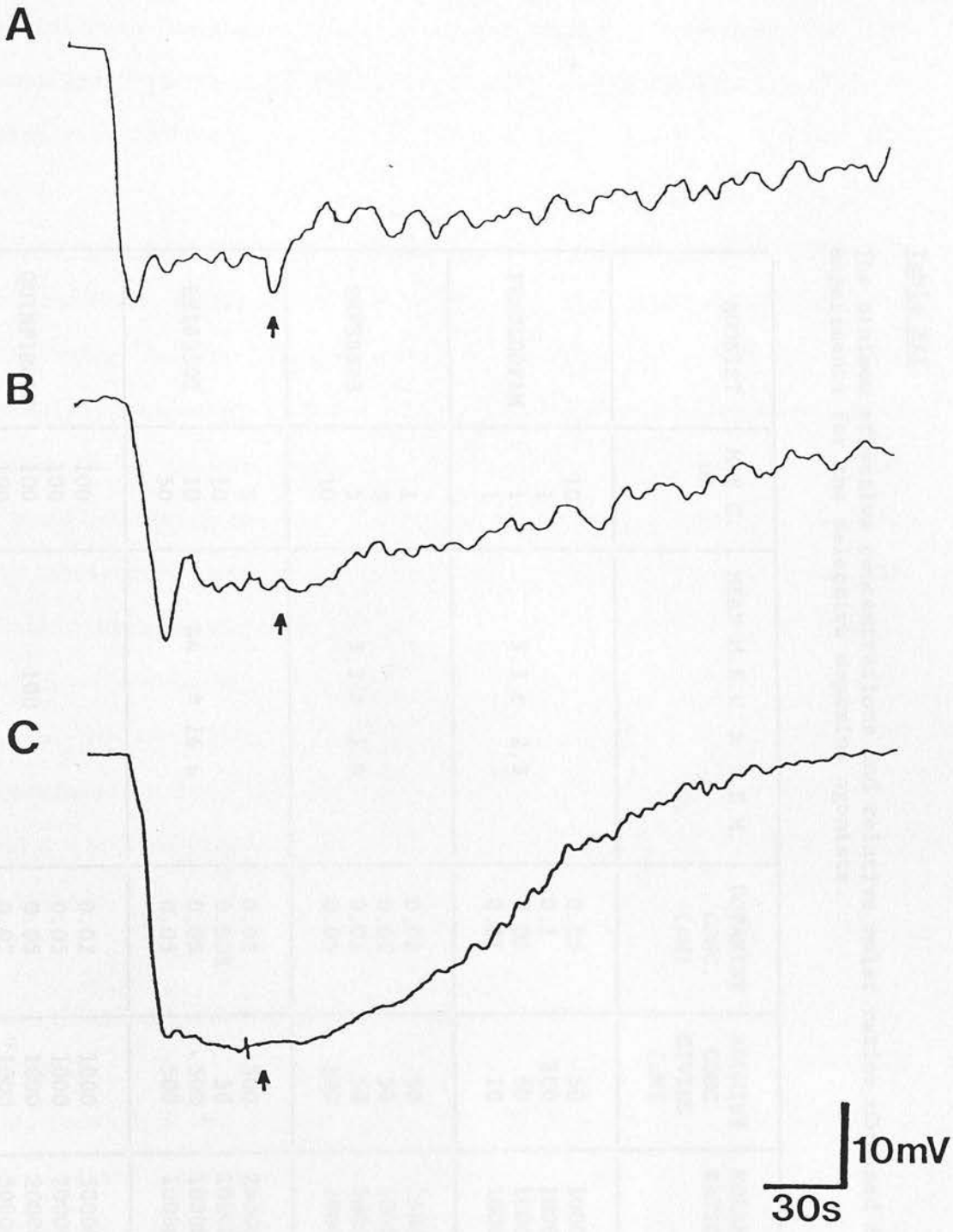

FIGURE 34 Direct comparison of the potency of quinpirole, LY163502 and SKF38393. A, B and C show the hyperpolarizing responses to 1mM quinpirole, B 500μM LY163502 and 50μM SKF38393 respectively. These responses were taken from a continuous recording from a single cell. Each agonist was added to the superfusate and was in contact with the preparation for 1min. The resting potential was -38mV. In A, B and C the arrow marks the beginning of the wash.

Table XII

The minimum effective concentrations and relative molar ratios obtained in individual experiments for the selective dopamine agonists.

AGONIST	M.E.C. μM	MEAN M.E.C. ± S.E.M.	DOPAMINE CONC. (μM)	AGONIST CONC. GIVING (μM)	MOLAR RATIO	MEAN MOLAR RATIO ± S.E.M.
FENOLDOPAM	10	3.3 ± 2.3	0.05	50	1000	1000
	1		0.1	100	1000	
	1		0.05	50	1000	
	1		0.01	10	1000	
SKF38393	1	5.3 ± 1.8	0.02	50	2500	3500 ± 866
	5		0.01	50	5000	
	5		0.01	50	5000	
	10		0.05	100	2000	
LY163502	5	44 ± 16.6	0.02	500	25000	13750 ± 3750
	10		0.005	50	10000	
	10		0.05	500	10000	
	50		0.05	500	10000	
QUINPIROLE	100	100	0.02	1000	50000	35000 ± 8660
	100		0.05	1000	20000	
	100		0.05	1000	20000	
	100		0.02	1000	50000	

M.E.C. = minimum effective concentration
CONC. = concentration
S.E.M. = standard error of the mean

as follows (relative value in parentheses): dopamine (1) >> fenoldopam (1,000) > SKF38393 (3,500) > LY163502 (13,750) > quinpirole (35,000).

Effect of quinpirole on responses to locally applied dopamine

The response produced by 1mM quinpirole was considerably smaller than would be the maximal response to dopamine (-110mV). As quinpirole appeared to have effects on secretion consistent with those of a partial agonist, the effect of quinpirole on the hyperpolarization to locally applied dopamine was studied. Figure 35 illustrates the inhibition by quinpirole of the response to locally applied dopamine (0.5μM, 35kPa). A and B show control responses to dopamine applied by two different durations of pressure pulse, 70 and 100ms. On addition of 100μM quinpirole to the superfusate a 20mV hyperpolarization was induced, and, as shown in C and D, the responses to the 70ms and 100ms pulse of dopamine were inhibited.

The effect of (±)sulpiride and (+)SCH23390 on responses to the agonists Although both D_1 and D_2 receptor agonists hyperpolarized the acinar cells, the D_1 agonists were more potent. This raised the possibility that the D_2 agonists were, in fact, acting on a D_1 receptor. For this reason, a study was made of the effects of selective D_1 dopamine receptor antagonist, SCH23390 and the selective D_2 dopamine receptor antagonist, (±)sulpiride, on the responses to locally applied agonists (1mM, 35kPa). Figure 36 illustrates the effect of dopamine antagonists on the hyperpolarization to fenoldopam. A shows the response to a 300ms pulse of fenoldopam in antagonist free solution. B shows the

response to an identical stimulus in the presence of 100μM (±)sulpiride after a 30 min exposure. C shows the control response of a different cell to a 50ms pulse of fenoldopam. D shows the response to an identical stimulus in the presence of 10μM (+)SCH23390 after a 30 min incubation. The effects of (±)sulpiride and (+)SCH23390 on responses to SKF38893, LY163502 and quinpirole are shown in a corresponding way in figures 37, 38 and 39. It is evident that the D_1 antagonist (+)SCH23390 inhibited the response to all four agonists, while (±)sulpiride did not. The inhibition by (+)SCH23390 of these responses could be surmounted by increasing the duration of the pulse of agonist and was reversed on washing with antagonist free solution.

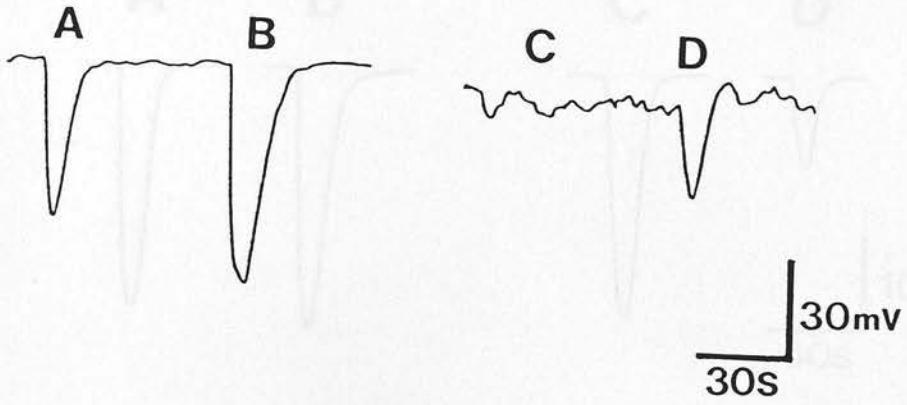

FIGURE 35 Inhibition of the hyperpolarizing response to pressure ejected dopamine (500nM; 35KPa), by the minimum effective concentration of quinpirole. A and B show control responses to 70ms and 100ms pulses of dopamine, respectively. C and D show the hyperpolarization induced by identical stimuli after the addition of 100μM quinpirole to the superfusate. The resting potential was -32mV.

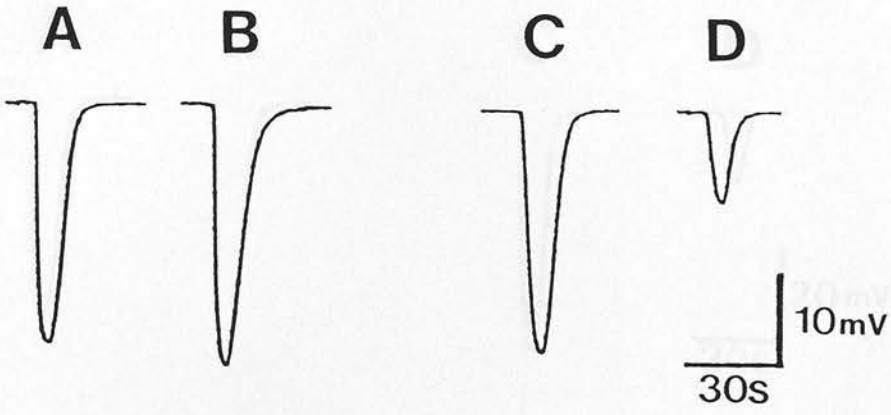

FIGURE 36 The effect of (±)sulpiride and (+)SCH23390 on the hyperpolarizing response to pressure ejected fenoldopam (1mM; 35KPa). A shows the response to a 300ms pulse of fenoldopam in antagonist free solution. B shows the response to an identical stimulus in the presence of 100μM (±)sulpiride after a 30min exposure. The resting potential was -50mV. C shows a control response of a different cell to a 50ms pulse of fenoldopam. D shows the response to an identical stimulus in the presence of (+)SCH23390 after a 30 min exposure. The resting potential of this cell was -40mV.

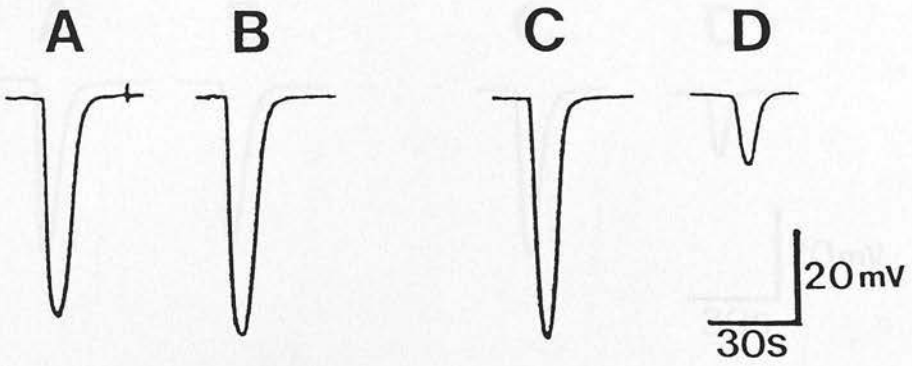

FIGURE 37 The effect of (±)sulpiride and (+)SCH23390 on the hyperpolarizing response to pressure ejected SKF38393 (1mM; 35KPa). A shows the response to a 150ms pulse of SKF38393 in antagonist free solution. B shows the response to an identical stimulus in the presence of 100μM (±)sulpiride after a 30min exposure. C shows the response of the same cell after a wash and D shows the response in the presence of (+)SCH23390 after a 30 min exposure. The resting potential of this cell was -44mV.

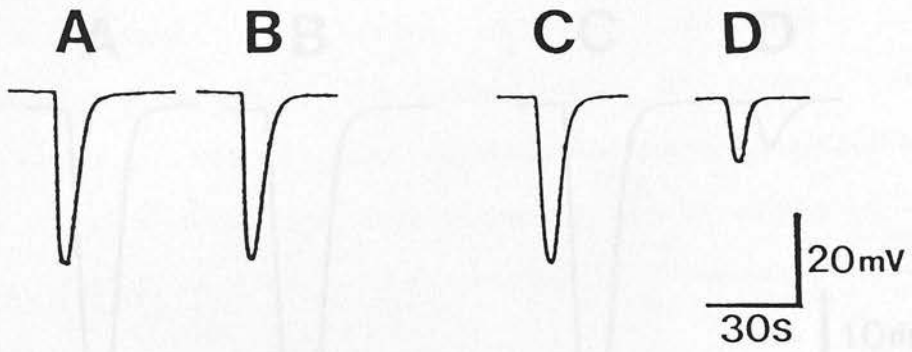

FIGURE 38 The effect of (±)sulpiride and (+)SCH23390 on the hyperpolarizing response to pressure ejected quinpirole (1mM; 35KPa). A shows the response to a 100ms pulse of quinpirole in antagonist free solution. B shows the response to an identical stimulus in the presence of 100μM (±)sulpiride after a 30min exposure. C shows the response of the same cell after a wash and D shows the response in the presence of (+)SCH23390 after a 30 min exposure. The resting potential of this cell was -52mV.

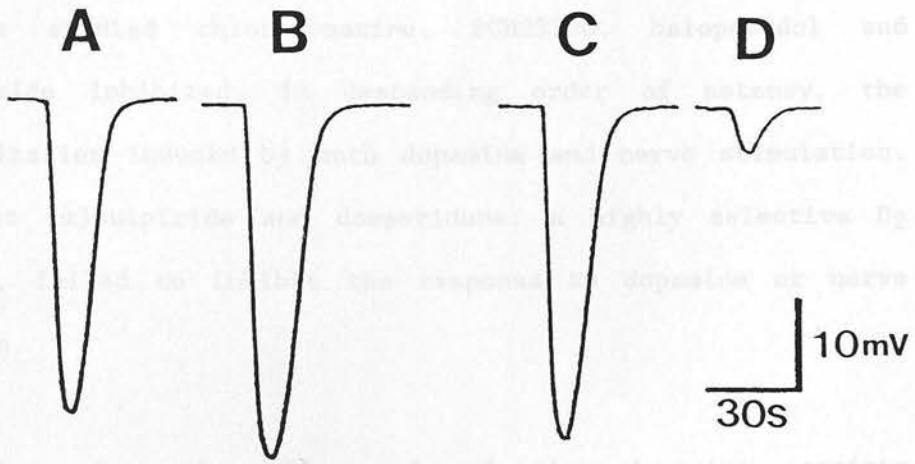

FIGURE 39 The effect of (±)sulpiride and (+)SCH23390 on the hyperpolarizing response to pressure ejected LY163502 (1mM; 35KPa). A shows the response to a 170ms pulse of LY163502 in antagonist free solution. B shows the response to an identical stimulus in the presence of 100μM (±)sulpiride after a 30min exposure. C shows the response of the same cell after a wash and D shows the response in the presence of (+)SCH23390 after a 30 min exposure. The resting potential of this cell was -40mV.

DISCUSSION

Both exogenously applied dopamine and stimulation of the suboesophageal nerve hyperpolarize the cockroach salivary gland acinar cells (House, 1973; Blackman *et al*, 1979). Of the antagonists studied chlorpromazine, SCH23390, haloperidol and metoclopramide inhibited, in descending order of potency, the hyperpolarization induced by both dopamine and nerve stimulation. In contrast (±)sulpiride and domperidone, a highly selective D_2 antagonist, failed to inhibit the response to dopamine or nerve stimulation.

Investigations into the effect of selective dopamine agonists established that the selective D_1 agonists, fenoldopam and SKF38393, and the selective D_2 agonists, LY163502 and quinpirole, induced, in descending order of potency, a hyperpolarization of the acinar cells. The responses to all four agonists were inhibited by (+)SCH23390, but not by (±)sulpiride.

The findings of this investigation are in good agreement with those of the investigation into the pharmacology of the secretory receptors, which is described in Chapter III. From Table XIII it is evident that the rank order of potency for both the antagonists and agonists is similar for the receptors mediating the secretory and electrical responses of the salivary gland to dopamine. Moreover, the K_Ds obtained for each antagonist in these two studies are virtually identical, and both the secretory and electrical response to the selective agonists were inhibited by the selective D_1 antagonist SCH23390, but not by the D_2 antagonist (±)sulpiride.

Table XIII

Comparison of the rank order of potency of dopamine in relation to receptor antagonists and agonists obtained in relation to secretion and the hyperpolarization.

RESPONSE		RANK ORDER OF POTENCY
HYPERPOLARIZATION	AGONISTS	DOP > FEN > SKF > LY > QUIN
	ANTAGONISTS	CLZ > SCH = HAL >> MET >> DOM = (±)SUL
SECRETION	AGONISTS	DOP > FEN > SKF > QUIN
	ANTAGONISTS	CLZ > SCH > HAL >> MET >> DOM = (±)SUL

CLZ = chlorpromazine; SCH = SCH23390; HAL = haloperidol; MET = metoclopramide;
DOM = domperidone; (±)SUL = (±)sulpiride; DOP = dopamine; FEN = fenoldopam;
SKF = SKF38393; LY = LY163502; QUIN = quinpirole

It thus seems highly likely that a single receptor, similar to the mammalian D_1 sub-type, subserves both the electrical and secretory response to dopamine. This conclusion contrasts with the view of Gray *et al* (1984), who suggested that a different receptor mediated each response. They reported that c-AMP, the levels of which are increased on activation of the dopamine receptor (Grewe and Kebabian, 1982), acts as an intermediate in the secretory, but not the electrical response to dopamine. This result however would be consistent with the idea that two second messengers are linked to a single receptor, c-AMP mediating the secretory response and the other subserving the hyperpolarization.

The conclusion that a single receptor might mediate independent responses is of considerable interest. D_1 receptors were originally classified as those dopamine receptors which are positively coupled to adenylyl cyclase; D_2 receptors were classified as those which did not influence the activity of this enzyme (Kebabian, 1978; Kebabian and Calne, 1979; Spano *et al*, 1978), although subsequent investigations established that many D_2 receptors were actually negatively coupled to adenylyl cyclase (Stoof and Kebabian, 1981; De Camilli *et al*, 1979; Cote *et al*, 1982). This classification, together with the idea that it was unlikely that a single receptor could be coupled to more than one second messenger system, have complicated the current understanding of different types of dopamine receptor. This was evident most recently in a review by Anderson *et al* (1990), who cite the finding that c-AMP does not always mimic D_1-receptor-mediated events and the variation in efficacy of D_1 agonists between physiological responses

and the biochemical response (activation of adenylyl cyclase) of the same preparation (Johansen and White, 1987) as evidence for the sub-division of D_1 receptors.

The question which remains relates to the nature of the second messenger subserving the hyperpolarization. It is highly probable that a calcium-dependent potassium conductance underlies the hyperpolarization and that both the electrical and secretory responses to dopamine rely, at least in part, on the release of calcium from intracellular stores (see Ginsborg and House, 1980). It is thus possible that an increase in inositol 1,4,5 triphosphate (IP_3) underlies the response, as IP_3 is now well established as a second messenger responsible for receptor-activated release of calcium from the endoplasmic reticulum (Berridge and Irvine, 1984). In support of this idea, D. MacEwen and I have shown that IP_3 and protein kinase-C are present in the salivary gland, and activation of the dopamine receptors translocates protein kinase-C from the cytosol to the membrane fraction of the cell, a process which is known to require diacyl glycerol, a bi-product of IP_3 formation (Nishizuka, 1986). In preliminary experiments I have also found that NaF potentiates the hyperpolarization induced by dopamine, while LiCl inhibits the hyperpolarization to dopamine in a non-competitive manner. These results might be explained by the action of NaF as a G-protein activator (Freissmuth *et al*, 1989), and LiCl as an antagonist of the phosphoinositide cycle (Avissar *et al*, 1988; Drummond, 1987). Further support of a role for IP_3 may be taken from recent findings which suggest that D_1 receptors in the rat striatum and renal cortex are coupled to phospholipase C (Baldi *et al*, 1988; Felder *et al*, 1988; Mahan *et al*, 1990).

CHAPTER V

CHARACTERIZATION OF THE DOPAMINE RECEPTORS SUBSERVING THE DEPOLARIZATION

INTRODUCTION

As has been mentioned previously, the hyperpolarization induced by dopamine and nerve stimulation is occasionally followed by a depolarization. It has been suggested (Ginsborg and House, 1976) that the hyperpolarizing and depolarizing components are mediated by two separate receptors. This chapter describes the ability of dopamine agonists to evoke a depolarization, and the effect of antagonists on the depolarization to dopamine. The methods are as described in Chapter IV.

RESULTS

Agonists

Fenoldopam (1 & 50μM), SKF38393 (10 & 50μM), LY163502 (10 & 100μM) and quinpirole (0.1 & 1mM) were all found to induce a depolarization when superfused for 1 min. These observations were made in at least two experiments with each agonist. However, the depolarizing component was most clearly seen when the agonists were applied locally by pressure ejection, rather than by superfusion. Figure 40 illustrates examples of responses to the four different agonists applied by pressure ejection. A shows the depolarization to a 200ms pulse of fenoldopam (n = 1). B shows the depolarization to a 50ms pulse of SKF38393 (n = 3). C shows the depolarization to a 150ms pulse of LY163502 (n = 2). D shows the depolarization to a 100ms pulse of quinpirole (n = 3). The simultaneous occurrence of the hyperpolarizing and depolarizing components in the response to all the agonists tested provides no support for the idea that these components are mediated by separate receptors.

Antagonists

Chlorpromazine (0.5 & 5μM), SCH23390 (20 & 50μM) and haloperidol (10, 20 & 100μM) inhibited the depolarization to dopamine and to nerve stimulation. Figure 41 illustrates the action of chlorpromazine. In A1 the depolarization induced by a 300ms pulse of dopamine in the absence of chlorpromazine is shown; A2 shows the depolarization to an identical stimulus in the presence of 5μM chlorpromazine after a 5 min exposure. In B1 the depolarization to

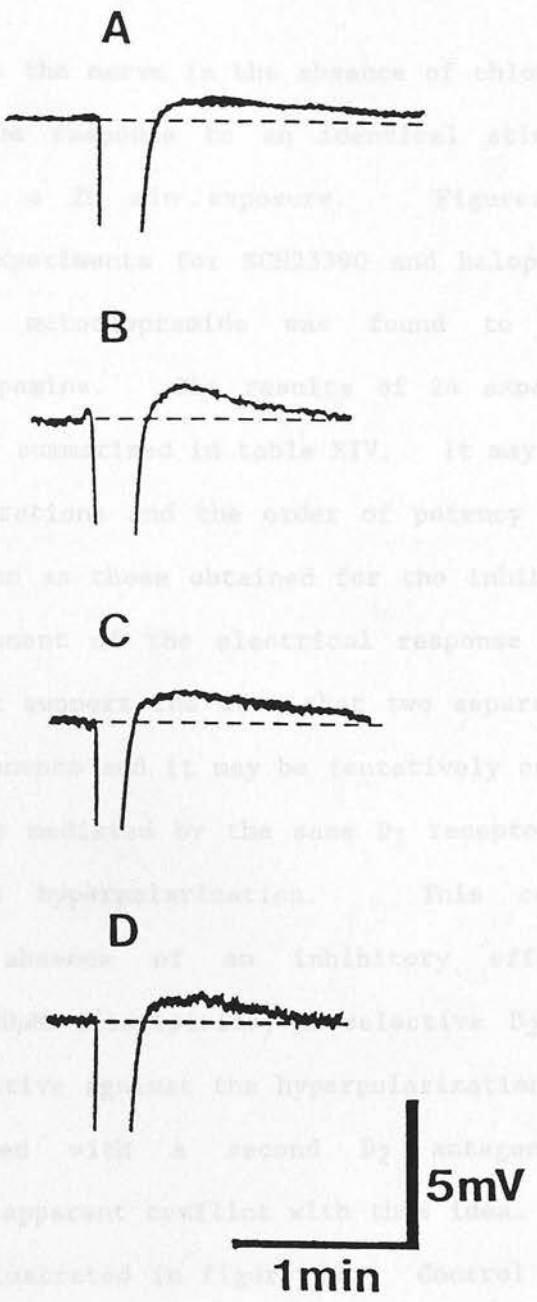

FIGURE 40 The depolarization induced by D_1 and D_2 dopamine receptor agonists. Each agonist was applied locally by pressure ejection (1mM; 35KPa). A shows the response to a 200ms pulse of fenoldopam, B the depolarization induced by a 70ms pulse of SKF38393, C the response to a 100ms pulse of quinpirole and D the response to a 150ms pulse of LY163502. The resting potentials of the cells represented in A, B, C and D were -65mV, -30mV, -44mV and -50mV. The level of the resting potential is marked by the broken line. In this and all subsequent figures the depolarization is upwards.

4 impulses applied to the nerve in the absence of chlorpromazine is shown; B2 shows the response to an identical stimulus in 5μM chlorpromazine after a 20 min exposure. Figures 42 and 43 illustrate similar experiments for SCH23390 and haloperidol. In one experiment 1mM metoclopramide was found to inhibit the depolarization to dopamine. The results of 24 experiments with these antagonists are summarized in table XIV. It may be seen that the range of concentrations and the order of potency are the same for the depolarization as those obtained for the inhibition of the hyperpolarizing component of the electrical response (see Chapter IV). This does not support the idea that two separate receptors mediate the two components and it may be tentatively concluded that the depolarization is mediated by the same D_1 receptor type as is responsible for the hyperpolarization. This conclusion is supported by the absence of an inhibitory effect on the depolarization of 100μM (±)sulpiride, a selective D_2 antagonist, which is also ineffective against the hyperpolarization. However, the results obtained with a second D_2 antagonist, namely domperidone, were in apparent conflict with this idea. The action of domperidone is illustrated in figure 44. Control responses to nerve stimulation are shown in A1 and A3. In A2 it can be seen that the depolarization was abolished by 50μM domperidone. The small degree of inhibition of the hyperpolarization was due to a pre-synaptic effect since domperidone potentiates rather than inhibits the hyperpolarizing effect of dopamine; this will be discussed in greater detail in Chapter VI. Figure 44B shows a further distinction between the inhibitory action of domperidone and the antagonists discussed previously. A control response to nerve stimulation is shown in B1. The response shown in B2 was obtained

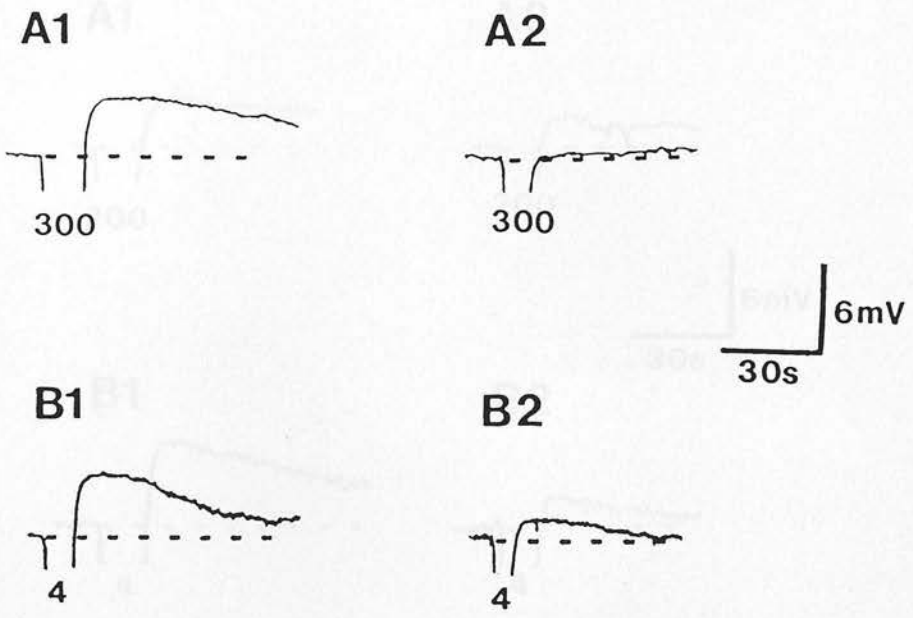

FIGURE 41 The inhibition by chlorpromazine of the depolarization to pressure ejected dopamine (500nM; 35KPa), and to nerve stimulation (100V; 20Hz). A1: depolarization induced by a 300ms pulse of dopamine in the absence of chlorpromazine. A2: the response to an identical stimulus in the presence of 5μM chlorpromazine after a 5min exposure. B1: the response of the same cell to 4 impulses applied to the nerve in the absence of chlorpromazine. B2: the depolarization induced by an identical stimulus in the presence of 5μM chlorpromazine after a 20min exposure. The resting potential was -40mV. In A and B the level of the resting potential is marked by the broken line.

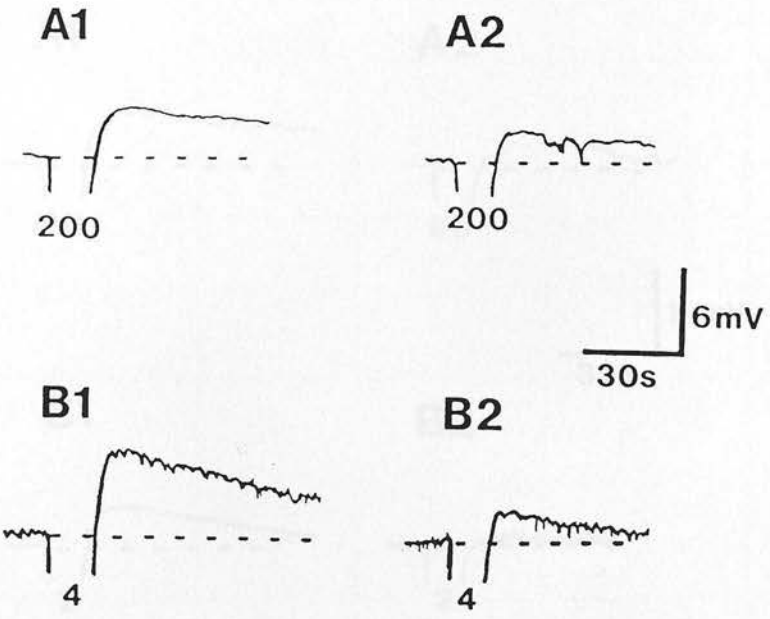

FIGURE 42 The inhibition by SCH23390 of the depolarization to pressure ejected dopamine (500nM; 35KPa), and to nerve stimulation (100V; 20Hz). A1: depolarization induced by a 200ms pulse of dopamine in the absence of SCH23390. A2: response to an identical stimulus in the presence of 20μM SCH23390 after a 10min exposure. The resting potential was -40mV. B1: the response of a different cell to 4 stimuli applied to the nerve in the absence of SCH23390. B2: the depolarization induced by an identical stimulus in the presence of 20μM SCH23390 after a 20min exposure. Resting potential was -30mV. In A and B the level of the resting potential is marked by the broken line.

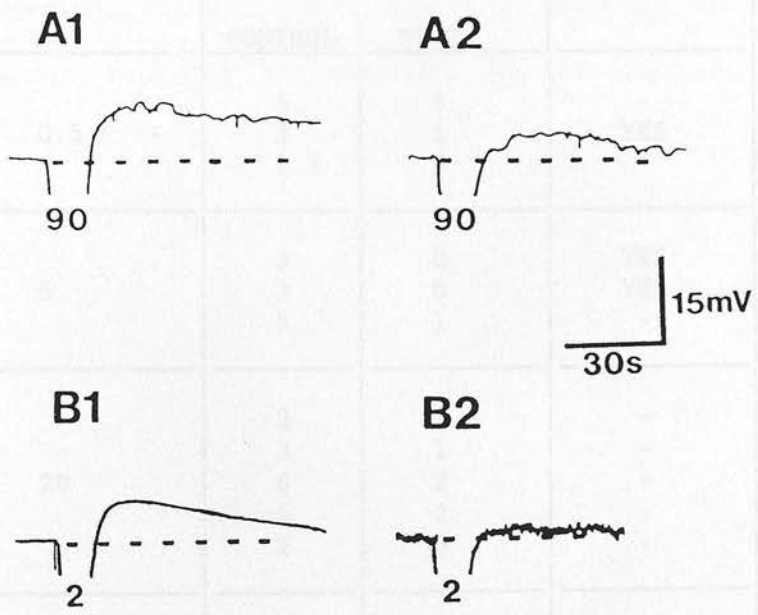

FIGURE 43 The inhibition by haloperidol of the depolarization to pressure ejected dopamine (500nM; 35KPa), and to nerve stimulation (100V; 20Hz). A1: depolarization induced by a 90ms pulse of dopamine in the absence of haloperidol. A2: the response to an identical stimulus in the presence of 100μM haloperidol after a 10min exposure. The resting potential was -50mV. B1: the response of a different cell to 2 impulses applied to the nerve in the absence of haloperidol. B2: the depolarization induced by an identical stimulus in the presence of 100μM haloperidol after a 25min exposure. The resting potential was -42mV. In A and B the level of the resting potential is marked by the broken line.

Table XIV

The effects of antagonists on the depolarization to dopamine and nerve stimulation.

ANTAGONIST	CONCENTRATION (μM)	MEAN DEPOLARIZATION (mV)		SURMOUNTED	REVERSED ON WASHING
		CONTROL	TEST		
CLZ	0.5	5	5	-	-
		3	1	YES	YES
		2.5	0	-	-
	5	3	0	YES	YES
		3	0	YES	NO
		5	1	-	-
SCH	20	2	0.5	-	-
		3	1	-	-
		6	2	-	YES
		4	2	-	-
		2	0	-	-
	50	5	0	YES	NO
		3.5	1	YES	YES
		3.25	0	-	-
HAL	10	3	1	-	NO
		2.3	2.3	-	-
		6.5	0	-	YES
	20	2.5	0	YES	NO
		2.5	0	YES	NO
		2	0	NO	NO
	100	2.5	0	NO	-
		7.7	0	NO	-
		3.5	0	NO	-
MET	1000	3	0	YES	YES

CLZ = chlorpromazine; SCH = SCH23390; HAL = Haloperidol; MET = Metoclopramide

to an identical stimulus fifteen minutes after the addition of 50μM domperidone to the superfusate. The depolarization has been abolished whilst the hyperpolarization has been potentiated. After a further fifteen minutes (B3) the hyperpolarization was also inhibited. A similar result was obtained in one other experiment. In another experiment, 100μM domperidone was found to inhibit the depolarization in response to pressure ejected dopamine whilst potentiating the hyperpolarization.

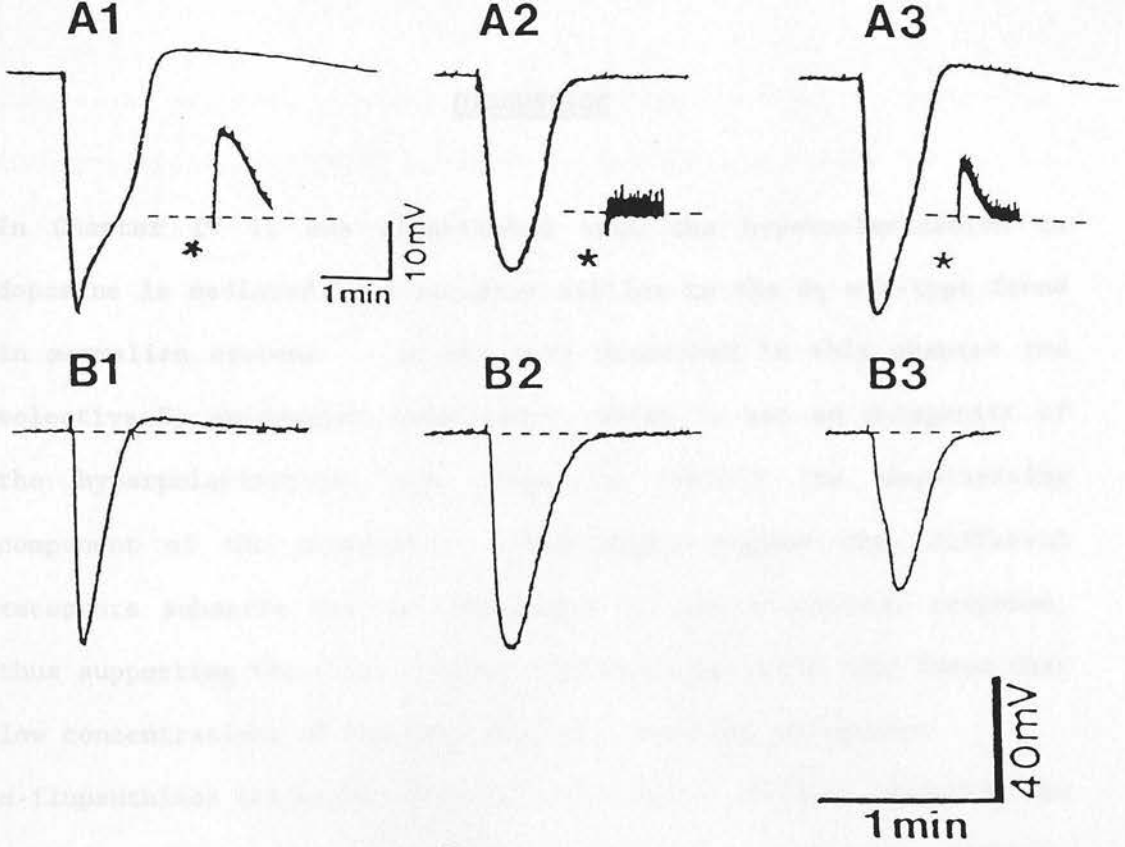

FIGURE 44 The inhibition by domperidone of the depolarization induced by nerve stimulation. A1 shows the hyperpolarization and the subsequent depolarization induced by 10 impulses applied to the nerve (100V; 20Hz) in the absence of domperidone. The inset (*) shows the depolarization at a higher gain, with the level of the resting potential marked by the broken line (-----). A2 shows the response to an identical stimulus in the presence of 50μM domperidone (20min exposure). A3 shows the response after washing. The resting potential was -38mV. Section B, from a different cell, illustrates the more rapid time course for the inhibition of the depolarization than of the hyperpolarization. B1 shows the response to 10 impulses applied to the nerve in the absence of domperidone. B2 shows the response to an identical stimulus after a 15min exposure to 50μM domperidone. The increased duration of the hyperpolarization is increased and the depolarization inhibited. B3 shows the response after a futher 15min exposure on. The level of the resting potential is marked by the broken line, and was -50mV.

DISCUSSION

In Chapter IV it was established that the hyperpolarization to dopamine is mediated by a receptor similar to the D_1 sub-type found in mammalian systems. In the work described in this chapter the selective D_2 antagonist domperidone, which is not an antagonist of the hyperpolarization, was found to inhibit the depolarizing component of the response. This might suggest that different receptors subserve the two components of the electrical response, thus supporting the view of House and Ginsborg (1976) who found that low concentrations of the non-selective dopamine antagonist α-flupenthixol inhibited the hyperpolarization without affecting the depolarization, an action which is shared by ergometrine (Bowser-Riley *et al*, 1978). This hypothesis becomes more attractive when one considers that prolonged exposure to dopamine can lead to a reduction in the hyperpolarization to nerve stimulation, without inhibiting the depolarization (Ginsborg and House, 1976). However, it is clear that if a second receptor is involved it is certainly not a classical D_1 or D_2 sub-type because, as has been shown, a selective D_1 antagonist inhibited the depolarization to dopamine, whilst another selective D_2 antagonist did not. Thus the "twin-receptor" hypothesis implies the existence of a hitherto unrecognized dopamine receptor whose pharmacology included activation by all dopamine agonists so far tested, inhibition by the D_2 antagonist domperidone and by the D_1 antagonist SCH23390, but not by the D_2 antagonist (±)sulpiride or by α-flupenthixol. Two other sets of results make the "twin-receptor" theory unattractive. First, the finding that both the selective D_1 agonists, SKF38393 and fenoldopam, and the selective D_2 agonists, quinpirole and LY163502,

induce a hyperpolarization and a depolarization. Secondly, chlorpromazine, SCH23390, haloperidol and metoclopramide inhibit the depolarization over the same range of concentrations in which they inhibit the hyperpolarization. These results taken by themselves strongly suggest that a single receptor mediates both components of the electrical response. The action of domperidone and α-flupenthixol would then be attributed to effects, not on the receptor, but on the processes following receptor activation.

CHAPTER VI

AN INVESTIGATION INTO THE EFFECTS OF DOMPERIDONE ON THE HYPERPOLARIZATION TO DOPAMINE AND NERVE STIMULATION

INTRODUCTION

As the selectivity of dopamine receptor ligands is not absolute, it was not surprising to find that as in the mammal the selective D_2 antagonists, haloperidol (Iversen, 1975; Snyder *et al*, 1975) and metoclopramide (Hope *et al*, 1978; Munemura *et al*, 1980; Seeman *et al*, 1987) were, in sufficiently high concentrations, effective in blocking the responses to dopamine (see Chapters III-V). However domperidone and (±)sulpiride, which are also selective D_2 antagonists (Laduron and Leyson, 1979; Jenner and Marsden, 1981), were found to potentiate rather than inhibit the hyperpolarization to dopamine. In an attempt to clarify the nature of the potentiation, the effects of domperidone have also been studied on the electrical responses to nerve stimulation and to 5-HT, an agonist that acts on distinct receptors from dopamine in this preparation (Bowser-Riley *et al*, 1978). The results suggest that the potentiation is due to a post-synaptic action independent of any dopamine receptor. The methods are as described in Chapter IV.

RESULTS

The effects of domperidone (1-100μM) and (±)sulpiride (1-100μM) on the electrical response of the salivary gland were studied. Addition of 1 (n=4) and 10μM (n=4) domperidone and 1μM (±)sulpiride (n=4) to the superfusate had no effect on the hyperpolarization to either nerve stimulation or dopamine. However, 50 (n=3) and 100μM (n=4) domperidone and 100μM (±)sulpiride (n=3) potentiated the hyperpolarization induced by dopamine. Figure 45 illustrates the effects of these two compounds after a 30min exposure. A1 shows the response to a 50ms pulse of dopamine in antagonist free solution. A2 shows the response to an identical stimulus in the presence of 100μM domperidone and, A3 the response after a 30min wash in drug free solution. The effect of (±)sulpiride is illustrated in a corresponding way in parts B1, B2 and B3. One can see that both an increase in the amplitude and the duration of the responses to dopamine resulted from the presence of domperidone or (±)sulpiride, and that the potentiation decreased on washing. It is also evident that the potentiation obtained in the presence of (±)sulpiride was much weaker than that obtained in the presence of domperidone. Due to this fact, the effects of (±)sulpiride were not studied further. Figure 46 illustrates the effects of 100μM domperidone on the response to dopamine and to nerve stimulation. The upper trace (A) shows control responses to two pulses of dopamine (DA7 & DA10) together with a response to nerve stimulation (NS). The lower trace (B) shows the corresponding responses in the presence of domperidone. It is evident that both the amplitude and duration of the responses to dopamine have been increased, whereas the response to nerve stimulation has been reduced. From this one

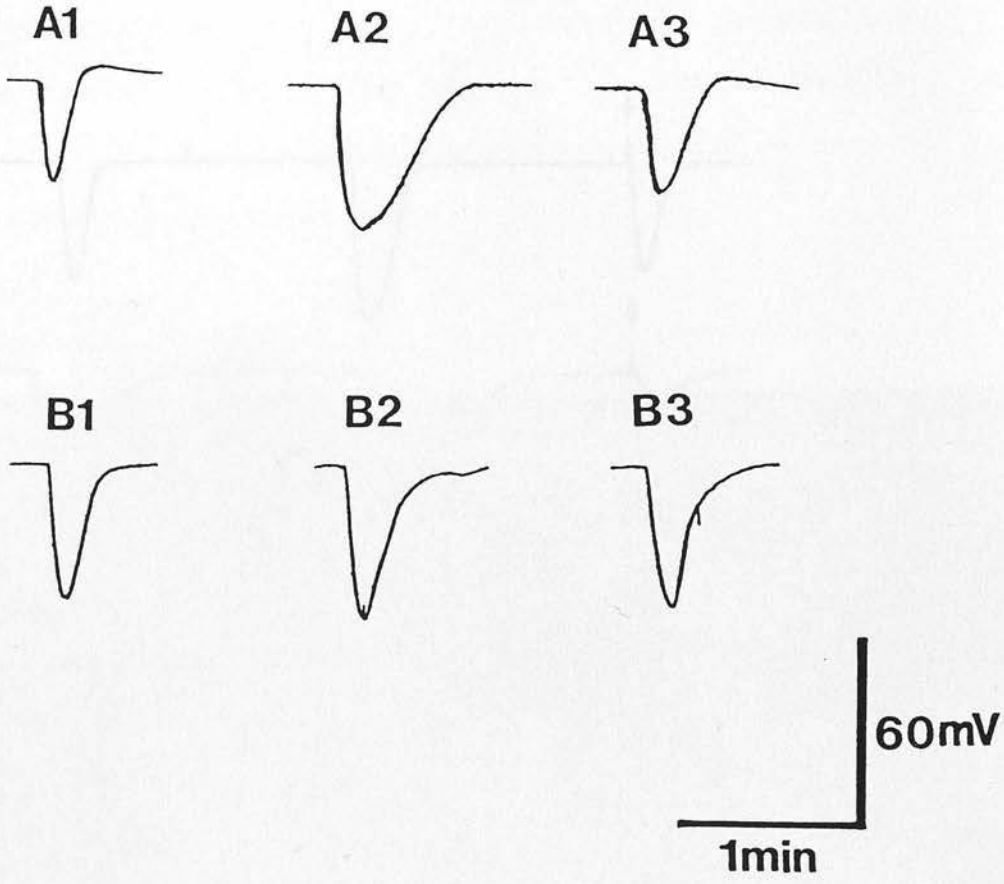

FIGURE 45 The potentiation by domperidone and by (±)sulpiride of the electrical response of the acinar cells to pressure ejected dopamine (500nM; 35KPa). A1 shows the hyperpolarization induced by a 50ms pulse of dopamine in the absence of domperidone. A2 shows the response to an identical stimulus in the presence of 100μM domperidone after a 30min exposure, and A3 the response after a wash. The resting potential was -30mV. B1 shows the control response of a different cell to a 150ms pulse of dopamine. B2 shows the response to an identical stimulus in the presence of 100μM (±)sulpiride after a 30min exposure. B3 shows the response after washing. The resting potential of this cell was -52mV.

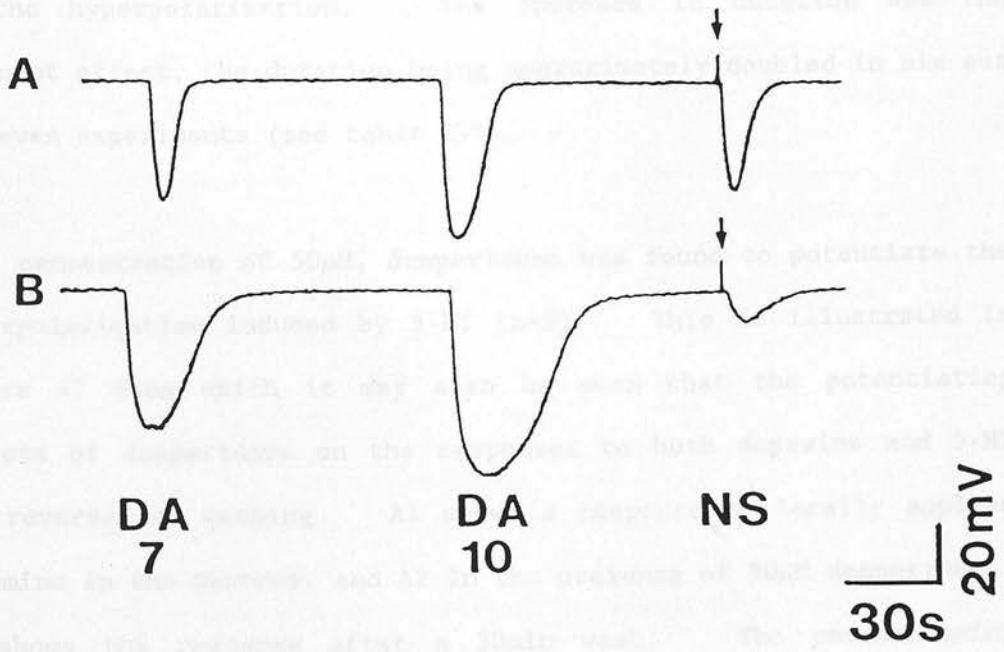

FIGURE 46 The effect of domperidone on responses of a single cell to dopamine and to nerve stimulation. A: two succesive responses to dopamine applied locally by pressure ejection (1μM; 35KPa) with a duration of 7ms (DA7) and 10ms (DA10), followed by a response to a train of 10 stimuli (100V; 20Hz) applied to the nerve at the point marked by the arrow (NS). B: responses of the same cell to stimuli identical to those in A in the presence of 100μM domperidone. The period of exposure was 30min. The resting potential was -36mV.

might be tempted to conclude that the transmitter at this synapse was not, in fact, dopamine. However, the inhibition of the response to nerve stimulation resulted from a secondary action (see below). The potentiation of the dopamine-induced hyperpolarization was maximal at 50μM domperidone, and in general, as mentioned above, was manifest as both an increase in the duration and the amplitude of the hyperpolarization. The increase in duration was the dominant effect, the duration being approximately doubled in six out of seven experiments (see table XV).

At a concentration of 50μM, domperidone was found to potentiate the hyperpolarization induced by 5-HT (n=2). This is illustrated in figure 47 from which it may also be seen that the potentiating effects of domperidone on the responses to both dopamine and 5-HT are reversed on washing. A1 shows a response to locally applied dopamine in the absence, and A2 in the presence of 50μM domperidone; A3 shows the response after a 30min wash. The corresponding responses to 5-HT are illustrated in parts B1, B2 and B3.

As mentioned above, the action of domperidone on the response to nerve stimulation was more complex than that on the responses to applied agonists. As illustrated in figure 48, the decline in the response was preceded by a phase of potentiation. Figure 48A shows a control response to nerve stimulation, parts B and C show the response after exposure to 50μM domperidone for 15min and 30min, respectively. Similar results were obtained in two other experiments. The reduction in the response to nerve stimulation was concentration-dependent, and at a concentration of 100μM domperidone abolished the response in two out of four cells. In

Table XV

Potentiation of the dopamine-induced hyperpolarisation by domperidone

DOMPERIDONE CONCENTRATION (μM)	DOPAMINE-INDUCED HYPERPOLARISATION					
	AMPLITUDE (mV)			DURATION (s)		
	CONTROL	TEST	% OF CONTROL	CONTROL	TEST	% OF CONTROL
50	42	57.5	136	10	23.5	233
	49.3	36.5	74	14.4	38.2	265
	23.3	38.3	164	21.8	22.2	102
100	34	43	126	8.1	19.6	242
	37	45	122	7.2	20.7	288
	23	24	104	4.8	13.2	275
	26	24	92	7.2	15.0	208

Each value quoted represents the mean of four responses from a single experiment.

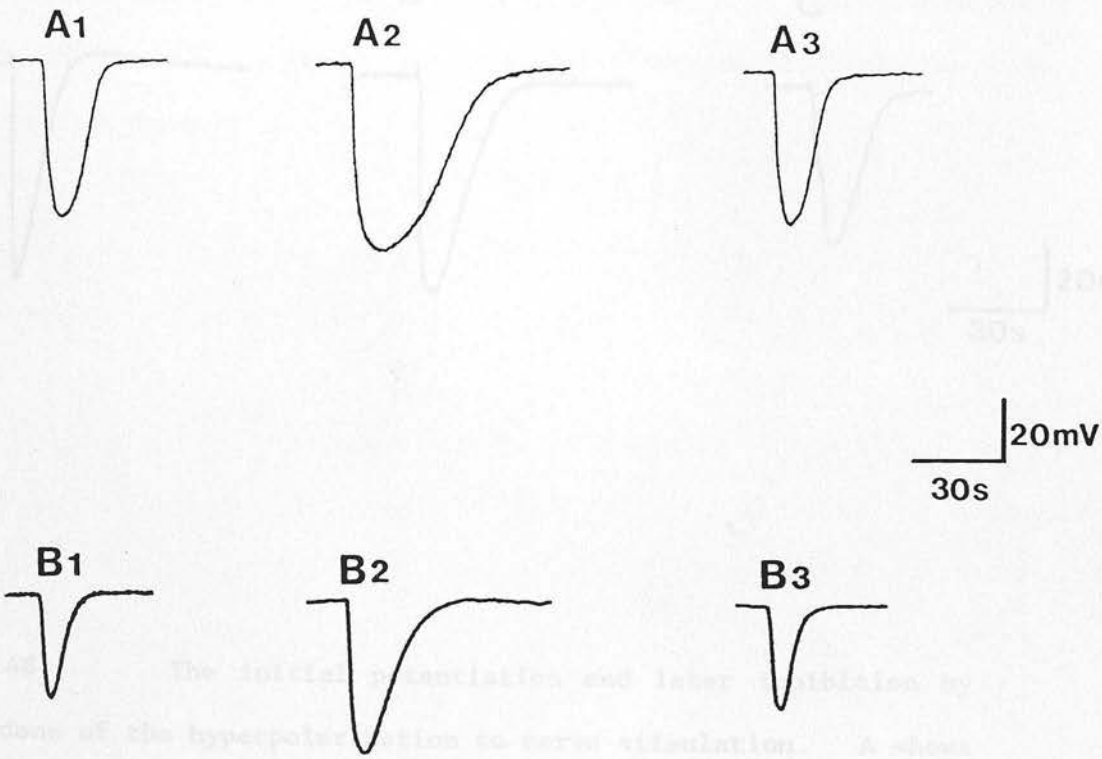

FIGURE 47 Potentiation by domperidone of the hyperpolarization induced by pressure ejection of dopamine (A; 1μM; 35KPa) and 5-HT (B; 10μM; 35KPa). A1: response to a 40ms pulse of dopamine in the absence of domperidone. A2: the response of the same cell to an identical stimulus in the presence of 50μM domperidone. A3: after a 30min wash. The resting potential was -36mV. B1 shows a control response of a different cell to a 100ms pulse of 5-HT. B2 shows the response to an identical stimulus in the presence of 50μM domperidone and B3 shows the response after a 10min wash. The resting potential was -52mV. In A and in B the period of exposure to domperidone was 10min.

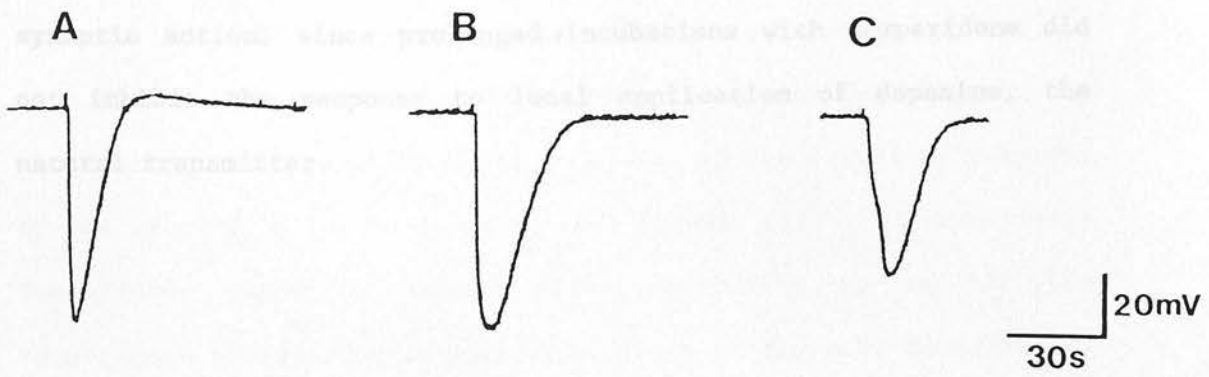

FIGURE 48 The initial potentiation and later inhibition by domperidone of the hyperpolarization to nerve stimulation. A shows the response to 10 stimuli applied to the nerve (100V; 20Hz) in the absence of domperidone. The responses to identical stimuli after a 15min exposure and a 30min exposure to 50μM domperidone are shown in B and C respectively. A, B and C were obtained from the same cell. The resting potential was -50mV.

the cells where the hyperpolarization was reduced but not abolished, the increased duration of the response relative to control was still apparent. Following abolition of the response to nerve stimulation, an increase in the number of stimuli in the train failed to overcome the blockade. The inhibition of the hyperpolarization to nerve stimulation was evidently due to a pre-synaptic action, since prolonged incubations with domperidone did not inhibit the response to local application of dopamine, the natural transmitter.

DISCUSSION

Domperidone potentiated the hyperpolarization induced by locally applied dopamine and by locally applied 5-HT. It also initially potentiated the hyperpolarization produced by nerve stimulation. One explanation for the potentiation might be that the responses to dopamine and 5-HT are normally limited by some reuptake process and that this is antagonised by domperidone. Against this argument is the finding that domperidone has no effect on the secretory response of the salivary gland to dopamine (see Chapter III). Alternatively domperidone might be acting post-synaptically to amplify the consequence of receptor activation: Since as shown by Bowser-Riley *et al* (1978), the receptors for dopamine and 5-HT in this preparation are distinct, post-synaptic potentiation of the response to both agonists is most easily explained by supposing that domperidone acts on some process occurring after receptor activation. Although domperidone has not, as far as I am aware, been shown to have any effect on possible intermediates between receptor activation and response, other dopamine antagonists have been shown to inhibit a number of these including protein kinase-C- (Petty, 1989), phospholipase A2- (Kahle *et al*, 1989) and calmodulin-dependent processes (Norman and Staehelin, 1982). It seems likely that the inhibition of calmodulin may be involved as the potentiating effect was common to (±)sulpiride, which is known to inhibit calmodulin (Norman and Staehelin, 1982). However a firm conclusion as to the mechanism of action of domperidone would require more direct biochemical assays.

CHAPTER VII

AN INVESTIGATION INTO THE ROLE OF CALMODULIN IN STIMULUS-SECRETION COUPLING

INTRODUCTION

A role for calcium in the mediation of cellular processes was first proposed by Ringer (1883). However the general view of calcium as an intracellular messenger remained unfocused until the discovery of calmodulin (Cheung, 1970; Cheung *et al*, 1975). Calmodulins are calcium-binding proteins which, on activation by calcium, interact with a variety of target proteins to regulate a number of cellular events.

The structure of calmodulins

Over 160 structurally related calcium-binding proteins have been identified. These have now been grouped into ten sub-families and ten unrelated single proteins (for review see Persechini *et al*, 1989). Calmodulins are proteins of low relative molecular mass (16 KDa), comprising a single polypeptide chain of 148 amino acid residues. They are unique in that they are present in all eukaryotes studied to date (Klee and Vanamen, 1982; Waisman *et al*, 1975 & 1977; Yamanaka and Kelly, 1981). The amino acid sequence of calmodulins is well conserved throughout evolution (Chafouleas *et al*, 1979) and many invertebrate calmodulins differ from that isolated from bovine brain in only 5 to 7 amino acid residues (Klee and Vanaman, 1982). Calmodulins are members of the family of 'EF-hand homolog' calcium-binding proteins (Persechini *et al*, 1989). The amino acid chain is arranged in the form of a dumbell, with two hemispheres positioned at either end of a long central helix. Each hemispheric portion of the molecule contains two EF-hands, in which the structure is orientated in the form of a helix loop helix. The

loop between the helices of the EF-hand represents the calcium-binding region of the molecule. Thus calmodulins bind a maximum of four calcium molecules, two within each hemisphere (Kretsinger and Barry, 1975). Nuclear magnetic resonance spectroscopy has shown that conformational changes occur in the EF-hand portion of the molecule during the process of calcium-binding (Ikura *et al*, 1983; Krebs and Carafoli, 1982; Iida and Porter, 1986). These changes result in an increase in affinity of the vacant calcium-binding sites. Calcium-dependent changes in the conformation of calmodulins also occur in the backbone connecting the two hemispheres. This alteration is complete after the binding of two calcium ions (Xu *et al*, 1983); however the changes in the orientation of the amino acids directly involved in the binding of calcium are not complete until all four sites are occupied (Seaton *et al*, 1983). It is generally accepted that the conformational changes around the calcium-binding sites expose hydrophobic surfaces in the hemispheric lobes of calmodulin, with which many target proteins interact (Manalan and Klee, 1984; Means and Klee, 1988). Many subcellular proteins have been recognized as calmodulin-binding proteins, of which a number have been identified as calcium-calmodulin regulated enzymes. Several of these proteins have been implicated in the control of secretion and their role will now be discussed.

Subcellular distribution of calmodulin and calmodulin-binding proteins

In rat submandibular glands (Singh *et al*, 1989) calmodulin is found predominantly in the cytosolic fraction of the cell. Less than 1% of the total calmodulin content of the rat submandibular acinar cells is found in the fraction associated with the mitochondria, golgi bodies, endoplasmic reticulum and plasma membranes. However, after activation by calcium, calmodulin is found predominantly in the particulate fractions (Singh *et al*, 1989). The reduction of calmodulin in the cytosolic fraction appears to reflect the binding of calmodulin, on activation of the cell, to target proteins in the mitochondria, golgi bodies, endoplasmic reticulum, plasma membrane and nuclei in addition to the cytosol (Singh *et al*, 1989). The wide distribution of calmodulin-binding proteins suggests that calmodulin may regulate many cellular functions. These include secretion, muscle contraction, cell motility, cell growth and gene expression (for review see: Klee and Vannaman, 1982; Persechini *et al*, 1989; Rasmussen and Means, 1989).

Calmodulin and secretion

Calmodulin has been reported to be responsible, at least in part, for the control of calcium-dependent secretion from the rat pituitary gland (Das *et al*, 1989; Schettini *et al*, 1984, 1985, 1987; Kile and Amos, 1988), bovine adrenal chromaffin cells (Burgoyne *et al*, 1982; Kenigsberg and Trifaro, 1985), rat and rabbit gastric mucosa (Black *et al*, 1989; Seidler and Sewing, 1989), rat intestine (Fedorak *et al*, 1989), rat hypothalamic cells

(Lewis *et al*, 1987), rat peritoneal mast cells (Izushi and Tasaka, 1989), rat submandibular and parotid salivary glands (Singh *et al*, 1989; Tojyo *et al*, 1989) and sea urchin eggs (Steinhardt and Alderton, 1982). In all these cases the secretory response was inhibited by prior incubation with calmodulin antagonists, or anti-calmodulin antibodies. In their study on the rat parotid salivary gland Tojyo *et al* (1989) found that both isoprenaline- and dibutyryl c-AMP-stimulated secretion was inhibited by the calmodulin antagonist W7 and the inhibition was reversed by exogenous calmodulin. In addition electron micrographic studies established that in the presence of W7, agonist-activated cells lost microvilli from their luminal surface, retained the majority of secretory granules within the cytoplasm and retained the discharged secretory material within the lumen of the cell. These effects were not mimicked by W5, a close analogue of W7 which possessed only limited anti-calmodulin activity. These findings suggest that calmodulin may be of importance in the regulation of several aspects of calcium-dependent secretion, including cytoskeletal reorganization, aggregation of secretory granules and fusion of the secretory granules with the plasma membrane. In support of this idea, a structurally different calmodulin antagonist, trifluoperazine, mimics the effect of W7 on the parotid cells (Tojyo *et al*, 1989) and has been shown to inhibit the formation of secretory vacuoles in the adrenal chromaffin cell plasma membrane and the fusion of secretory granules with the plasma membrane (Burgoyne *et al*, 1982). Furthermore calmodulin-binding proteins and substrates for calmodulin-dependent enzymes have been identified on secretory granules and cytoskeletal structures of a number of cell types (see Burgoyne and Geisow, 1981; Howe *et al*, 1983; Geisow and Burgoyne,

1987; Fournier and Trifaro, 1988; Parsons and Creutz, 1986; Harper, 1988).

Calmodulin antagonists

Two of the most potent and selective calmodulin antagonists presently available are W7 and calmidazolium. Calmidazolium is particularly useful as, unlike the neuroleptic calmodulin antagonists, it appears to be free of any activity at cell surface receptors at concentrations which inhibit calmodulin (Gietzen *et al*, 1981). The structure of these compounds conforms to the classical model proposed by Weiss *et al* (1982): this consists of a bulky hydrophobic region, formed by two aromatic rings, which allows interaction with the non-polar regions within the hemispheric lobes of calmodulins, and a positively charged group, essentially a basic nitrogen atom, not less than three carbon atoms from the hydrophobic rings. Of the two compounds W7 has been most extensively studied. Both high and low affinity binding sites for W7 have been identified within calmodulin (Hidaka *et al*, 1981). The low affinity sites are more numerous and are thought to be of little importance to the activity of the antagonist. Three high affinity sites have been located, all of which are centered around the hydrophobic clefts exposed on calcium-binding to the hemispheric lobes (Hidaka *et al*, 1981; Tanaka *et al*, 1982 & 1983). It is now accepted that the inhibition of calmodulin-dependent events by these antagonists results from competition with calmodulins target proteins for these binding sites (Ovadi, 1988). In-vitro biochemical assays have established that the IC_{50} for inhibition of calmodulin-dependent activation of a variety of enzymes by W7 and calmidazolium lies

within the range 0.01-10μM and 25-100μM respectively (Asano, 1989; Van Belle, 1984; Brumley and Wallace, 1989; Hidaka *et al*, 1981; Tanaka *et al*, 1982; Gietzen *et al*, 1981). Although both W7 and calmidazolium have been shown also to inhibit the activity of protein kinase-C and calpain I, the IC_{50} values are between 5 and 600 fold higher than those quoted for calmodulin-dependent enzyme activities (Brumley and Wallace, 1989). Except for the inhibition of protein kinase-C and calpain I, all actions common to both W7 and calmidazolium are, at least at present, considered to be specific to calmodulin (for review see Ovadi, 1989).

The aim of the present investigation is to study the involvement of calmodulin in the responses of the cockroach salivary gland to dopamine. The methods were as described in Chapters III and IV.

Secretion

The addition of W7 to the solution bathing the gland caused submaximal secretion. The results of two experiments are given in Table XVI. It can be seen that increases in the concentration of W7 up to 10μM caused increased rates of secretion but a further increase in concentration caused a reduced rate of secretion. Figure 49 compares the responses over a range of concentrations to W7 and dopamine from the same preparations. The maximum rate of secretion induced by W7 was less than 50% of the maximum response to dopamine. The period required for full equilibration with W7 varied within the range 5 - 25 min. Figure 50 shows the response to (A) 0.1μM and (B) 0.3μM dopamine; C shows the secretory response to 100μM W7 alone, D, the response to 100μM W7 and 0.3μM dopamine in combination and, E, the effect of 3μM dopamine with W7 still present. It can be seen that W7 inhibited the secretory response to dopamine. This inhibition was not overcome by an increase in the concentration of dopamine even to a level of 3μM, but was reversed after washing with W7 free solution. A similar experiment in which the effect of calmidazolium was studied is illustrated in Figure 51. Three additional experiments were performed for calmidazolium and for W7. It might be supposed that W7 and calmidazolium are partial agonists of the dopamine receptor, however to my knowledge neither compound has been shown to interact with cell surface receptors (see also Gietzen *et al*, 1981).

Table XVI

The secretory responses obtained to a range of concentrations of W7.

CONCENTRATION OF W7 (μM)	DIAMETER (DIV)		VOLUME (nl)		TIME (min)		RATE nl/min	
	E X P E R I M E N T							
	1	2	1	2	1	2	1	2
1.0	0	0	0	0	5	5	0	0
3.0	9.5	16.0	7.54	33.5	5	5	1.5	6.7
10.0	23.0	25.0	99.5	127.83	5	5	19.9	25.5
100.0	11.0	8.0	10.9	4.19	5	5	2.2	0.84

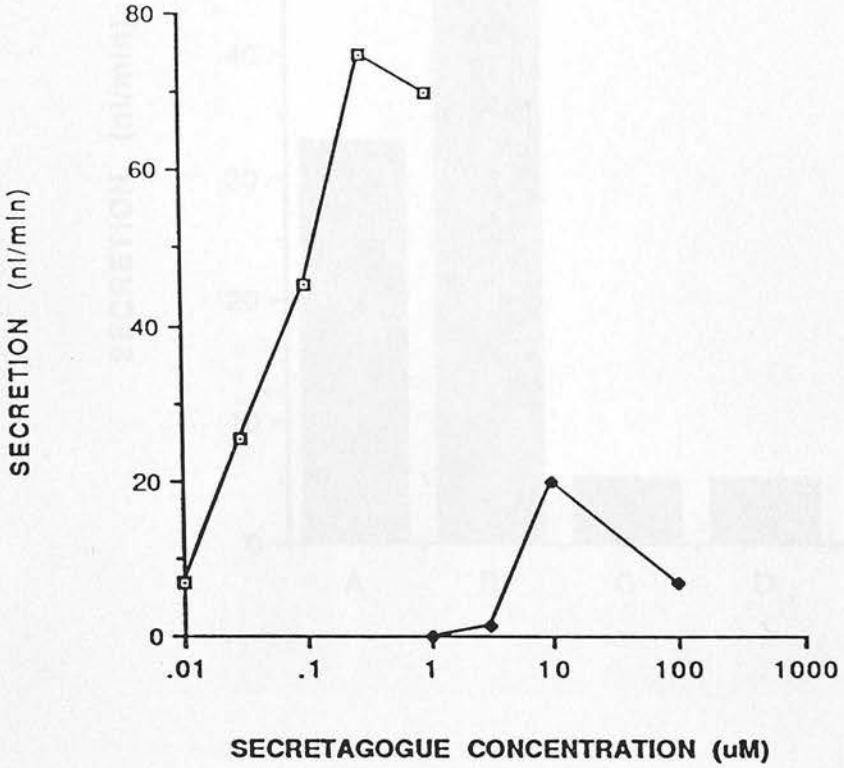

FIGURE 49 Comparison of the log-concentration-response curves for dopamine and W7. Abcissa: Secretagogue concentration (μM; logarithmic scale). Ordinate: rate of secretion in nl/min. Open squares represent the response to dopamine. Closed squares represent the response to W7. These data were obtained from a single experiment.

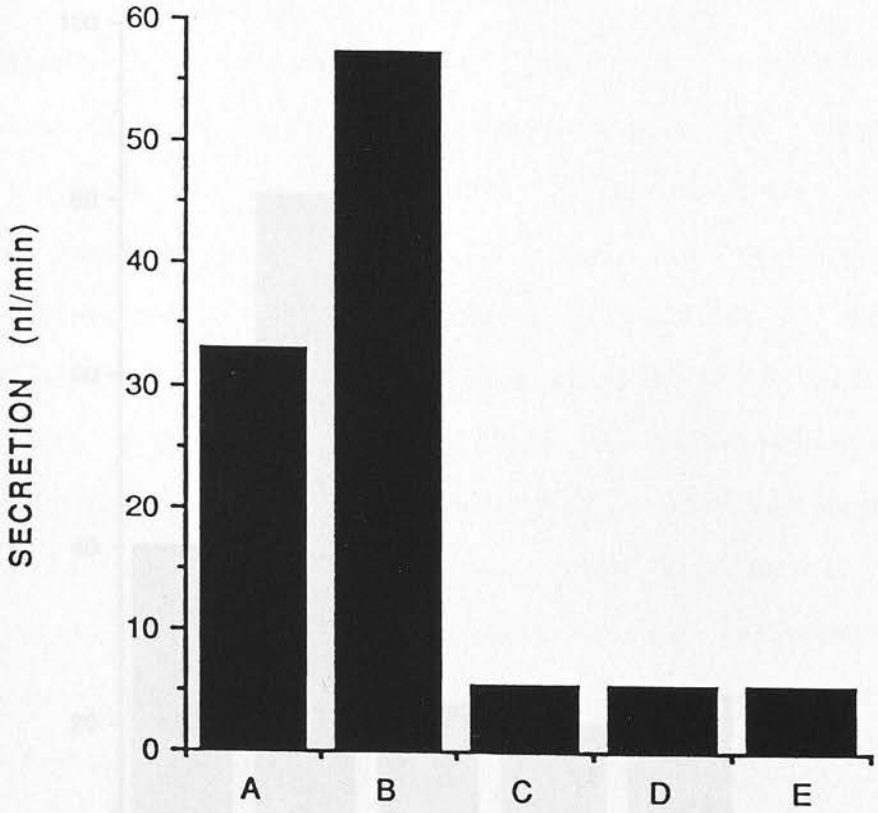

FIGURE 50 Block diagram illustrating the inhibition by W7 of the secretory response to dopamine. Ordinate: rate of secretion in nl/min. The columns represent the secretory responses to A, 0.1μM and B, 0.3μM dopamine in the absence of W7. C: the secretory response to 100μM W7. D: the response to 100μM W7 in combination with 0.3μM dopamine. E: the response to 100μM W7 in combination with 3μM dopamine.

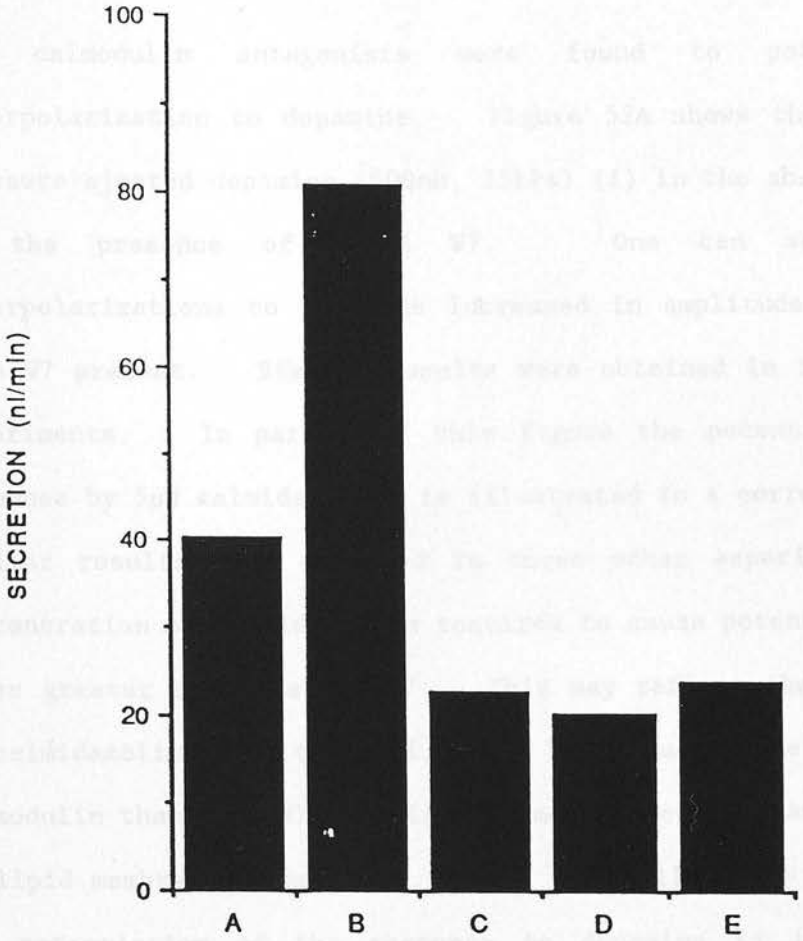

FIGURE 51 Block diagram illustrating the inhibition by calmidazolium of the secretory response to dopamine. Ordinate: rate of secretion in nl/min. The columns represent the secretory responses to A, 0.1μM and B, 0.3μM dopamine in the absence of calmidazolium. C: the secretory response to 10μM calmidazolium. D: the response to 10μM calmidazolium in combination with 0.3μM dopamine. E: the response to 10μM calmidazolium in combination with 3μM dopamine.

The calmodulin antagonists were found to potentiate the hyperpolarization to dopamine. Figure 52A shows the response to pressure ejected dopamine (500nM, 35kPa) (i) in the absence and (ii) in the presence of 0.1μM W7. One can see that the hyperpolarizations to dopamine increased in amplitude and duration with W7 present. Similar results were obtained in two additional experiments. In part B of this figure the potentiation of the response by 5μM calmidazolium is illustrated in a corresponding way. Similar results were obtained in three other experiments. The concentration of calmidazolium required to cause potentiation was 50 times greater than that of W7. This may reflect the slower entry of calmidazolium into the cell, as it has a much higher affinity for calmodulin than does W7, but is much more lipophilic and accumulates in lipid membranes (see Ovadi, 1989). Equally it is possible that the potentiation of the response to dopamine is independent of calmodulin, as one would always expect W7 to be less potent if the blockade of calmodulin was involved. It may be that longer periods of exposure to lower concentrations of calmidazolium should be attempted.

Higher concentrations of the calmodulin antagonists induced a hyperpolarization on their own. Figure 53 illustrates the response to addition of (A) 100μM W7 and (B) 10μM calmidazolium to the superfusate. The hyperpolarization to each compound was close to the maximum (-110mV) obtained to dopamine under these conditions (KCl 1mM, see Ginsborg *et al*, 1974). The period of exposure required to elicit a hyperpolarization varied. In some cells the

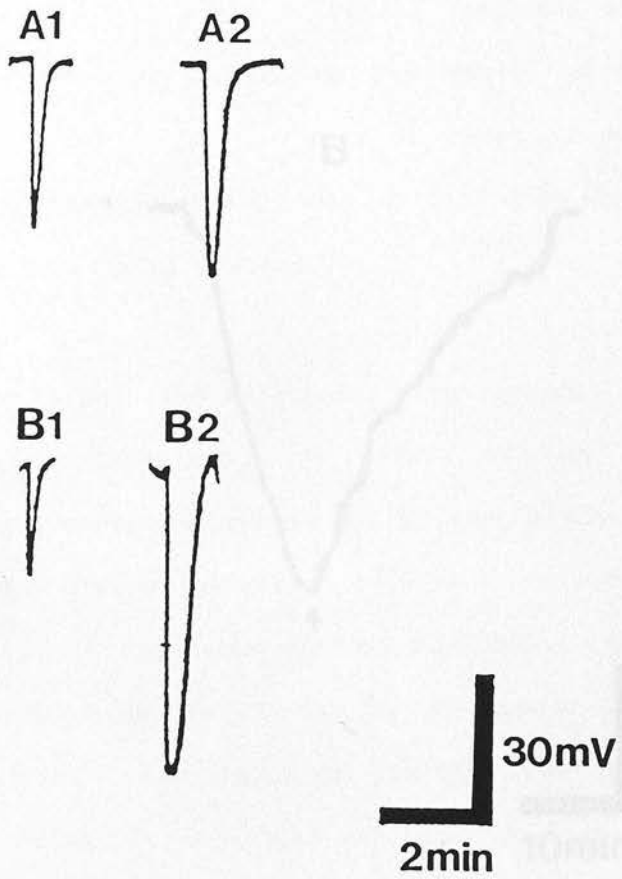

FIGURE 52 Potentiation by W7 and by calmidazolium of the hyperpolarization induced by locally applied dopamine (500nM; 35KPa). A1: response to a 50ms pulse of dopamine in the absence of W7. A2: hyperpolarization to an identical stimulus in the presence of 100nM W7 after a 15min exposure. The resting potential was -46mV. B1: control response of a different cell to a 120ms pulse of dopamine. B2: response to an identical stimulus in the presence of 5μM calmidazolium after a 10min exposure. Resting potential -50mV.

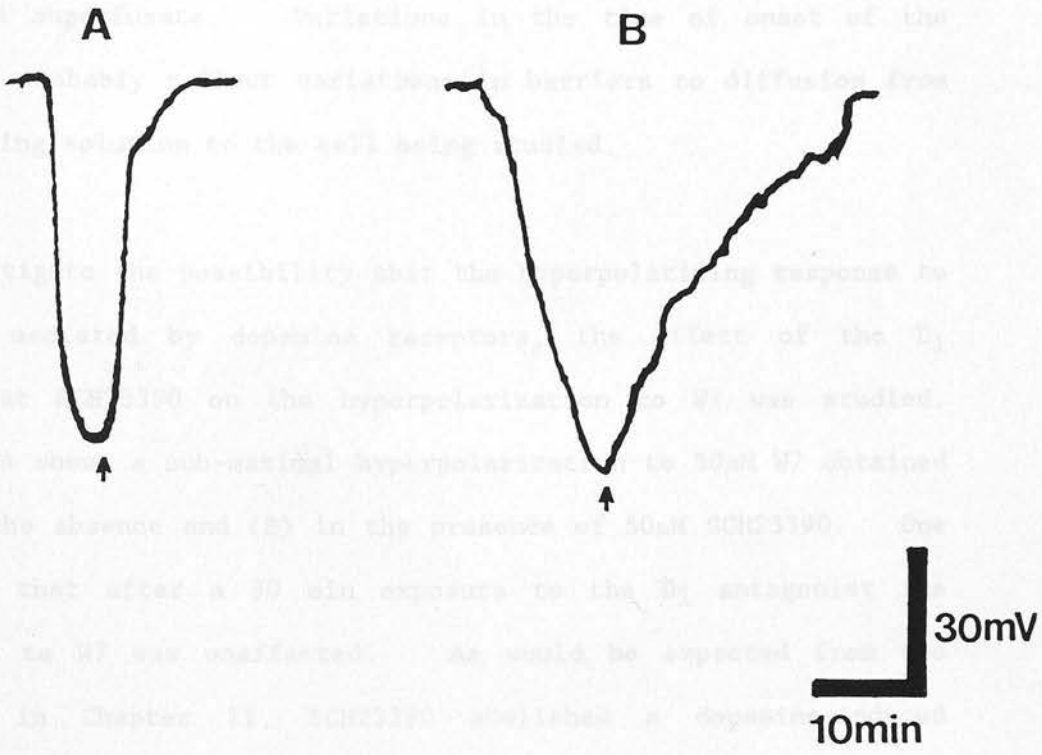

FIGURE 53 The hyperpolarizing response to W7 and to calmidazolium. A: the hyperpolarization to 100μM W7. W7 was in contact with the preparation for 7min before the onset of the response. The resting potential was -32mV. B: the hyperpolarization of a different cell to 10μM calmidazolium. This compound was in contact with the preparation for 5min before the onset of the response. Resting potential was -36mV. In each case the arrow marks the begining of the wash.

response was almost immediate (30 s), while in the majority of cases no effect was observed until 5-15 min after addition of these compounds to the solution. Figure 53.1 shows the response of a cell to 100μM W7 on an expanded time scale. This illustrates the slow onset of the response and slow decline on the removal of W7 from the superfusate. Variations in the time of onset of the response probably reflect variations in barriers to diffusion from the bathing solution to the cell being studied.

To investigate the possibility that the hyperpolarizing response to W7 was mediated by dopamine receptors, the effect of the D_1 antagonist SCH23390 on the hyperpolarization to W7 was studied. Figure 54 shows a sub-maximal hyperpolarization to 50μM W7 obtained (A) in the absence and (B) in the presence of 50μM SCH23390. One can see that after a 30 min exposure to the D_1 antagonist the response to W7 was unaffected. As would be expected from the studies in Chapter II, SCH23390 abolished a dopamine-induced hyperpolarization equal in amplitude to that induced by W7. Similar results were obtained in two other experiments. It may thus be concluded that the calmodulin antagonists have a post-synaptic action independent of the dopamine receptor. In addition the greater potency of calmidazolium than that of W7 suggests that inhibition of calmodulin may underlie the hyperpolarization.

Ginsborg *et al* (1980) established that the hyperpolarization to dopamine was a calcium-dependent event, which probably relies, at least in part, on the release of calcium from intracellular stores. Thus it seemed important to investigate whether or not the hyperpolarization to W7 was (i) calcium-dependent and (ii) due to

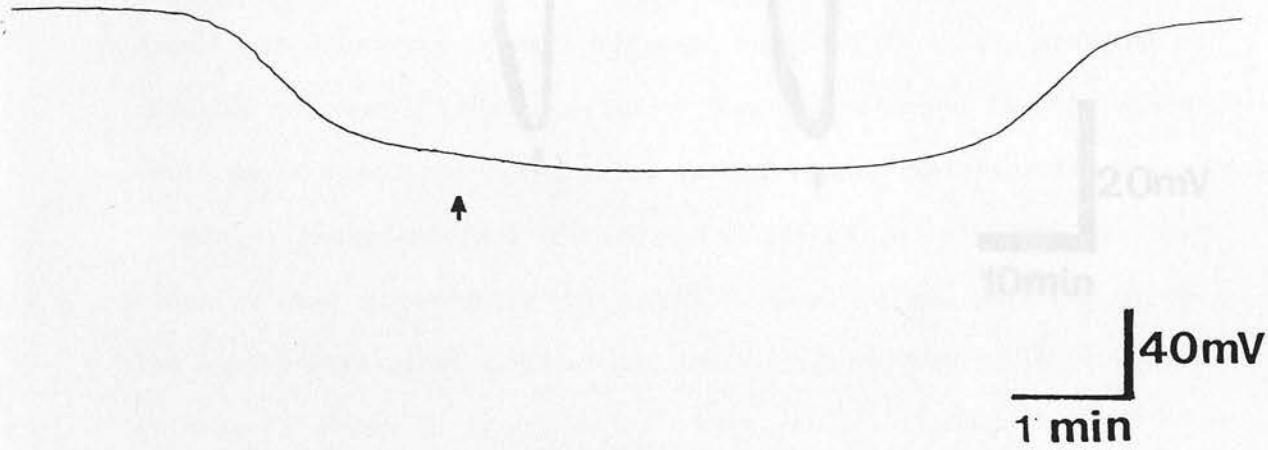

FIGURE 53.1 The hyperpolarizing response to 100 uM W7. W7 was in contact with the preparation for 7 min before the onset of the response. The resting potential was - 32 mV. The arrow marks the beginning of the wash.

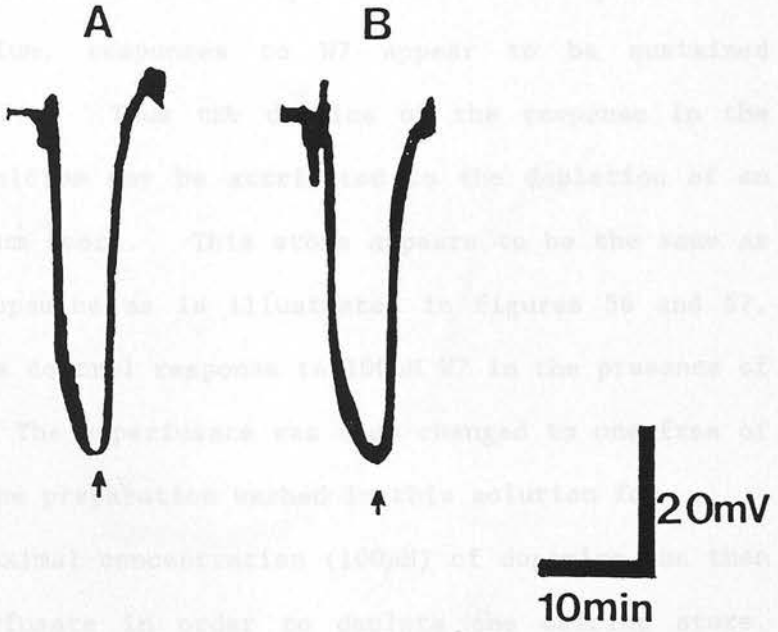

FIGURE 54 The effect of SCH23390 on the hyperpolarization induced by W7. A: the response to 50μM W7 in antagonist free solution. B: the response to an identical stimulus in the presence of 50μM SCH23390 after a 30min exposure. The resting potential was -52mV. In A and B the arrow marks the beginning of the wash. In each case W7 was in contact with the preparation for 30s before the onset of the response.

the release of calcium from the same cellular source as that accessed by dopamine. Figure 55 shows the hyperpolarization evoked by W7 in the absence of added extracellular calcium. As can be seen, the response to W7 declined after about 60 min. Similar results were obtained in one other experiment. In the presence of extracellular calcium, responses to W7 appear to be sustained indefinitely (n = 7). Thus the decline of the response in the absence of added calcium may be attributed to the depletion of an intracellular calcium store. This store appears to be the same as that accessed by dopamine as is illustrated in figures 56 and 57. Figure 56A shows the control response to 100μM W7 in the presence of external calcium. The superfusate was then changed to one free of added calcium and the preparation washed in this solution for 10 min. A supramaximal concentration (100μM) of dopamine was then added to the superfusate in order to deplete the calcium store. The hyperpolarization induced and subsequent decline of the response to zero is shown in figure 56B. This decline indicates a fall in cytoplasmic calcium concentration and, as dopamine was present throughout, the depletion of the calcium source. The superfusing fluid was then changed to one which contained 100μM W7, but no added calcium or dopamine. Figure 56C showed that this solution had no effect on the membrane potential. Thus following the depletion of the source of calcium on which the response to dopamine was dependent, no further response could be obtained to W7. The response to W7 and to dopamine recovered on readmission of calcium to the superfusate. Similar results were obtained in four other experiments. It would appear therefore that the hyperpolarization to W7 is calcium-dependent and relies on the same source of calcium as does dopamine. This conclusion is supported by the finding that

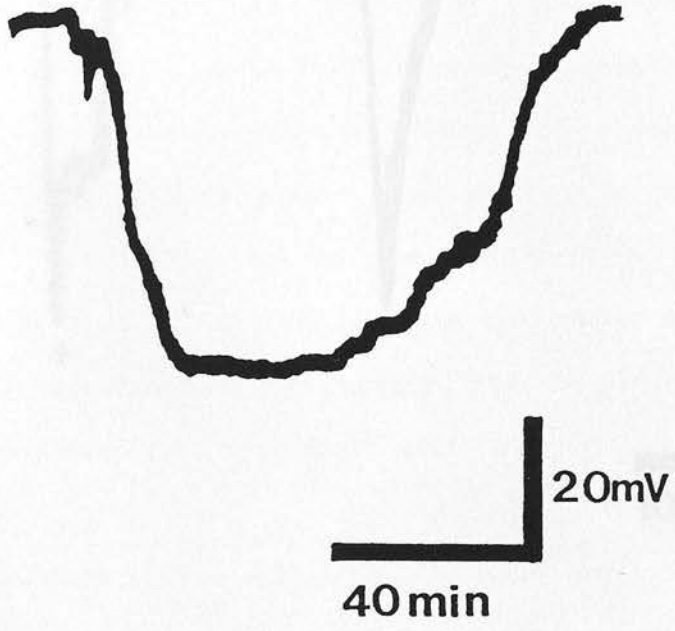

FIGURE 55 The hyperpolarization induced by 100μM W7 in the absence of external calcium after a 10min exposure to calcium free solution (no EGTA added). W7 was in contact with the preparation for 8min before the onset of the response. The resting potential was -44mV.

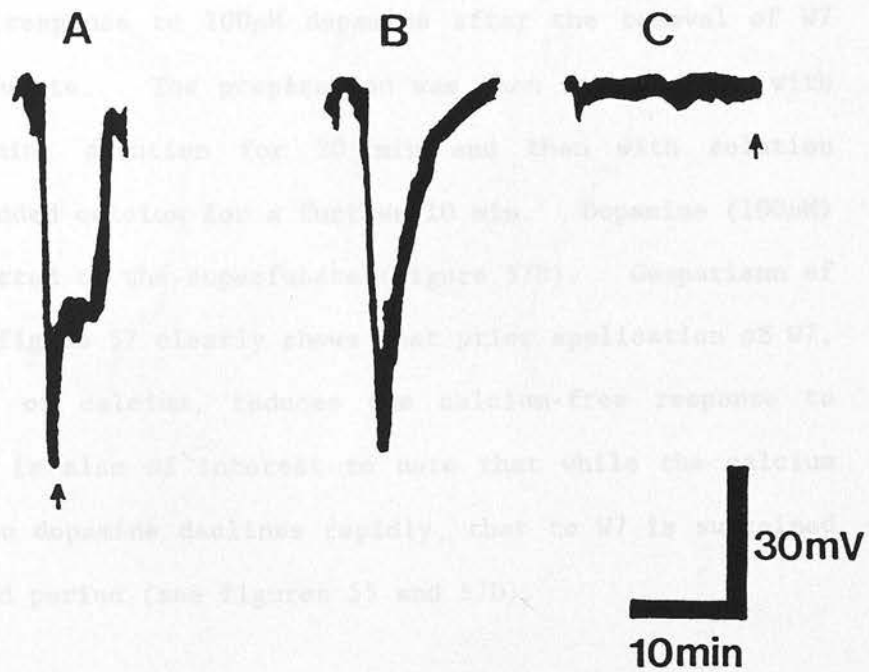

FIGURE 56 The abolition of the response to W7 by prior stimulation with dopamine in the absence of external calcium. A: the hyperpolarization induced by 100μM W7 in the presence of calcium. W7 was in contact with the preparation for 30s before the onset of the response. B: the hyperpolarization to 100μM dopamine with calcium absent after a 10min exposure to calcium free solution (no EGTA added). Note the decline of the response to the resting potential level in the presence of dopamine. C: the effect of subsequent superfusion of 100μM W7 with calcium still absent. In A and B the arrow marks the begining of the wash. These responses were taken from a continuous recording from a single cell. The resting potential was -45mV. In A and C the arrow marks the beginning of the wash.

exposure of the preparation to W7 in the absence of calcium reduces the response to dopamine. Figure 57A shows the response to 100μM dopamine in the presence of calcium. The preparation was then washed for 10 min in calcium free solution. Figure 57B shows the response to 100μM W7 with calcium still absent. Figure 57C shows the subsequent response to 100μM dopamine after the removal of W7 from the superfusate. The preparation was then washed first with calcium containing solution for 20 min and then with solution containing no added calcium for a further 10 min. Dopamine (100μM) was then readmitted to the superfusate (figure 57D). Comparison of parts C & D of figure 57 clearly shows that prior application of W7, in the absence of calcium, reduces the calcium-free response to dopamine. It is also of interest to note that while the calcium free response to dopamine declines rapidly, that to W7 is sustained over a prolonged period (see figures 55 and 57D).

A study was also made of the effect of prolonged exposure to W7 on submaximal responses to locally applied dopamine. After up to 30min in 100μM W7, submaximal responses to dopamine were potentiated, as would be expected from the effect of low concentrations of W7 on the response to dopamine (see figure 52A). However, after a more prolonged exposure to W7 subsequent responses to dopamine were inhibited as shown in figure 58. A shows a control response to locally applied dopamine (5×10^{-7}M, 35kPa). B shows the effect of an identical stimulus after 60min exposure to 100μM W7 and a washout to allow recovery of the resting potential. C shows that the inhibition caused by W7 was partly surmountable.

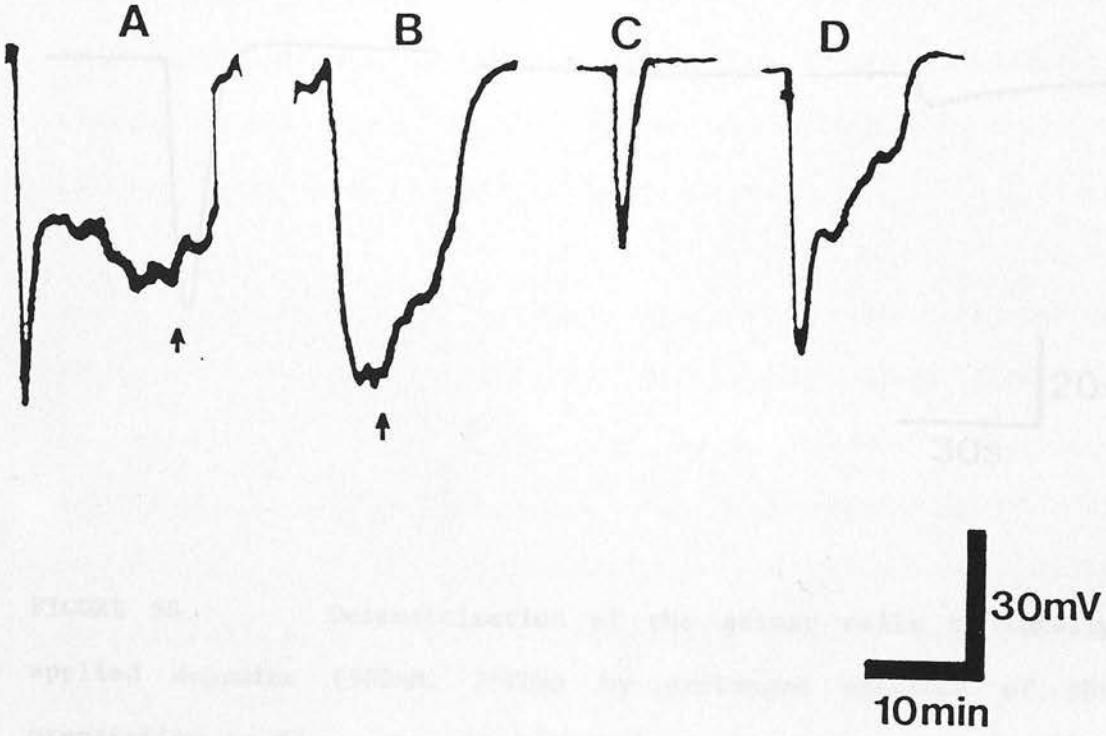

FIGURE 57 The inhibition of the response to dopamine by prior stimulation with W7 in the absence of calcium. A: the response induced by 100μM dopamine in the presence of calcium. B: the response to 100μM W7 with calcium absent after a 10min exposure to calcium free solution (no EGTA added). W7 was in contact with the preparation for 6min before the onset of the response. In A and B the arrows mark the beginning of the wash. C: the effect of subsequent superfusion of 100μM dopamine with calcium still absent. D: the response to 100μM dopamine in calcium free solution after exposure to normal calcium containing solution for 20 min. All five responses were taken from a continuous record from a single cell. The resting potential was -40mV.

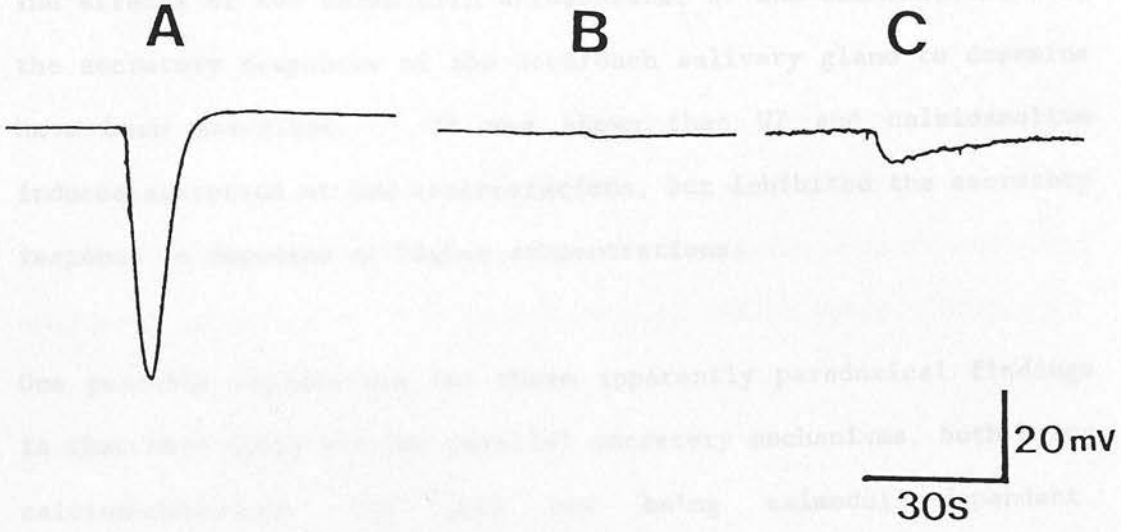

FIGURE 58 Desensitization of the acinar cells to locally applied dopamine (500nM; 35KPa) by prolonged exposure of the preparation to W7. A: the hyperpolarization induced by a 100ms pulse of dopamine before exposure to W7. B: the effect of a 100ms pulse of dopamine after a 1h exposure to 100μM W7. C: subsequent response to a 2s pulse of dopamine. Resting potential -40mV.

DISCUSSION

Secretion

The effects of two calmodulin antagonists, W7 and calmidazolium, on the secretory responses of the cockroach salivary gland to dopamine have been described. It was shown that W7 and calmidazolium induced secretion at low concentrations, but inhibited the secretory response to dopamine at higher concentrations.

One possible explanation for these apparently paradoxical findings is that that there are two parallel secretory mechanisms, both being calcium-dependent, but only one being calmodulin-dependent. Inhibition of calmodulin, since the electrophysiological data suggests that this would increase the level of free calcium within the cell, would therefore activate the calmodulin-independent component of secretion, but prevent any calmodulin-dependent contribution to secretion. A likely candidate for the regulation of the calmodulin-independent pathway is protein kinase-C, which has been shown to be an intermediate in secretion from gastric mucosal cells (Siedler and Sewing, 1989), platelets (Yamanashi *et al*, 1983) and rat pituitary cells (Das *et al*, 1989). In preliminary experiments D. MacEwen and I have shown that protein kinase-C is present in the cytosol of homogenates of cockroach salivary glands, but is found in higher concentrations in the particulate fractions in homogenates of glands that have been exposed to dopamine.

As the affinity of calmodulin for its target proteins varies (see Ovadi, 1989), an alternative explanation is that calmodulin antagonists inhibit selectively one calmodulin-dependent event, such as calcium sequestration, without affecting another, such as secretion.

Another possibility, namely that W7 and calmidazolium are partial agonists of the dopamine receptor, is highly unlikely, as to my knowledge they are without agonist or antagonist activity at cell surface receptors (see also Geitzen *et al*, 1981; Ovadi, 1989).

Electrophysiology

In contrast to their effects on secretion, the calmodulin antagonists were stimulatory with respect to the electrical response of the salivary gland acinar cells. Thus low concentrations of these compounds potentiated the hyperpolarization to dopamine, while higher concentrations of W7 and calmidazolium hyperpolarized the acinar cells on their own. Additional studies with W7 established that the hyperpolarization resulted from a post-synaptic action independent of dopamine receptor activation, as SCH23390 failed to inhibit a sub-maximal response. Furthermore, the hyperpolarization to W7 was found to rely on the release of calcium from the same cellular source as that accessed by dopamine, as depletion of the calcium store by dopamine or by W7 inhibited any further response to either compound. This suggests that W7 and calmidazolium inhibit a process which opposes increases in cytosolic calcium levels at rest. It seems likely that this process is calmodulin-dependent as

calmidazolium was more potent in this respect than was W7. Moreover the concentrations used were within the range known to inhibit calcium-calmodulin-dependent processes (see introduction).

Model

It is proposed that calmodulin, on combining with calcium, activates systems which reduce cytoplasmic free calcium concentration. Figure 59 illustrates a more complex model in which calmodulin-independent processes coupled to protein kinase-C are incorporated. It is postulated that in the resting cell the concentrations of free and bound calcium are in dynamic equilibrium. IP_3, which is present in high concentrations within the acinar cells at rest and may be an intermediate in the hyperpolarization to dopamine (see Chapter IV), promotes an increase in free calcium, and calmodulin/protein kinase-C promotes a decrease. Inhibition of calmodulin/protein kinase-C would evidently result in an increase in free calcium and hence a hyperpolarization. It is also of interest to note that with appropriate kinetic parameters the model would be expected to give rise to oscillations in free calcium concentration since the system has both positive and negative feedback. Although calcium oscillations such as occur in hepatocytes (Woods *et al*, 1987; Walker *et al*, 1986), pituitary gonadotrophes (Shanghold, 1988) and endothelial cells (Jacob *et al*, 1988) have not been observed in the cockroach salivary gland acinar cells, the oscillations seen in the hyperpolarizing response to dopamine suggest their existence (see figures 34 & 57A). In general, upon agonist-induced calcium release, the oscillations are maximal once the threshold for release is breached, the frequency of the

oscillations increasing as the agonist concentration is increased (Berridge, 1988; Rink and Jacob, 1989). Woods *et al* (1987) suggested that calcium-mediated inhibition of phospholipase-C and the subsequent reduction in IP_3 may be responsible for the oscillations. However, this theory was abandoned when intracellular injection of a stable analogue of IP_3 was shown to induce oscillations (Berridge, 1988; Parker and Miledi, 1986; Capoid *et al*, 1987; Wakui *et al*, 1989). The model currently favoured relies on the existence of two separate non-mitochondrial calcium stores, one insensitive and the other sensitive to IP_3. The calcium released from the IP_3-sensitive pool acts as a stimulus for release from the pool insensitive to IP_3 (Thevenod *et al*, 1989; Goldbeter *et al*, 1990). The existence of oscillations in this model relies upon the complete depletion of an IP_3 sensitive pool and its subsequent replenishment by transmembrane influx of calcium. It is postulated that during replenishment no calcium release from the IP_3-sensitive pool, and hence from the IP_3-insensitive pool, can occur until a threshold concentration of stored calcium is reached. During this period, the free calcium concentration falls. Against this idea is the finding that oscillations can be generated by direct intracellular injection of calcium or by calcium influx stimulated by the ionophore A23187 (Osipchucka *et al*, 1990). The model proposed here (see Figure 59) would be expected to result in oscillations in all the circumstances in which they have been observed, namely on stimulation of cell surface receptors, G-protein activation, microinjection of IP_3 and of calcium, and calcium influx induced by the ionophore A23187.

Calmodulin-dependent processes The underlying mechanism relies on the reduction of intracellular free calcium by three separate calmodulin-dependent processes, namely (A) activation of IP_3 kinase (Colca *et al*, 1988; Morris *et al*, 1987; Ryu *et al*, 1987; Biden *et al*, 1987; Tallant and Wallace, 1985), (B) activation of plasma membrane calcium pumps (James *et al*, 1989; Enyedi *et al*, 1989; Eggermont *et al*, 1989; Petruniaka *et al*, 1989) and (C) activation of calcium pumps in the endoplasmic reticulum (Eggermont *et al*, 1989; James *et al*, 1989; Wegener *et al*, 1986; Burgoyne *et al*, 1989). They evidently imply that an increase in calcium concentration by activating calmodulin may reduce the concentration of IP_3 and increase the rate of calcium sequestration. These processes would be inactivated as the calcium level falls, allowing a subsequent rise and thus oscillations.

Calmodulin-independent processes Although the findings of the present investigation together with those of other workers support such a negative feedback, it is unlikely that the calcium-calmodulin complex is solely responsible. Other candidates include calcium activated protein kinase-C which has been shown to close calcium channels (Marchetti and Brown, 1988; Limas, 1980) induce the metabolism of IP_3 by IP_3 phosphatase (Connolly *et al*, 1986; Nishizuka, 1986) and which may also be an activator of endoplasmic reticulum calcium ATPase (Petruniaka *et al*, 1989). As mentioned above (p.160) protein kinase-C is present in the gland and both calmidazolium and W7 inhibit its activity (Brumley and Wallace, 1989).

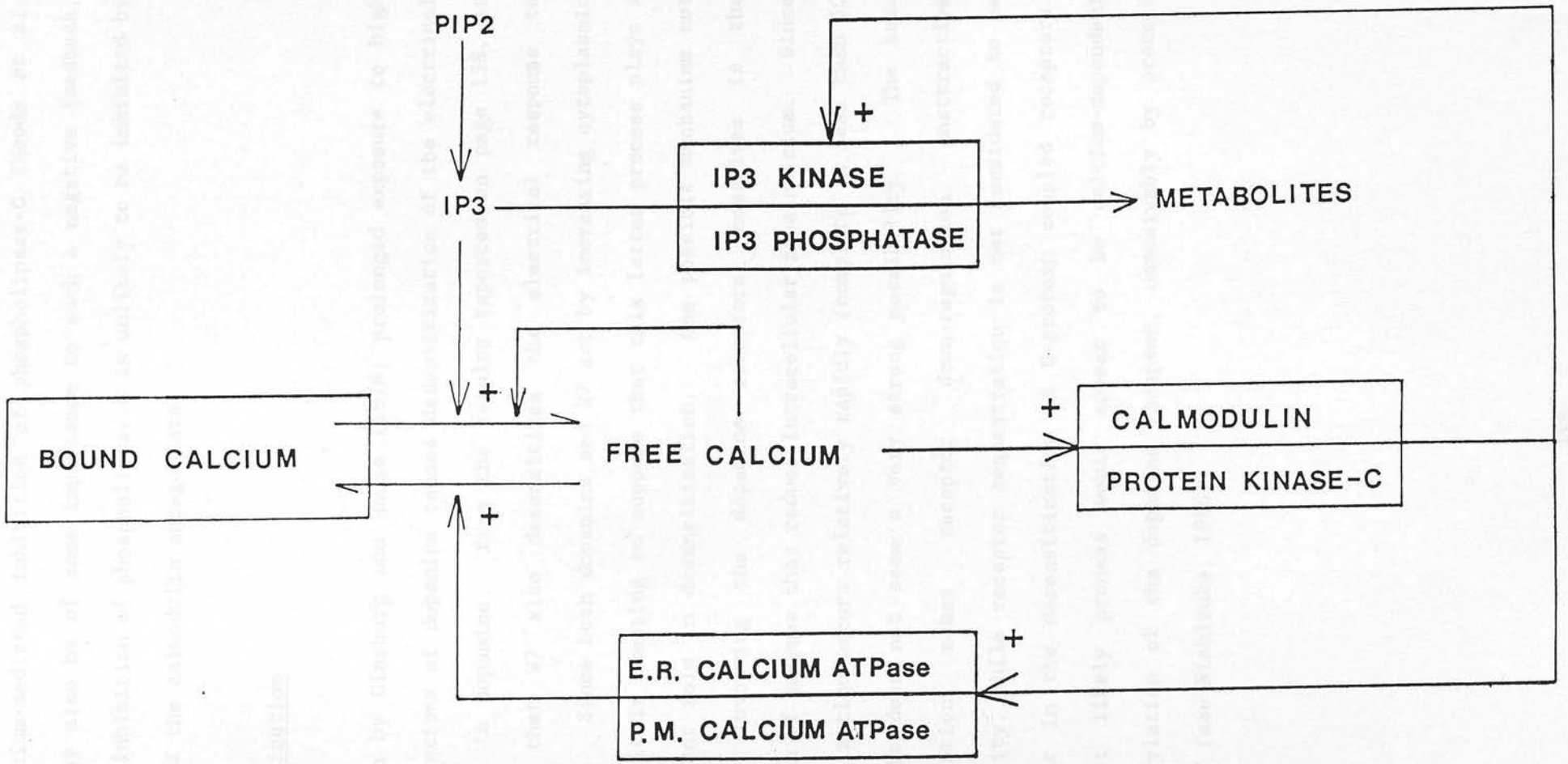

FIGURE 59 Proposed 'model' for the negative feedback system controlling the level of cytosolic free calcium concentration at rest and during stimulus-secretion coupling. P.M. = plasma membrane; E.R. = endoplasmic reticulum; + = activation.

The calcium-mediated inhibition of phospholipase-C (Woods *et al*, 1987) may also be of some importance to such a negative feedback, but the inhibition of phospholipase-C is unlikely to be inhibited by either of the calmodulin antagonists.

Desensitization

As shown by Ginsborg and House (1976) prolonged exposure to high concentrations of dopamine causes desensitization of the electrical response to dopamine; from the results reported on page 158, it appears that W7 also desensitizes the electrical response to dopamine. Since both dopamine and W7 act by increasing cytoplasmic calcium it is tempting to suppose that this latter process plays a significant role in desensitization. One possible mechanism not directly involving the dopamine receptors themselves is the induction of enzymes that reduce intracellular free calcium. Since desensitization occurs relatively rapidly (complete in less than 60 min) this does not seem a very strong possibility. The same consideration makes receptor down-regulation unattractive possibility, while receptor sequestration is not considered to be important in the desensitization of G-protein coupled receptors. The most likely process would appear to be calcium-dependent phosphorylation of the dopamine receptor, conceivably by protein kinase-C (see Nishizuka, 1986).

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APPENDIX I

Drugs and chemicals

Dopamine (3-hydroxy tyramine HCl), haloperidol (4-[4-(4-chlorophenyl)-4-hydroxypiperidino]-4'-fluorobutyrophenone), calmidazolium (compound R24571; (1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)-methoxyl]-ethyl]-1H-imidazolium chloride)) and W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide) were obtained from SIGMA (Poole, Dorset, U.K.). Chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)phenothiazine HCl) was obtained from Aldrich Chemical Company (Gillingham, Dorset, UK). (±)Sulpiride (5-(aminosulphonyl)-n-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide) was obtained from Semat (St. Albans, Herts. UK). SCH23390 (8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol maleate) was a gift from Dr. V.G. Daniels, Kirby Warrick Pharmaceuticals (Bury St. Edmunds, Suffolk, UK). Domperidone (5-chloro-1-{1-[3-(2-oxobenzimidazolin-1-yl)propyl]-4-piperidyl}]benzimidazolin-2-one) was a gift from Janssen Pharmaceutical (Marlow, Bucks, UK). Metoclopramide (4-amino-5-chloro-n-(2-diethylaminoethyl)-2-methoxybenzamide HCl) was a gift from Beecham Pharmaceuticals (Harlow, Essex, UK). Quinpirole (trans-(-)-4aR-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-2H-pyrazolo-(3,4-g)quinoline monohydrochloride) and LY163502 (trans-(-)-5,5a,6,7,8,9,9a,10-octahydro-6-propylpyrimido[4,5-g]quinolin-2-amine dihydrochloride dihydrate) were donated by Lilly laboratories (Indianapolis, IN, USA). Fenoldopam (SKF 82526-J, 6-chloro-7,8-dihydroxy-1-(*p*-hydroxyphenyl)-2,3,4,5-tetrahydro-(1H)-3-benzazepine) was supplied by Dr. J Skidmore, SK&F Laboratories, Welwyn, Herts, UK. (+)SCH23390 (8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-

3-benzazepin-7-ol maleate) and SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine HCl) were obtained from Semat, St Albans, Herts, UK.

Dopamine was dissolved in 1mg ml^{-1} ascorbic acid with the final dilution being made in physiological saline. The maximum concentration of ascorbic acid applied to the preparation was 0.1mg ml^{-1}, and when tested alone this had no effect on the preparation.

Domperidone, (±)sulpiride and haloperidol were dissolved in 5mg ml^{-1} tartaric acid and dilutions made from this in physiological saline. The pH of the solution was adjusted to control values (pH 7.7-7.8) with 1M NaOH; in this condition tartaric acid was found to have no effect on the preparation.

Calmidazolium was dissolved in DMSO and dilutions made from this in physiological saline. The highest concentration of DMSO applied to the preparation was 1%, and when applied alone this had no effect on the responses to dopamine.

APPENDIX II

PROGRAM WRITTEN BY PROFESSOR B.L. GINSBORG

```
ca: mark.f
      PROGRAM MARKF
C     PARALLEL DOSE RESPONSE FIT
C     PRINTOUT VERSION NO INTERMEDIATE RESULTS
C     E04CCF EXAMPLE PROGRAM TEXT MODIFIED
C     MARK 11.5(F77) REVISED.  NAG COPYRIGHT 1985.
C     .. Parameters ..
      INTEGER          N, IW
      PARAMETER        (N=4,IW=N+1)
      INTEGER          NOUT
      PARAMETER        (NOUT=6)
C     ****************************************************************
C
      CHARACTER*12 C1,C2
C
C     ****************************************************************
C     ARRAYS IN COMMON
      DOUBLE PRECISION T(200), Y(200),TT(200),YY(200)
C     .. Scalars in Common ..
      INTEGER          IMONIT
C     .. Local Scalars ..
      DOUBLE PRECISION F, R, TOL
      INTEGER          I, IFAIL, MAXCAL
C     .. Local Arrays ..
      DOUBLE PRECISION SIM(IW,N), W1(N), W2(N), W3(N), W4(N), W5(IW),
     *                 X(N)
C     .. External Functions ..
      DOUBLE PRECISION X02AAF
      EXTERNAL         X02AAF
C     .. External Subroutines ..
      EXTERNAL         E04CCF, FUNCT, MONIT
C     .. Intrinsic Functions ..
      INTRINSIC        SQRT
C     .. Common blocks ..
      COMMON           /OUTP/IMONIT
      COMMON Y, T,M,YY,TT,MM
      DOUBLE PRECISION P,KCO,KAN,AMP
      COMMON P,KCO,KAN,AMP
111      WRITE(6,99990)
99990 FORMAT('     HOW MANY CONT PAIRS? ')
      READ(5,*) M
C     OBSERVATIONS  CONTROL 7 AGONIST 8
C     (I = 1, 2, . . . , M)
C     CONCENTRATION T RESPONSE Y
C
C     ****************************************************************
C
      CALL GETARG(1,C1)
      CALL GETARG(2,C2)
      OPEN (7, FILE=C1)
      OPEN (8, FILE=C2)
C
C     ****************************************************************
C
      WRITE(6,99991)
99991 FORMAT(' HOW MANY ANTAG PAIRS')
      READ(5,*) MM
      DO 11 I =  1,N
      X(I) = 1.0D0
   11 CONTINUE
```

```
      WRITE(6,33)
   33  FORMAT('  DATA TO BE FITTED WITH LOGOID')
   34  FORMAT(' CONTROL')
   35  FORMAT(' WITH ANTAG PRESENT')
       WRITE(6,34)
         READ (7,*) (T(I), Y(I),I = 1,M)
      READ(8,*) (TT(I),YY(I),I =1,MM)
      DO 20 I = 1,M
      WRITE(6,19999) T(I),Y(I)
19999 FORMAT(2F12.5)
   20 CONTINUE
       WRITE(6,35)
       DO 21 I=1,M
      WRITE(6,19999) TT(I),YY(I)
   21 CONTINUE
C     ** SET IMONIT TO 1 TO OBTAIN MONITORING INFORMATION ***
      IMONIT = 0
      TOL = SQRT(X02AAF(R))
      MAXCAL = 5000
      IFAIL = 0
      WRITE(6,99989)
99989 FORMAT('   guess values:p,maxresponse,ec50cont,ec50ant')
      READ(5,*) P,AMP,KCO,KAN
      CALL E04CCF(N,X,F,TOL,IW,W1,W2,W3,W4,W5,SIM,FUNCT,MONIT,MAXCAL,
     *            IFAIL)
      WRITE (NOUT,FMT=99998) F
      WRITE (NOUT,FMT=99997) P*X(1),KCO*X(2),KAN*X(3),AMP*X(4)
      WRITE (NOUT,FMT=99996) IFAIL
C
99998 FORMAT (' FINAL FUNCTION VALUE IS ',F16.8)
99997 FORMAT (' P,KCONT,KANT,AMP',4F16.8)
99996 FORMAT (' THIS HAS ERROR NUMBER',I3)
      END
C
C
C
      SUBROUTINE FUNCT(N,XC,FC)
      DOUBLE PRECISION FC
      INTEGER          N
      DOUBLE PRECISION XC(N)
      INTRINSIC        DEXP
      DOUBLE PRECISION T(200), Y(200),TT(200),YY(200)
C     ..
      INTEGER I
      DOUBLE PRECISION EB, A,B
C     ..
      COMMON Y, T,M,YY,TT,MM
      DOUBLE PRECISION P,KCO,KAN,PW,KCOW,KANW,AMP,AMPW
      COMMON P,KCO,KAN,AMP
      PW = P*XC(1)
      KCOW = KCO*XC(2)
      KANW = KAN*XC(3)
      AMPW = AMP*XC(4)
      IF ( PW .LE. 0) GO TO 33
      IF (KCOW .LE. 0) GO TO 33
      IF (KANW .LE. 0) GO TO 33
      FC = 0.0
      DO 20 I=1,M
      A=T(I)**PW
      FA = (AMPW*A)/(A + KCOW**PW) - Y(I)
```

```
      FC = FC +FA*FA
 20   CONTINUE
      DO 50 I =1,MM
      EB = TT(I)**PW
      B = (AMPW*EB)/(EB + RANW**PW) - YY(I)
            FC = FC + B*B
C
   50 CONTINUE
      RETURN
   33 FC = 1.0D12
      RETURN
      END
C
C
C
      SUBROUTINE MONIT(FMIN,FMAX,SIM,N,N1,NCALL)
C     .. Parameters ..
      INTEGER          NOUT
      PARAMETER        (NOUT=6)
C     .. Scalar Arguments ..
      DOUBLE PRECISION FMAX, FMIN
      INTEGER          N, N1, NCALL
C     .. Array Arguments ..
      DOUBLE PRECISION SIM(N1,N)
C     .. Scalars in Common ..
      INTEGER          IMONIT
C     .. Local Scalars ..
      INTEGER          I, J
C     .. Common blocks ..
      COMMON           /OUTP/IMONIT
C     .. Executable Statements ..
      IF (IMONIT.NE.0) THEN
         WRITE (NOUT,FMT=99999) NCALL, FMIN
         WRITE (NOUT,FMT=99998) ((SIM(I,J),J=1,N),I=1,N1)
      END IF
C     END OF PRINT ROUTINE
      RETURN
C
99999 FORMAT (' AFTER',I5,'   CALLS,  F  VALUE IS',F16.9,'  WI',
     *  'TH SIMPLEX')
99998 FORMAT (4(3F12.4,/))
      END
C** END OF E04CCFEXPT

%
```

```
  cat kmdatc
0.001 0
0.003  0.6
0.01 8.96
0.03  25.69
0.1 41.03
0.3  82.17
1.0 89.78

%

  cat kmdata
0.01  0
0.03 1.4
0.1 2.68
0.3 7.2
1.0 16.29
3.0 65.81
10.0 76.52
% log
```

```
  epcf77 -ats -fpa -o marko mark.f -lnag
EPC Fortran77/386                          Version 2.4.11 (1167)
Copyright (c) 1987, 1989 EPCL.              All Rights Reserved.
mark.f
   program    MARKF
   subroutine FUNCT
   subroutine MONIT

   163 Lines Compiled
%
  marko kmdatc kmdata
     HOW MANY CONT PAIRS?
7
 HOW MANY ANTAG PAIRS
7
  DATA TO BE FITTED WITH LOGOID
 CONTROL
     0.00100      0.00000
     0.00300      0.60000
     0.01000      8.96000
     0.03000     25.69000
     0.10000     41.03000
     0.30000     82.17000
     1.00000     89.78000
 WITH ANTAG PRESENT
     0.01000      0.00000
     0.03000      1.40000
     0.10000      2.68000
     0.30000      7.20000
     1.00000     16.29000
     3.00000     65.81000
    10.00000     76.52000
   guess values:p,maxresponse,ec50cont,ec50ant
1
100
0.2
2.5
 FINAL FUNCTION VALUE IS     374.55377954
 P,KCONT,KANT,AMP      1.28644657      0.09137569      2.18635886     93.74675660
 THIS HAS ERROR NUMBER  0
%
```

APPENDIX III

Published Papers

1. Characterization of the dopamine receptor mediating the hyperpolarization of cockroach salivary gland acinar cells *in vitro*. A.M. Evans & K.L. Green (1990) Br.J.Pharmacol. 101, 103-108. (see overleaf).

2. The action of dopamine receptor antagonists on the secretory response of the cockroach salivary gland *in vitro*. A.M. Evans & K.L. Green (1990) Comp.Biochem.Physiol., (In press).

3. The effect of domperidone on the electrical response of cockroach salivary gland acinar cells. A.M. Evans & K.L. Green. Eur.J.Pharmacol., (In press).

Br. J. Pharmacol. (1990), **101**, 103–108

Characterization of the dopamine receptor mediating the hyperpolarization of cockroach salivary gland acinar cells *in vitro*

[1]A.M. Evans & K.L.Green

Department of Pharmacology, University of Edinburgh, Edinburgh EH8 9JZ

1 Intracellular recordings have been made of the hyperpolarization of cockroach salivary gland cells induced by nerve stimulation and dopamine.

2 The relative potency of a number of dopamine antagonists in inhibiting the dopamine- and nerve-mediated hyperpolarization was studied. SCH23390 (10–50 μM), chlorpromazine (0.1–5 μM), haloperidol (10–100 μM) and metoclopramide (1 mM) inhibited the hyperpolarization.

3 In contrast, domperidone and (±)-sulpiride potentiated the hyperpolarization induced by both nerve stimulation and dopamine.

4 Apparent dissociation constants ($K_{D_{app}}$) were obtained for the blockade of the dopamine-induced hyperpolarization. The rank order of potency ($K_{D_{app}}$ in parentheses) was as follows: chlorpromazine (0.2 μM); haloperidol (3.3 μM); SCH23390 (4.1 μM); metoclopramide (265 μM); domperidone and (±)-sulpiride (inactive).

5 It is concluded that the receptor subserving the dopamine-induced hyperpolarization of the salivary gland acinar cells is the same as that mediating the secretory response to dopamine. In addition these data support our findings, which suggested that this receptor is similar to the D_1 dopamine receptor, but distinct from the D_2 receptor found in mammalian systems.

Introduction

Stimulation of the suboesophageal nerve evokes secretion from the cockroach (*Periplaneta americana* and *Nauphoeta cincerea* Olivier) salivary gland (Whitehead, 1971; Smith & House, 1977), which is accompanied by a hyperpolarization of the acinar cells (House, 1973). Previous studies have established that the neurotransmitter involved is dopamine (Bland *et al.*, 1973; Fry *et al.*, 1974; Kapoor *et al.*, 1983). Activation of the dopamine receptor leads to an increase in a calcium-dependent potassium conductance, which underlies the hyperpolarizing response (Ginsborg *et al.*, 1974; 1980), and an increase in cytosolic adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Grewe & Kebabian, 1982) which appears to be an intermediate in the secretory, but not the electrical response to dopamine (Gray *et al.*, 1984). More recently the rank order of potency of a number of dopamine antagonists was used to characterize the receptor mediating the secretory response of the salivary gland. It was concluded that the receptor was similar to the D_1 receptor found in the mammalian CNS and periphery (Evans & Green, 1990). It is therefore of some interest to establish whether or not the same order of potency is obtained for the receptor mediating the electrical response of the acinar cells to dopamine. To enable as direct a comparison as possible the present investigation follows closely the protocol used in the study of the secretory response. Thus the rank order of potency for the selective D_1 antagonist SCH23390, the selective D_2 antagonists domperidone, (±)-sulpiride, haloperidol and metoclopramide (for review see Clark & White, 1987), and the non-selective dopamine antagonist chlorpromazine (O'Boyle & Waddington, 1984) was obtained. The results suggest that the receptor mediating the dopamine-induced hyperpolarization is the same as that mediating the secretory response to dopamine, and is similar to the D_1 receptor found in mammalian systems.

[1] Author for correspondence.

Methods

Paired salivary glands and associated ducts were dissected from adult cockroaches (*Nauphoeta cinerea* Olivier) as described previously (House, 1973). The preparation was pinned to the base of a shallow perspex chamber lined with silicone resin (Sylgard, Dow Corning). The main ducts of the gland were drawn into a suction electrode to allow stimulation of the encapsulated suboesophageal nerve. The preparation was kept at room temperature (18–25°C) and perfused at a rate of 5 ml min^{-1} with a solution of the following composition (mM): NaCl 160, $CaCl_2$ 5, KCl 1, glucose 20, Tris buffer 2.5, pH adjusted to between 7.70 and 7.80 with HCl. Recording microelectrodes were filled with 3M KCl and had resistances between 20 and 40 MΩ. Membrane potentials were recorded on a pen recorder (Gould, Brush 220) and on video tape (Sony SL-F 30; with a digital audio processor, Sony PCM-701 ES) via a pre-amplifier (Dagan 8900 patch clamp). Sub-maximal responses to nerve stimulation were obtained with a train of 1–10 impulses, 0.5 ms pulse width, 1–100 V, 20 Hz. Dopamine (500 nM) was applied to the preparation locally by pressure ejection from a broken microelectrode (pulse of 35 kPa, 0.003–5 s; Picospritzer II, General Valve Corporation). An interval of 90 s was allowed between any two stimuli. Antagonists were added to the perfusate after four consistent control responses had been obtained to each stimulus applied.

Estimation of apparent K_D *values*

When applied by pressure ejection the absolute concentration of the agonist at the acinus is, of course, unknown and a function of time. However, it was assumed that the effective concentration of agonist at the acinus was proportional to the duration of a pulse of fixed pressure (McCaman *et al.*, 1977; Sakai *et al.*, 1979). This assumption was supported by the fact that over a considerable range of pulse durations, the $\log_{10}$-duration response curve was linear (Figure 1). Therefore, throughout the present investigation the duration of the pressure pulse used for agonist application, and not agonist concentration, was used in the estimation of dose-ratios.

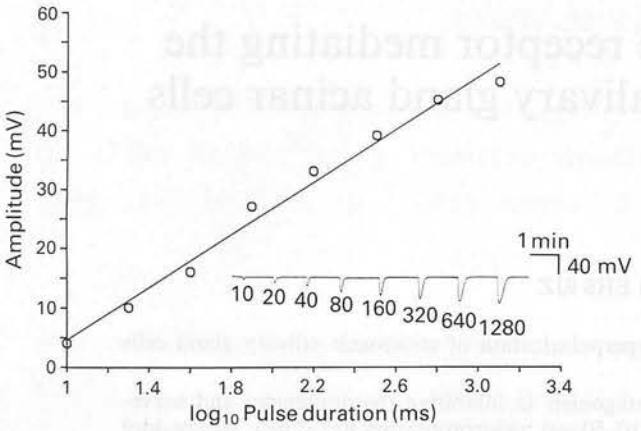

Figure 1 Dose–response curve for the dopamine-induced hyperpolarization of the cockroach salivary gland cells. The results are from a single experiment. Dopamine was applied by pressure ejection (35 kPa; duration of pulse as shown). The inset shows the experimental record, with the duration of the pulse marked below each response. Recordings of the membrane potential were made intracellularly. The initial resting potential was −55 mV. In this and all subsequent records the hyperpolarization is downwards.

The rate of equilibration of all active antagonists studied was inconsistent and often full equilibration was not obtained even during incubations of up to 1 h, with the dose-ratio increasing during this period. These considerations make it impossible to determine absolute values for the K_D. However, it seemed likely that useful estimates (at least for comparative purposes) would be obtained from the increase in stimulus required, after a fixed period of incubation in the presence of the antagonist, to match a response in antagonist-free solution. This assumption seems justified by the degree of agreement between the results in this paper and those obtained by more conventional methods in relation to the secretory response of this gland (Evans & Green, unpublished observation).

In practice, values for the apparent K_D (K_{Dapp}) were obtained from 'dose-ratios' estimated in a 'three point assay' (Edinburgh Staff, 1968); the time of exposure to the antagonist was 30 min. The antagonists studied were: SCH23390 (1–100 μM); chlorpromazine (0.1–5 μM); haloperidol (1–100 μM); metoclopramide (0.1–1 mM); (±)-sulpiride (1–100 μM); domperidone (1–100 μM). Dose-ratios were estimated independently on separate preparations for three concentrations of each antagonist (except metaclopramide; see results). A separate value for the $\log_{10} K_{Dapp}$ was estimated for each experiment (see Waud & Parker, 1971), and the geometric means of the K_{Dapp} together with confidence limits was calculated (Sokal & Rohlf, 1969).

Drugs and chemicals

Dopamine (3-hydroxytyramine HCl) and haloperidol (4-[4-(4-chlorophenyl) - 4 - hydroxypiperidino] - 4′ - fluorobutyro - phenone) were obtained from Sigma (Poole, Dorset). Chlorpromazine (2 - chloro - 10 - (3 - dimethylaminopropyl)phenothiazine HCl) was obtained from Aldrich Chemical Company (Gillingham, Dorset). (±)-Sulpiride (5-(aminosulphonyl)-n-[(1-ethyl - 2 - pyrrolidinyl)methyl] - 2 - methoxybenzamide) was obtained from Semat (St Albans, Herts). SCH23390 (8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol maleate) was a gift from Dr V.G. Daniels, Kirby Warrick Pharmaceuticals (Bury St Edmunds, Suffolk). Domperidone (5 - chloro - 1 - {1 - [3 - (2 - oxobenzimidazolin - 1 - yl)propyl] - 4 - piperidyl}]benzimidazolin-2-one) was a gift from Janssen Pharmaceutical (Marlow, Bucks). Metoclopramide(4-amino-5-chloro-n-(2-diethylaminoethyl)-2-methoxybenzamide HCl) was a gift from Beecham Pharmaceuticals (Harlow, Essex).

Dopamine was dissolved in ascorbic acid 1 mg ml^{-1}, with the final dilution being made in physiological saline. The maximum concentration of ascorbic acid in the ejection pipette was 0.1 mg ml^{-1}, and when tested alone this had no effect on the preparation.

Domperidone, (±)-sulpiride and haloperidol were dissolved in tartaric acid, 5 mg ml^{-1}, and dilutions made from this in physiological saline. The pH of the solution was adjusted to control values (pH 7.7–7.8) with 1 M NaOH; in this condition tartaric acid was found to have no effect on the preparation.

Results

Stimulation of the suboesophageal nerve evokes a stimulus-dependent hyperpolarization of the cockroach (*Nauphoeta cinerea* Olivier) salivary gland acinar cells *in vitro*, which is occasionally followed by a depolarization (House, 1973). When applied locally by pressure ejection, dopamine mimicked the effect of nerve stimulation in a concentration-related manner (Figure 1). It should be noted that throughout this study the external potassium concentration was 1 mM. Thus it was not unusual for the hyperpolarization induced by dopamine to exceed a potential of 100 mV (see House, 1973; Ginsborg *et al.*, 1974).

The effects of SCH23390, haloperidol, chlorpromazine and metoclopramide

At high concentrations some of the antagonists had non-specific effects. The inhibition of the hyperpolarization by chlorpromazine at concentrations >5 μM was insurmountable, while SCH23390 destabilized the acinar cells membrane at concentrations ≥100 μM. It was necessary therefore to study the effect of these compounds over a limited concentration range.

SCH23390 (10–50 μM), chlorpromazine (0.1–5 μM), haloperidol (10–100 μM) and metoclopramide (1 mM) blocked the hyperpolarization induced by both dopamine and nerve stimulation. The onset of inhibition of the response to dopamine was immediate, while in the case of nerve stimulation there was a delay of between 3–10 min. This is illustrated in Figure 2 which shows a typical record of the onset of blockade following addition of SCH23390 (50 μM) to the bath solution.

Nerve stimulation

Figure 3 illustrates the action of the antagonists on the response to nerve stimulation. Figure 3a (i) shows a control response; (ii) shows the response after addition of SCH23390 (20 μM) to the perfusate; (iii) shows the effect of increasing the number of impulses in the train on the response after the onset of blockade; and (iv) shows the degree of recovery on washing. Parts b, c and d of Figure 3 illustrate the corresponding results for chlorpromazine (5 μM), haloperidol (20 μM) and metoclopramide (1 mM). The inhibition of the

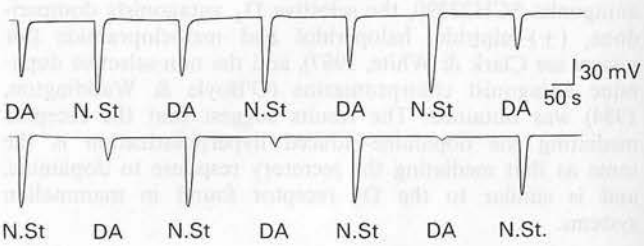

Figure 2 The time course for blockade of the hyperpolarization induced by locally applied dopamine (DA; 35 kPa; 10 ms) and nerve stimulation (NSt; 100 V; 20 Hz; 0.5 ms pulse width; 2 impulses in the train) respectively, following addition of SCH23390 (50 μM); (b) is continuous with (a). The initial resting potential was −42 mV.

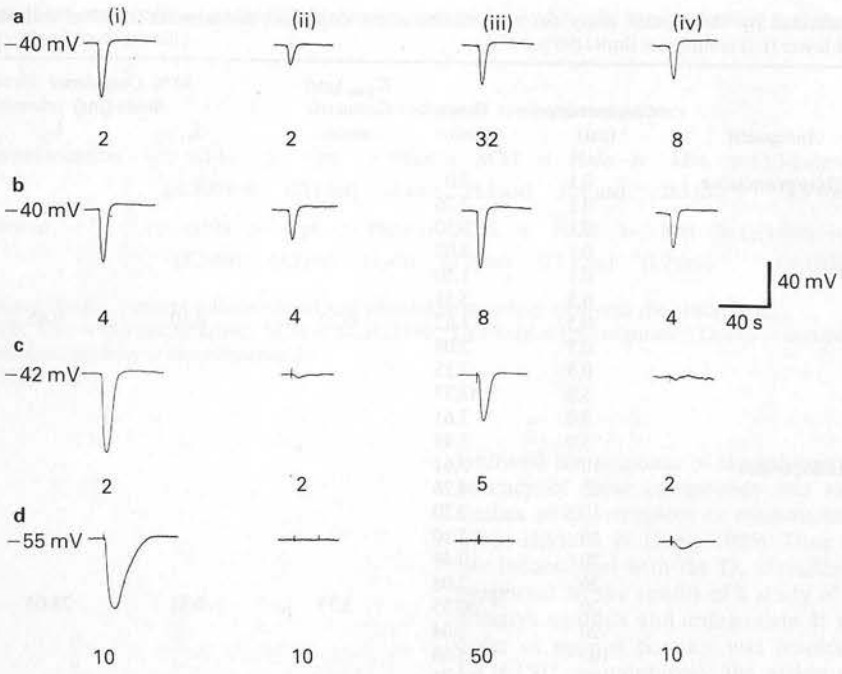

Figure 3 The blockade of the hyperpolarization induced by nerve stimulation following addition of (a) SCH23390 (20 μM), (b) chlorpromazine (5 μM), (c) haloperidol (20 μM) and (d) metoclopramide (1 μM). (i) Control response to nerve stimulation (100 V; 20 Hz; 0.5 ms pulse width; number of impulses in the train as indicated below each response), (ii) response following the addition of the antagonist, (iii) the effect of increasing the number of impulses in the train after 30 min incubation with the antagonist and (iv) recovery following a 30 min wash. The initial resting potential for each experiment was as shown.

response by SCH23390 (10–50 μM) and chlorpromazine (0.5–5 μM) was readily surmounted and was reversed on washing, although full recovery was not always obtained. In contrast, the blockade of the hyperpolarization by haloperidol (10–100 μM) and metoclopramide (1 mM) was insurmountable, and the inhibition by the former was not reversed on washing. The inhibition of the response to dopamine by these compounds was found to be surmountable and was reversed on washing (see below). Thus haloperidol and metoclopramide must have an additional presynaptic action.

Dopamine

As stated in the methods, a more quantitative analysis of the effects of the antagonists was undertaken with respect to the dopamine-induced hyperpolarization. Figure 4a illustrates the

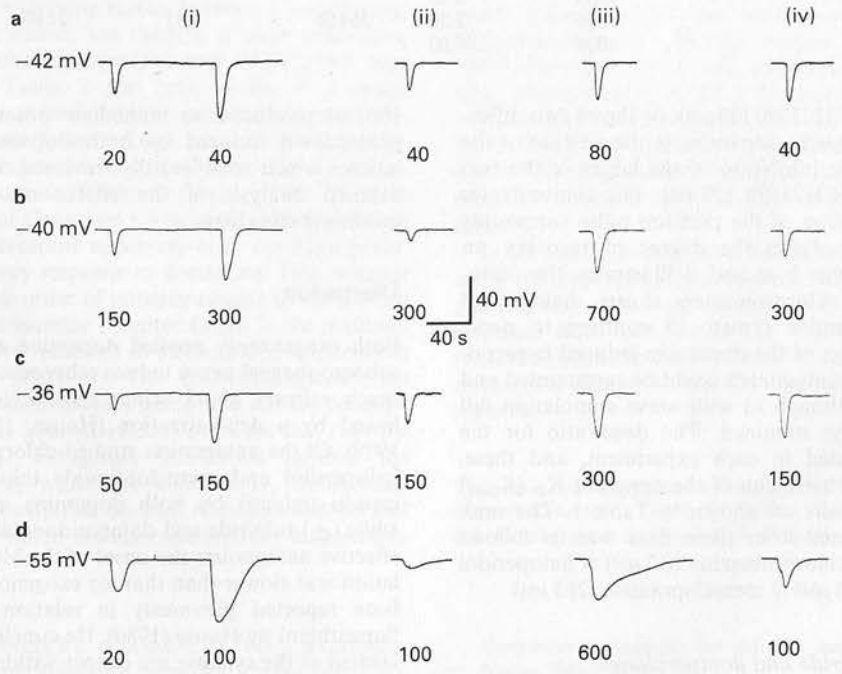

Figure 4 The blockade of the dopamine-induced hyperpolarization by (a) SCH23390 (20 μM), (b) chlorpromazine (5 μM), (c) haloperidol (20 μM) and (d) metoclopramide (1 mM). (i) Paired, submaximal control responses to dopamine (DA; 35 kPa; duration of pulse in ms as shown), (ii) the larger of the two control responses following addition of the antagonist, (iii) response to increased duration of dopamine application after a 30 min incubation, and (iv) the degree of recovery of the response following a 30 min wash. The initial resting potential for each experiment is shown.

Table 1 Dose-ratios estimated by 'three point assay' for each of the active dopamine antagonists together with the geometric mean $K_{D_{app}}$ and upper (L_2) and lower (L_1) confidence limits (95%)

Antagonist	*Concentration* (μM)	*Dose-ratio*	$K_{D_{app}}$ (μM) *Geometric mean*	*95% Confidence limits* (μM) L_1	L_2
Chlorpromazine	0.1	2.0			
	0.1	2.76			
	0.1	2.50			
	0.1	3.03			
	0.1	1.50			
	0.5	5.51			
	0.5	1.82	0.22	0.10	0.45
	0.5	2.08			
	0.5	2.15			
	5.0	43.37			
	5.0	7.61			
	5.0	3.49			
Haloperidol	10	5.61			
	10	4.76			
	10	2.20			
	10	2.10			
	20	10.48			
	20	2.08			
	20	50.35	3.77	0.51	28.05
	20	5.04			
	100	5.66			
	100	46.21			
	100	39.24			
	100	55.32			
SCH23390	10	1.45			
	10	3.48			
	10	1.70			
	10	4.80			
	10	1.45			
	20	6.19			
	20	14.27			
	20	2.35	4.06	0.49	33.67
	20	20.00			
	20	3.30			
	20	1.59			
	50	275.69			
	50	222.21			
	50	22.85			
	50	7.08			
Metaclopramide	1000	3.90			
	1000	3.50			
	1000	2.30	264.66	0.03	21.80
	1000	16.60			

'three point assay' for SCH23390 (20 μM): (i) shows two different sub-maximal responses to dopamine in the absence of the antagonist; (ii) shows the inhibition of the larger of the two control responses by SCH23390 (20 μM); (iii) demonstrates that increasing the duration of the pressure pulse surmounts the blockade; and (iv) shows the degree of recovery on washing. Figure 4 sections b, c and d illustrates the corresponding results for chlorpromazine (5 μM), haloperidol (20 μM) and metoclopramide (1 mM). In contrast to nerve stimulation, the inhibition of the dopamine-induced hyperpolarization by all of these antagonists could be surmounted and reversed on washing, although as with nerve stimulation full recovery was not always obtained. The dose-ratio for the antagonists was calculated in each experiment, and these, together with the geometric mean of the apparent K_D ($K_{D_{app}}$) with 95% confidence limits are shown in Table 1. The rank order of potency obtained from these data was as follows ($K_{D_{app}}$ in parentheses): chlorpromazine (0.2 μM) > haloperidol (3.3 μM) = SCH23390 (4.1 μM) > metoclopramide (265 μM).

The effect of (±)-sulpiride and domperidone

(±)-Sulpiride (1–100 μM) and domperidone (1–100 μM) failed to antagonize the hyperpolarization induced by either dopamine or nerve stimulation. On the contrary, higher concentrations of (±)-sulpiride (100 μM) and domperidone (50 and 100 μM) produced an immediate potentiation of the hyperpolarization induced by both dopamine and nerve stimulation, which was readily reversed on washing. A more detailed analysis of the effects of domperidone will be published elsewhere.

Discussion

Both exogenously applied dopamine and stimulation of the suboesophageal nerve induce a hyperpolarization of the cockroach salivary gland acinar cells, which is occasionally followed by a depolarization (House, 1973; Blackman *et al.*, 1979). Of the antagonists studied chlorpromazine, SCH23390, haloperidol and metoclopramide inhibited the hyperpolarization induced by both dopamine and nerve stimulation, while (±)-sulpiride and domperidone did not. For each of the effective antagonists the onset of the blockade of nerve stimulation was slower than that for exogenous dopamine. This has been reported previously in relation to the action of α-flupenthixol by House (1980). He concluded that the receptors located at the synapse are deeper within the acinus than those activated by the exogenously applied agonist. This may offer a further barrier to diffusion, resulting in the delayed onset of blockade.

The rank order of potency for the blockade of the hyperpolarizing response to locally applied dopamine was: chlor-

Table 2 Comparison of the rank order of potency of dopamine antagonists against the hyperpolarizing and the secretory response of the cockroach salivary gland to dopamine

Response to dopamine	*Antagonist rank order of potency*
Hyperpolarization	α-Flu ≧ Cpz > Phen > SCH = Halo ≫ Met ≫ (±)-Sulp = Domp
	(< 500 nM) (215 nM) (1 μM) (4.1 μM) (3.8 μM) (265 μM) (> 100 μM)
*Secretion	α-Flu > Cpz > Phen > SCH > Halo ≫ Met ≫ (±)-Sulp = Domp
	(3.3 nM) (0.2 μM) (1 μM) (2.2 μM) (17.5 μM) (1.2 mM) (> 100 μM)

Values in parentheses are the $K_{D_{app}}$ except α-flupenthixol and phentolamine which represent the absolute K_D.
α-Flu = α-flupenthixol; Cpz = chlorpromazine; SCH = SCH23390; (±)-Sulp = (±)-sulpiride; Domp = domperidone; Halo = haloperidol; Phen = phentolamine; Met = metoclopramide.
* Evans & Green, (1990).

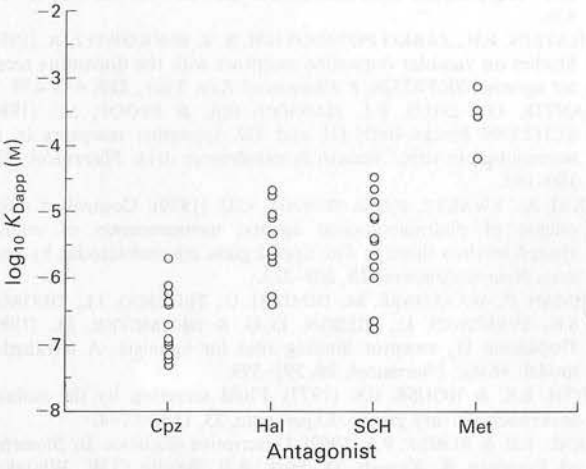

Figure 5 The range of $\log_{10} K_{D_{app}}$ for each antagonist studied. Cpz = chlorpromazine; Hal = haloperidol; SCH = SCH23390; Met = metoclopramide.

promazine > haloperidol = SCH23390 ≫ metoclopramide ≫ (inactive) (±)-sulpiride and domperidone. As illustrated in Figure 5, there is a considerable scatter between K_Ds obtained from individual experiments, yet there is a clear separation between chlorpromazine, haloperidol and SCH23390, and metoclopramide. In Table 2 the rank order of potency obtained in this study, extended by the addition of phentolamine and α-flupenthixol (Bowser-Riley *et al.*, 1978; House & Ginsborg, 1976), is compared with that obtained for the secretory response to dopamine (Evans & Green, 1990). Thus, in contrast to the view of Gray and co-workers (1984), it would appear that a single receptor subserves both the hyperpolarization and the secretory response to dopamine. This receptor has an antagonist rank order of potency similar to the D_1 but distinct from the D_2 dopamine receptor found in the mammalian CNS and periphery (Hilditch & Drew, 1985; Anderson *et al.*, 1985; Seeman *et al.*, 1987). The similarity between the receptor on the cockroach salivary gland and the D_1 receptor in mammalian systems is most evident from the fact that the selective D_1 receptor antagonist SCH23390 inhibits the response to dopamine, while the selective D_2 antagonists domperidone and (±)-sulpiride do not. Although haloperidol and metoclopramide, which are also selective D_2 antagonists, inhibited the response of the salivary gland to dopamine, the potency of these compounds was similar to that found in studies of D_1 receptors in mammalian systems (Flaim *et al.*, 1985; Hilditch & Drew, 1985). Thus this inhibitory action is not inconsistent with the D_1 classification. This conclusion is supported by the results of a study of the interaction between selective agonists and antagonists. It was found that the rank order of agonist potency was fenoldopam > SKF38393 > LY163502 > quinpirole; the action of all four agonists was blocked by SCH23390, but not by (±)-sulpiride (Evans & Green, unpublished observations).

It must be noted that both in the present investigation and with respect to the dopamine-induced secretion of the salivary gland (Evans & Green, 1990), the $K_{D_{app}}$ for SCH23390 was between 2 and 5 orders of magnitude higher than that found in most functional studies of D_1 receptors in mammalian preparations (Hilditch & Drew, 1985; Anderson *et al.*, 1985). The pharmacology of all D_1 receptors is not however identical (see Plantje *et al.*, 1984; Niznik *et al.*, 1988; Ohlstein *et al.*, 1984; Anderson *et al.*, 1985; Fleminger *et al.*, 1983; Hilditch & Drew, 1985; Goldberg *et al.*, 1983) and the receptor on the cockroach salivary gland may thus represent a separate subtype.

As with D_1 receptors in other preparations, both invertebrate and mammalian (Lafon-Cazal Bockaert, 1984; Kebabian & Calne, 1979), the dopamine receptor in the cockroach salivary gland has been shown to be linked to a dopamine-sensitive adenylyl cyclase (Grewe & Kebabian, 1982). However, Gray and co-workers (1984) demonstrated that although cyclic AMP is an intermediate in the secretory response, it is not involved in the electrical response to dopamine. Thus there must be two distinct second messengers linked to this dopamine receptor, cyclic AMP mediating the secretory response and the other subserving the hyperpolarization.

In summary, it appears that the dopamine receptor mediating the hyperpolarizing response of the cockroach salivary gland acinar cells is the same as that mediating the secretory response. Both here and in the investigation of the secretory response (Evans & Green, 1990) we have established that this receptor is similar to the D_1 receptor found in mammalian systems.

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