THE EFFECTS OF DOPAMINE AND DOPAMINERGIC AGENTS ON AN IDENTIFIED COCKROACH MOTONEURONE

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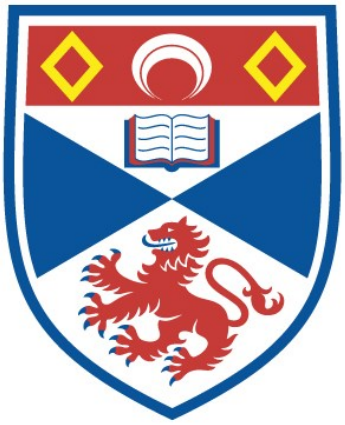

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BY

JULIAN P L DAVIS

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ABSTRACT

Dopamine is one of a number of neurotransmitter candidates found in the insect CNS. It has been localised in a number of neurones, and others have been shown to respond to the application of dopamine. This study investigates the response of the common inhibitory motoneurone D_3 to dopamine, and its ionic basis. The response to dopamine is distinguished pharmacologically from responses to noradrenaline, octopamine and acetylcholine, all neurotransmitter candidates within the insect CNS, and it is concluded that a receptor specific for dopamine is present on this cell. Finally, this putative dopamine receptor is characterised pharmacologically, and its position relative to the extant classification schemes for mammalian receptors, and its similarity or otherwise to other invertebrate dopamine receptors is discussed.

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SECTION 3 – EXPERIMENTAL RESULTS

SECTION 1 - GENERAL INTRODUCTION

1.1 INSECTS - ECONOMIC IMPORTANCE & USE AS A MODEL FOR STUDY

Insects are ecologically one of the most successful groups of animals on earth, accounting for over 75% of all known species. They are supremely adaptable, and have colonised practically every available niche, except those in marine environments. Here they faced competition from the phylogenetically related crustacea, which replace them in these habitats.

As a consequence of their extreme abundance and opportunist ecology, insects have become economically important. They carry diseases which affect both man and man's domesticated animals. Malaria, caused by the parasitic micro-organism *Plasmodium*, and spread by the bite of female mosquitoes of the genus *Anopheles*, is a major cause of death in the tropical third world. Trypanosomiasis, spread by flies of the genus *Glossina*, not only affects humans causing extreme lassitude, and eventually coma and death, but also produces high mortality rates among cattle and other domestic animals in affected areas.

Historically little could be done to prevent the ravages of insects. More recent advances in technology provided chemical agents which killed one or more stages of the life-cycle of many insects. Some of

those early insecticides, however, proved dangerous to man and the environment.

DDT (p-dichlorodiphenyltrichlorethane) was used effectively as an insecticide during the 1950s and '60s. More recently it has been found to have undesirable side-effects. DDT is fat soluble, and chemically persistent, allowing it to be stored in fatty tissues once ingested. When animals are eaten by those further up food chains, and in many cases ultimately by man, these high concentrations of DDT can produce liver damage, CNS depression, weakness and eventually coma and death.

The need now is for the development of pesticides which are specifically targeted at particular species. This requires a detailed knowledge of the ways in which such agents can effectively be used to exploit differences in the neurochemistry and neuropyhsiology of pest species.

The insect CNS has been the subject of much intensive study, but even now the neuropharmacology is poorly understood in most species. Insects represent easily maintained subjects for research, and allow investigations into neuropharmacology and neurophysiology to be performed on a nervous system which is much simpler than that of a mammal. It still, however, retains many of the characteristics of more complex systems in terms of neuronal interactions.

1.2 TRANSMITTERS

An important goal in the study of the insect CNS is the understanding of synaptic transmission within it. Chemical synapses permit much greater modification of information than electrical synapses, and it is these which have received the majority of attention in recent years.

The agents responsible for chemical neurotransmission have been more closely studied in vertebrates than in insects. The majority of classical neurotransmitters found in vertebrates are also found in insects (see Pitman, 1985). Determination of the identity and role of neuropeptides within the insect CNS, however, is relatively limited.

Before an agent can be accepted as the neurotransmitter at a particular synapse, it must satisfy basic criteria. The substance must be present and detectable in the cells from which it is released, and enzymes and precursors for its synthesis must also be present. Calcium dependent release of the substance to the extracellular space must be demonstrable, and mechanisms for the inactivation or removal of the candidate must exist in the immediate vicinity of the release site. Application of the prospective transmitter by artificial means on to the post-synaptic area should mimic the action of the transmitter released as a result of pre-synaptic stimulation. In addition, agents which mimic or block the actions of the endogenous transmitter must also mimic or interfere with the action of the putative transmitter substance when it is

added. Finally, biochemical demonstration of specific receptors for the transmitter substance in the post-synaptic membrane is necessary.

Release of transmitter is classically thought to be calcium dependent. Dudel et al (1983), however, working on the crayfish, reported evidence which they took to indicate that release could be produced by the depolarisation of the presynaptic terminal alone, without the necessity for the influx of calcium. However, this theory remains controversial, and has been refuted by Zucker & Lando (1986) who believe that release under these circumstances is probably caused by calcium influx in a portion of the terminal area not voltage-clamped by the experimental pipette. Release may also be regulated by pre-synaptic factors, such as receptors for the released tranmsitter which may be present on the presynaptic terminal and act as a feedback mechanism to restrict the further release of transmitter (Langer, 1974; Raiteri et al, 1978; Langer, 1981).

Acetylcholine, known to be the excitatory transmitter at the vertebrate skeletal neuromuscular junction, has also been shown to exist in the insect CNS (see Klemm, 1976; Pitman, 1985; Sattelle, 1985). It has been shown to mimic epsp's in the cockroach 6th abdominal ganglion, and both the epsp and the response to iontophoretically applied acetylcholine were enhanced by eserine (Pitman & Kerkut, 1970). In addition, acetylcholine is suspected to act as the neurotransmitter at the synapse between the trochanteral hairplate and the motoneurone D_{∞} (Carr & Fourtner, 1980).

Acetylcholine receptors are divided broadly into two types, nicotinic and muscarinic, according to the preferential binding of nicotine or muscarine. There is evidence for the existence of both types in the insect nervous system. However, the nicotinic receptor found in the insect CNS appears to differ pharmacologically from vertebrate models (Benson, 1988) and presynaptic muscarinic receptors may be involved in autoregulation of transmitter release (Breer & Sattelle, 1987).

Of the amino-acid transmitters, that which has received most attention is γ-aminobutyric acid (GABA). It is an important inhibitory transmitter in the vertebrate CNS (Duggan, 1985), and has been shown to have similar properties in both the central and peripheral nervous systems in insects (Kerkut, Pitman & Walker, 1969a; 1969b; Pitman & Kerkut, 1970; Pitman, 1985; Benson, 1988; Moss & Miller, 1988). However, there appear to be important differences between the pharmacology of the vertebrate receptors and those found in insects (Sattelle et al, 1986; Benson, 1988).

Glutamate has been identified as an excitatory transmitter at the insect neuromuscular junction (Usherwood et al, 1968; Daoud & Usherwood, 1978; Usherwood, 1978), a role which it also appears to fulfil in mammals (Mayer, Westbrook & Guthrie, 1984).

Of more direct relevance to the present study are the biogenic amines. These are the catecholamines dopamine, noradrenaline, and adrenaline, the indolalkylamine 5-hydroxytryptamine, and the monophenolamine octopamine. Histamine is also a neurotransmitter candidate in insects.

Biochemical assays have allowed assessment and quantification of these amines present within a variety of insect preparations besides the cockroach. Dopamine has been shown to be present in the nervous system of the caddis fly *Anabolia nervosa* (Klemm & Björklund, 1971), in the locust, *Schistocerca gregaria*, (Klemm & Axelsson, 1973; Robertson, 1986), in the locust *Locusta migratoria*, (Hiripi & S-Rosza, 1973), and in the hemipteran, *Rhodnius prolixus* (Flanagan, 1986). Noradrenaline has been found in the nervous system of *Schistocerca* (Klemm & Axelsson, 1971; Robertson, 1986), in *Locusta migratoria* (Hiripi & S-Rosza, 1973), and in *Rhodnius* (Flanagan, 1986). Octopamine has been found in locusts (Hoyle, 1975; Hoyle & Barker, 1975; Robertson, 1986; Konings *et al*, 1989, also see Axelrod & Saavedra, 1977; Orchard, 1982 for reviews). Serotonin (5-HT) has been shown to exist in *Schistocerca* (Klemm & Axelsson, 1973) and *Rhodnius* (Flanagan, 1986; Orchard, 1989). Histamine has also been found in insect nervous systems, especially in the optic lobes of the brain (Elias & Evans, 1983). Many studies have attempted to find adrenaline, but so far, it has been undetectable in most (see Pitman, 1985).

In the cockroach *Periplaneta americana*, dopamine has been found in many parts of the CNS. Frontali & Haggendal (1969) showed both dopamine and noradrenaline to be present in the brain, and Dymond & Evans (1979) detected dopamine throughout the nervous system. They also found noradrenaline, in quantities approximately 10 times less than those of dopamine, (0.29 pmole/ganglion noradrenaline to 2.61 pmole/ganglion dopamine), while adrenaline was again undetectable.

Evans (1978a; 1980) detected octopamine in the cockroach CNS in quantities of 5.28 pmoles/ganglion and Martin et al (1984) used a high performance liquid chromatography (HPLC) technique to assay all the amines present, including octopamine. They found that the levels of amines in the thoracic ganglia were 4.79 pmole octopamine to 2.44 pmole dopamine, noradrenaline not being assayed in these ganglia.

Dopamine, noradrenaline, adrenaline, and 5-HT can be localised at a cellular level by the aldehyde fluorescence technique of Falck & Hillarp (Falck, 1962). This technique has been improved by the substitution of glyoxylic acid for formaldehyde, which results in brighter and more easily detected fluorescence (Lindvall et al, 1974), and has been further refined for use on unsectioned insect material (Fleming & Pitman, 1982; Baker & Pitman, 1989).

Fluorescence histochemical techniques have been used in vertebrate preparations, and was used to localise dopamine in neurones of the gastropod *Planorbis corneus* by Powell & Cotrell[c] (1974). Frontali (1968) found neurones within the cockroach brain which fluoresced with the characteristics of amines, and Fleming & Pitman (1982) and Baker & Pitman (1989) produced definite evidence of the presence of dopamine or noradrenaline, but not of 5-HT, in the thoracic and abdominal ganglia.

Octopamine can be localised by extraction and high-voltage electrophoresis (Hoyle & Barker, 1975; Evans, 1980). There is evidence that in the locust, the dorsal unpaired median neurones contain and release octopamine on stimulation (Hoyle, 1975; Evans,

1980). The quantity of octopamine present in the cockroach CNS is slightly larger than the amount of dopamine (Evans, 1980).

In the mammalian CNS, dopamine and noradrenaline are synthesised in a common pathway. The amino acid tyrosine is first converted to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase. L-DOPA is then decarboxylated by DOPA-decarboxylase to give dopamine. In noradrenergic cells, dopamine is then further converted by dopamine-ß-hydroxylase to noradrenaline (Blaschko, 1973).

The breakdown of these amines in vertebrates is predominantly by means of monoamine oxidase, which converts amines to aldehydes, and catechol-O-methyl transferase, which deactivates the molecule by methylation.

In insects, Maxwell et al (1978) showed that while dopamine appears to be synthesised from tyrosine in the moth *Manduca sexta*, noradrenaline is not produced from this precursor. It appears likely therefore that there are significant differences between the biosynthetic processes for biogenic amines in insects and mammals.

The enzymes involved in deactivation of amines in vertebrates do not appear to play any major role in insects (Evans, 1980). Sekeris & Karlson (1966) showed that certain N-acetylated amine derivatives are involved in cuticular tanning following ecdysis, and more recent evidence suggests that N-acetylation is one possible way in which amines may be recycled (Vaughan & Neuhoff, 1976). Uptake of amines by a variety of tissues has been demonstrated. Evans (1978b) showed

that cockroach nervous tissue had a sodium-dependent uptake system for the uptake of octopamine, and Orchard (1989) has shown that *Rhodnius* neurohaemal tissue possesses a high-affinity uptake mechanism for serotonin. In the cockroach, an uptake system for dopamine has been characterised in vesicles prepared from brain extracts (Gifford, 1989).

There is, therefore, evidence that biogenic amines are present in the insect CNS, and that the criteria for their consideration as neurotransmitters are satisfied at least in part.

Dopamine and noradrenaline are known to be involved in synaptic transmission in the mammalian CNS, where they exert predominantly inhibitory influences on their target neurones, although biogenic amines, especially dopamine, have been shown to increase excitability in rat spinal neurones (Barasi & Roberts, 1977). Lesions within the dopaminergic tracts of the basal ganglia are thought to be associated with Parkinsonism. This condition can in some cases be alleviated by injection of either dopamine itself or its metabolic precursors. In addition, 5-HT has been implicated in the supraspinal control of spinal pain processing (Duggan, 1985).

In molluscs, biogenic amines have been shown to exert both excitatory and inhibitory effects on neurones. 5-HT elicits an excitatory response from cells in the abdominal ganglion of *Aplysia*, which shows an unusual voltage-dependency, increasing in size at depolarised potentials (Pellmar & Wilson, 1977). The basis of this response was

shown to be a 5-HT induced calcium current (Pellmar & Carpenter, 1979; 1980).

Klein & Kandel (1978; 1980) found that 5-HT simulated presynaptic facilitation, and suggested that this process may form the basis for the simple learning process involved in the gill withdrawal reflex of *Aplysia*.

Recently, it has been demonstrated that 5-HT, through actions which modulate intracellular levels of cyclic AMP, suppresses a steady-state calcium-activated potassium current in tail sensory neurones of *Aplysia* (Walsh & Byrne, 1989; Baxter & Byrne, 1989).

Dopamine has both excitatory and inhibitory effects on molluscan neurones. Berry & Cottrell (1975) showed that stimulation of a giant dopaminergic neurone in *Planorbis corneus* produced excitatory, inhibitory or biphasic responses in a number of follower neurones.

Osborne (1977) found that cyclic AMP levels were raised in neurones of *Helix pomatia* following application of dopamine. Similarly, Juel (1981) showed that presynaptic function in *Helix pomatia* was altered by changes in the intracellular level of cyclic AMP, brought about by stimulation of dopamine receptors on the presynaptic cell. He further established that these effects were mediated by autoreceptors for dopamine (Juel, 1982). These effects were susceptible to a range of dopaminergic agonists and antagonists (Juel, 1983).

Stoof, de Vlieger & Lodder (1985) went further and attributed the opposing excitatory and inhibitory responses to dopamine in growth hormone producing cells (GHC's) of *Lymnaea stagnalis* to distinct dopamine receptor types. These receptors corresponded to mammalian classification.

However, more recent work has suggested that at least a proportion of the receptors present on molluscan neurones do not conform. Bokisch & Walker (1988) have shown that the dopamine induced hyperpolarisation of *Helix* central neurones was mediated via receptors which could not be clearly classified as belonging to either of the two accepted vertebrate classes.

In *Lymnaea*, Audesirk (1989) investigated the effects of a range of dopaminergic agonists and antagonists, and concluded that the receptors mediating the hyperpolarising response of buccal and pedal ganglion neurones to dopamine could not be readily reconciled with either vertebrate class of dopamine receptor.

In insects, biogenic amines have been shown to have a wide variety of roles. Outside the nervous system, the luminescence produced by the light organ of the larval firefly (*Photinus*) has been found to be induced by intra-ganglionic injection of a variety of amines, including adrenaline, noradrenaline, synephrine and dopamine (Carlson, 1968). More recently, it has been shown that octopamine is the transmitter in this system, since octopaminergic antagonists can block the light response, and an octopamine-sensitive adenylate cyclase has been isolated from the light organ (Orchard, 1982).

In the cockroach *Periplaneta americana*, Schofield & Treherne (1986) showed that the potassium sensitivity of the basolateral membrane of the glial cells which make up the blood-brain barrier was reduced by octopamine. In contrast, other neurohumoral substances, including dopamine and 5-HT were found to enhance this sensitivity. Orchard et al (1988) have found evidence that there is a serotonergic nerve supply to the epidermis of *Rhodnius prolixus*, and they conclude that 5-HT plays a role in the integration of feeding behaviour by acting as a plasticising factor, allowing distension of the gut wall during feeding. Octopamine has been implicated in the mobilisation of lipids and sugars following stress induced by handling or exercise in the cricket *Acheta domesticus* (Woodring et al, 1989).

Evidence for the aminergic modulation of the neuromuscular junction was first obtained by Evans & O'Shea (1977), who found that an octopaminergic dorsal unpaired median (DUM) neurone innervated the extensor-tibiae muscle in the locust *Schistocerca gregaria*. This neurone has been termed DUMETi. By stimulation of this neurone, they produced a potentiation of the epsp and the twitch tension resulting from the stimulation of an identified motoneurone SETi. Since local application of octopamine to the area of the neuromuscular junction reproduced this effect, they concluded that the modulation of the synaptic efficacy in this system was the result of the release of octopamine from the modulatory neurone.

Evans (1981; 1982) has since characterised the receptors mediating this effect, and the modulation of a myogenic rhythm in specialised muscle fibres, and has classified these receptors as specific for

octopamine. More recently (Evans, 1984a; 1984b), it has also been shown that, in this same preparation, octopamine released at the neuromuscular synapse causes an elevation of cyclic-AMP levels within the muscle cells, indicating how octopamine may achieve its potentiating effect. Orchard & Lange (1985) have also provided evidence that octopamine is responsible for the modulation of the activity of the muscles of the oviduct in *Locusta*.

In other preparations, octopamine has also been shown to enhance neuromuscular transmission. Klaasen & Kammer (1985) and Klaasen, Kammer & Fitch (1986) found that the magnitude of the ejp evoked in the dorsal longitudinal muscle of the moth *Manduca sexta* by stimulation of the motor nerve, was enhanced by low concentrations of octopamine (10^{-10} to 10^{-6} M). Other biogenic amines, including dopamine, were found to be ineffective in this system.

In the cockroach *Periplaneta americana*, octopamine has been found to suppress proctolin or glutamate induced contractions of the hyperneural muscle (Moss & Miller, 1988), but the authors do not make any comment on the possible physiological role of the agents tested.

In the peripheral nervous system, biogenic amines, most notably dopamine, have been shown to cause a hyperpolarisation of the acinar cells of the salivary glands in the cockroach *Nauphoeta cinerea* (House, 1973). The magnitude of the hyperpolarising effect of dopamine on this system is concentration-dependent, and dopamine is the most potent of the biogenic amines tested. Consequently, Bowser-Riley & House (1976) have suggested that dopamine is the transmitter

at this neuroglandular synapse. In 1978, Bowser-Riley, House & Smith further characterised the receptors mediating this response, and concluded that the adrenergic antagonist phentolamine discriminated between two receptors in the preparation. One of these receptors binds 5-HT and the other binds catecholamines. The neurotransmitter, suspected to be dopamine, is bound by this catecholamine receptor. Since then, further work has shown that dopamine and its rigid analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) are the most potent stimulators of the acinar cell response (House & Ginsborg, 1982).

In the locust *Schistocerca gregaria*, the innervation of the salivary gland is similar to that of the cockroach (Baines, Tyrer & Mason, 1989). Secretion can be elicited by stimulation of the salivary nerve, and this effect can be mimicked by the application of dopamine and 5-HT (Baines & Tyrer, 1989). They have not, however, been able to obtain evidence as to which of the amines is the transmitter in this system.

The central effects of dopamine and other catecholamine transmitters were first noted when Twarog & Roeder (1957) noted that adrenaline and noradrenaline both caused the production of bursts of activity which could be recorded from nerves of the cockroach *Periplaneta americana*. In addition, they reported that adrenaline appeared to enhance transmission at the cercal nerve/giant interneurone synapse.

Gahery & Boistel (1965) found that amines, including dopamine, produced bursts when applied generally to the sixth abdominal

ganglion of the cockroach, but could not find any evidence of modulation of synaptic transmission. Kerkut et al (1969b) showed that the dorsal unpaired median (DUM) neurones of the cockroach sixth abdominal ganglion were excited by application of adrenaline, noradrenaline and dopamine. In contrast, when applied by iontophoresis to neurones within the ant brain, Steiner & Pieri (1969) found dopamine to inhibit the spontaneous activity of these neurones.

Sombati & Hoyle (1984) demonstrated that iontophoretic application of octopamine into the neuropile of the metathoracic ganglion of the locust could initiate a repeated flexion-extension-flexion rhythm in the musculature of the tibia. In addition, application of octopamine to the 6th abdominal ganglion suppressed oviposition digging behaviour. They concluded that octopamine might be acting as a neuromodulator in this system.

Further central effects of amine transmitters were demonstrated by Claasen & Kammer (1986), who found that pressure injection of dopamine, octopamine and 5-HT into thoracic ganglia of the moth *Manduca sexta* altered production of a flight motor pattern producing rhythmic wing flapping. They suggested that dopamine, octopamine and 5-HT may be involved in initiating, maintaining and terminating flight behaviour in the intact animal.

Pitman & Baker (1989) have found that the common inhibitory motoneurone D_3 (Iles, 1976) is depolarised by application of dopamine, noradrenaline and octopamine, octopamine being the least

potent of these agents. They also found that responses to dopamine could be blocked by either α- or β-adrenergic antagonists, but that these agents (phentolamine & propranolol respectively) also suppressed responses to acetylcholine, suggesting non-specificity of action.

In the visual lamina of the eye of the fly *Musca*, Hardie (1987) has suggested that histamine may act as the neurotransmitter. Other transmitter substances, such as acetylcholine, GABA and glutamate, while producing responses, did not mimic the effect of light, while histamine application evoked a hyperpolarising response very similar in all respects to the light response.

1.3 DOPAMINE RECEPTORS AND THEIR CLASSIFICATION

Biogenic amines, and in particular dopamine, have been shown to be present and active in the insect CNS. It is likely therefore that receptors specific for amines may exist on insect central neurones.

Many amine transmitters are thought to have their effects by the alteration of the intracellular levels of cyclic AMP. These levels are altered by the activation of an adenylate cyclase enzyme. Different adenylate cyclases which respond specifically to a particular amine such as octopamine or 5-HT (Nathanson & Greengard, 1973; 1974), as well as dopamine have been isolated.

Receptors for dopamine have been identified in many mammalian preparations (Kebabian et al, 1972; Clement-Cormier et al, 1974; Karobath & Leitich, 1974; Miller et al, 1974; Iversen, 1975; Seeman et al, 1975; Kebabian & Saavedra, 1976; Caron et al, 1978; Gorissen & Laduron, 1979; List et al, 1980; Goldberg & Kohli, 1983; Leff & Creese, 1983; Hahn & MacDonald, 1984). These receptors have been identified in two distinct anatomical areas. The peripheral vascular system has dopamine receptors which mediate effects linked to the control of vascular dilation, and the central nervous system where receptors mediate effects concerned with communication between neurones.

A lot of studies have shown that dopamine receptors are present in invertebrate nervous systems. This has broadly been found by two

methods - biochemical (and binding) assays showing adenylate cyclase presence and activity, and physiological-pharmacological studies.

Bodnaryk (1979), working on the moth *Mamestra configurata* produced evidence for a dopamine-sensitive adenylate cyclase using *in vitro* biochemical assays of the cAMP content of neurones. Osborne (1977) found that excitatory dopamine receptors in neurones of the snail *Helix pomatia* mediated increases in intracellular levels of cAMP by linkage to an adenylate cyclase. Schmidt et al (1981) found biochemical evidence for the presence of dopamine-sensitive adenylate cyclase and also physiological evidence of the presence of a D_1 dopamine receptor in the salivary glands of the Lone Star tick, *Amblyomma americanum*.

Sakharov & Salànki (1982) showed that dopamine, acting on D_2 receptors, was responsible for the inhibition of locomotory rhythms in the snail *Lymnaea stagnalis*. They also showed that D_2 antagonists interfered with normal locomotion, by allowing indiscriminate activation of muscles, out of the normal strict sequence.

Stoof et al (1985; 1986) and de Vlieger et al (1986) also used *Lymnaea* in studies on the roles of dopamine and dopamine receptors in growth hormone producing cells (GHCs). In 1985, Stoof et al showed that the hyperpolarising effect of dopamine on these cells was mimicked by the D_2 agonist LY 141865, and suppressed by the D_2 antagonist YM 09151-2. Conversely, they showed that the D_1 agonist SK&F 38393 depolarised the GHCs. This effect was mimicked by intracellular injection of cAMP, and suppressed by the D_1 antagonist

SCH 23390. They concluded that both D_1 and D_2 receptors regulated different aspects of the activity of GHCs.

In 1986, de Vlieger et al provided further evidence that the hyperpolarising response to dopamine of the GHCs was potassium-dependent, and that the response was the result of the modulatory action of dopamine on a potassium conductance.

The pharmacology of the dopamine receptors involved in the responses of buccal neurones and the pedal giant neurone of *Lymnaea* have been further investigated by Audesirk (1989). Both types of neurone showed a hyperpolarising response to the direct application of dopamine to the cell surface, associated with an increase in membrane conductance. The response of the pedal giant neurone was unaffected by the D_1 antagonist SCH23390 (up to 5×10^{-5} M), and was not significantly suppressed by the D_2 antagonist metoclopramide (5×10^{-5} M). In addition, both the D_1 agonist SK&F 38393 (5×10^{-5} M) and the D_2 agonist LY 171555 (5×10^{-5} M) were ineffective in mimicking the dopamine response. Dibutyryl cyclic AMP produced no response, suggesting that cyclic AMP is unlikely to act as a second messenger in this system. The same agents had similar effects on the buccal (B-2) neurones, with the exception that SK&F 38393 caused a hyperpolarisation, mimicking the effects of dopamine. From these results, Audesirk concluded that the pharmacological profile of the receptors mediating these responses was different from that of mammalian receptors, and also from that of the receptors on the GHC's investigated by Stoof et al (1985).

The first classifications of these two types of receptors were performed on vertebrate central receptors by Kebabian & Calne (1979) and on peripheral receptors by Goldberg & Kohli (1983).

Although the seminal paper of Goldberg & Kohli was not published until 1983, and should therefore be discussed after that of Kebabian & Calne, the investigations leading to the conclusions expressed in these papers were undertaken at roughly the same time. Since this study is primarily concerned with central receptors, a brief summary of Goldberg & Kohli's work will be presented first and the conclusions and implications of Kebabian & Calne's studies will then be discussed in greater detail.

The initial experiments on the classification of vascular receptors were performed in the 1960s and '70s (Goldberg, Sonneville & McNay, 1968; Goldberg, Volkman & Kohli, 1978). Goldberg & Kohli (1983) identified two subtypes of peripheral dopamine receptors (DA_1 and DA_2). Stimulation of the DA_1 receptor subserves the relaxation of vascular smooth muscle, while stimulation of the DA_2 receptor subserves the inhibition of noradrenaline release from postganglionic sympathetic nerves. The pharmacological profile of the two types was different. One of the most important distinctions was that apomorphine acted only as a partial agonist of the dopamine response (in the case of the DA_1 receptor), while in the case of the DA_2 receptor, it was a full potent agonist. The results of this study are summarised in Table 1

TABLE 1

CLASSIFICATION OF PERIPHERAL DOPAMINE RECEPTORS

CRITERION	DA_1	DA_2
Functional model	Vascular relaxation	Neuronal inhibition
AGONISTS		
Prototype agonist	Dopamine	Apomorphine
Ergot derivatives	Inactive	Active
Apomorphine	Weak, partial agonist	Full, potent agonist
ANTAGONISTS		
Sulpiride	(R) 2-4 times more potent than (S)	(S) 100 times more potent than (R)
Domperidone	Inactive	Potent antagonist

(Adapted from Goldberg & Kohli, 1983)

Since the present study is concerned with the identification of putative dopamine receptors in the cockroach CNS, the subdivision of central dopamine receptors, by Kebabian & Calne (1979) is perhaps most relevant. Their classification rests mainly on the presence and quality of a link between the receptor and adenylate cyclase. Their D_1 subclass shows a stimulatory link, the binding of dopamine leading to an increase in adenylate cyclase activity and hence to an elevation of the intracellular level of cAMP. D_2 receptors, however, show either no link, or an inhibitory one, resulting in either no effect on, or a lowering of, the level of cAMP.

Kebabian et al (1972) isolated a dopamine-sensitive adenylate cyclase from homogenates of the caudate nucleus of rat brain. This was also stimulated by similar concentrations of apomorphine, and was blocked by low concentrations of haloperidol, a known antagonist of dopamine-mediated events *in vivo* in mammalian brain.

The prototype receptors for Kebabian & Calne's D_1 and D_2 receptors were found in the bovine parathyroid and anterior pituitary respectively. Stimulation of those in the parathyroid results in the release of parathyroid hormone, while stimulation of those in the anterior pituitary results in the inhibition of the release of prolactin.

They showed that secretion of prolactin was under dopaminergic control. Kebabian et al (1972) extracted a dopamine-sensitive adenylate cyclase from the rat caudate nucleus, and provided evidence that this cyclase had many characteristics similar to the putative

dopamine receptor. A dopamine-sensitive adenylate cyclase has also been extracted from the rat caudate nucleus by Clement-Cormier et al (1974), and from the rat striatum by Miller et al (1974; 1975) and Seeman et al (1975).

The classification was strengthened by a pharmacological profile showing clear distinctions between the two sub-classes. Apomorphine was shown to be a partial agonist or an antagonist to the D_1 receptor, and a full, potent agonist to the D_2. In addition, the antagonists metoclopramide and sulpiride were found to specifically suppress D_2-mediated events, having no effect on those mediated by D_1 receptors. The results of this study are sumarised in Table 2.

TABLE 2

CLASSIFICATION OF CENTRAL DOPAMINE RECEPTORS

CRITERION	D_1	D_2
Cyclase linkage	Yes	No
Prototype receptor	Bovine parathyroid gland	Mammotroph of anterior pituitary
Dopamine	Agonist (μM)	Agonist (nM)
Apomorphine	Partial agonist or antagonist	Agonist (nM)
Ergots	Potent antagonist (nM) or weak agonist (μM)	Agonist (nM)
Selective antagonist	None known	Metoclopramide Sulpiride

(Adapted from Kebabian & Calne, 1979)

At the time of their study (1979), no specific D_1 antagonist existed. Since that time, specific agents have been developed, notably SCH 23390. This benzazepine derivative was shown by Iorio et al (1983) to block D_1-mediated effects in rats, and to suppress dopamine-sensitive adenylate cyclase activity *in vitro* at concentrations some 2000 times lower than those required to block the binding of spiroperidol (an indicator of D_2 specific activity). This has now become an accepted addition to the classification of Kebabian & Calne.

Radio-labelling techniques, using labelled ligands for both D_1 and D_2 receptors, have been used to provide supportive evidence for this classification (Iversen, 1975; Seeman et al, 1975).

Since then, other workers have proposed a third sub-class of dopamine receptor (List et al, 1980). This has been identified by binding studies, and is a receptor showing high affinity for dopamine. It has been proposed that this should be called the D_3 receptor, though little further work has been done on it.

The linkage between a receptor and the ionophore which it controls can be either direct, such as in the electroplax acetylcholine receptor (Stevens, 1985) or indirect, in which case it may involve a secondary substance. This may form a link between the receptor and the ionophore, and is usually termed a second messenger. A common example is cyclic adenosine-3',5'-monophosphate (cyclic AMP). This has been shown to be instrumental in the mediation of several post-synaptic responses (Nathanson, 1979). Although the precise mechanism

of the involvement of cyclic AMP is variable, it is possible to set out a general scheme.

A transmitter molecule binding to a receptor on the outside of the cell membrane causes conformational changes in the receptor protein. This in turn is now thought to activate a G protein, situated within the membrane itself, which obtains energy for conformational change from the conversion of guanine tri-phosphate (GTP) to guanine di-phosphate (GDP) and inorganic phosphate (Casey et al, 1988). G proteins may regulate receptor-coupled ion channels directly, as is the case with adenosine receptors (Sasaki & Sato, 1987), or they may then activate an adenylate cyclase, an enzyme which produces cyclic AMP and P_i from adenosine triphosphate (ATP).

It has been suggested that this adenylate cyclase is in certain cases synonymous with the receptor (Kebabian et al, 1972; Nathanson & Greengard, 1974), and its presence has been demonstrated in many preparations (Karobath & Leitich, 1974; Clement-Cormier et al, 1974; Nathanson & Greengard, 1973; Kebabian & Saavedra, 1976; Osborne, 1977; Bodnaryk, 1979; Schmidt et al, 1981; Uzzan & Dudai, 1982; Orr et al, 1987; O'Donnell & Singh, 1988).

Cyclic AMP then activates a protein kinase, (an enzyme which phosphorylates proteins and causes conformational change), and this interacts with the proteins which make up the ion channel in the membrane. In general, the inactive, or closed form of the channel is converted to the active, or open form by phosphorylation. After use,

cyclic AMP is degraded, by phosphodiesterase, to 5'AMP, which is ultimately recycled into ATP.

While cyclic AMP is perhaps the best understood of the second messengers, there are other compounds which may act in this way. One of these is inositol trisphosphate (IP_3). This has been shown to be produced in the blowfly salivary gland as a result of the binding of 5-HT to membrane receptors. Berridge & Irvine (1984) have suggested that the binding of 5-HT to its receptor activates a G protein in the membrane. This, by means of GTP/GDP in turn activates a phosphodiesterase within the cytoplasm. This converts phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol, both of which can function as second messengers. It is suggested that IP_3 then binds to a receptor on the membrane of the endoplasmic reticulum, known to function as an intracellular store for calcium (Taylor, 1986; McBurney & Neering, 1987), and causes release of calcium, with concomitant activation of calcium-dependent processes within the cell. Diacylglycerol, however, is thought to remain within the plasma membrane, where it may have effects upon protein conformation.

1.4 AIMS OF THE PROJECT

If pesticides are to be made sufficiently specific that they only affect target species, then a clearer understanding of the neurophamacology of such species is essential.

This project focussed on the nervous system of the ubiquitous pest species, the American cockroach (*Periplaneta americana*), and in particular on the possible role of dopamine in the central nervous system.

One of the aims was to investigate the response to dopamine (already demonstrated by Fleming,. 1986; Pitman & Baker, 1989; Pitman & Davis, 1988) of an identified common inhibitory motoneurone in the prothoracic ganglion of the ventral nerve cord. This neurone was designated D_3 by Iles (1976) in his review of the anatomy of the cockroach CNS. It was hoped to characterise the response in terms of its ionic basis and pharmacology, and to indicate whether specific receptors for dopamine existed in this preparation, or whether dopamine might be acting on receptors for other amines. Since noradrenaline and octopamine had been shown to be present in the CNS (see Pitman, 1985), the experiments were planned particularly to distinguish between the actions of these amines and those of dopamine.

If evidence was forthcoming for a dopamine receptor, then further pharmacological characterisation would be undertaken, in the context

of existing classification schemes for dopamine receptors in vertebrates.

The choice of pharmacological agents in the study was determined largely by references to the vertebrate classification schemes in the literature. Some (for example, apomorphine and haloperidol) were cited as benchmark dopaminergic agents, while others (such as, SCH 23390, SK&F 82526, LY 171555, and YM 09151-2) were experimental compounds reported to have dopaminergic activity in a variety of preparations.

SECTION 2 - METHODS AND MATERIALS

2.1 THE PREPARATION

These experiments were performed on a neurone in the prothoracic ganglion of the ventral nerve cord of the adult male cockroach *Periplaneta americana*. A section of the cord, comprising the three thoracic ganglia and their connectives, was dissected out, placed, ventral side uppermost, on a small plastic slide, and attached to it by small rubber bands around the slide and over the connectives (Figure 1).

To allow easier access to the neurones under investigation (which lie towards the posterior end of the ventral surface of the ganglion), the preparation was arranged such that the prothoracic ganglion was on a slightly raised portion of the slide, with the anterior of the cord at the bottom of the bath as viewed through the microscope. The prothoracic ganglion was desheathed to facilitate impalement of the cells, and to allow applied agents access to the surface membrane. If the sheath were not removed, its role as a blood/brain barrier would restrict access of at least some of the experimental agents (Treherne & Pichon, 1972).

In order to allow freer access for drugs and other agents circulating in the saline, the cell body of the neurone was cleaned, and glial tissue removed from around it, especially below the soma. This was

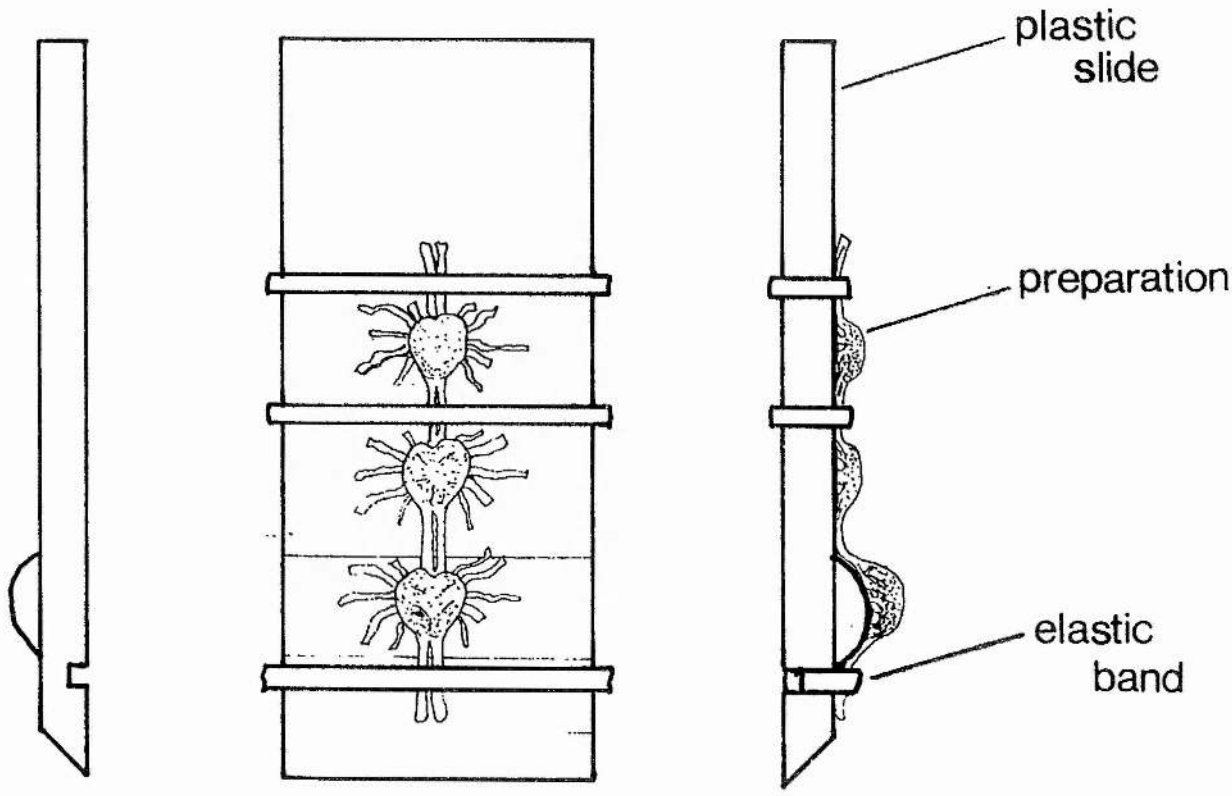


Figure 1 The ventral nerve cord mounted on the plastic slide. The ganglion is 1,5 - 2 mm in diameter, and the slide is approximately 1 cm wide and 2 cm long.

achieved using a saline-filled microelectrode, the tip of which was broken back to allow ejection, under pressure from a syringe, of a directable jet of saline.

In some experiments, a broken and fire-polished microelectrode, connected by tubing to a mouthpiece, was used to provide a vacuum to raise the cell body above the general level of the ganglion. This improved access for agents which were carried in the bath circulation, but the technique proved difficult to standardise, and it was latterly abandoned in favour of comprehensive cleaning with a saline jet.

2.2 THE EXPERIMENTAL BATH

For these experiments, the preparation was mounted in a Perspex bath, with a volume of 3 ml. Provision was made for circulation, oxygenation and replacement of the saline (detailed compositions of all saline solutions used are given in Appendix 1), as well as direct injection of drug solutions (Figure 2). The preparation within the bath was viewed through an Olympus zoom binocular dissecting microscope, fitted with x20 eyepieces, giving a range of magnification of 14 to 80 times. This allowed accurate positioning under visual control of microelectrodes and drug pipettes with respect to the preparation.

The preparation chamber was physically isolated from that containing the bath electrodes, and the two were connected electrically using a glass bridge filled with an agar/KCl gel. This prevented the generation of variable junction potentials by alterations in the ionic environment of the bath electrodes during experiments.

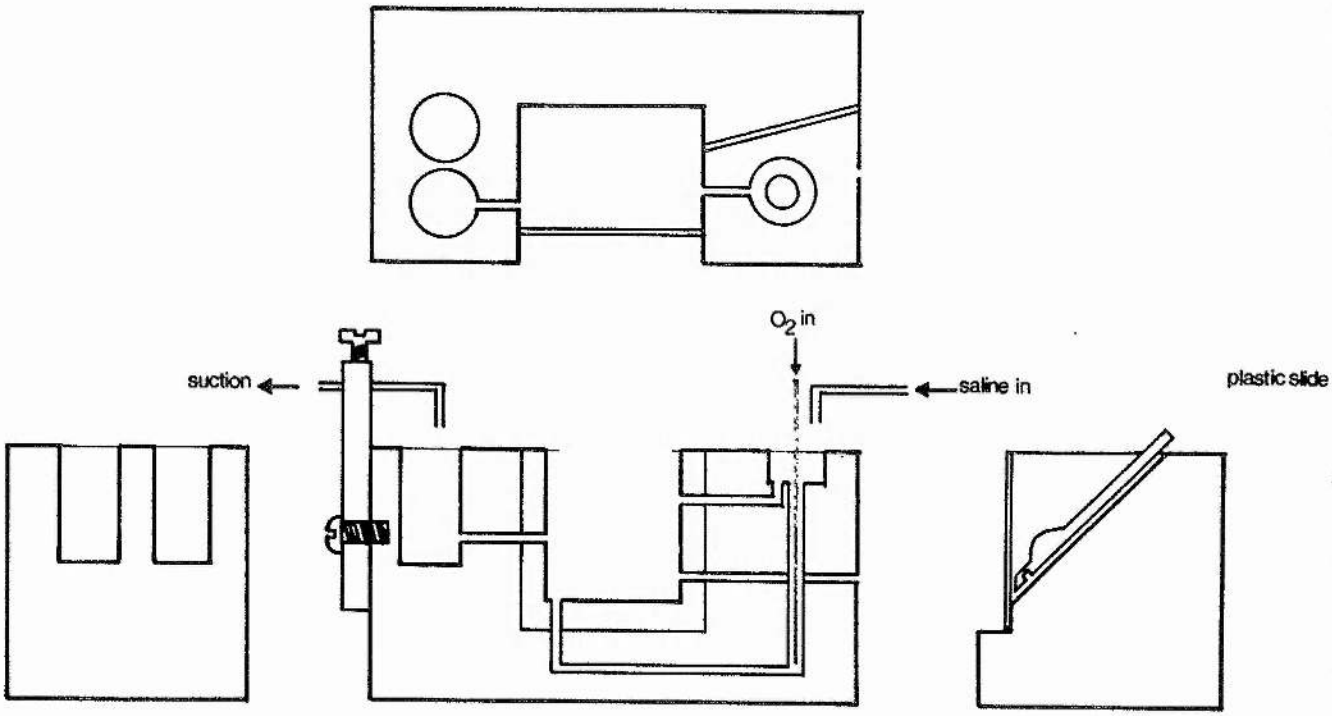


Figure 2 The Perspex experimental chamber. Provision is made for the addition of agents to the circulation, and for the continuous superfusion and oxygenation of the circulating solution.

2.3 INTRACELLULAR RECORDING

Intracellular recordings were made from the neurone soma by single or dual impalement. Glass microelectrodes were pulled using a Narishige PE2 vertical puller from 1.5 mm external diameter thin-walled filament glass capillary tube, supplied by Clark Electromedical Instruments. Recording microelectrodes, filled with 1 or 2 M potassium acetate, had tip resistances between 20 and 30 MΩ.

Potassium acetate was chosen as the filling solution since it has been noted that some insect neurones show exceptional sensitivity to changes in internal chloride levels (Pitman, personal observation). In addition, this solution was less likely to affect the resting properties of the cell, as potassium concentrations inside are high, and acetate is not readily transported by physiological mechanisms and cannot travel through chloride channels.

2.3.1 Bevelling Microelectrodes

In experiments using two microelectrodes, whether under current- or voltage-clamp conditions, the current-passing microelectrode was frequently bevelled to between 8 and 15 MΩ, using a slowly revolving (≃ 1 rev/s) fine abrasive disc. This was viewed through a binocular microscope under angled illumination from a lamp so positioned as to cast a shadow of the microelectrode tip onto the surface of the disc, thus allowing the microelectrode to be lowered until its tip was just in contact with the abrasive.

2.3.2 Microelectrode Holders

The microelectrodes were located in holders made from flexible silicone tubing, through the centre of which a silver wire was passed. This in turn was directly soldered onto a 4 mm plug, to allow for connection to the probe stage of the recording system. To eliminate junction potentials and base-line drift associated with polarisation, the silver wire was coated with silver chloride by electrolysis in a bath of 1 M potassium chloride.

2.3.3 Amplification

The probe stages of the amplifiers were held in Prior micromanipulators fitted with 10 to 1 reduction drives to give finer control in orthogonal horizontal planes.

Each microelectrode was connected to a laboratory-built high impedance, unitary gain amplifier. A bridge circuit was incorporated into this amplifier to enable the membrane potential of the cell to be monitored, and at the same time to allow polarisation of the cell membrane by current injection. The amplifiers had provision for capacitance compensation, microelectrode resistance check, and calibration (preset 10 and 50 mV output to CRO or chart recorder). A complete circuit diagram is given in Appendix 4.

2.3.4 Generation of Current Pulses

Square-wave pulses, of variable amplitude and duration were provided by a Grass SD9 stimulator, the output of which was not isolated. For use in impaling the cell, a pulse of hyperpolarising current, of 25 nA amplitude and 20 mS duration, was used to disrupt the membrane sufficiently to allow the microelectrode tip to penetrate.

Once an impalement had taken place, a measure of the input resistance of the cell could be obtained using pulses, at a frequency of 1 Hz, at an amplitude sufficient to produce 10 mV hyperpolarising excursions of membrane potential at the start of the experiment.

2.3.5 Current-Clamp Recording

2.3.5.1 Single Microelectrode Recording

In preliminary investigations into the nature of the response to dopamine, single-microelectrode current-clamp recordings were made. A single microelectrode, filled with 1 or 2 M potassium acetate was used, connected as detailed above. This gave a measure of the resting membrane potential of the cell and any effects of dopaminergic agents on it. It was also possible to apply repeated hyperpolarising pulses to the cell through the bridge of the amplifier to gain some idea of any changes in the input resistance of the cell.

The basis of this technique is that channels may be opened or closed by the binding of transmitter molecules to receptors, altering the membrane resistance. A constant current pulse applied to the cell through an intracellular electrode would result in a smaller or larger voltage excursion of the membrane, thus giving an estimate of the change in input resistance of the cell. Any change in microelectrode resistance during an experiment (eg due to partial blockage of the tip), would cause the bridge circuit to become unbalanced. The result of this was that any estimations of input resistance made by this method were of necessity approximate.

2.3.5.2 Double Microelectrode Recording

In order for more accurate recordings of membrane potential to be made simultaneously with the injection of current, the neurone was impaled with two microelectrodes, connected to separate amplifiers. One could now be used to monitor the absolute value of the membrane potential, while the other was used to inject current (either depolarising or hyperpolarising) into the cell. Since no current was being injected through the bridge of the recording amplifier, there was no interference with the monitoring of the membrane potential. As a consequence, this technique allowed a more accurate determination of the input resistance of the cell.

2.3.6 Voltage-Clamp Recording

If a depolarising response is of a particular magnitude at a certain membrane potential and that magnitude changes in a voltage-dependent

manner, then the alteration of membrane potential caused by the depolarisation itself will alter the ultimate size of the response. For this reason the magnitude of a depolarisation does not therefore directly reflect the size of the currents underlying it.

It was often necessary to investigate the effects of current or pharmacological agents on the currents flowing across the membrane. The voltage-clamp technique allowed the membrane potential to be kept constant at a predetermined level (the holding potential). Any changes in membrane conductance elicited by the application of a drug would now be manifested not as a shift in membrane potential, but as a change in the flow of current across the membrane, provided that the holding potential chosen was not equal to the reversal potential of the response under investigation. In this case, since the driving force on the causal ions would be zero, no current would flow through channels opened by the application of drugs to the membrane. This current was measured as the amount of current flowing back to earth through the current monitor, a current-to-voltage convertor in the return circuit (see Appendix 4).

The voltage-clamp was achieved by using two intracellular microelectrodes. One monitored the membrane potential. This potential was fed into the input of a laboratory-built high-voltage (± 100 V) inverting amplifier with adjustable gain and frequency response (Figure 3). The output of this amplifier fed back to the second microelectrode. Under stable conditions, a constant current, the magnitude of which was dependent on the difference between the

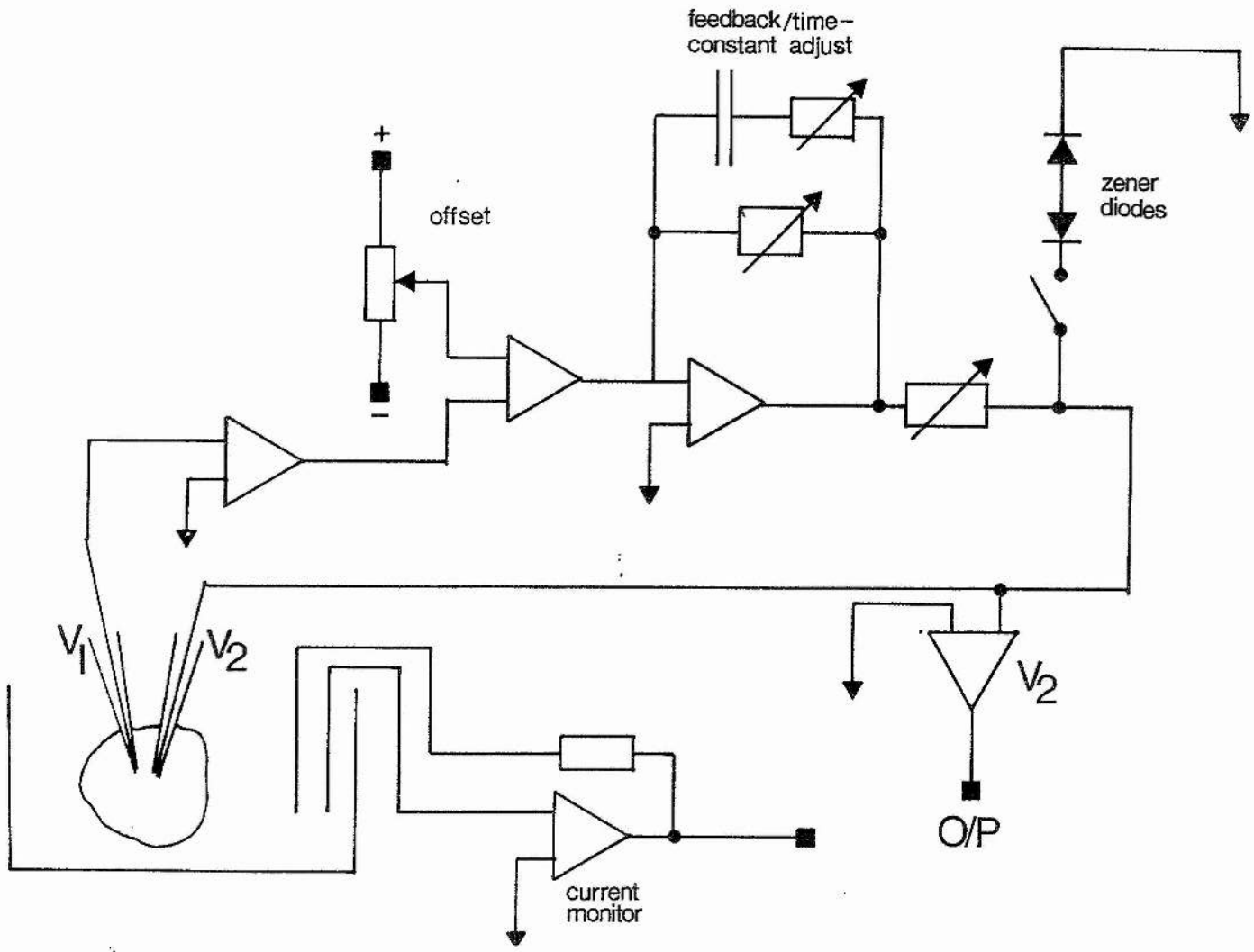


Figure 3 A schematic diagram of the recording circuit.

resting membrane potential and the chosen holding potential, flowed around this system.

Any change in membrane conductance which tended to cause the membrane potential of the cell to change was sensed by the voltage-clamp amplifier, which automatically sent an equal and opposing current through the second microelectrode into the cell, thus counteracting the change of membrane potential. It was the amount and polarity of this correcting current which was measured by the current monitor and displayed.

Under these conditions an event which would normally be seen as a depolarisation, was now seen as an inward current (by convention displayed in a downward direction on a CRO screen). The holding potential could be controlled to any desired level. By the application of external pulses the membrane could be stepped briefly from the holding potential to different command potentials.

2.3.6.1 Long-Pulse Experiments

Initial voltage-clamp experiments were performed using a long-pulse technique. In this, the length of the command step was longer than the duration of the drug response. This presented problems of electrical stability, since prolonged applications of polarising current (up to 30 seconds for a typical dopamine response) caused damage to the cell, or altered the conductance (presumably by activating voltage-dependent currents) which caused electrical instability of the cell membrane. In addition this level of

stimulation may result in the intracellular accumulation of various ions, which would affect trans-membrane gradients and contribute to this electrical instability. This was especially the case when the cell membrane potential was being stepped to values positive to the natural resting potential.

2.3.6.2 Short-Pulse Experiments

In these experiments, the duration of command pulses was reduced to 500 ms. The drug application was made, and a brief command step applied to the cell at the peak of the response. The command step and the Picospritzer II were triggered from the same waveform generator, and a small amount of variable delay allowed exact synchronisation of the command step with the peak of the response. This method largely overcame the problems associated with maintaining the cell membrane potential at values far from the resting potential for prolonged periods, and allowed repeated measurements to be taken of the amplitude of a response at a full range of command potentials, including those positive to the resting potential, without any damage to the cell.

2.3.7 Computer-Driven Experiments

A Tandon PCX computer, linked to a Cambridge Electronic Devices 1401 interface unit, produced a series of incrementing command pulses. The software allowed the pulse width, inter-pulse interval, holding potential, and command potential increment to be preset. The resultant series of command pulses from the holding potential

positive to +40 mV at intervals of 2 seconds, gave a current-voltage plot on which the effects of dopamine could be assessed.

Data were stored on a Racal Store 4DS FM tape recorder, which allowed photographic records to be made of relevant parts of the experimental run, after the experiment had been concluded.

2.3.8 Data Display

Data were displayed on a Tektronix 5103N dual-beam oscilloscope, fitted with one differential (type 5010) and one dual-beam plug-in amplifier (type 5018). This allowed for the simultaneous display of two voltage channels, from the two microelectrodes, and of current passing through the experimental bath. Hard copies of responses were obtained on a Gould 220 pressure-ink recorder.

2.3.9 Current Monitoring

The current passing through the experimental bath was monitored using a current-to-voltage converter with a DC offset and switchable feedback resistors (10 KΩ, 100 KΩ, & 1 MΩ) providing variable gain. This, after calibration, allowed accurate measuring of any current injected into the cells, or of current applied to the bath via such devices as iontophoretic current pumps. The two silver/chloride bath electrodes had a large surface area to maximise contact with the saline, increase the response speed, and minimise electrode polarisation.

2.4. DRUG APPLICATION

2.4.1 Microiontophoresis

The ejection of a compound from a microelectrode is governed by four processes. These are iontophoresis, diffusion, bulk flow due to hydrostatic pressure, and bulk flow due to electro-osmosis. Each of these processes has an effect on its own, but the combined effect of all is often not as simple as the algebraic sum of the components.

Iontophoresis itself is the movement of ions along a gradient of electrical potential. The outcome of iontophoresis, in terms of drug release, can be expressed by :

$$q = \frac{-nI}{zF}$$

where q is the efflux in moles per second, I is the current applied to the microelectrode, z is the valence number of the ion, and F is Faraday's constant. The constant 'n' is the transport number, which takes account of the fact that only a proportion of the applied current will be carried by any one species of ion, and is different for each ion species. This is important when a drug is dissolved, since other ions present, for example H^+ or H_3O^- in water, and K^+, Na^+ and Cl^- in saline may carry a significant proportion of the ejecting current. If the drug solution is not a concentrated one, this may mean that very little drug is actually ejected, even with a relatively large ejection current.

The rate of release, and the speed at which the steady-state release is attained, depend on the value of retaining current applied to the microelectrode in order to reduce spontaneous efflux. The larger the retaining current, the longer it takes for the steady-state to be achieved during an ejection pulse. Thus for very short pulses, much less drug may be ejected if a retaining current is employed (Purves, 1984; Stone, 1985).

Iontophoresis has been used to apply a number of different transmitters and other drugs to insect neurones (for example Kerkut et al, 1969a; 1969b; Steiner & Pieri, 1969; Pitman & Kerkut, 1970; Hardie, 1987). However, the results obtained by microiontophoresis in the present study proved to be less repeatable than those from pressure-ejection experiments. It was also difficult when recording under voltage-clamp conditions, to reduce to an acceptable level the large current artefacts generated by the iontophoretic current pumps.

Experiments using microiontophoresis of drug solutions were performed using one of two pieces of equipment - a laboratory-built mains-operated current pump, the output of which was not isolated from mains earth, and a World Precision Instruments Microinotophoresis Programmer, powered by dry cells, and with an isolated output. The WPI Programmer was used for experiments performed under voltage-clamp conditions, since an independent circuit return wire could be employed. This reduced interference with recordings of trans-membrane currents during voltage-clamp experiments. Both of these devices allowed constant monitoring of the current being passed through the iontophoretic microelectrode.

In most cases, however, pressure-ejection was used for the routine application of agonists.

2.4.2 Pressure Ejection of Agonists

Pressure-ejection has advantages over iontophoresis in experiments to study the actions of drugs with low ionic mobility, solubility, or ionisation. The quantity ejected can also be gauged visually, under a microscope, and the volume of the droplet formed can be estimated.

In these experiments, a Picospritzer II unit (General Valve Corporation) was used. This provided internally timed pulse durations from 2 to 999 ms (longer duration pulses could be provided by a manually operated external trigger). Pressure was provided by a garden spray unit which allowed pressures between 1 and 25 pounds per square inch to be generated.

Microelectrodes, similar to those used for intracellular recording, were filled with a solution of the appropriate agonist drug. The microelectrode was then 'bumped' against the Perspex bath to break the tip until a pressure-pulse produced a 'puff' of solution which could be seen in the saline of the bath under the microscope. The diameter of the tip could not be carefully controlled by this method, but practice allowed a tip diameter to be gauged that would prevent serious spontaneous gravity-induced efflux of drug solution, and yet allow sufficient to escape during a pressure pulse.

The quantity of solution ejected from a micropipette is dependent, in a linear manner, on the ejection pressure. It has been shown (Stone, 1985) that the amount of ^{3}H sucrose released from a micropipette was a linear function of the ejection pressure. It was therefore necessary to regulate this carefully throughout the experiment. The ejection volume is also directly dependent on the diameter of the pipette tip. Very small fluctuations in this diameter can cause very large fluctuations in the ejected volume. For a change in tip diameter of only 0.3 μm, form 0.9 to 1.2 μm, the volume ejected may change tenfold (Stone, 1985).

In addition, the actual concentration of drug in the fluid ejected may not be the same as that in the microelectrode. This can result from electrical interactions between the drug molecules and the glass of the microelectrode, and also the effects of surface tension. The volume ejected may also vary depending on the ejection environment. For instance, less drug is ejected into mammalian brain tissue than into saline (Dray et al, 1983).

2.4.3 Bath Application

Under certain conditions, it was desirable to know the concentration of drug reaching the preparation and to eliminate artefacts which might possibly result from current (using microiontophoresis) or pressure (using pressure-ejection) application. The drug solution could be injected directly into the circulating saline. Since the volume of saline in the bath and the concentration of the drug

solution were accurately known, it was possible to determine more precisely the concentration of drug reaching the cell.

2.5 DRUG SOLUTIONS

In general, drugs that were not sufficiently soluble in saline to give the required stock concentrations were dissolved first in a few drops of 0.1 M hydrochloric acid (or exceptionally dimethyl sulphoxide - DMSO), and then diluted to the required volume with saline. The pH was checked before use, and if necessary corrected, to 7.2.

Controls were performed using a solution of hydrochloric acid or DMSO in saline. This was injected into the bath circulation. Correction for pH was carefully carried out at all stages of the process to eliminate the possibility of any pH artefact being mistaken for a true response.

Details of drug solutions are given in Appendix 3.

SECTION 3 - EXPERIMENTAL RESULTS

3.1 THE NATURE AND IONIC BASIS OF THE RESPONSE TO DOPAMINE

3.1.1 Introduction

Amines in general, and catecholamines and dopamine in particular, have been shown to have a wide variety of cellular actions in molluscan and arthropod nervous systems. However, with the exception of the report by Steiner & Pieri (1969) that dopamine applied iontophoretically caused inhibition in the ant brain, catecholamines have generally been shown to excite insect central neurones. A detailed review of these studies has already been given in the General Introduction.

The cell membrane is selectively permeable to different ions by virtue of its possession of channels which may be selective in terms of which ions they allow to pass (eg Na^+ - Catterall, 1985; K^+ - Thompson, 1977). This permeability, and any net difference in the concentration of permeable ions inside and outside the cell will result in an electrochemical gradient, and a potential difference will result.

In a cell at rest, $[K^+]_i$ is usually much greater than $[K^+]_o$, and likewise, $[Na^+]_o \gg [Na^+]_i$, and $[Cl^-]_o \gg [Cl^-]_i$. These gradients are maintained by ion transport processes, which may require metabolic

energy (from the breakdown of ATP), such as the electrogenic sodium-potassium ATPase, also known as the sodium pump, which transports K^+ into, and Na^+ out of the cell with a ratio of 3 : 2 (Thomas, 1969; 1972). There is also currently controversy over the existence of an active pump for chloride ions (Gerencser et al, 1988). Other systems include various forms of co-transport, such as sodium-glucose transport and the (Na-K-Cl) cotransporter (see Haas, 1989), and ion exchange mechanisms such as Cl^-/HCO_3 exchange (Gerstheimer et al, 1987), many of which may also contribute to the regulation of internal pH. The dynamic equilibrium for an ion will be determined by the rate at which it is pumped into/out of the cell, and the rate at which it passes back across the membrane through ion channels.

In a situation where the permeability to one ion (and hence the conductance of the membrane for that ion) is relatively large, the resting potential will tend to be close to the equilibrium potential for that ion, ie the potential at which those ions are in a state of dynamic equilibrium across the membrane. If the resting conductance of any ion is increased, then the resting potential will tend towards the equilibrium potential for that ion.

Hodgkin & Katz (1949), using the squid giant axon as a model, showed that the rising phase of the action potential was attributable to rapid changes in gNa following stimulation. By removing Na^+ from the external medium, they abolished the action potential. The effect depended directly on degree of reduction of $[Na^+]_o$. This is not universally the case, however, it having been shown that in some arthropod skeletal muscle, for example, the action potential is

carried by calcium or magnesium ions (Hagiwara & Naka, 1964; Wood, 1957) instead of by sodium.

As well as action potentials, sodium ions have also been shown to carry synaptic currents, the most clearly understood being the current induced by the action of acetylcholine on its nicotinic receptor, which is held to be closely coupled to a membrane channel which allows the passage of both sodium and potassium ions. These receptors, and their associated sodium currents, form the basis of excitatory neuromuscular transmission in vertebrate skeletal muscle first demonstrated by del Castillo and Katz (1955). Acetylcholine receptors, and sodium-mediated synaptic events have also been shown to occur in insects (Harrow, David & Sattelle, 1982; Breer & Sattelle, 1987).

The role of K^+ in excitable cells was first demonstrated by Hodgkin & Huxley (1953), who, using $^{42}K^+$, demonstrated its efflux from squid giant axon following stimulation, which resulted in an increase in gK, whereupon K^+ flows down its concentration gradient, and the membrane potential approaches E_K, thus repolarising the membrane.

Because, in the majority of tissues, $[K^+]_o$ is much less than $[K^+]_i$, E_K is usually negative, and an increase in gK results in an efflux of K^+, producing an outward current. Increasing gK will result in a hyperpolarisation of the cell if other ions contribute conductance at the resting potential, and the same criteria apply as mentioned above for gNa. In a system with both Na^+ and K^+ conductances, an increase in excitability can be brought about either by increasing gNa, and

depolarising the cell as a result of influx of Na^+, or by reducing gK, thus slowing the efflux of K^+, and producing a net depolarisation. A wide variety of K^+ currents have been found (eg Thompson, 1977).

Hydrogen ions are of course central to the regulation of intracellular pH (Meech & Thomas, 1987), and have been shown to carry rapidly-activating membrane currents in neurones of the snail *Lymnaea stagnalis* (Byerley et al, 1984).

Chloride is essentially the only anion which contributes to synaptic potentials in excitable cells. In some tissues, notably skeletal muscle, Cl^- is assumed to equilibrate on both sides of the membrane, such that the equilibrium potential for chloride is equal to the membrane potential, since the membrane is assumed to be freely permeable to Cl^- (Hodgkin & Horowicz, 1959). In mammalian neurones, and in other excitable tissues, such as smooth muscle, $[Cl^-]_i$ is substantially higher than would be expected by passive diffusion, and this is explained by the facilitated inward movement of Cl^- across the cell membrane by a variety of mechanisms. These may include Cl^-/HCO_3 exchange and forms of co-transport (see Haas, 1989). There is, however, debate at present as to whether an active chloride pump exists (Gerencser et al, 1988). It is not clear if these systems exist in insect neurones, although some mechanism for the transport of ions must be present.

Chloride has been shown to be the carrier ion for currents induced by the binding of γ-amino butyric acid (GABA) with its receptor. GABA

is involved in the segmental inhibition of pain transmission (Duggan, 1985), and in molluscs, chloride has been shown to carry current components gated by both GABA and L-glutamate (King & Carpenter, 1989), and in insects, chloride has been implicated in the response to GABA (Kerkut et al, 1969b; Benson, 1988).

Calcium is an extremely important ion in the metabolism of neurones. It is known that small increases in $[Ca^{2+}]_i$ (normally around 1×10^{-7} M) can cause severe damage to neuronal integrity, and that elevation of $[Ca^{2+}]_i$ appears to play a role in the damage of neurones following ischaemia or hypoglycaemia in the mammalian brain (McBurney & Neering, 1987). Clearly neurones must be capable of maintaining these low intracellular levels of calcium, and at the same time allowing changes in these levels which permit calcium ions to function as intracellular regulators.

Neurones possess sophisticated mechanisms for buffering intracellular calcium (Taylor, 1986; McBurney & Neering, 1987). Calcium ions may be taken up by cell organelles such as the mitochondria and endoplasmic reticulum, from where they may also be released back into the cytosol. In addition, calcium ions may be bound to proteins, such as calmodulin, within the cytosol itself. The intracellular level of calcium ions is therefore the result of equilibrium between free, bound and sequestered calcium

In neurones, Ca^{2+} influx has been known for a long time to be a prerequisite for transmitter release. More recently, however, Ca^{2+} has also been shown to have a direct role as a current carrying ion

in several preparations. Calcium ions were shown to be the carriers of a voltage-dependent current induced by 5-HT in *Aplysia* neurones (Pellmar & Wilson, 1977; Pellmar & Carpenter, 1979; 1980). Klein & Kandel (1978; 1980) demonstrated that behavioural sensitisation of the gill-withdrawal reflex of *Aplysia* resulted from the enhancement of a Ca^{2+} current underlying the action potential of sensory neurones. This Ca^{2+} component was itself enhanced by the suppression of a K^+ current, probably by 5-HT. In addition, Goodman & Heitler (1979) showed that in the locust *Schistocerca nitens*, action potentials in the neurone DUMETi were blocked both by TTX and low $[Na^+]_o$, and also by external applications of cobalt or lanthanum ions, which would indicate the involvement of Ca^{2+} in the action potential. They found, however, that to block the action potential it was necessary to remove the influence of both Na^+ and Ca^{2+}.

Insect motoneurone cell bodies do not normally support or generate action potentials. In some cockroach motoneurones, depolarisation activates an outwardly directed K^+ current (rectifier) and causes the membrane potential to oscillate, falling short of initiating an action potential (Pitman, 1979).

In the presence of TEA, depolarisation produced long duration action potentials, which were abolished by reduced $[Ca^{2+}]_o$, thus suggesting that a large proportion of the action potential was carried by Ca^{2+}. These action potentials could also be elicited following intracellular injection of Ca^{2+}-chelators, such as EGTA, or tri-potassium citrate. It was concluded (Pitman, 1975; 1979) that TEA blocked voltage-dependent K^+ channels, and thereby unmasked voltage-

dependent Ca^{2+} channels, while by chelating internal Ca^{2+}, EGTA and citrate were supposedly both increasing the inwardly-directed gradient for Ca^{2+}, and also suppressing the powerful outwardly-directed Ca^{2+}-dependent K^+ current and short-circuiting inward currents across the membrane, thus preventing the action potential from occurring.

The effects of the flow of ions will be to alter the trans-membrane potential of the cell, causing either excitation or inhibition. If these changes take place in post-synaptic cells as the result of information transfer across the synapse, they are known as post-synaptic potentials. Excitatory post-synaptic potentials (epsp's) have their effect by causing depolarisation of the post-synaptic membrane, and thus bringing it closer to its spike-producing threshold. Conversely, inhibitory post-synaptic potentials (ipsp's) can have their effects either by directly hyperpolarising the post-synaptic cell, or by causing an increase in the conductance of the post-synaptic membrane, thus short-circuiting any conductances which would tend to depolarise the cell. However, some preparations exhibit depolarising ipsp's.

The voltage amplitude of a post-synaptic potential is dependent on the resistance of the post-synaptic membrane. If the resistance falls, then the change in the membrane potential of the post-synaptic cell produced by a psp is reduced, and conversely increased if the resistance is raised. For these 'classical' synaptic events, the magnitude of the post-synaptic current is dependent on the driving

force upon the particular permeating ion species in an ohmic manner. The driving force is, in turn, dependent on the membrane potential.

While many psp's are 'classical' in nature, there are other cases in which the channels themselves are voltage-sensitive, only allowing current flow at particular potentials, and thus causing changes in the excitability of the cell which vary with the membrane potential.

An example of a voltage-sensitive transmitter operated channel is that involved in the mediation of the M-current. The M-current, so called because of its sensitivity to the cholinergic agonist muscarine, has been extensively studied. It was first demonstrated, by Weight & Votava (1970), that muscarine and other agonists, including ACh, produced an increase in input resistance and an enhancement of repetitive firing in sympathetic ganglion cells. They showed that this was due to the inactivation of a potassium conductance.

Later research indicated the voltage-sensitivity of the M-current, it being activated between potentials of -60 and -10 mV, and contributing to a small extent to the resting membrane potential (Brown & Adams, 1980; Brown, Constanti & Adams, 1981). It is not activated by calcium, since it is unaffected by calcium antagonists and was shown to be carried by potassium ions. In addition, it could be inhibited pharmacologically (Adams, Brown & Constanti, 1982).

Since the M-current is a potassium current, and is thus outwardly directed at the normal resting potential, it will tend to

hyperpolarise the membrane. The voltage-sensitivity of the channels involved, however, means that as the membrane potential becomes more negative, the current is inactivated, the feedback system serving to maintain the resting membrane potential. Stimulation of the muscarinic ACh receptors which gate these potassium channels causes suppression of the M-current, and thus depolarisation. In sympathetic ganglion cells, therefore, this results in a sustained depolarisation which allows repetitive firing of the neurone.

Pellmar & Wilson (1977) have shown that 5-HT induces a voltage-sensitive current in *Aplysia* neurones. The voltage-dependence of the response is such that a region of negative slope resistance is induced in the current-voltage relationship of the neurone. Pellmar & Carpenter (1979; 1980) later showed that this current was in fact carried by calcium ions, and therefore mediated through voltage-sensitive calcium channels.

A voltage-dependent response to 5-HT has also been demonstrated in snails of the genus *Helix* (Cottrell, 1982). Activation of a giant 5-HT containing neurone produced a voltage-dependent response in follower cells, and this response was mimicked by the application of 5-HT. The small depolarisation produced was studied further under voltage-clamp conditions, and was found to be markedly voltage-dependent. At resting potential (in these cells, -40 to -50 mV), the response was small, but increased markedly at more depolarised potentials. Further investigation showed that this current was probably carried by calcium ions.

Baxter & Byrne (1989) demonstrated that 5-HT modulates a complex system of membrane currents in *Aplysia* neurones, at least two components of which are carried by potassium. One of these, which was blocked by 4-aminopyridine and tetraethylammonium, was markedly voltage-dependent, and was activated significantly only at potentials more positive than -10 mV.

The voltage-dependent effects of the actions of glutamate at N-methyl-D-aspartate (NMDA) receptors have been studied in detail (Mayer, Westbrook & Guthrie, 1984; Nowak et al, 1984). If glutamate is applied at potentials at or near the normal resting potential of the cell, its depolarising effect is comparatively small. If, however, the cell is depolarised (presumably, *in vivo*, this would be the result of other synaptic inputs, but can be achieved *in vitro* by artificial means), then the ability of glutamate to depolarise is greatly enhanced. It has been shown that at the normal resting potential of the cell, the ion channel is blocked by magnesium ions. As the cell is depolarised, these are dislodged, allowing other ions to cross the membrane and enhance the depolarising action.

It has previously been demonstrated that the cell D_3 responds to the application of dopamine with a depolarisation and a modest fall in input resistance consistent with the opening of membrane channels (Pitman & Baker, 1989). It was important to be able to reproduce these findings routinely, to study the response under both current-clamp and voltage-clamp conditions, and to investigate the underlying ionic mechanism and voltage-dependence, if any, before the response

could be characterised pharmacologically. The experiments described in this section were performed in order to answer questions such as:-

- Is the depolarising response of neurone D_3 a repeatable phenomenon, with recognisable characteristics ?

- Which ions underlie the response, and is calcium necessary for its initiation ?

- Could a similar mechanism to the modulation of the 'M' current underlie the response, ie the inhibition of an outward current, and if so, is this the only component of the response ?

- Does the response show any voltage-dependence, and if so, is it similar in nature to that of any other responses described in the literature ?

- What could be the physiological role of this type of response ?

The dopamine response itself was investigated first, under both current-clamp and voltage-clamp conditions, and using a variety of methods for the application of dopamine. Secondly, the effect of alterations in $[Na^+]_o$, $[K^+]_o$, $[Cl^-]_o$, $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$; cadmium ions, and the calcium antagonist verapamil on the response and its voltage-dependence was studied. A set of experiments was performed using a computer-generated pulse-train and bath-applied dopamine, to

investigate the effect on the overall, stimulus-induced outward currents seen in this neurone.

3.1.2 Current-Clamp Response

3.1.2.1 Pressure Application

The response to pressure-applied dopamine was a depolarisation, associated with a modest fall in the input resistance of the cell. This was monitored by the application of hyperpolarising pulses to the cell at a frequency of 1 Hz via a second microelectrode (Figure 4). The normal resting potential of the cell was between -50 and -60 mV. Typical responses had an amplitude of the order of 4 to 9 mV, and lasted for some 25 to 30 seconds. The response showed quite marked desensitisation (Figure 5) when repeated applications of dopamine were made. The resting input resistance of D_{ca} was of the order of 0.5 to 5 Megohms (mean value 1.79 Megohms ± 1.3 SD, n = 11), determined from the magnitude of hyperpolarisations produced by current pulses of known magnitude. This fell by approximately 20% during a typical response.

Under current-clamp, the response to dopamine showed some voltage-dependency. At potentials negative to the normal resting potential of the cell, the magnitude of the depolarisation increased gradually. At a point around -120 mV, it then increased more suddenly. At potentials more positive than the normal resting potential, the response reduced in size, and finally at potentials around 0 mV,

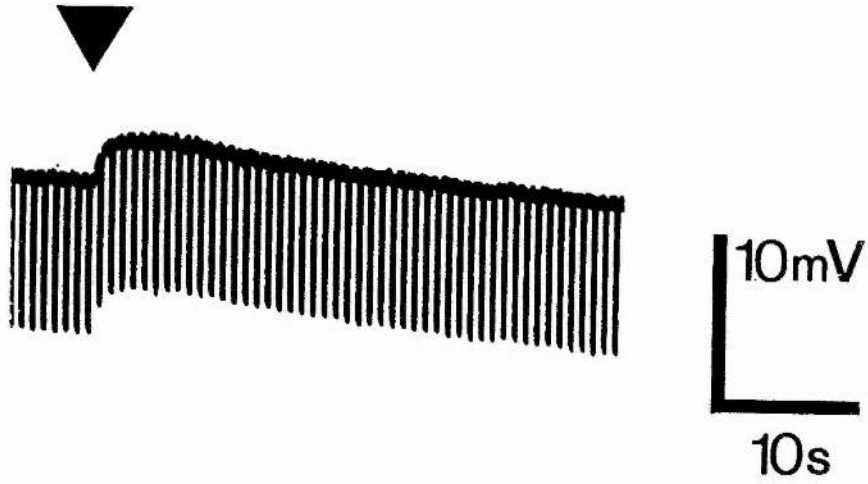


Figure 4 A single current-clamp response to pressure-applied dopamine. This recording was made using two microelectrodes, and constant current pulses were applied in order to indicate any changes in input resistance during the application of dopamine. Resting potential was -55 mV.

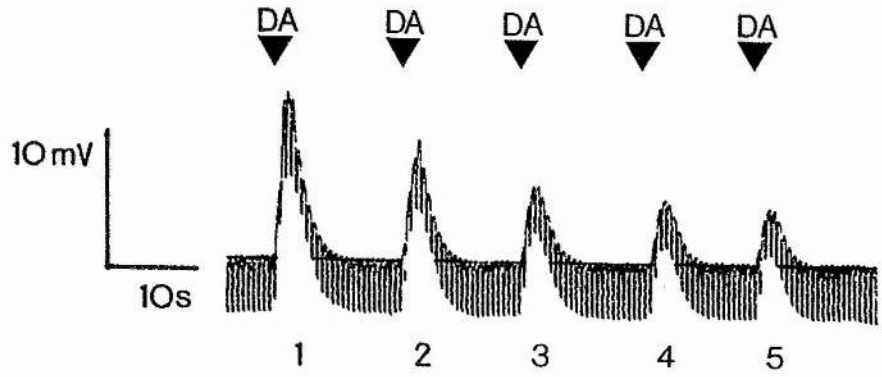


Figure 5 Desensitisation of the current-clamp response to pressure-applied dopamine. Each pulse was of equal duration and equal ejection pressure. Desensitisation was absent as long as at least 1 minute was allowed to elapse between pressure pulses. Resting potential was -61 mV.

became virtually indistinguishable from the background noise level. It did not appear to reverse.

It was not possible to determine the magnitude of the response at potentials more positive than this, since it was difficult to hold the membrane potential constant, owing to the activation of membrane currents which tended to have a de-stabilising effect, and to the tendency of the current-passing microelectrode to block during prolonged pulses. This problem was also encountered when performing experiments under voltage-clamp using the 'long-pulse' method, and necessitated the development of the 'short-pulse' technique in order to allow stable recordings at these more positive potentials.

3.1.2.2 Microiontophoresis

For these experiments, solutions of dopamine in distilled and deionised water, at various concentrations and pH values were tested, but consistent results were best obtained with 0.6 M dopamine, at a pH of 3.5. Ejection currents were between 20 and 500 nA, and retaining currents between 5 and 25 nA. Figure 6 shows a typical response obtained with the tip of the microionophoretic electrode in very close proximity to the cell body. As the quantity of dopamine released was much smaller than in the case of pressure application, this distance was critical.

There does not appear to be any significant difference between either the nature or voltage-dependency of the responses obtained by

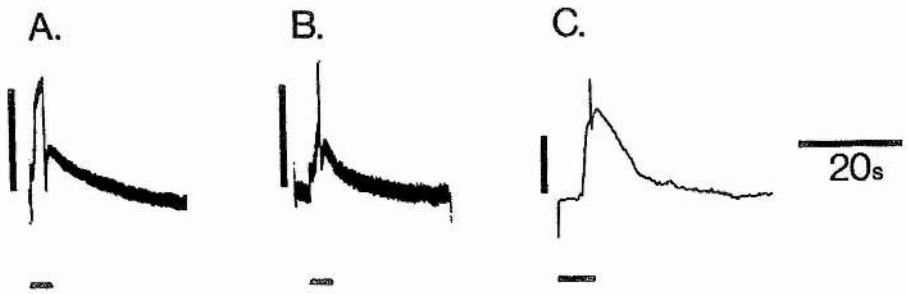


Figure 6 Representative current-clamp responses to the microiotophoresis of dopamine. Ejection current was 150 nA and the retaining current was 15 nA. In traces A and B, pulse duration was 5 s, while in trace C, it was 10 s. Note that the current pulses produced artefacts. The vertical scale bars are 10 mV in each case. The horizontal bars under the traces represent the duration of the ejection pulses.

pressure application and by microiontophoresis, recorded under current-clamp conditions.

3.1.2.3 Bath Application

In these experiments, a solution of dopamine in saline, at concentrations between 0.5 and 50 mM, both in the presence and absence of stabiliser, was added to the bath circulation, and recordings made of the response at different membrane potentials. Although, as mentioned above, it proved difficult to hold the membrane at a given potential for the relatively long period required for the dopamine solution to mix with the circulating saline in this method, several experiments were performed, and measurements were made, albeit at relatively few potentials. The threshold concentration at which a response could be obtained by bath application was of the order of 2 mM (final concentration), although this response was unreliable. A reliable response could be obtained by the use of 5 mM dopamine.

3.1.3 Voltage-Clamp Response

3.1.3.1 Pressure Application

Under voltage-clamp conditions, at a holding potential close to the normal resting potential of the cell (-55 mV to -60 mV), the response was seen as a small inward current, of similar duration to the depolarisation of the current-clamp response, and of the order of 0.5 nA in magnitude. In order to obtain an indication of the voltage-

dependency of the response, current values were taken at a range of command potentials.

Figure 7 below shows individual current traces produced at different command potentials. These responses were obtained by the 'long-pulse' method (see Section 2), and as such, only provided clear traces at membrane potentials more negative than about -20 mV.

As the membrane potential was stepped to progressively more negative potentials, the current initially showed very little increase in magnitude. A point was eventually reached, however, normally at membrane potentials between -120 mV and -150 mV, at which the magnitude of the inward dopamine-induced current increased dramatically and reversibly, and within 20 to 30 mV of the initiation of the increase, may have reached magnitudes of up to 80 nA, though values of up to 40 nA were more common. When the IV curve was re-plotted for the same neurone, these values were similar, although responses recorded from different preparations could differ by a few nA. The point at which the dramatic increase occurred was also variable between preparations.

The 'short-pulse' method (see Section 2) provided a means of measuring dopamine-induced current values at potentials more positive than -20 mV, since the cell membrane was only stepped to the new command potentials for 500 ms, not long enough for sustained membrane currents to have any detrimental effect on the stability of the recording. The disadvantage of this method was that no actual current traces were obtained. Instead, two pulses were recorded in

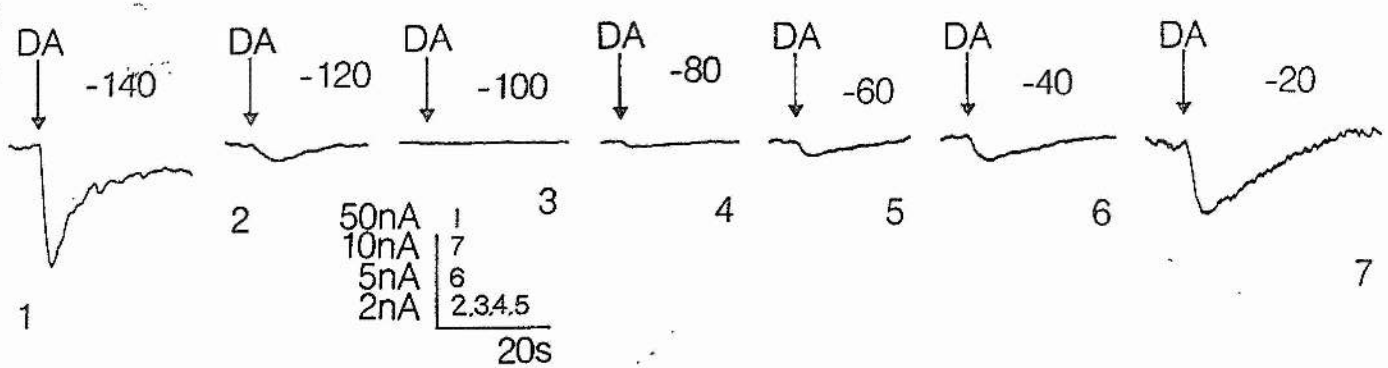


Figure 7 Individual current traces produced under voltage-clamp conditions, using the 'long-pulse' protocol, at a range of command potentials by pressure-applied dopamine. Holding potential was -60 mV.

the absence and presence of dopamine, and the difference between the magnitudes of these was an indication of the size of the dopamine-induced current which flowed during the application of the command potential. Figure 8 shows a typical series of pulse pairs and the resultant graph.

The values from the pulse pairs were fed into a computer, which was then used to generate curves indicating the IV relationship of the cell. Figure 9 illustrates a typical set of data in which the three curves indicate the IV relationship of the cell in the presence and absence of dopamine, and the difference between the two, which represents the magnitude of the dopamine-induced current at each of a range of command potentials. For routine use, only the resultant dopamine-induced currents were plotted, a typical example is shown in Figure 10

At potentials positive to the normal resting potential of the cell, the magnitude of the dopamine-induced inward current increased. It passed through a maximum of about 5 to 15 nA at a command potential of around -20 mV, declined, and finally reversed at around 0 mV, to be seen as an increasing outward current. If the response current had been mediated through non-voltage-dependent channels, it would have been expected to decrease.

3.1.3.2 Microiontophoresis

For these experiments the conditions were the same as those employed for the current-clamp experiments. The inward dopamine-induced

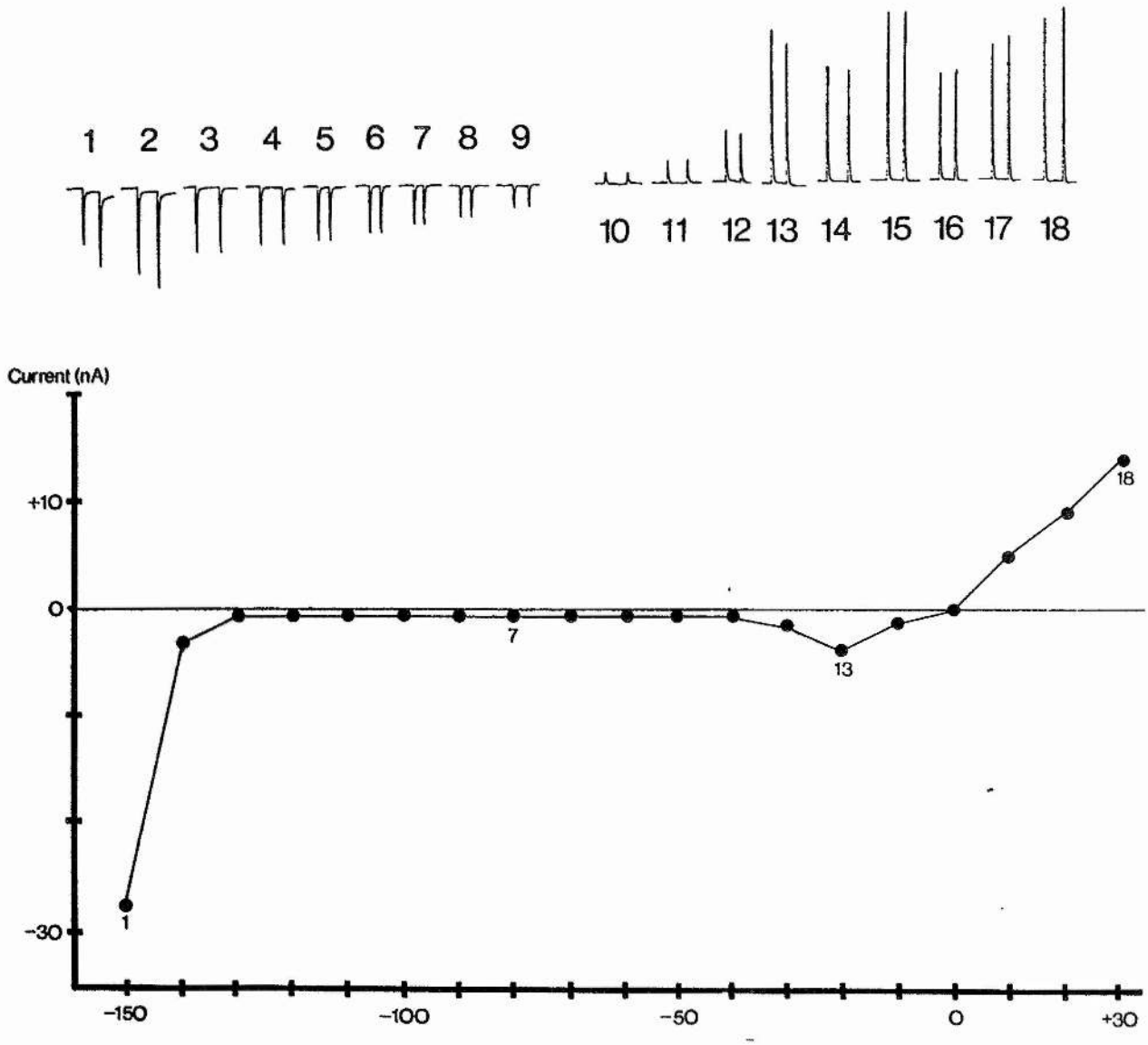


Figure 8 A representative series of pulse pairs produced in the presence and absence of dopamine by the 'short-pulse method'. The values obtained are plotted in the lower section. Calibrations refer to the traces shown at the top of the figure.

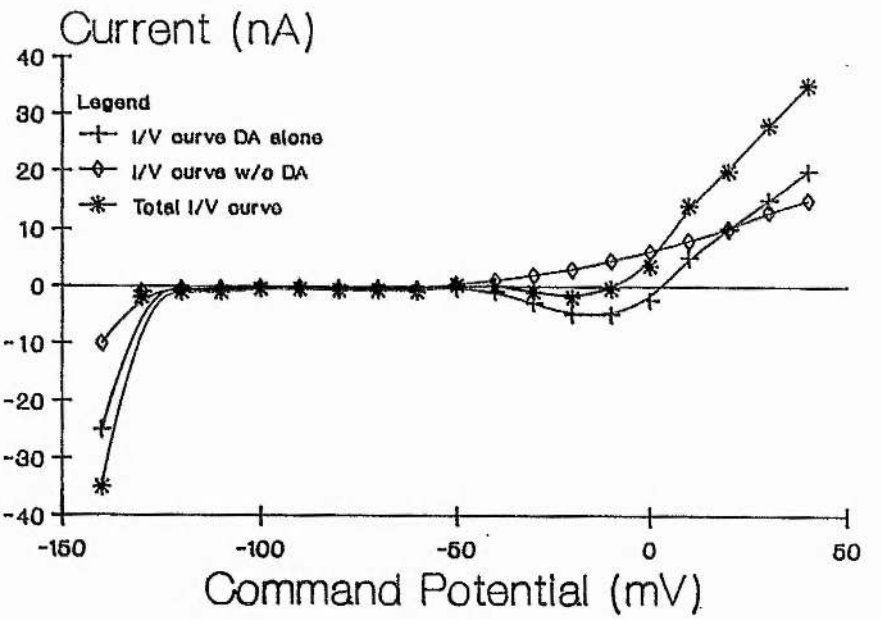


Figure 9 Representative data indicating the current-voltage relationship of the cell under voltage-clamp in the presence and absence of dopamine, and the voltage-dependence of the resultant dopamine-induced current, represented as a difference curve. Data were obtained by the 'short-pulse' method.

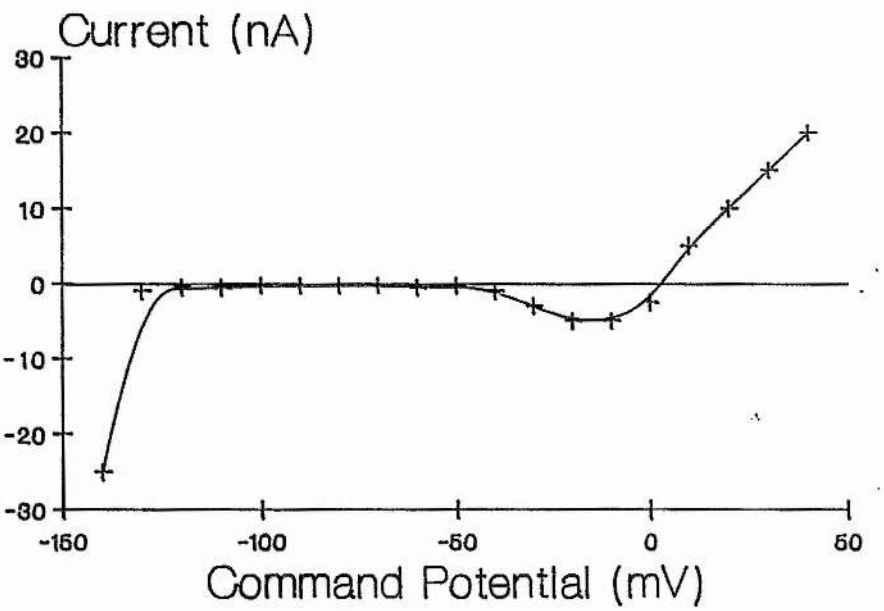


Figure 10 A difference curve indicating the voltage-dependence of the voltage-clamp response current to pressure-applied dopamine, obtained by the 'short-pulse' protocol.

current response resulting from the microiontophoresis was not materially different from that obtained by pressure application, and the voltage-dependency of the response was also similar. Figure 11 shows a typical voltage-clamp responses.

3.1.4 Control Experiments

To ensure that the unusual shape of the IV curve for dopamine was not artefactual, three different factors were investigated. These were the presence of the stabiliser (1% sodium metabisulphite) in the drug solution, the pH of the solution, and the physical effect of the pressure-pulse on the cell membrane.

3.1.4.1 Control for Stabiliser

In order to eliminate the stabiliser as a cause of artefacts, experiments were performed using a solution of dopamine in saline, without a stabiliser. These experiments were of necessity rapid, since the dopamine solution became tinged with pink very soon after it had been made up, indicating that oxidative decomposition of the dopamine had begun. After 20 to 30 minutes, the solution no longer elicited any response even when applied in long pulses, suggesting that most of the dopamine present had decomposed.

In terms of the size and time course of individual responses, and of the voltage-dependency of responses over a range of command potentials, the results of these experiments, under both current- and

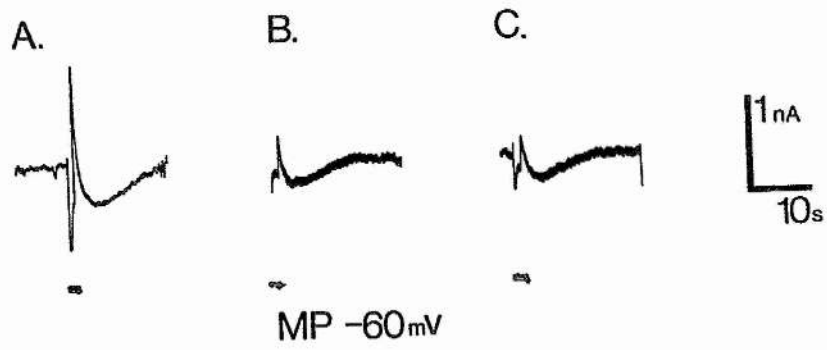


Figure 11 Voltage-clamp responses to the microiontophoresis of dopamine. Dopamine was applied using an ejection current of 250 nA (trace A) or 100 nA (traces B & C), and a retaining current of 20 nA. Pulse duration was 1 s, and holding potential -60 mV. It proved impossible to eradicate completely the large current artefact resulting from the ejection current pulse, due to the interference this produced with the clamp current.

voltage-clamp, were indistinguishable from those obtained using a dopamine solution containing the stabiliser (Figure 12).

Experiments were also performed in which the stabiliser solution alone (1% sodium metabisulphite in saline) was pressure applied onto the surface of the neurone soma, while recordings were made under current-clamp or voltage-clamp conditions. These experiments produced no evidence of any response, either depolarising or hyperpolarising, or of any effect on the input resistance of the cell.

3.1.4.2 Control for pH

Great care was taken (within experimental limitations) to correct the pH of all drug solutions. This was done in the preparation of the stock solutions, such as the stabiliser, either by the use of a carefully calibrated pH meter, or by the use of pH paper and standard buffer solutions. In the case of very small amounts of solution, pH was measured by pH paper, since a micro-pH meter was not available. The solution was corrected to a pH as near to 7.2 as possible (equal to that of normal physiological saline).

To eliminate the remote possibility that a drift in the pH of drug solutions could have been responsible for the responses obtained, some experiments were also performed using a solution of saline, with added HCl or NaOH, to alter the pH to either 6.7 (0.5 units less than normal), or 7.7 (0.5 units more than normal). A typical current-

Figure 12 A representative trace of a current-clamp response produced by pressure-application of a solution containing dopamine without stabiliser. Current pulses were applied through a second microelectrode, and resting potetial was -65 mV.

clamp experiment illustrates that no detectable effect was seen (Figure 13).

3.1.4.3 Control for Pressure Artefacts

In order to test for the effects of pressure on the neurone, saline was ejected onto the surface of the cell.

Under both voltage- and current-clamp conditions, a deflection in the resting state of the cell membrane was seen only when high ejection pressures were used (> 15 psi), or when the ejection pipette was very close to the cell surface. As indicated in Figure 14, the change in membrane potential caused by a pressure ejection was essentially of the same duration as the pressure pulse. It showed none of the voltage-dependency associated with the dopamine response. Since the normal time course of a single dopamine response was of the order of 30 seconds, and pressure pulses were rarely longer than 1 second, it was usually possible to eliminate these artefacts in normal experiments. In cases where it did prove impossible, the preparation was rejected.

The experiments involving the microiontophoresis and bath application of dopamine also served as controls for this, since responses were obtained in both cases which clearly could not be attributed to the effects of pressure.

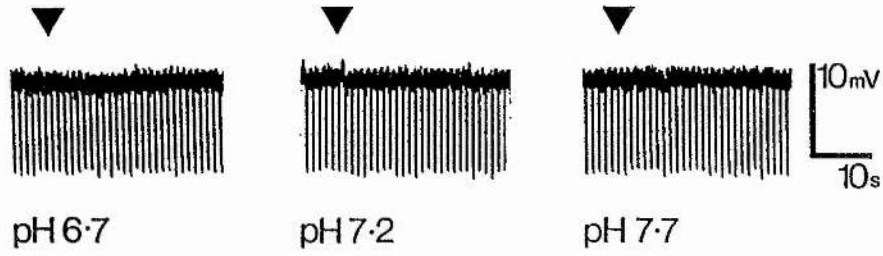


Figure 13 An illustration of the lack of membrane electrical response to solutions of saline at different pH values. Current pulses were applied through a second microelectrode, and there was no evidence of any alteration in membrane resistance. Resting potential was -62 mV.

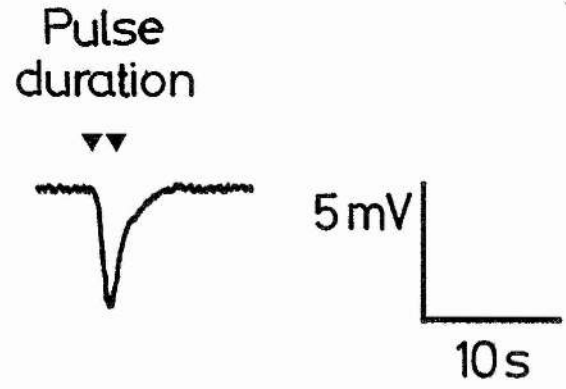


Figure 14 A typical current-clamp pressure artefact. The transient change in membrane potential (resting potential -59 mV) is of the same duration as the pressure pulse.

3.1.5 Ion Substitution Experiments

The experiments detailed here took the form of ion-substitutions in which external concentrations of Na^+, K^+, Cl^- or Ca^{2+} were altered. In addition, internal concentrations of Cl^- and Ca^{2+} were changed by impalement with microelectrodes containing KCl and tri-potassium citrate respectively. The calcium antagonists cadmium and verapamil were applied, to discover the extent to which Ca^{2+} was involved either in the initiation of the response or in the carrying of current during it. These two agents were chosen since it has been shown that there are differences in the ways in which organic and inorganic calcium channel blockers act (Oyama et al, 1986). The constitutions of the replacement salines used are given in Appendix 1.

Finally, experiments were performed using a computer-driven pulse generator with fast time resolution, which enabled the effects of dopamine on overall membrane currents to be assessed.

3.1.5.1 Effect of Alteration of $[Na^+]_o$

Experiments were performed in which the preparation was incubated in a solution in which $[Na^+]_o$ was reduced from a normal value of 214 mM, to 42.8 mM, a five-fold reduction. In three experiments, after incubation in 42.8 mM Na^+ saline for up to 15 minutes, the inward current component seen at command potentials in the region of -20 mV was suppressed, while in four others it was unaffected. Figure 15

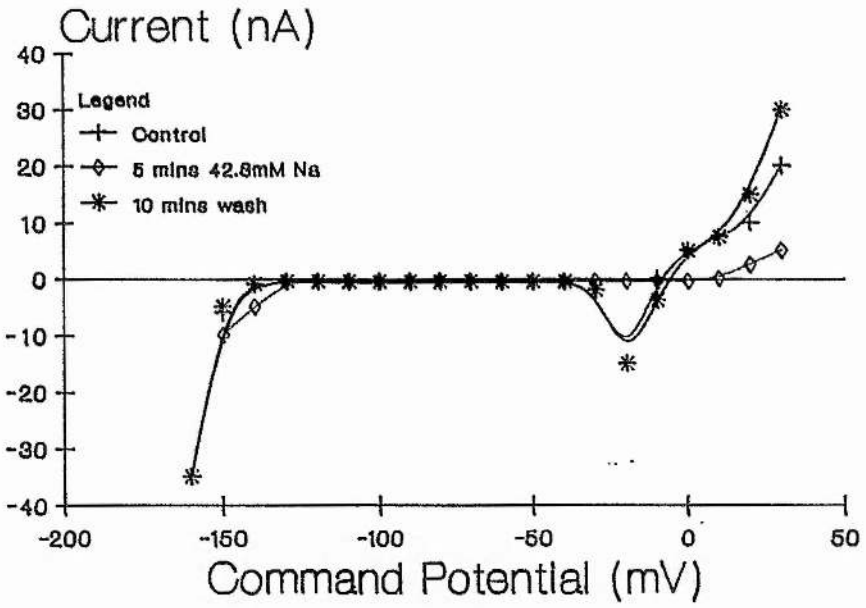


Figure 15 The effect of a five-fold reduction in $[Na^+]_o$, from 214 mM to 42,8 mM, on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV). Data were obtained by the 'short-pulse' protocol. This illustrates an experiment in which the inward current at positive potentials was altered by the ion replacement. In other experiments, no effect was seen.

illustrates an experiment in which suppression occurred in low sodium solution. No effect was observed upon the increase in magnitude of the currents at more negative command potentials.

3.1.5.2 Effect of Alteration of $[K^+]_o$

Experiments were performed in which $[K^+]_o$ was increased from a normal value of 3.1 mM to 15.5 mM. In five experiments this substitution caused the reduction or abolition of the inward current kink, and one of these is illustrated in Figure 16. However, in three other experiments this reduction was not observed. Like low Na^+ solutions, it did not, however, appear to have any effect on the magnitude of the inward current at more negative potentials.

3.1.5.3 Effect of Alteration of $[Cl^-]_o$ and $[Cl^-]_i$

In these experiments, $[Cl^-]_o$ was reduced from a normal level of 235 mM to a level of 1/5 normal, 47 mM. Microelectrodes filled with 3 M KCl were used in order to raise $[Cl^-]_i$, although it was not possible to estimate by how much $[Cl^-]_i$ was being altered. The results of both sets of experiments were the same - in some cases, (3 out of 7 for changes in $[Cl^-]_o$ and 3 out of 8 for changes in $[Cl^-]_i$), the point at which the magnitude of dopamine induced currents increased dramatically was shifted in the depolarising direction. Figure 17 shows a typical record in which $[Cl^-]_o$ was altered. In three further experiments, no effect was observed. No effect was seen on the magnitude of currents at potentials positive to the resting potential.

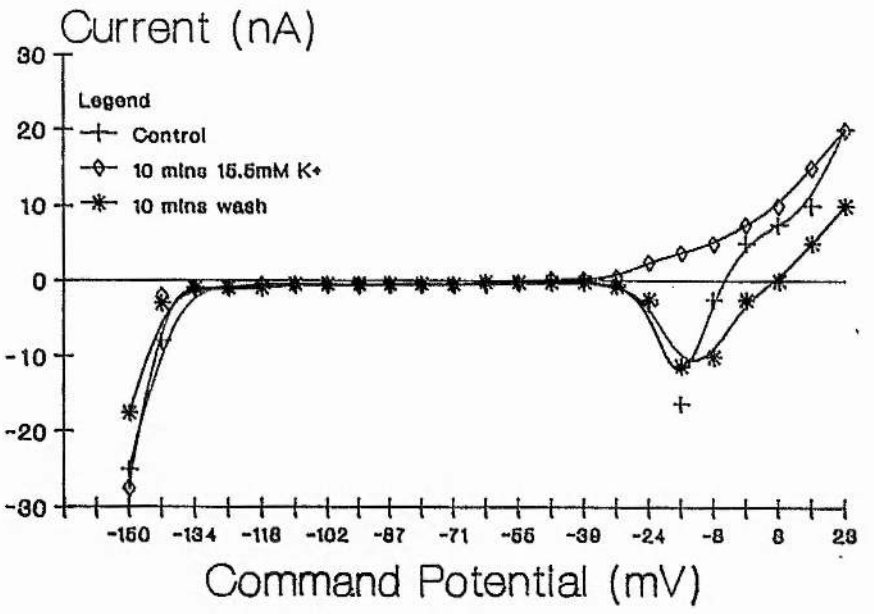


Figure 16 The effect of a five-fold increase in $[K^+]_o$, from 3,1 to 15,5 mM, on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV). In this experiment, this change reduced the inward current at positive potentials. In other experiments, however, no effect was observed.

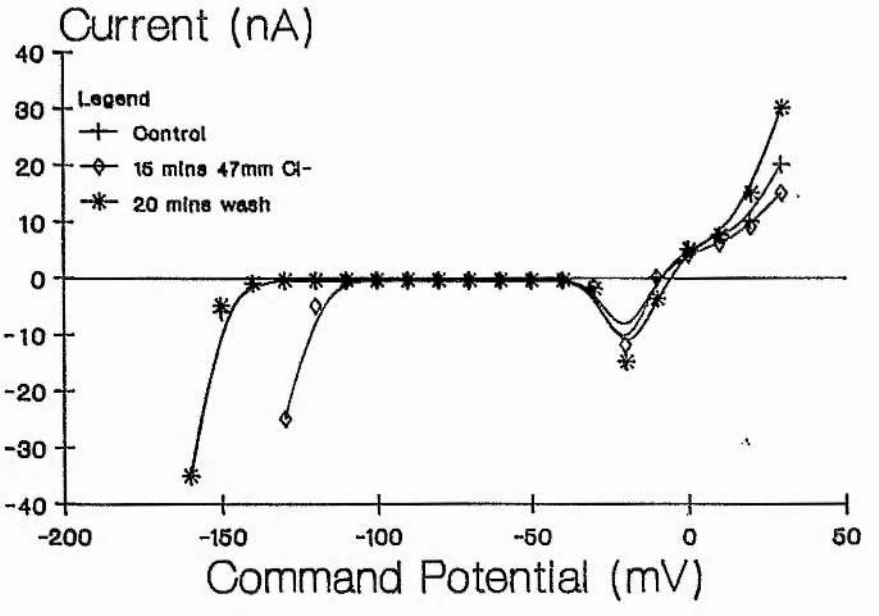


Figure 17 The effect of a five-fold reduction in $[Cl^-]_o$, from 235 mM to 47 mM, on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV). In none of the experiments was any effect observed on the positive end of the curve, but in some experiments, including that illustrated, the current at negative potentials was altered.

3.1.5.4 Effect of Alteration of $[Ca^{2+}]_o$ and the Use of Calcium Antagonists

In a series of experiments, $[Ca^{2+}]_o$ was reduced to 1/3 normal (3 mM) and 1/10 normal (0.9 mM) (3 and 5 experiments respectively), and zero (2 experiments). The Nernst equation would predict shifts of E_{Ca} of 13.8 & 29 mV respectively for concentrations of 3 and 0.9 mM Ca^{2+}, at 18° C.

Under current-clamp conditions, the response size was reduced, to a progressively greater extent, in 3 mM, 0.9 mM and nominally Ca^{2+}-free saline respectively. In 3 mM Ca^{2+}, the response was reduced to approximately 75% of its original value: in 0.9 mM Ca^{2+}, to around 25%, and abolished completely in Ca^{2+}-free. In all three cases, the effects were reversible on washing with normal saline (Figure 18).

Under voltage-clamp conditions, the response showed little reduction in 3 mM Ca^{2+}. In 0.9 mM Ca^{2+}, the response was severely depressed being reduced by around 75 - 80% of its original value across the full range of command potentials studied (Figure 19).

In Ca^{2+}-free saline, no response could be observed, even at more negative potentials. Again, in all the voltage-clamp experiments, the effects of low Ca^{2+} were completely reversed on washing with normal saline.

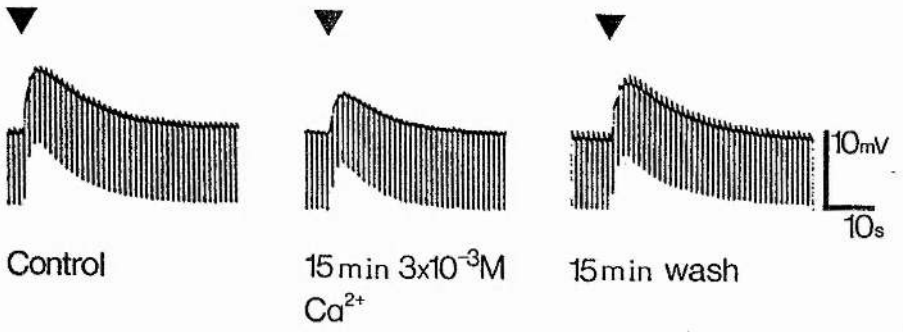


Figure 18 The effect of a three-fold reduction in $[Ca^{2+}]_o$, from 9 mM to 3 mM, on the current-clamp response to pressure-applied dopamine. The response was reversibly suppressed. Resting potential was -54 mV, and current pulses were applied through a second microelectrode.

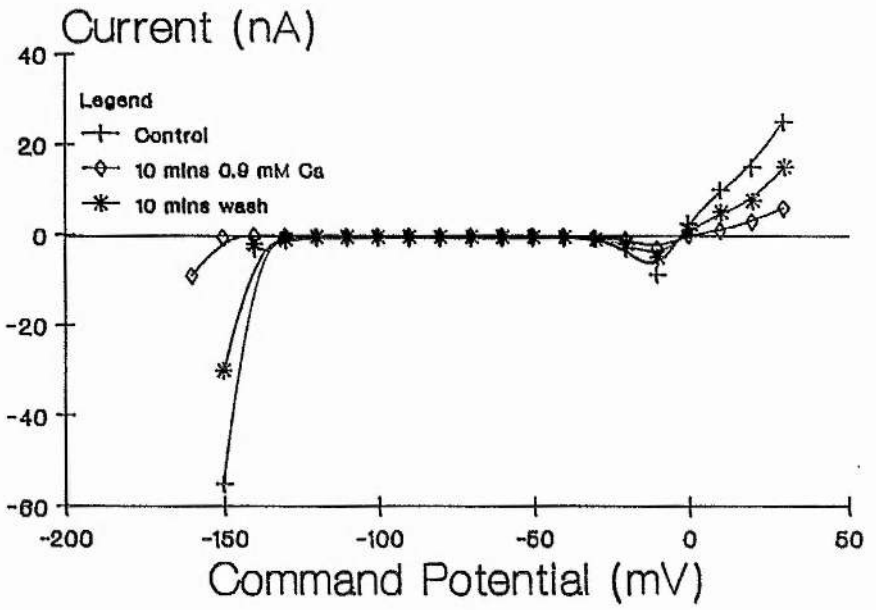


Figure 19 The effect of a ten-fold reduction in $[Ca^{2+}]_o$, from 9 mM to 0,9 mM, on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV).

In five experiments under voltage-clamp, $[Ca^{2+}]_o$ was increased to 2 and 4 times normal (18 & 36 mM). These changes would shift E_{Ca} by 8.7 and 17.5 mV respectively, at 18° C. In both cases an enhancement of the response was observed. The maximal response value was increased by up to 25% in 36 mM Ca^{2+} (Figure 20). The effect was reversible on washing with normal saline.

Pharmacological agents which block Ca^{2+} channels were also used to investigate the effect of Ca^{2+} on the response. Blockade of Ca^{2+} channels would be expected to have a similar effect to the reduction of $[Ca^{2+}]_o$, since both will reduce the amount of Ca^{2+} crossing the membrane in response to a stimulus.

Experiments were performed using the organic Ca^{2+} channel blocker verapamil, and the inorganic competitive antagonist ion cadmium. This choice was made since it has been suggested (Oyama et al, 1986) that organic and inorganic Ca^{2+} antagonists can have differing effects on certain characteristics of Ca^{2+} currents, Verapamil appearing to block the Ca^{2+} channel from the intracellular end (Fleckenstein, 1988). Cadmium ions are known to block Ca^{2+} channels by competition, since they are of a similar size and charge to Ca^{2+}.

In twelve experiments (5 under current-clamp, 7 under voltage-clamp) verapamil reversibly reduced the response size. A final bath concentration of 1×10^{-3} M verapamil gave rise, after incubation times up to 25 minutes, to a reversible and virtually complete abolition of the response current over the full range of command potentials tested under voltage-clamp (Figure 21). This also gave

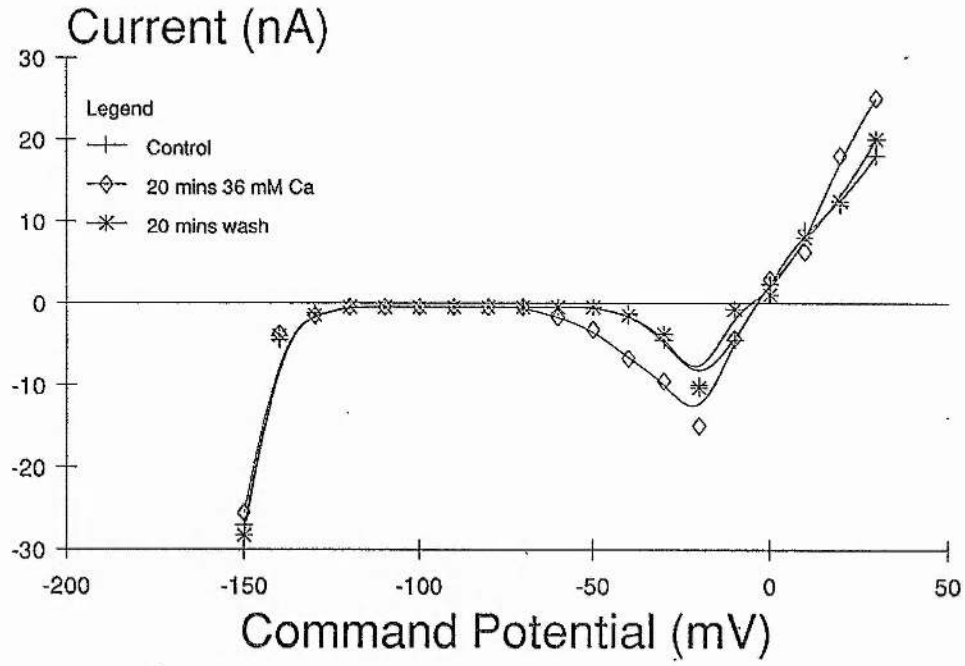


Figure 20 The effect of a four-fold increase in $[Ca^{2+}]_o$, from 9 mM to 36 mM, on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV).

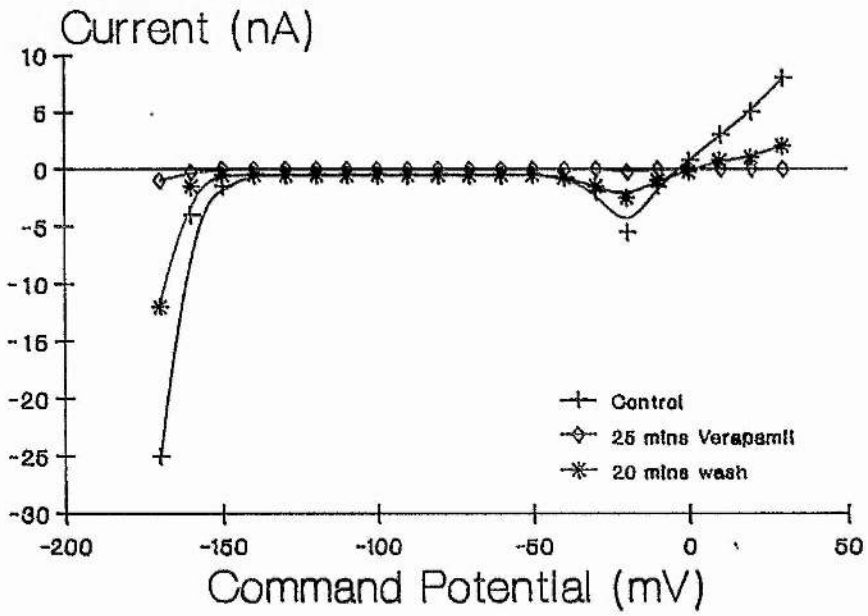


Figure 21 The effect of 1×10^{-3} M verapamil on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV).

rise to a reversible reduction of the magnitude of the depolarising response, to about 10% of its original size, under current-clamp.

In nine experiments (3 under current-clamp, 6 under voltage-clamp), a final concentration of 1 x 10^{-3} M cadmium resulted, after 10 - 15 minutes incubation in a reversible suppression of the DA-induced currents seen under voltage-clamp (Figure 22). Under current-clamp, the size of the depolarising response was also reversibly reduced. The reversal of the effects of cadmium was in all cases achieved within 20 to 40 minutes.

3.1.5.5 Effect of Alteration of $[Ca^{2+}]_i$

Experiments were also performed using microelectrodes filled with tri-potassium citrate, which chelates free calcium. It was assumed that impalement would thus reduce $[Ca^{2+}]_i$, since this chelation would cause a fall in the cytosolic calcium concentration. In turn, this would be expected to cause stored calcium to move down its concentration gradient into the cytosol, and so progressively deplete calcium stores. Given the fact that raising $[Ca^{2+}]_o$, and hence increasing the trans-membrane gradient for calcium enhanced the size of the response to dopamine, chelating internal calcium would be expected to have the same effect.

Of the six experiments performed under voltage-clamp, three showed some enhancement of the response size. In a typical control experiment, the peak inward current value at a command potential of -20 mV might be 10 nA. The mean peak current at -20 mV for the six

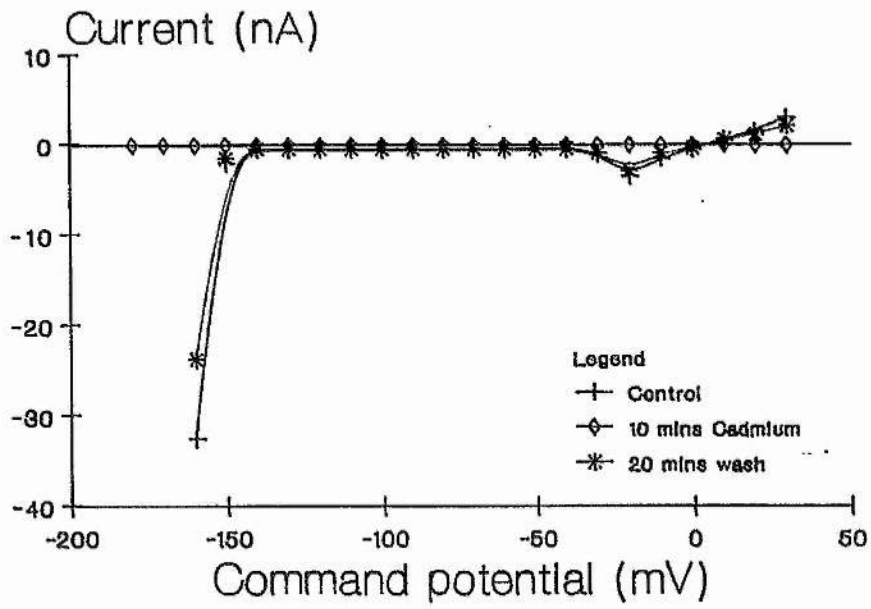


Figure 22 The effect of 1 x 10^{-3} M cadmium on the dopamine-induced response current at a range of command potentials (holding potential was -60 mV).

experiments performed with tri-potassium citrate was 17.8 nA (± 2.04 SD) compared with 9.1 nA (± 2.29 SD, n = 7, $p<0.001$) for experiments performed with potassium acetate. However, this increase in size occurred predominantly at potentials positive to the normal resting potential. Little or no effect was seen in any of the four experiments under current-clamp (Figure 23).

3.1.5.6 Computer-Driven Experiments

A series of experiments was performed using a computer-driven step-pulse system, and a high-speed, high-compliance voltage-clamp amplifier, to investigate the effect of exogenous dopamine on the net outward currents seen during pulses under voltage-clamp.

Four experiments were performed and of these three showed a clear suppression of the net outward current by 5 mM dopamine (in 1% Na/MBS saline) in the bath circulation. In only one experiment (Figure 24), however, was this effect fully reversible. The reduction was of the order of 10% of the maximal value, over the full range of command potentials tested (-70 to 0 mV).

3.1.6 Discussion

In the experiments reported here, the response under current-clamp at the resting potential of the cell (-50 to -60 mV) was a depolarisation, associated with a modest increase in membrane conductance. This much was demonstrated by Fleming (1986) and Pitman & Baker (1989). The magnitude of the response was relatively small

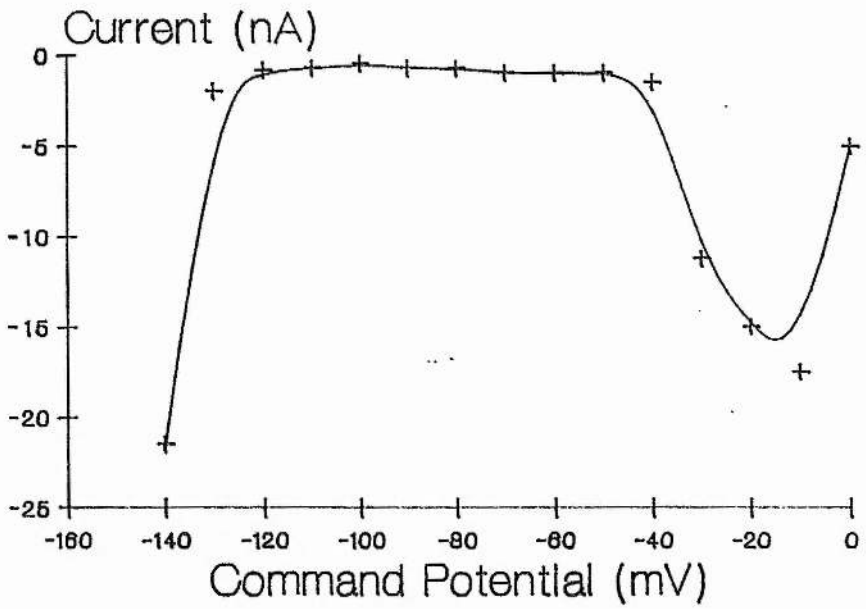


Figure 23 The response to dopamine after intracellular injection of citrate anions. This would be expected to reduce $[Ca^{2+}]_i$ by chelation. By comparison with a control experiment, the maximum inward current at around -20 mV was enhanced by approximately 50%. Holding potential was -60 mV.

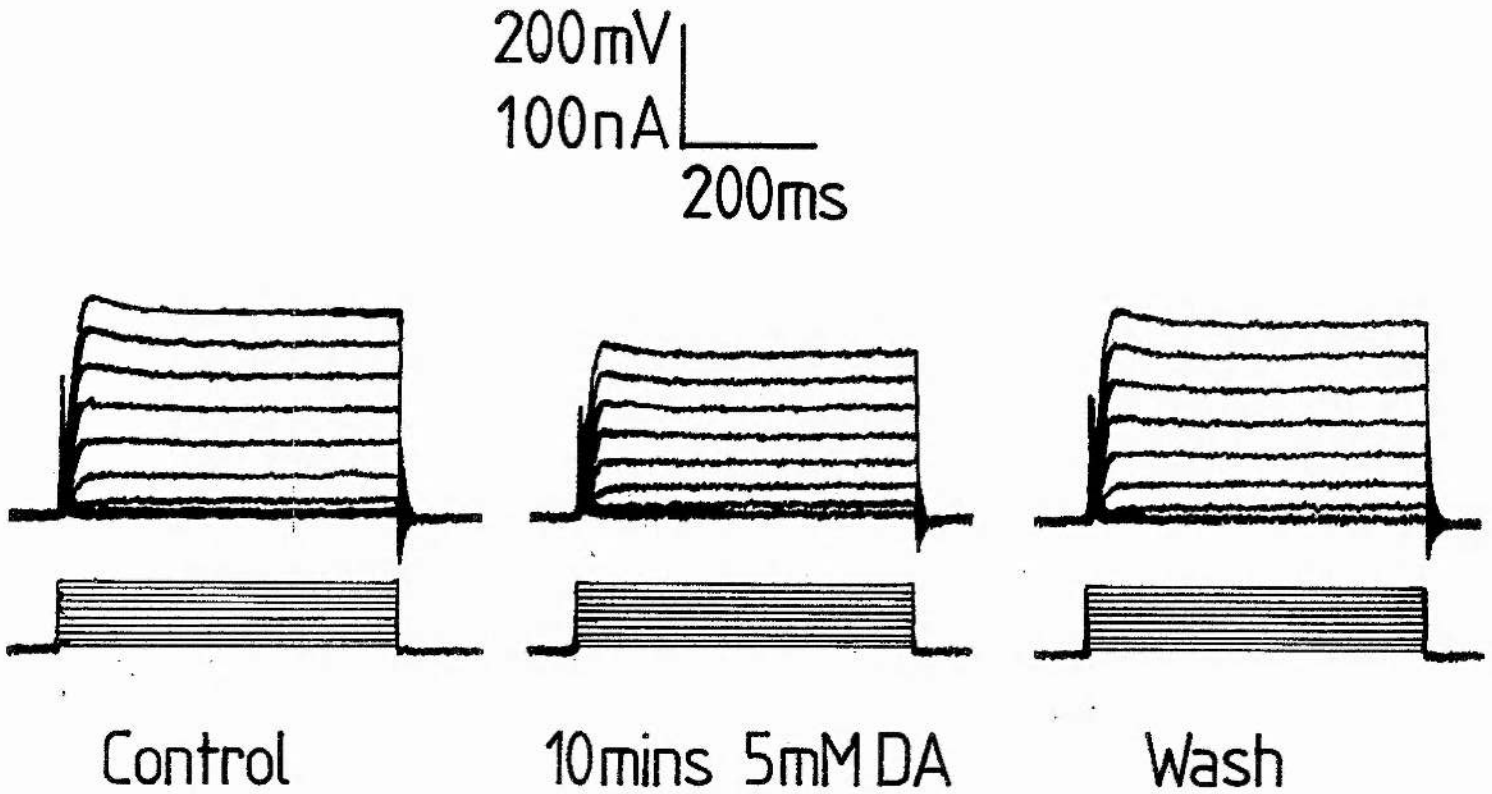


Figure 24 The effect of bath-application of 5 mM dopamine on the magnitude of the net outward current produced by depolarising voltage steps in multiples of 10 mV from a holding potential of -70 mV to a maximum of +30 mV.

(approx. 10 mV), and this, coupled with the small change in conductance, might suggest that the principal action of dopamine was not being observed at these potentials.

The response to dopamine was susceptible to desensitisation, unlike the hyperpolarising response of the acinar cells of the cockroach salivary gland (Bowser-Riley & House, 1976). This distinction between the two types of response might suggest that the two distinct receptor types thought to mediate the responses in molluscan (Berry & Cottrell, 1975; Stoof, de Vlieger & Lodder, 1985; Bokisch & Walker, 1988; Audesirk, 1989) and insect neurones may have different affinities for dopamine, and that there may be differences in the rate of dissociation of the receptor-transmitter complex.

Under voltage-clamp conditions, at or near the resting potential of the cell (≃-60 mV), the response was evident as a net inward current, usually no larger than 0.5 nA. At potentials between -50 and -100 mV, the response was of a relatively constant magnitude, but as the membrane potential was stepped to progressively more negative levels, the magnitude of the inward currents induced by dopamine increased rapidly, this change occurring at around -140 mV.

When the membrane potential was stepped to potentials positive to RP, a marked increase in the magnitude of inward currents was seen. Under these conditions, the response current increased progressively at potentials from RP (≃-60 mV) to -30 mV, reached a peak, usually approximately 10 nA, at around -20 mV, and then declined, reversing

at around 0 mV, to be seen as an increasing outward current at more positive potentials.

The unusual nature of the voltage-dependency of the response required that control experiments be performed to preclude the possibility that these results were artefactual. In fact, there was no significant difference between responses obtained by microiontophoresis, bath application and pressure ejection of dopamine. In addition experiments using the vehicle alone and altering $[pH]_o$ indicated that the response could not be attributed to these.

There are two possible reasons for this voltage-dependency. First, the process of binding and dissociation of dopamine to receptors may itself be voltage-dependent. In other words, the affinity of the receptors for dopamine may change with membrane potential. Although receptors of different affinities for dopamine have been isolated, and this has formed a part of the basis for their classification, (Kebabian & Calne, 1979; List et al, 1980; Creese et al, 1983), no evidence has yet been published to suggest that these differences result from voltage-dependency of the binding-dissociation process.

Second, and more likely, is the possibility that the ion channels linked to the dopamine receptors which mediate the response current may themselves be voltage-dependent. Voltage-sensitive ion channels have been known for many years. For example, Hodgkin & Katz (1949) and Hodgkin & Huxley (1953) showed that the sodium and potassium components of the action potentials in the squid axon were mediated

by voltage-dependent channels, which allowed the separate components to be activated at appropriate membrane potentials for their function, and voltage-dependent potassium channels are responsible for the modulation of excitability in amphibian sympathetic ganglion cells (Brown & Adams, 1980). The NMDA receptor has been shown to modulate a mixed ion channel, normally blocked by magnesium ions at resting potential. Depolarisation dislodges these magnesium ions and permits larger currents to flow (Mayer, Westbrook & Guthrie, 1984; Novak et al, 1984). In a variety of molluscan neurones, 5-HT receptors have been shown to modulate calcium currents (Pellmar & Carpenter, 1979; 1980; Cottrell, 1982; Baxter & Byrne, 1989). More recently, the modulation of voltage-gated calcium channels by G-protein-mediated systems has been demonstrated in a number of tissues (Tsien et al, 1988; Rosenthal et al, 1988; Brown et al, 1981).

It is perhaps unlikely that the dramatic increase in current magnitude at the more negative potentials has any physiological significance, since potentials as negative as this are unlikely to occur *in vivo*. Since in most neurones, E_{Cl} is negative (around -60 mV), it was important to investigate the possible contribution of chloride ions to the response in this region of the IV curve. However, alteration of $[Cl^-]_o$ or $[Cl^-]_i$ produced only an unreliable shift of the IV curve in response to a predicted shift of E_{Cl}. A 5-fold reduction in $[Na^+]_o$ also occasionally suppressed currents at the more negative end of the IV relationship, which was not affected by changes in $[K^+]_o$. Reduction of $[Ca^{2+}]_o$ routinely suppressed the response across the full range of comand potentials, as did the calcium channel blockers cadmium and verapamil. It therefore remains

unclear whether, or to what extent, sodium, potassium, or chloride ions contribute to the response current at these more negative potentials, although the reliability of the effect of changing calcium handling suggests an involvement of this ion in the mediation of the response, either as part of a gating mechanism for other ion currents, or as current-carrying ions.

If, as seemed likely, the inward current component seen at potentials around -20 mV was physiologically the more important, then an understanding of its ionic basis was essential. Alterations of $[Na^+]_o$ and $[K^+]_o$ would be expected to shift the reversal potential of the response (and to shift the IV curve under voltage-clamp). In practice, the results of these ion-substitution experiments were equivocal. In some cases, but not in all, increasing $[K^+]_o$ from 3.1 to 15.5 mM reversibly abolished the inward current peak at around -20 mV. Moreover, a 5-fold reduction in $[Na^+]_o$ occasionally suppressed this inward current component, and sometimes also exerted an influence at the more negative end of the IV relationship, which was not affected by changes in $[K^+]_o$.

Similar observations have been made in other invertebrate preparations. For example, Wilson & Wachtel (1974; 1978) demonstrated that prolonged cholinergic and dopaminergic inhibition in *Aplysia* neurones caused a region of negative-slope resistance to appear on the current voltage curve. This was altered by ion-replacement in the bathing solution, sodium-free sea water abolishing the region of negative-slope resistance. These results show similarities with the effect seen in the present study, in which the

inward current peak was abolished (albeit unreliably) by reduction of $[Na^+]_o$.

The source of variability of the results obtained in the present study by ion substitutions is unclear. It is possible however that local variations in ion concentration in the immediate vicinity of the soma may be responsible, and these may result from variation in cleaning of the cell undertaken at the beginning of each experiment. It is also possible that infolding of the cell membrane is responsible for trapping ions close to the membrane, thus locally altering the concentration. This has been suggested as a reason for the deviation of the experimentally determined reversal potential from that predicted for a calcium-activated potassium conductance in cell 28 of the cockroach metathoracic ganglion (Thomas, 1984). It is known that the insect CNS possesses an efficient blood-brain barrier (Treherne & Pichon, 1972), and the extent to which this may contribute to the restriction of access to the soma remains unknown.

Ion substitution experiments demonstrated to Pellmar and Wilson (1977) that while the magnitude of the current induced in response to 5-HT was unaffected by increasing $[K^+]_o$, it was consistently reduced by lowering $[Na^+]_o$, and they also concluded that sodium ions were involved in the mediation of the response at these depolarised potentials. It was later demonstrated, however, that this current was carried, at least in part, by calcium ions (Pellmar & Carpenter, 1979; 1980).

Cottrell (1982) found a synaptically generated hyperpolarising response in a follower cell (the A neurone) of a giant 5-HT containing neurone in *Helix*, which was mimicked by the application of 5-HT to the A neurone. The response was unaffected by replacement of extracellular sodium or potassium, but appeared either directly or indirectly to involve calcium ions, and showed a definite voltage-dependency, increasing in magnitude at more depolarised potentials.

Juel (1984) has shown that long-lasting synaptic inhibition of a neurone in *Helix pomatia* was the result of inhibition of a voltage-dependent calcium current. The synaptic events leading to this suppression were shown to be mediated by dopamine (Juel, 1983).

Cottrell (1988) has also demonstrated a suppressive effect of 5-HT on a voltage-dependent potassium current in *Helix* C1 neurones. This current appears not to be modulated by cyclic AMP, but by protein kinase C and phosphorylation, perhaps activated by a G protein. Further evidence in support of this has been provided by the actions of the phosphatase inhibitor, okadaic acid (Cottrell, 1989; Cohen et al, 1990), which potentiated the response to 5-HT (Cohen et al, 1989)

A steady-state calcium-activated potassium current modulated by 5-HT has been observed in tail sensory neurones of *Aplysia* (Walsh & Byrne, 1989; Baxter & Byrne, 1989). When sensory neurones were depolarised by application of pulses, the magnitude of the elicited outward current was reduced by around 30% by 5-HT. This effect is apparently mediated by alteration of the intracellular level of cyclic AMP.

The results obtained in the present study have a number of similarities with those reported above. Reduction of $[Na^+]_o$ reduced the size of the inward currents, suggesting that sodium ions might play a part in the mediation of the response. In addition, the results of the computer-controlled experiments indicated that the effect of dopamine was to reduce the magnitude of the overall outward currents induced by voltage-steps. However, the result of increasing $[K^+]_o$, which suppressed the inward current peak, was inconsistent with this, since increase of $[K^+]_o$ would be expected to suppress outward currents by reducing the electrochemical gradient. Alterations of chloride concentrations, both inside and out, also caused shifts of the IV relationship for dopamine, but none of these effects were reliably reproducible, and it is not possible at present to establish in any detail the ionic basis of the response to dopamine with respect to sodium, potassium or chloride.

The results of experiments involving alteration of calcium concentrations, however, provided reproducible results, and it appears that calcium may play an important role in the response to dopamine in this preparation. This role may take two forms. Firstly calcium may actively carry a proportion of the response current, or secondly calcium may be responsible for the activation of other currents, for example calcium-activated potassium currents. Reduction of $[Ca^{2+}]_o$ suppressed the response, and its removal abolished it altogether. Equally, increasing $[Ca^{2+}]_o$ enhanced the response, as did reducing $[Ca^{2+}]_i$ with tri-potassium citrate. Moreover, the calcium channel blockers cadmium and verapamil both abolished the response. The suppression of the response by the

reduction of external calcium does not indicate whether calcium acts as a trigger or a current carrier. However, since lowering internal calcium or raising external calcium both enhanced the response, it is less likely that calcium is activating a current carried by another ion.

Calcium ions modulate or control many intracellular metabolic processes, and the concentration of free intracellular calcium is tightly controlled by a complex homeostatic system. Calcium may be bound by cytosolic proteins, such as calmodulin, or stored in organelles such as the endoplasmic reticulum. Calcium channels exist in both the cell membrane and in the boundary membranes of such organelles, and their variety and control has been widely studied (McBurney & Neering, 1987; Tsien et al, 1988). It has been shown that the response to dopamine of the neurone D_3 is not mediated by changes in the intracellular concentration of cyclic AMP (Fleming, 1986; Pitman & Baker, 1989).

In this preparation, however, it is not clear whether calcium ions actually carry a proportion of the dopamine-evoked current, or whether they serve as a trigger, perhaps activating some form of calcium-sensitive current mediated by sodium or potassium ions. Sufficient data is not presently available to allow this question to be adequately addressed.

It is not certain, however, that the voltage-dependence of this response is the result of the activation of voltage-dependent channels of the classical type, which function as a result of

voltage-induced conformational change, which may then alter the ability of the ionophore to conduct.

The depolarising action of glutamate on N-methyl-D-aspartate (NMDA) receptors has also been shown to be voltage-dependent (Mayer et al, 1984; Nowak et al, 1984). In this case, at the normal resting potential of the cell, the ionophore is blocked by magnesium ions. The depolarising response of cultured embryonic mouse spinal neurones to glutamate was small at potentials near the normal resting potential of the cell. If, however, the cell was depolarised by other synaptic input, magnesium ions were dislodged from the ion channels and the depolarising effect of glutamate was greatly enhanced. It remains a possibility that a similar mechanism might operate to enhance the action of dopamine at more depolarised potentials. However, since the normal saline solution in which the voltage-dependence of the response was observed was nominally magnesium-free, it is not likely that a precisely similar mechanism would be present.

Functional roles for aminergic transmission have been demonstrated in a number of preparations. Dopamine antagonists heve been shown to interfere with the motor program generation responsible for the locomotor waves in the snail, *Helix pomatia*. Injection of antagonists caused abnormalities in the rhythms of contraction, and also 'hyperactivity', leading to the conclusion that dopamine was acting as an inhibitory modulator in this system (Sakharov & Salánki, 1982). In 1983, Wieland & Gelperin, working on the snail *Limax maximus* observed that the application of dopamine to salivary

neurones caused excitation, and led to the initiation of a feeding motor program.

In the crustacea, the stomatogastric ganglion of the lobster *Panulirus interruptus* contains pyloric neurones which have been shown to depolarise when exposed to dopamine, and which alter the pattern of output from the rhythm generator responsible for stomach movement during digestion (Harris-Warrick & Flamm, 1986).

The octopaminergic modulation of the locust neuromuscular junction was first shown by Evans & O'Shea (1977), who found that an octopaminergic neurone inervated the extensor-tibiae muscle in the locust *Schistocerca gregaria*. By stimulation of this neurone, they produced a potentiation of the epsp and the twitch tension resulting from the stimulation of an identified motoneurone. More recently (Evans, 1981; 1984a; 1984b), the receptors mediating this response have been characterised, and it has also been shown that, in this same preparation, octopamine released at the neuromuscular synapse causes an elevation of cyclic-AMP levels within the muscle cells, indicating how octopamine may achieve its potentiating effect. Octopaminergic modulation of neuromuscular transmission has also been demonstrated in other insect preparations (Klaasen & Kammer, 1985; Klaasen, Kammer & Fitch, 1986).

House (1973), Bowser-Riley & House (1976) and House & Ginsborg (1982) have shown that dopamine consistently causes a hyperpolarising response in acinar cells of the salivary gland of the cockroach *Nauphoeta cinerea*. In the intact gland, the application of dopamine

was followed by an increase in secretion. The hyperpolarisation was insensitive to reduction of extracellular calcium, but secretion, and hence neuroglandular transmission, was suppressed.

In the honey bee *Apis mellifera*, MacMillan & Mercer (1987) concluded that dopamine inhibited neurones which were involved in the conditioning of the bee to respond to a stimulus of sugar by extending its proboscis. Dopamine suppressed this conditioning, although the authors stressed that conclusions concerning the cellular actions of dopamine should not necessarily be drawn from these results.

A common inhibitory (CI) motoneurone may exert an influence over many follower cells at the same time, and the CI neurones of the cockroach may be involved in the walking cycle (Pearson & Bergman, 1969). If, as is demonstrated by the results of the present study, CI motoneurone D_{∞} responds to dopamine by depolarising, this would enhance any inhibitory effect it may have upon follower neurones by enhancing release of inhibitory transmitter at its terminals on muscle end-plates. If synaptic pathways existed whereby the degree of depolarisation in the excitatory nerve fibres or the muscle itself could be made to influence the membrane potential of the CI neurone, perhaps by the release of dopamine, then the voltage-dependency of the response would allow increased inhibition to be applied at correspondingly higher levels of depolarisation. In other words, the greater the degree of excitation, the greater the feedback signal to the CI neurone, and the larger the amount of inhibition applied to the muscle. Prolonged exposure of neurone D_{∞} to dopamine causes it

to exhibit bursting activity (Pitman & Baker, 1989). This is contrary to the observations of Wilson & Wachtel (1974; 1978) who showed that prolonged dopaminergic inhibition in neurones of *Aplysia* suppressed burst production. However, it is possible that dopamine may have a role in the control of motor programming.

Further work is needed to identify the ions involved in carrying the response current and the involvement of calcium in its mediation. In addition, the tracing of synaptic connections onto and from D_2 would allow the functional significance of the voltage-dependence of the response to be explained.

3.1.7 Summary

At the normal resting potential of the cell, the response to dopamine under current-clamp conditions was a depolarisation associated with a slight fall in the input resistance of the cell. Under voltage-clamp, this was seen as an inward current. In both cases, the response showed a marked voltage-dependency, increasing greatly in magnitude at more negative potentials (between -120 and -150 mV), and, under voltage-clamp, also increasing to a lesser extent at potentials around -20 mV. The current magnitude then fell towards zero and apparently reversed at around 0 mV. The response under current-clamp did not appear to reverse. The response could also be obtained by the use of microiontophoresis or bath application of dopamine.

The effects of changing external concentrations of Na^+, K^+, Cl^- and Ca^{2+} were investigated. The effects of changing Na^+, K^+ and Cl^- were inconsistent, sometimes causing abolition of the region of negative slope resistance induced at potentials around -20 mV, and sometimes having no effect. Alteration of $[Cl^-]_i$ had similarly unpredictable effects. This variability may be due to difficulties of access, or the presence of an efficient blood-brain barrier.

Reducing $[Ca^{2+}]_o$ caused suppression of the response, and chelation of internal Ca^{2+} by tri-potassium citrate enhanced the response undr voltage-clamp, but not under current-clamp. Similarly, raising $[Ca^{2+}]_o$ enhanced the response. The calcium antagonists verapamil and cadmium both suppressed the response.

The net outward currents produced by depolarising voltage steps were depressed by dopamine.

3.2 DISTINCTIONS BETWEEN RESPONSES TO DOPAMINE, NORADRENALINE, OCTOPAMINE, & ACETYLCHOLINE

3.2.1 Introduction

Having determined the characteristics of the response of neurone D3 to dopamine, and gained some insight into the ionic events which might underlie it, the next important step was to demonstrate that the observed effects were not the result of the actions of dopamine on receptors for other transmitter candidates.

Dopamine, noradrenaline, octopamine and acetylcholine are all present in the cockroach CNS (see Section 1.- General Introduction). Noradrenaline and octopamine are both sufficiently similar in structure to dopamine for it to be a possibility that dopamine might be acting either on receptors for one of these amines, or on a generalised amine receptor.

Nathanson and Greengard (1973) suggested that the effects of noradrenaline on cyclic AMP levels in CNS neurones might be mediated by the interaction of noradrenaline with receptors for dopamine coupled to adenylate cyclase. Noradrenaline receptors that are not linked to adenylate cyclase may, however, be present.

Octopamine has been localised within the insect CNS (eg Dymond & Evans, 1979; Fleming & Pitman, 1982), and has been shown to be involved in the control of leg muscles in the locust (Evans, 1980;

1981; 1982). Evans showed that dopamine was significantly less potent in eliciting the observed effects, and it was concluded that they were the result of activation of specific octopamine receptors. Furthermore, it was shown (Evans, 1981) that there were three classes of octopamine receptors, octopamine$_1$, octopamine$_{2A}$ and octopamine$_{2B}$, and that it was possible to distinguish pharmacologically between them. In addition, he was able to distinguish pharmacologically between the actions of octopamine and dopamine.

Acetylcholine, although not a structurally similar molecule to the amines, is a common transmitter in insect systems, and as such it was important to be able to rule out the possibility that dopamine might be acting on acetylcholine receptors. This was especially so since previous work had shown that responses to both dopamine and acetylcholine were suppressed by the adrenergic antagonists phentolamine and propranolol (Pitman & Baker, 1989). This suggested the possibility that dopamine might also be acting on cholinoceptors.

In order to assess the likelihood that dopamine might not be acting at a receptor site specific for itself, it was decided to observe the effects of antagonists to dopamine, noradrenaline, octopamine, and acetylcholine respectively, on responses to all four transmitters. These compounds were tested under both voltage-clamp and current-clamp conditions.

The choice of antagonists was made in order to exploit the selectivity of certain compounds for specific receptors. The dopamine antagonist SCH 23390 has been shown to block D_1 dopamine

receptors in the vertebrate brain (Iorio et al, 1983). The ergot derivatives ergometrine and ergotamine have been used as adrenergic antagonists in many preparations (for example, Ascher, 1972). Metoclopramide has been used to distinguish between forms of the octopamine receptor (Evans, 1981), and is normally considered to be a potent D_2 dopamine receptor antagonist (Kebabian & Calne, 1979). However, preliminary experiments using metoclopramide on this preparation revealed that it apparently possessed no dopaminergic activity, and could therefore be used to distinguish between receptors for octopamine and dopamine. The nicotinic acetylcholine antagonist α-bungarotoxin is commonly used in insect preparations (Harrow et al, 1979), and has not been shown to suppress responses to unrelated transmitter compounds.

In all these experiments, the recordings made under current-clamp were taken at the normal resting potential of the cell (usually between -50 & -60 mV), whereas voltage-clamp recordings were made over a range of command potentials.

3.2.2 Responses to Noradrenaline, Octopamine, & Acetylcholine

3.2.2.1 The Response to Noradrenaline

Noradrenaline, at a concentration of 100 mM (in the ejection microelectrode), applied under current-clamp conditions, depolarised the cell, and caused a fall in input resistance (Figure 25). The magnitude of the response, (commonly between 2 and 5 mV), was smaller than that of the response to dopamine.

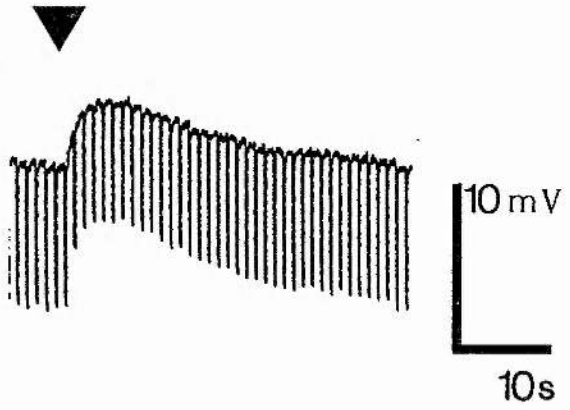


Figure 25 A single current-clamp response to pressure-applied noradrenaline. Resting potential was -66 mV, and current pulses were applied through a second microelectrode.

The response under current-clamp showed a degree of voltage-dependency. As the membrane was stepped to potentials progressively more negative with respect to the normal resting potential, the size of the response varied in a manner similar to that of the response to dopamine, increasing dramatically at potentials more negative than -120 mV. However, at potentials positive relative to the resting potential, the response size reduced, and became virtually undetectable, but showed no apparent reversal.

Under voltage-clamp, the voltage-dependency of the response to noradrenaline was, at potentials negative to the normal resting potential, very similar to that of the response to dopamine (Figure 26). At potentials close to the resting potential, the response was seen as a modest inward current. As the membrane potential was stepped progressively more negative than the resting potential, the magnitude of the induced current showed little change until a point was reached (usually between -120 mV and -150 mV), at which the magnitude of the induced current increased dramatically. At potentials more positive than the resting potential, however, the voltage-dependency was different from that of the response to dopamine. The response did not show the increase in inward current characteristic of responses to dopamine. Instead, the response reversed, and was seen as an outward current, increasing progressively in magnitude as the membrane potential was stepped to progressively more positive potentials.

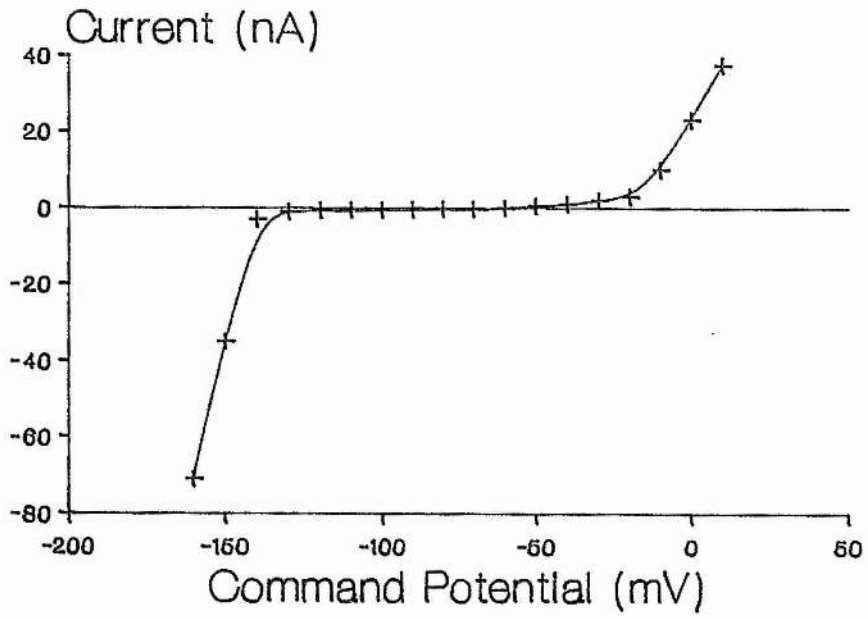


Figure 26 The voltage-dependence of the voltage-clamp response to noradrenaline. The illustration shows a difference curve produced from data obtained by the 'short-pulse' method in the presence and absence of noradrenaline. Holding potential was -60 mV.

3.2.2.2 The Response to Octopamine

To eliminate the possibility that the actions of dopamine in the present study were mediated via receptors for octopamine, the actions of exogenous octopamine on the preparation were determined. These were compared with those of dopamine.

Under current-clamp, pressure-application of 100 mM octopamine onto the cell surface, exactly as performed for dopamine, produced a depolarisation, associated with a small fall in input resistance (Figure 27). In general, the magnitude, both of the depolarisation, and of the fall in input resistance, were smaller than those induced by the application of dopamine, but not as small as those induced by the application of noradrenaline.

The response under current-clamp showed a degree of voltage-dependency (Figure 28). At potentials negative with respect to the normal resting potential, the response size increased in magnitude gradually as pregressively more negative command potentials were applied, but this rate increased once a particular point was reached, usually at around -130 mV. At potentials positive with respect to the resting potential, the response reduced in size, ultimately becoming undetectable, but did not appear to reverse, even at potentials as positive as +30 mV.

Under voltage-clamp, the current-voltage curve for the response to octopamine was essentially similar to that of the response to dopamine (Figure 29). At potentials close to the normal resting

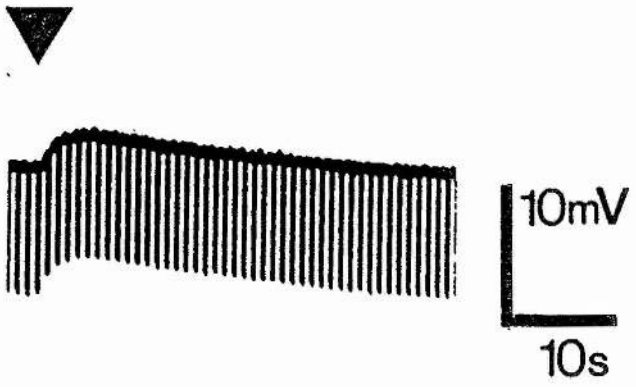


Figure 27 A single current-clamp response to pressure-applied octopamine. Resting potential was - 57 mV, and current pulses were applied through a second microelectrode.

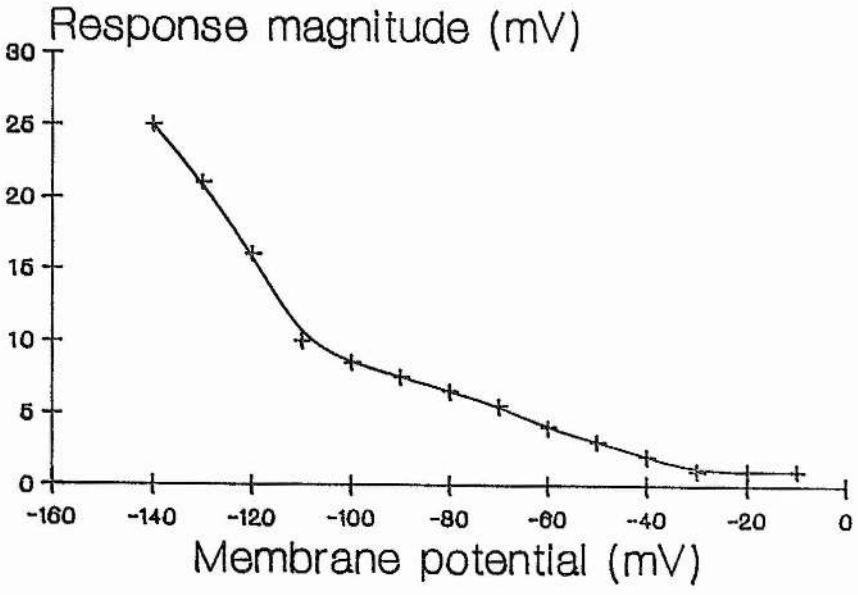


Figure 28 The voltage-dependence of the current-clamp response to octopamine. The membrane potential was stepped to a range of values by the passage of current through a second microelectrode. Resting potential was -56 mV.

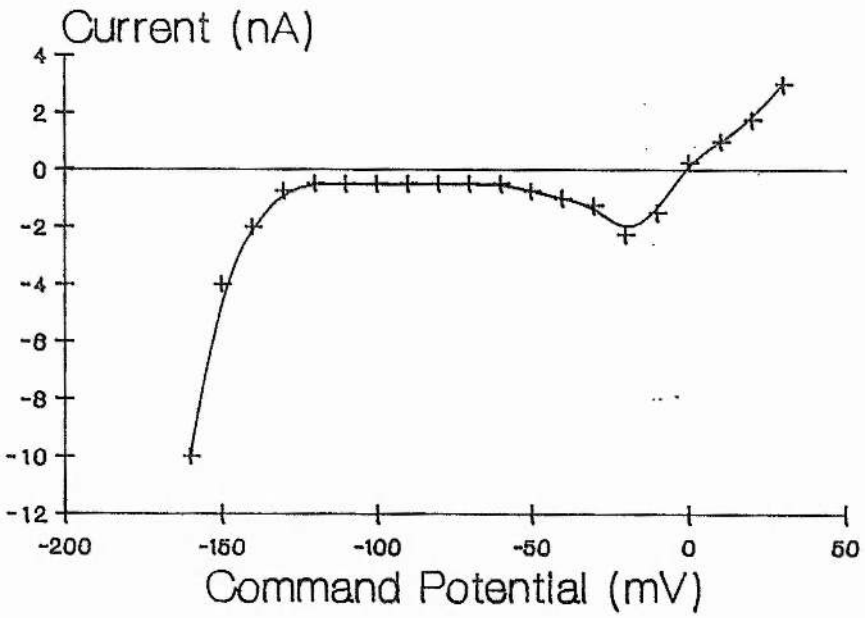


Figure 29 The voltage-dependence of the voltage-clamp response to octopamine. The curve was obtained from the difference between the magnitudes of current obtained by voltage pulses in the presence and absence of dopamine. Holding potential was -60 mV.

potential of the cell, the application of octopamine induced a small inward current. In general, the magnitude of this was smaller for a given application size than was the current induced by dopamine, commonly around 0.25 nA compared to 0.5 nA for an equivalent application of dopamine. At command potentials progressively more negative than the resting potential, the current remained at around 0.25 nA. A point was reached, however, usually between -130 mV and -160 mV, at which the magnitude of the induced inward current increased suddenly, in a similar fashion to that observed with the application of dopamine. In the case of octopamine, however, the magnitudes of the induced currents at given potentials were smaller, the rate at which it increased was usually less, and the point at which the increase commenced was often slightly more negative than for the response to dopamine.

At potentials more positive than the resting potential, the curve took a similar shape to that for dopamine. There was an increase in the magnitude of the inward current at potentials between -40 mV and -20 mV, the response reached a maximum at around -20 mV, and then reversed, at around 0 mV, to be seen as a net outward current. This situation was similar to that for dopamine, but, again, the overall magnitudes of the induced currents were generally smaller.

3.2.2.3 The Response to Acetylcholine

Under current-clamp conditions, acetylcholine applied by pressure-pulse (or in some cases by iontophoresis) caused a large depolarisation, up to 20 mV in amplitude, associated with a marked

fall in the input resistance of the cell, often to as little as 20% of the original value (Figure 30). The membrane conductance modulated by acetylcholine showed no voltage-dependency, the increase in response size with progressive hyperpolarisation being almost linear, as predicted if the response is mediated by channels which are not voltage-sensitive (Figure 31). By extrapolation, the reversal potential was estimated to be around -40 mV (mean value -44 mV, n = 8 ± 4.3 SD).

Under voltage-clamp, the response to pressure-applied acetylcholine, at potentials close to the normal resting potential of the cell, was seen as an inward current (Figure 32), the magnitude of which was substantially larger than that of the equivalent dopamine-induced current (around 6 nA compared with 0.5 nA for dopamine). As the cell was stepped to progressively more negative command potentials, the magnitude of the current increased in a more or less linear fashion. Conversely, as the membrane potential was stepped positive, the magnitude of the current fell, and the response reversed at a potential of around -40 mV, confirming the results obtained under current-clamp conditions (Figure 33).

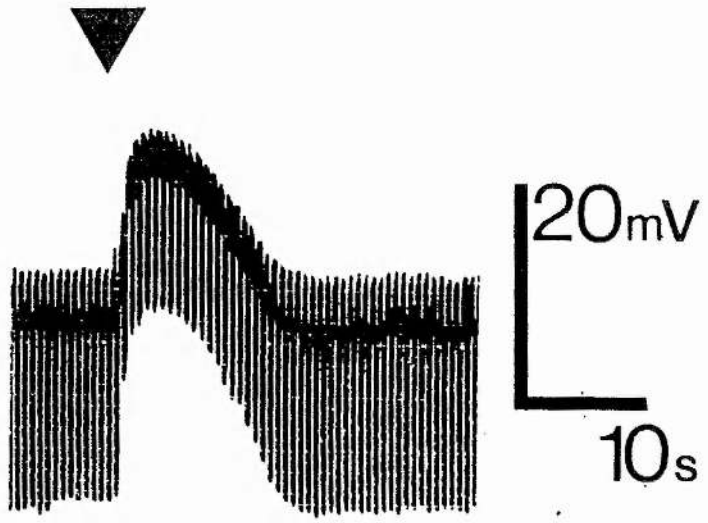


Figure 30 A single current-clamp response to pressure-applied acetylcholine. Resting potential was -63 mV, and current pulses were applied through a second microelectrode.

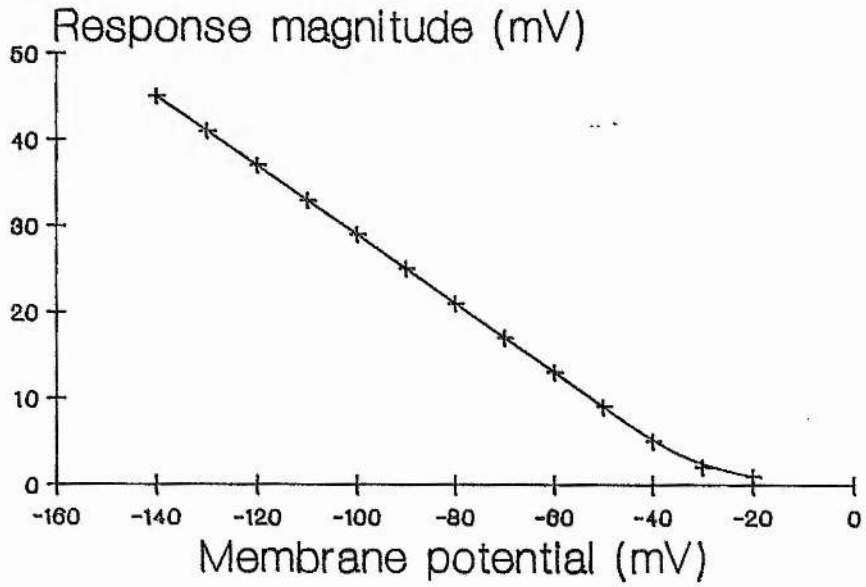


Figure 31 The voltage-dependence of the current-clamp response to acetylcholine. Resting potential was -59 mV, and the membrane potential was stepped to a range of values by the passage of current through a second microelectrode.

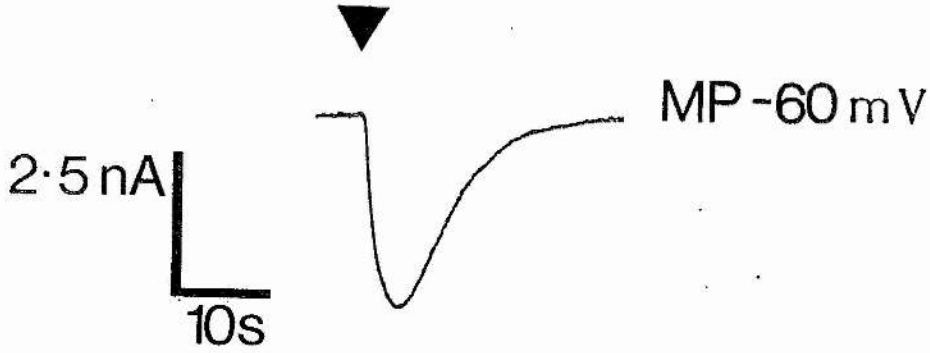


Figure 32 A single voltage-clamp response to pressure-applied acetylcholine. Resting potential was -60 mV.

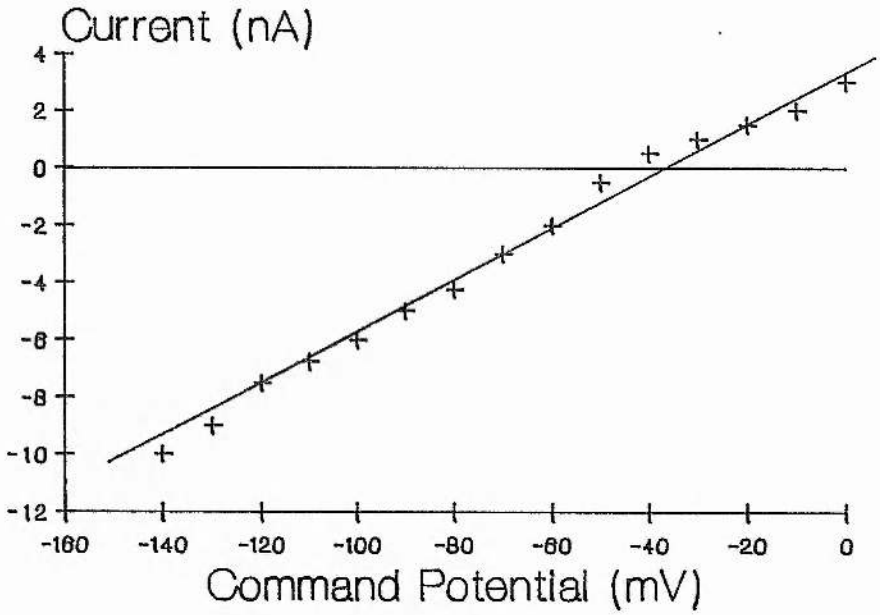


Figure 33 The voltage-dependence of the voltage-clamp response to acetylcholine. Data were obtained by the 'short-pulse' method, and the line drawn from the results of a linear regression analysis.

3.2.3 Pharmacological Distinction Between Responses to Dopamine, Noradrenaline, Octopamine, & Acetylcholine

3.2.3.1 Differential Effects of SCH 23390 & Ergotamine on Responses to Dopamine & Noradrenaline

The mammalian D_1 receptor antagonist SCH 23390 is a potent suppressor of the response to dopamine in this preparation. Under current-clamp conditions, at a concentration of 1 x 10^{-6}M, SCH reversibly suppressed the response to dopamine, and washed off completely after 10 to 20 minutes (Figure 34).

Under voltage-clamp conditions, the dopamine-induced currents were suppressed completely at a concentration of 1 x 10^{-6}M, indicating a total blockade of the response even at relatively negative command potentials, where the response current would be expected to be at its largest. In a typical experiment (Figure 35), before the application of SCH 23390, at a command potential of -160 mV, the magnitude of the induced current was -13 nA, -0.5 nA at -60 mV, and +15 nA at +30 mV. After 5 minutes exposure to 1 x 10^{-6} M SCH 23390, these values were reduced to 0 nA at all potentials. After 10 minutes wash, the response had been restored, giving response current magnitudes of -10 nA at -160 mV, -0.5 nA at -60 mV, and +10 nA at +30 mV. SCH 23390 had no apparent effect on the resting current-voltage relationship of the cell.

Conversely, 1 x 10^{-6} M SCH 23390 had no significant effect on the current-clamp response to noradrenaline, and the magnitude of the

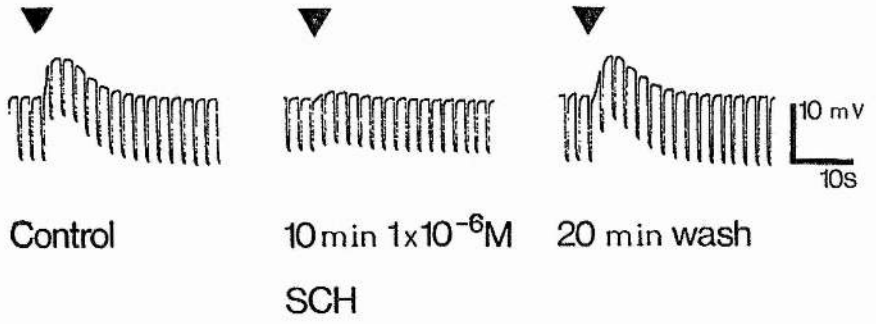


Figure 34 The application of 1 x 10^{-6} M SCH 23390 reversibly suppressed the current-clamp response to dopamine. Current pulses were applied through a second microelectrode, and resting membrane potential was -59 mV.

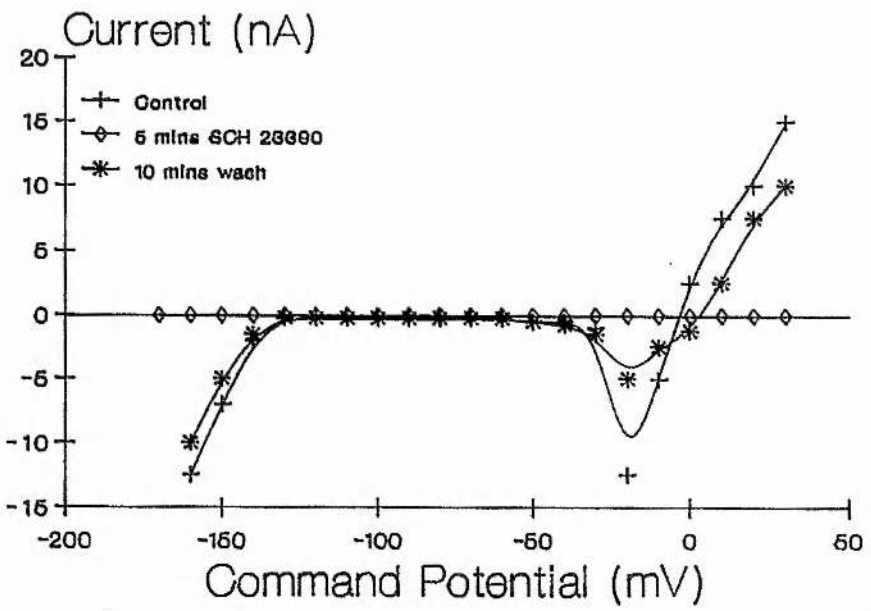


Figure 35 The application of 1 x 10^{-6} M SCH 23390 reversibly suppressed the voltage-clamp response to dopamine. Data were obtained by the 'short-pulse' method, and holding potential was -60 mV.

inward current seen in response to noradrenaline under voltage-clamp was not materially affected (Figure 36). In this example, at a command potential of -170 mV, the current magnitude was reduced from -56 nA before the application of 1×10^{-5} M SCH 23390, to -40 nA after 10 minutes incubation, but had increased to -50 nA again after 25 minutes incubation. At potentials positive to the normal resting potential, SCH 23390 had no detectable effect.

In contrast to the effects of SCH 23390, 1×10^{-5} M ergotamine had no detectable effect on the dopamine response current magnitude at any of a range of command potentials even after incubation times of up to 45 minutes (Figure 37). At potentials positive to the normal resting potential of the cell, no suppression of response was seen at all. At a command potential of -140 mV, the control experimental value of dopamine-induced current was -43 nA. Twenty minutes after the administration of 1×10^{-5} M ergotamine, the value was reduced to -25 nA. However, 15 minutes later, the value had once again increased to -31 nA, suggesting that any variation was not a result of the effects of ergotamine, but rather of the normal variability of the response current magnitude. No suppression was seen of the response under current-clamp conditions, and no effect was observed on the resting current-voltage relationship of the cell.

Some variability has also been seen in experiments using dopamine alone where successive runs of pulses have produced curves which are not identical, but which differ by a few nA between runs at a particular command potential.

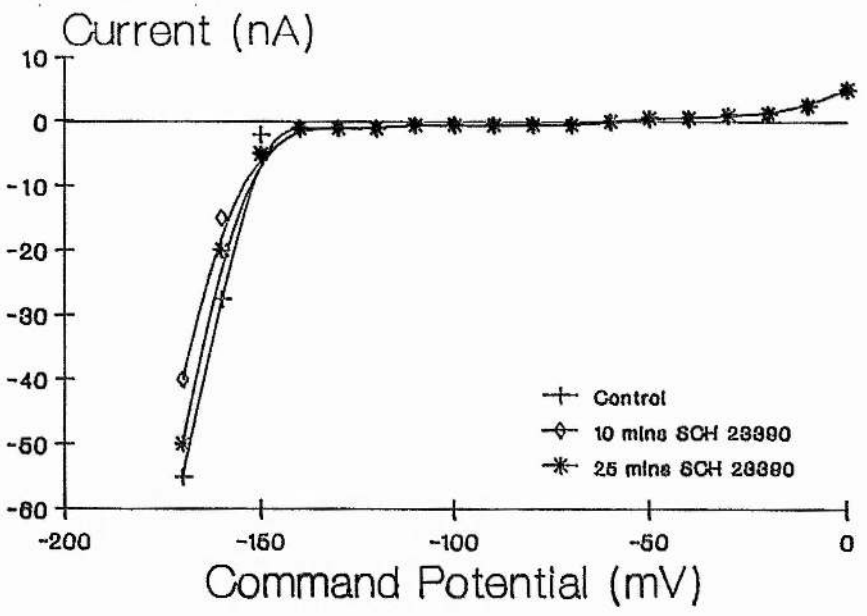


Figure 36 The inability of 1 x 10^{-6} M SCH 23390 to block the voltage-clamp response to noradrenaline. Holding potential -60 mV.

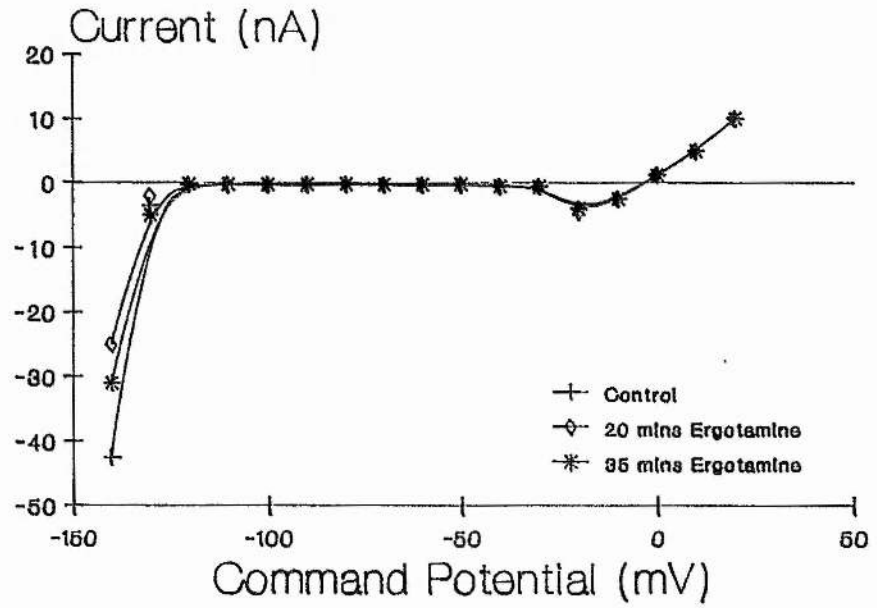


Figure 37 The inability of 1 x 10^{-6} M ergotamine to block the voltage-clamp response to dopamine. Holding potential -60 mV.

A typical voltage-clamp experiment, as illustrated in Figure 38, showed that 1 x 10^{-6} M ergotamine reversibly suppressed the response to noradrenaline after incubation times of between 25 and 40 minutes, across a range of command potentials. In the initial control run, the magnitudes of noradrenaline-induced currents were -33 nA at a command potential of -140 mV, -0.25 nA at -60 mV, and +10 nA at 0 mV. After 10, minutes exposure to 1 x 10^{-6} M ergotamine, these values were reduced to 0 nA at -140 mV and -60 mV and +1 nA at 0 mV. A current of -4 nA was observed however at the command potential of -180 mV. The response current magnitude recovered after 20 minute wash to values of -34 nA at -150 mV, -0.25 nA at -60 mV, and +8 nA at 0 mV. Under current-clamp conditions, the size and duration of the depolarising response was reduced, and returned to full size after 20 to 45 minutes wash-off. No effect was seen on the resting current-voltage relationship of the cell.

3.2.3.2 Differential Effects of SCH 23390 & Metoclopramide on Responses to Dopamine and Octopamine

The effective antagonism of dopamine responses by SCH 23390 has been described above (section 3.2.3.1).

Under both voltage- and current-clamp conditions, 1 x 10^{-5} M SCH 23390 had no detectable effect on the response to octopamine. It had no effect on the magnitude of octopamine-induced currents at any of the full normal range of command potentials, even after prolonged

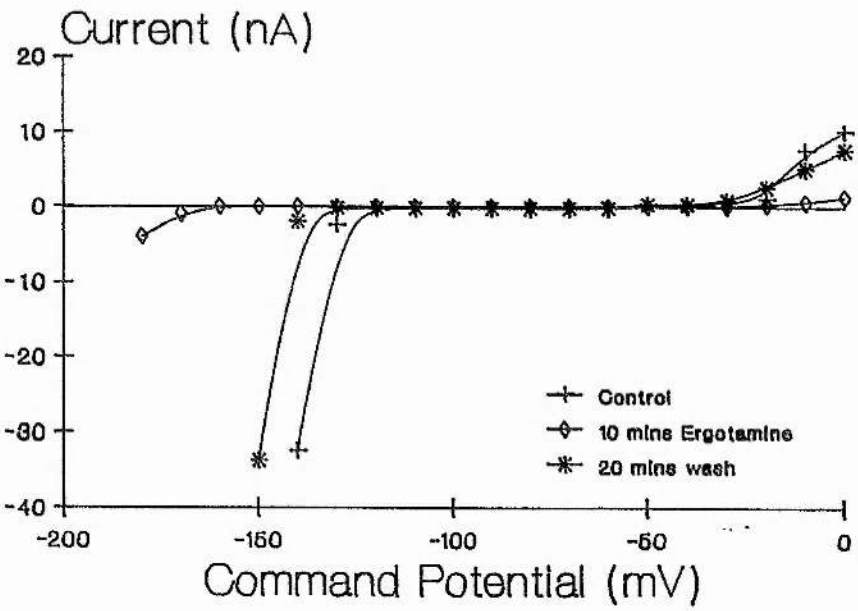


Figure 38 The application of 1 x 10^{-6} M ergotamine reversibly suppressed the voltage-clamp response to noradrenaline. Holding potential -60 mV.

incubation (in excess of 1 hour). The illustration shows the curves from a typical voltage-clamp experiment, in which the magnitude of induced currents was unaffected (Figure 39).

Metoclopramide is a mammalian dopaminergic antagonist, which has been used in the classification schemes for mammalian dopamine receptors as a specific D_2 antagonist (Kebabian & Calne, 1979). At a concentration of 1×10^{-5} M had no detectable effect on the response to dopamine under current-clamp conditions (Figure 40). Equally, in voltage-clamp experiments, no significant reduction in the magnitudes of the induced currents was observed. A typical voltage-clamp experiment is illustrated in Figure 41. The values obtained for the magnitudes of the induced currents were, -30 nA at a command potential of -140 mV, -0.5nA at -60 mV, and +10 nA at +30 mV. No detectable change was observed in these values after 10 and 25 minutes exposure to metoclopramide. This antagonist had no detectable effect on the resting current-voltage relationship of the cell.

In experiments under both current-and voltage-clamp conditions, exposure to 1×10^{-6} M metoclopramide reversibly suppressed the response to octopamine. In a representative voltage-clamp experiment (Figure 42), the octopamine-induced current was reversibly abolished, across the full range of command potentials, after 10 minutes exposure to metoclopramide. The response returned to full size after 15 minutes wash. In the control run, the response magnitude was -15 nA at a command potential of -150 mV, -0.5 nA at -60 mV, and +7 nA at +30 mV. After 10 minutes exposure to metoclopramide, the values were

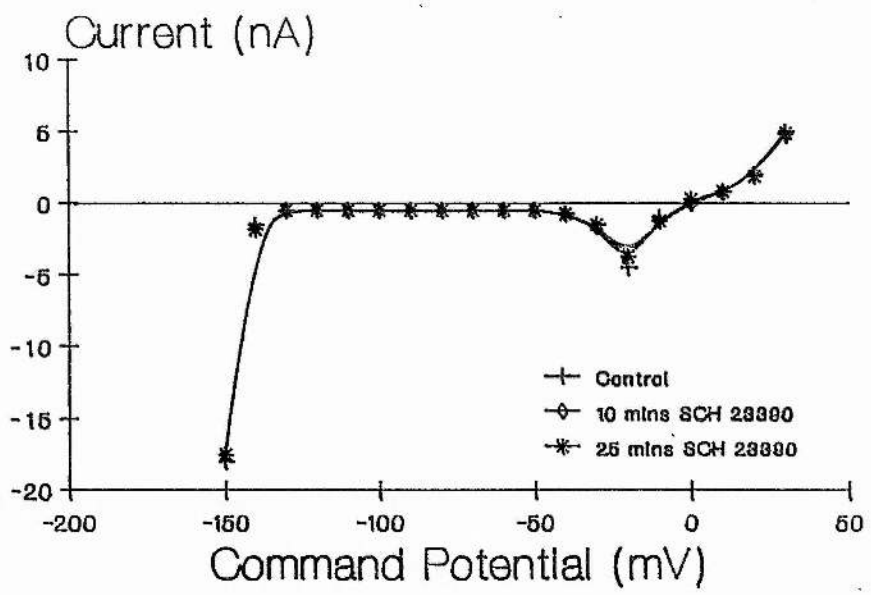


Figure 39 The inability of 1 x 10^{-6} M SCH 23390 to suppress the voltage-clamp response to octopamine. Holding potential was -60 mV.

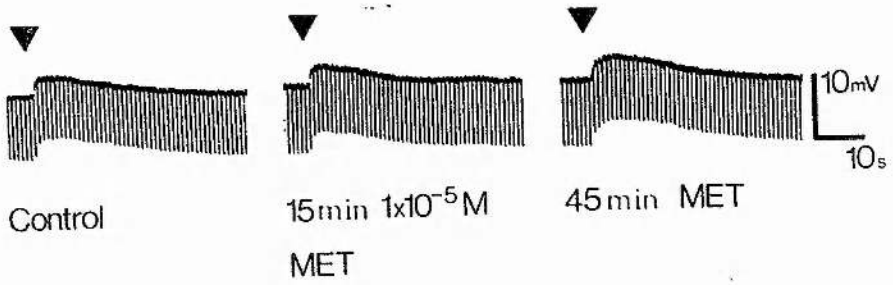


Figure 40 The inability of 1 x 10^{-6} M metoclopramide to suppress the current-clamp response to dopamine. Metoclopramide was also ineffective at a higher concentration (1 x 10^{-4} M). Resting potential -57 mV.

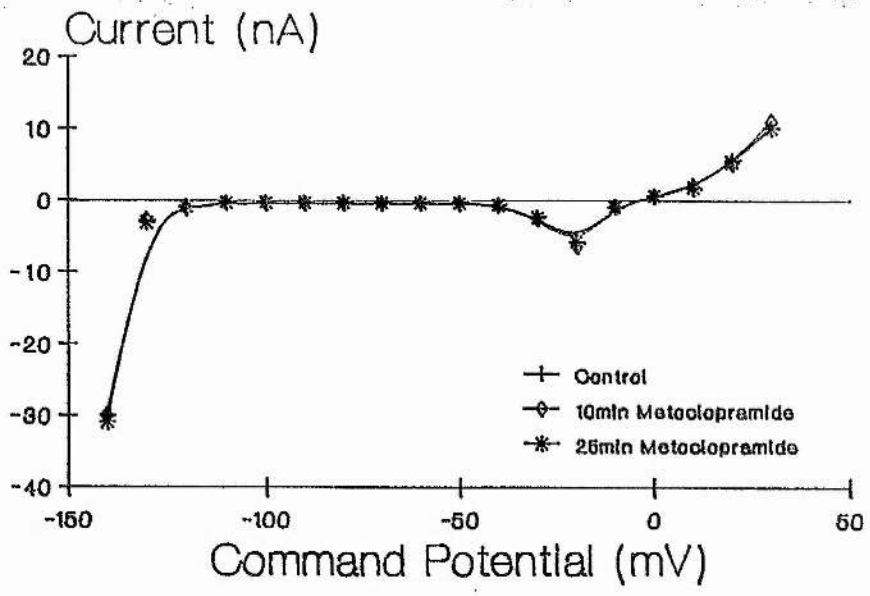


Figure 41 The lack of effect of 1 x 10^{-6} M metoclopramide on the voltage-clamp response to dopamine. Holding potential -60 mV.

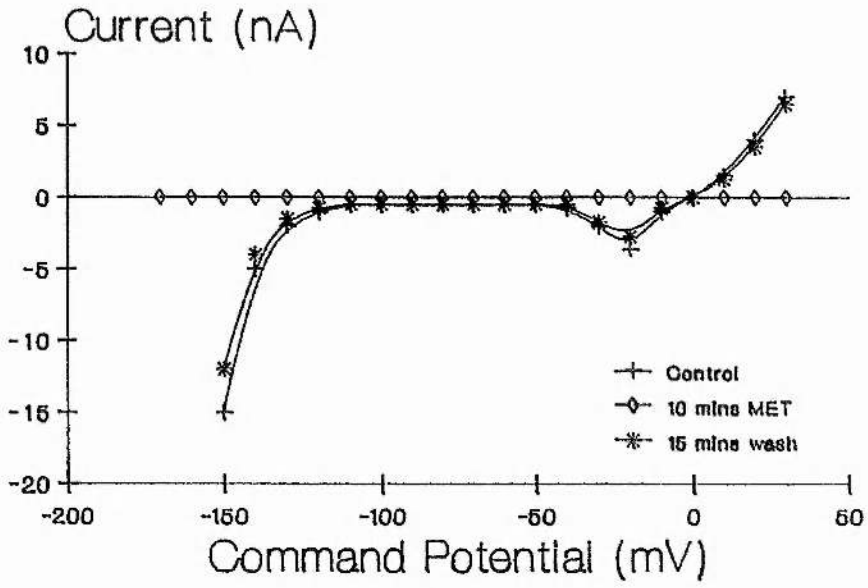


Figure 42 The application of 1 x 10^{-6} M metoclopramide reversibly suppressed the voltage-clamp response to octopamine. Holding potential -60 mV.

0 nA at all potentials. Following 15 minutes wash, the values returned to -12 nA at 150 mV, -0.5 nA at -60 mV, and +6.75 nA at +30 mV. Under current-clamp, the size of the depolarisation was reduced, but returned to normal on washing (Figure 43).

3.2.3.3 Differential Effects of SCH 23390 & α-Bungarotoxin on Responses to Dopamine & Acetylcholine

The effective antagonism of dopamine responses by SCH 23390 has been described above (section 3.2.3.1).

In experiments under current-clamp, no effect was seen on the response to dopamine after up to 35 minutes exposure to 1×10^{-6} M α-bungarotoxin. Under voltage-clamp conditions, the magnitude of the induced currents seen over a range of command potentials was unaffected. In the control run of a typical voltage-clamp experiment (Figure 44), the current values were -26 nA at -140 mV, -0.5 nA at -60 mV, and +20 nA at +30 mV. After 10 minutes exposure to α-bungarotoxin, dopamine-induced currents at these potentials were -21 nA at -140 mV, -0.5 nA at -60 mV, and +20.5 nA at +30 mV respectively. The subsequent values, after 35 minutes exposure to α-bungarotoxin were -27 nA at -140 mV, -0.5 nA at -60 mV, and +21 nA at +30 mV, indicating that α-bungarotoxin was having no significant blocking effect on the response to dopamine. This antagonist had no observable effect on the resting current-voltage relationship of the cell.

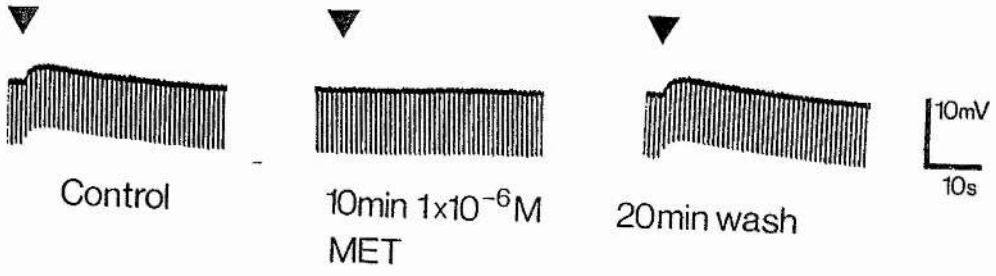


Figure 43 The suppressive effect of 1 x 10^{-6} M metoclopramide on the current-clamp response to octopamine was fully reversible on washing. Resting potential was -55 mV.

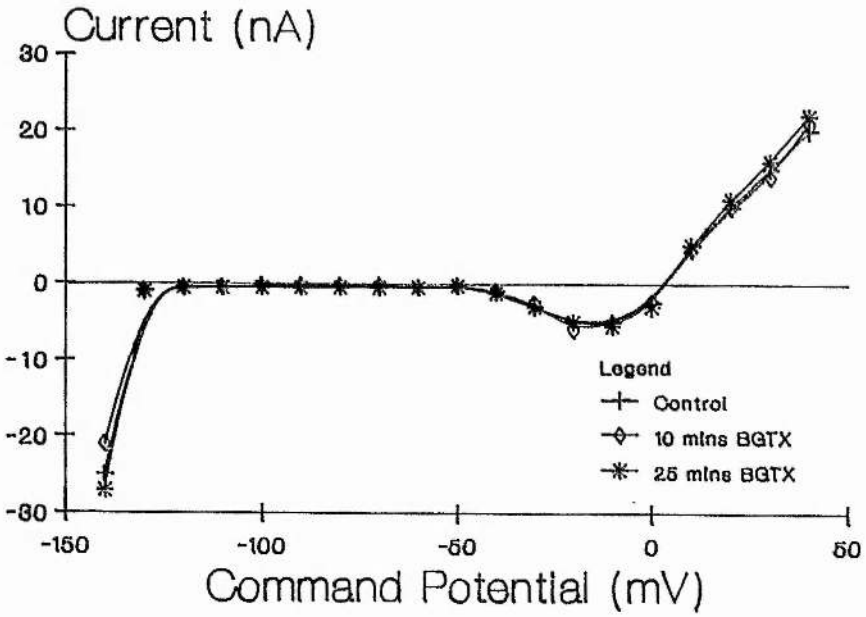


Figure 44 The inability of 1 x 10^{-6} M α-bungarotoxin to affect the voltage-clamp response to dopamine. Holding potential -60 mV.

Under both current-clamp and voltage-clamp conditions, exposure to 1 x 10^{-7} M α-bungarotoxin produced a marked and irreversible blockade of the response to acetylcholine. Under current-clamp, the size of the depolarisation was reduced, showing no recovery even after prolonged washing. Under voltage-clamp, the magnitude of the acetylcholine-induced currents was also suppressed (Figure 45). In the control run, the magnitude of the induced currents were -10 nA at -140 mV, -2 nA at -60 mV, and +3 nA at 0 mV. After 10, minutes exposure to α-bungarotoxin, the values were -5 nA at -140 mV, -1 nA at -60 mV, and +1.5 nA at 0 mV. Even after 45 minutes wash, the values had continued to decline, -1.5 nA at -140 mV, -0.1 nA at -60 nA, and +0.5 nA at 0 mV. Prolonged washing did not reverse the effect.

Figure 46 shows that 1 x 10^{-5} M SCH 23390 had no detectable effect on the acetylcholine-induced currents in a typical voltage-clamp experiment. At potentials positive to the normal resting potential of the cell, any change in current magnitude was undetectable, while at potentials negative to the normal resting potential of the cell, the variation was very small, -10 nA before, -11 nA after 15 minutes, and -10.5 nA after 35 minutes exposure to 1 x 10^{-5} M SCH 23390. Exposure times of more than 65 minutes produced no greater effect.

3.2.4 Discussion

The aim of these experiments was to determine whether dopamine was acting at receptors for other transmitters, or whether a specific dopamine receptor existed on this cell.

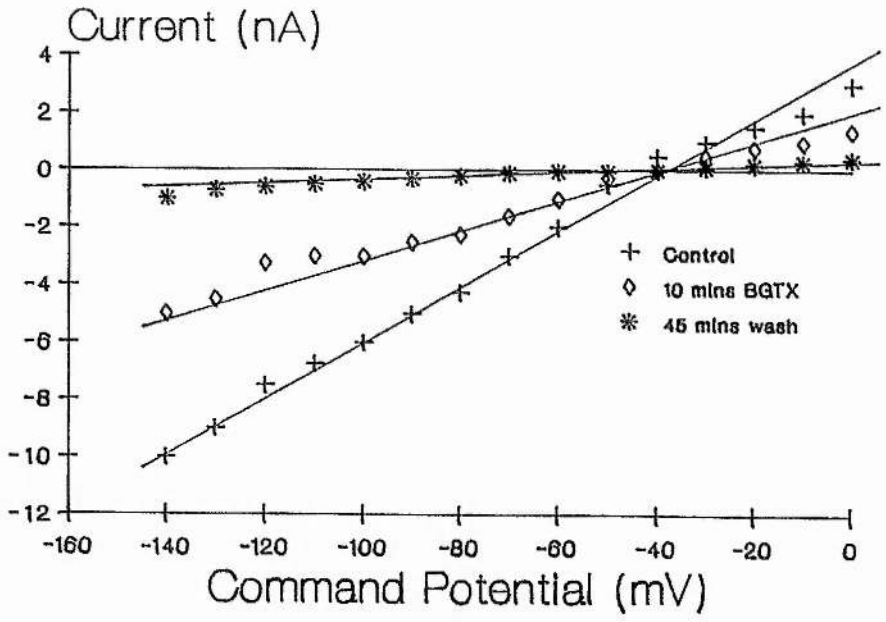


Figure 45 The application of 1 x 10^{-6} M α-bungarotoxin reversibly suppressed the voltage-clamp response to acetylcholine. Holding potential -60 mV.

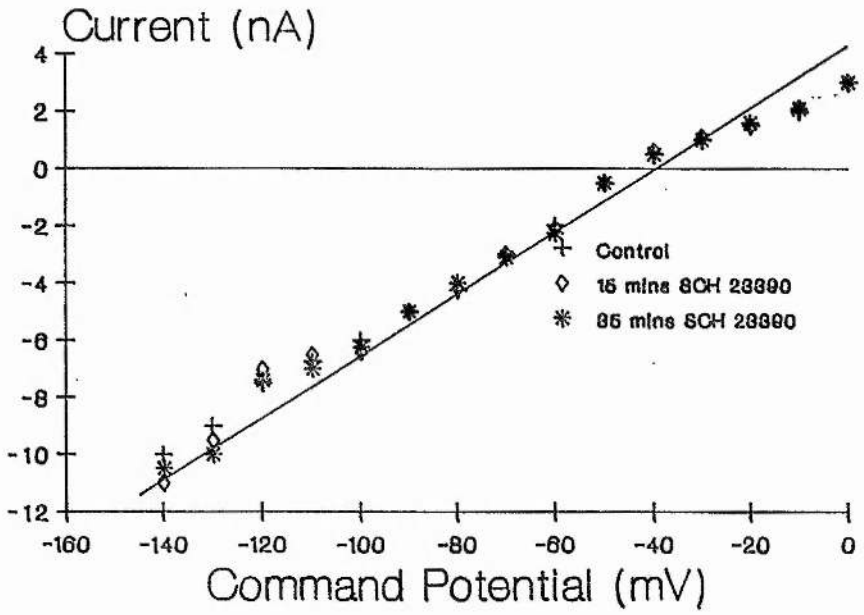


Figure 46 The inability of 1 x 10^{-6} M SCH 23390 to suppress the voltage-clamp response to acetylcholine. Holding potential -60 mV.

The amines dopamine, octopamine and noradrenaline have all been shown to be present in the cockroach CNS (Evans, 1980), and acetylcholine is an important neurotransmitter in the insect central nervous system (Sattelle, 1985).

Since dopamine, noradrenaline and octopamine are all comparatively similar molecules, it was possible that dopamine could be acting at receptors for octopamine or noradrenaline, or at a general amine receptor activated by all three ligands.

Acetylcholine, although structurally quite different, was involved because of the ability of the adrenergic antagonists phentolamine and propranolol to block acetylcholine responses as well as those to dopamine in this same preparation (Pitman & Baker, 1989). It therefore became necessary to eliminate the possibility that dopamine was acting at cholinergic receptors.

Initially, the responses of neurone D3 to the four transmitter candidates were investigated, to evaluate any differences in size, time-course, and voltage-dependency.

The current-clamp responses to dopamine, noradrenaline and octopamine all appeared similar, being relatively small depolarisations (maximum 10 mV, more often 5-6 mV), associated with a modest fall in input resistance. Responses elicited by pulses of 100 mM (in the pipette) dopamine were, however, slightly larger than responses to either noradrenaline or octopamine. The time-course of all three was

similar. Acetylcholine produced a much larger response, commonly 12-15 mV, associated with a more marked fall in input resistance.

Under voltage-clamp, the differences between the voltage-dependencies of the responses to the four agonists were sufficient to suggest that the responses were not mediated via the same receptors. The voltage-dependency of the dopamine response was only mimicked at all closely by that of octopamine, which showed a similar IV relationship, but in general with smaller current magnitudes at a given potential than was the case for dopamine. The response to noradrenaline showed a voltage-dependency similar to that of the dopamine response at potentials more negative than RP (-60 mV). At more positive potentials, however, no increase in inward current magnitude was observed, the response reversing at around -50 mV, and then increasing in size as an outward current. Although these responses were different, the close molecular similarity of dopamine, noradrenaline and octopamine admits of the possibility that the three agonists could all be acting on receptors for any one amine, or on a generalised amine receptor.

The response to acetylcholine showed none of the same voltage-dependency at any command potential, the IV relationship instead being approximately linear. By extrapolation of the IV relationship, it was possible to determine that the response reversed at around -40 mV, a value in agreement with the result of Pitman & Kerkut (1970). The differences in response characteristics strongly suggested that acetylcholine was operating ion channels which were distinct from those operated by the amines.

The results of experiments in which responses to dopamine, noradrenaline, octopamine and acetylcholine were suppressed only by specific antagonists, and not by antagonists of any of the other agonists, indicated that dopamine was most likely to be acting on a specific receptor or receptors, and not on receptors for other transmitter candidates.

These results also indicate that while dopamine is not acting at receptors for noradrenaline and octopamine, it is equally likely that receptors for these transmitters do exist, since responses to these were only abolished by agents with relatively good specificity for these receptors. However, it is beyond the scope of the present study to evaluate the contribution, if any, these receptors may be making to the function of neurone D3 *in vivo*.

Interestingly, metoclopramide, held to be a mammalian D_2 antagonist of good specificity (Kebabian & Calne, 1979), had no effect on the response to dopamine in this preparation. This may be taken to indicate that the dopamine receptor present here has the characteristics of a D_1 receptor. However, it has been shown that agents which alter cyclic AMP metabolism have no effect on dopamine reponses in neurone D3 (Fleming, 1986; Pitman & Baker, 1989). Since D_1 receptors are 'classically' described as being linked to adenylate cyclase, it may be that the dopamine receptor present here does not conform to mammalian classification schemes.

In conclusion, the results reported in this section indicate that a specific dopamine receptor is present on neurone D3, and that it

appears to mediate the observed voltage-dependent effects of dopamine.

3.2.5 Summary

The dopaminergic antagonist SCH 23390 reversibly suppressed the response to dopamine in this preparation, at a concentration of 1 x 10^{-6} M. However, at a concentration of 1 x 10^{-5} M, it had no detectable effect on responses to noradrenaline, octopamine or acetylcholine.

Ergotamine, a noradrenergic antagonist, reversibly suppressed the response to noradrenaline at a concentration of 1 x 10^{-6} M, but had no effect on responses to dopamine, octopamine or acetylcholine, at a concentration of 1 x 10^{-5} M.

Metoclopramide, a specific D_2 dopaminergic antagonist in mammalian systems, had no effect on the response to dopamine, noradrenaline or acetylcholine, at a concentration of 1 x 10^{-5} M, but reversibly suppressed the response to octopamine at 1 x 10^{-6} M.

α-bungarotoxin irreversibly blocked the acetylcholine response at 1 x 10^{-7} M, but at 1 x 10^{-5} M, had no effect on responses to dopamine, noradrenaline, or octopamine.

3.3 PHARMACOLOGICAL PROFILE OF THE PUTATIVE DOPAMINE RECEPTOR

3.3.1 Introduction

The history and development of the extant classification schemes for mammalian dopamine receptors have been set out in detail in the General Introduction. Having established, with reasonable certainty, that a dopamine receptor is present in the preparation, the experiments reported here were designed to test the effectiveness of a range of dopaminergic agents on the resting membrane properties of the cell, and on the response to dopamine, in order to compare the characteristics of the putative dopamine receptor present here with those found in other preparations.

The most relevant study is that of Kebabian & Calne (1979) and these experiments aimed specifically at assessing how well, if at all, this dopamine receptor fits into their scheme, which divides dopamine receptors into two sub-classes - D_1 and D_2. Their classification scheme is partially based on the presence and nature of the link between the receptor and adenylate cyclase. In the case of D_1 receptors, the relationship is excitatory, whereas in that of D_2 receptors, the link if any is inhibitory. D_1 and D_2 receptors can also be distinguished according to differences in their pharmacological profile.

It has already been demonstrated that dopamine responses in the insect motoneurone D_3 do not appear to depend upon an increase in

intracellular cyclic AMP, suggesting that the insect receptor does not resemble the mammalian D_1 receptor. In this study, a range of agonist and antagonist agents have been used, which have previously been shown to be effective in distinguishing between mammalian D_1 and D_2 receptors. Full chemical names and structural formulae are given in Appendix 5.

3.3.1.1 Agonists Active at D_1 Receptors

The substituted naphthalene, A-6,7-DTN (ADTN) is quoted in the literature as being a potent dopamine agonist, with the ability to stimulate dopamine-sensitive adenylate cyclase (Miller et al, 1974). Equally, it has been shown that ADTN stimulates the DA_1 receptor in the canine vascular bed (Crumly et al, 1976). In invertebrate systems, ADTN mimics the effect of dopamine in eliciting the feeding motor program in *Limax* (Wieland & Gelperin, 1983), and in the cockroach *Nauphoeta cinerea*, ADTN can evoke both electrical responses from the acinar cells of the salivary gland, and secretory responses from intact glands (House & Ginsborg, 1982). Thus ADTN is essentially a D_1 active compound.

Recently, compounds with engineered dopaminergic activity have become available. Generally, these have far greater specificity than naturally-derived compounds such as ADTN.

SK&F 82526 is a synthetic benzazepine derivative with D_1 agonist activity developed by Smith, Kline & French. The specific effects of this drug on dopamine-stimulated adenylate cyclase (Setler *et al*,

1978) and on mammalian central D_1 receptors (Hahn et al, 1983; Chipkin & Latranyi, 1987), have been studied.

This compound has also been shown to have specific activity on the DA_1 receptor (Weinstock et al, 1980; Goldberg & Kohli, 1983). However, its effects on peripheral dopamine receptors in dogs can be reversed competitively by dopaminergic antagonists such as metoclopramide and bulbocapnine (Hahn et al, 1982), suggesting some degree of activity at D_2 receptors.

SK&F 38393 is closely related chemically to SK&F 82526. In molluscs, it has been shown to mimic the effects of dopamine in increasing the excitability of growth hormone producing cells (GHC's) in *Lymnaea* (Stoof, de Vlieger & Lodder, 1985; Stoof, Werkman, Lodder & de Vlieger, 1986). It has also been demonstrated to elicit the feeding motor program in *Limax maximus*, stimulated *in vivo* by dopamine (Wieland & Gelperin, 1983). Both these actions are consistent with the activation of D_1 receptors, since Stoof et al have shown that the excitatory response of GHC's to dopamine is mediated via D_1 receptors, and Wieland & Gelperin demonstrated that the action of dopamine in *Limax* indicated that D_1 receptors were involved, although they concluded that these might have an antagonist profile different from that of mammalian receptors.

3.3.1.2 Agonists Active at D_2 Receptors

The morphine derivative apomorphine is produced from morphine by treatment with concentrated hydrochloric acid, and has been known to

chemists and physicians since the late 19th century. It has been used as a benchmark dopamine agonist in the classification schemes of both Kebabian & Calne (1979) and Goldberg & Kohli (1983) - ie in both CNS and peripheral tissues - and has been used clinically as an emetic.

Apomorphine is a specific D_2 (and DA_2) agonist, and although it has some agonist activity on D_1 (and DA_1) receptors, it has a very large potency differential, being a full, potent agonist at D_2 receptors, while being only a weak, partial agonist at D_1 receptors. This is one of the few points on which the classification schemes of central dopamine receptors (Kebabian & Calne) and peripheral dopamine receptors (Goldberg & Kohli) agree.

Apomorphine has been shown to mimic the actions of dopamine on various systems. Kebabian & Saavedra (1976) showed that apomorphine mimicked the effects of dopamine in the pituitary gland. Apomorphine has also been shown to act as a competitive antagonist of the dopamine response in the rat brain (Costall & Naylor, 1976). Goldberg & Kohli (1983) showed that apomorphine mimicked the actions of dopamine in causing a reduction in the release of noradrenaline from postganglionic sympathetic nerves.

MacDonald & Berry (1978) demonstrated that apomorphine was useful in distinguishing between the responses produced by dopamine in neurones of *Planorbis corneus*. House & Ginsborg (1982) found evidence that apomorphine could mimic the effects of dopamine in eliciting hyperpolarisation and secretion in cockroach salivary glands.

Another morphine-derived compound, bromocryptine is more soluble than apomorphine, more stable in solution and has similar agonist effects on dopamine receptors. It is used clinically in similar applications to apomorphine.

Ergometrine is one of a class of compounds known as the dopaminergic ergots, being extracted from the Wheat Rust fungus, or Ergot (*Claviceps purpurea*). The dopaminergic ergots are, according to Kebabian & Calne (1979), a useful group of compounds to use for distinguishing between classes of dopamine receptor. They are potent (nanomolar) antagonists of the D_1 receptor (Ascher, 1972; Sakharov & Salánki, 1982; Wieland & Gelperin, 1983), but are equally potent agonists of the D_2 receptor, allowing a relatively clear distinction between the two.

The compound LY 171555 (Hahn & MacDonald, 1984) is the (-) isomer of the partial ergoline LY 141865. LY 141865 has been shown to discriminate between the two peripheral dopamine receptors in the cardiovascular system (Hahn et al, 1983), and to stimulate selectively the D_2 central receptor (Tsuruta et al, 1981). LY 171555 has also been shown to selectively activate presynaptic dopamine receptors in the periphery, examples of the DA_2 receptor (Lokhandwala & Steenberg, 1984; Hahn & MacDonald, 1984).

In *Lymnaea*, LY 141865 has been observed to hyperpolarise GHC's, a similar effect to that exhibited by dopamine, in the same study in which the D_1 agonist SK&F 38393 was shown to depolarise the cells. It has been concluded that the two effects were mediated by D_2 and D_1

receptors respectively, since in addition, intracellular injection of cyclic AMP mimicked the depolarising response, confirming that it was mediated by D_1 receptors. (Stoof, de Vlieger & Lodder, 1985; Stoof, Werkman, Lodder & de Vlieger, 1986).

3.3.1.3 Antagonists Active at D_1 & D_2 Receptors

Fluphenazine, a phenothiazine derivative, is a broad-spectrum dopaminergic antagonist, suppressing activity at both D_1 and D_2 receptors. It is a neuroleptic drug used in the treatment of various neurological disorders in man, such as schizophrenia. In experimental studies, it and other phenothiazine derivatives have been used as characterising antagonists for the D_1 receptor, but does not have good selectivity (Leff & Creese, 1983). It has been used as an antagonist of dopamine-stimulated salivary gland adenylate cyclase activity in the tick *Amblyomma* (Schmidt et al, 1981). However, fluphenazine does not have good specificity for either class of dopamine receptor.

Butaclamol, an isoquinoline compound, is a relatively recently introduced neuroleptic drug, which shows selectivity in its activity. The (+) isomer is active as a dopaminergic antagonist, showing competitive binding with dopamine and other antagonists in rat brain preparations (Seeman et al, 1975), while the (-) isomer is inactive. It has not been shown to have a high degree of specificity for either of the two classes of dopamine receptor.

Flupenthixol is one of the thioxanthene group of compounds, and is found in two optically active forms, cis- and trans-flupenthixol. Trans-flupenthixol has been found to be inactive in dopaminergic systems, while the cis-isomer is active as a potent antagonist of dopamine-sensitive adenylate cyclase activity, and also of the dopaminergic response in the anterior pituitary (Miller et al, 1975). This suggests that it does not possess a particularly good selectivity for either class of dopamine receptor (Kebabian & Calne, 1979).

3.3.1.4 Antagonists Active at D_1 Receptors

Compounds with activity specific to D_1 receptors have historically been hard to find, but with developments in chemical synthesis, compounds have become available which do show good D_1 specificity. One such is SCH 23390, which was developed by the Schering Corp as a potential benzazepine antipsychotic drug. It was hoped that this compound might provide treatment by blocking dopaminergic transmission within the CNS without the side-effects, such as tremor, rigidity, and tardive dyskinesia, similar to Parkinsonism.

Experiments performed by workers in Schering's own laboratories provided evidence that SCH 23390 was a D_1 receptor antagonist with very good specificity (Iorio et al, 1983). Their results showed that while injection into experimental animals of effective doses of SCH 23390 produced none of the aforementioned side-effects, SCH 23390 blocked dopamine-sensitive adenylate cyclase *in vitro* (an assessment of D_1 activity) at concentrations some 2000 times lower than those

needed to block spiroperidol binding (an assessment of D_2 activity), thus suggesting a very high degree of D_1 specificity.

SCH 23390 has been demonstrated to act as an antagonist of the D_1 receptors mediating the depolarisation of GHC's in *Lymnaea* (Stoof, de Vlieger & Lodder, 1985; Stoof, Werkman, Lodder & de Vlieger, 1986)

3.3.1.5 Antagonists Active at D_2 Receptors

Compounds active at the D_2 receptor include haloperidol, a member of the butyrophenone group of compounds, which was found to antagonise dopamine responses in several mammalian tissues, in which dopamine-sensitive adenylate cyclase activity had been identified (Kebabian, Petzold & Greengard, 1972; Clement-Cormier et al, 1974), but at micromolar or greater concentrations.

Sakharov & Salánki (1982) have shown that haloperidol suppresses the effect of dopamine in their observations of *Helix pomatia*, in which they noted its ability to alter the characteristics of the locomotory waves of muscle contraction in the foot. Haloperidol has also been reported to be an antagonist of the effects of dopamine in the brain of the honey bee, *Apis mellifera*. Dopamine was shown to suppress the ability of bees to respond to a conditioning stimulus by extending its proboscis. Haloperidol blocked this suppressive effect, re-establishing the conditioning process (Macmillan & Mercer, 1987).

Spiroperidol (also known as spiperone), is best known from the literature as a ligand used in binding assays of dopamine receptors (Gorissen & Laduron, 1979). It is used as a competitive radiolabelled tracer in the isolation of receptors, and the localisation of receptors within physiological systems (Creese et al, 1983). It is supposedly selective in its binding, which is more potent to the D_2 receptor than the D_1. It is a butyrophenone, related to haloperidol, and is used as a neuroleptic drug.

The benzamide metoclopramide has been shown to block some dopamine receptors in different preparations (Jenner & Marsden, 1979), and is used clinically as an antiemetic. In the present study, metoclopramide has been shown to be ineffective in blocking dopamine responses (see Section 3.2.3.2). It supposedly has greater activity at D_2 receptors than at D_1 (Kebabian & Calne, 1979). Evans (1981, 1984a) has shown that while chlorpromazine preferentially blocks octopamine$_1$ (OA_1) receptors, metoclopramide preferentially blocks octopamine$_2$ (OA_2) receptors. Metoclopramide is also capable of distinguishing between OA_{2A} and OA_{2B} receptor sub-types, since metoclopramide is a much more potent blocker of OA_{2A} than of OA_{2B} receptors.

Another substituted benzamide, YM 09151-2 is an experimental compound found to be a potent dopaminergic antagonist (Stoof & Kebabian, 1984), but is not used clinically. It has been shown to be active on the D_2 receptor, but not on the D_1 receptor, competing for D_2 binding sites with other benzamides (Grewe et al, 1982). Stoof et al (1985)

demonstrated its ability to block the D_2 mediated hyperpolarisation of GHC's in *Lymnaea*.

The benzamide sulpiride is another of the dopaminergic agents that has an active and an inactive isomer. The positive isomer is inactive, while (-) sulpiride is an active antagonist of the D_2 receptor. (Stoof & Kebabian, 1984; Kebabian & Calne, 1979; Goldberg & Kohli, 1983). Sulpiride is used clinically as an antiemetic and tranquiliser. Both YM 09151-2 and sulpiride antagonise the dopamine-induced, D_2 mediated effects on GHC's in *Lymnaea* (Stoof et al, 1985; 1986).

Chlorpromazine is a phenothiazine, related to fluphenazine, whose clinical applications are essentially similar, as a neuroleptic and antipsychotic drug. It has been used as a competitive ligand for dopamine in biochemical models of the D_2 receptor in rat brain (Leff & Creese, 1983). Chlorpromazine has been shown to suppress the hyperpolarising response to dopamine of RB visceral neurones in *Aplysia* (Ascher, 1972), and to interfere with the dopaminergic control of locomotory motor programs in *Helix pomatia* (Sakharov & Salánki, 1982). In addition it is reported to be an antagonist of the effects of octopamine in the locust (Evans, 1980).

3.3.2 Results

3.3.2.1 Agonists Active at D_1 Receptors

ADTN was found to be an effective agonist, approximately equipotent with dopamine in this preparation. At a concentration of 1 x 10^{-1} M, pressure-applied ADTN produced a recognisable and repeatable depolarisation (6 experiments). The depolarisation was not, however, associated with as easily detectable a fall in the input resistance of the cell. Under voltage-clamp, the voltage-dependency of the response to ADTN was similar to that of dopamine, showing the increase in inward current magnitude at potentials between -20 mV and 0 mV (6 experiments). A typical voltage-clamp record (Figure 47) shows this. At a concentration in the picospritzing microelectrode of 1 x 10^{-4} M, ADTN still produced results very similar to those produced by dopamine at the same concentration.

Pressure-application of a solution of 1 x 10^{-4} M SK&F 82526 (the most concentrated obtainable in 0.1 M HCl, diluted with saline) elicited no response (11 experiments). The illustration (Figure 48) shows traces from a typical current-clamp experiment. There is no evidence of any depolarisation or change in the input resistance of the cell.

3.3.2.2 Agonists Active at D_2 Receptors

Apomorphine was used under precisely the same experimental conditions as was dopamine, both in pressure-ejection and bath-application experiments, under both current- and voltage-clamp (9 experiments for

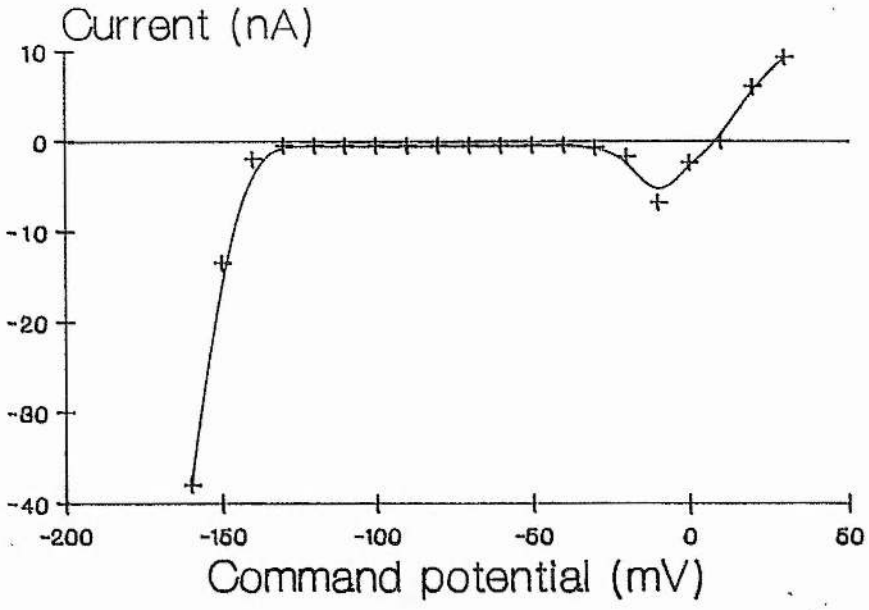


Figure 47 The voltage-dependence of the voltage-clamp response to pressure-applied ADTN. Data were obtained by the 'short-pulse' method, in the presence and absence of ADTN, to yield a difference curve. Holding potential was -60 mV.

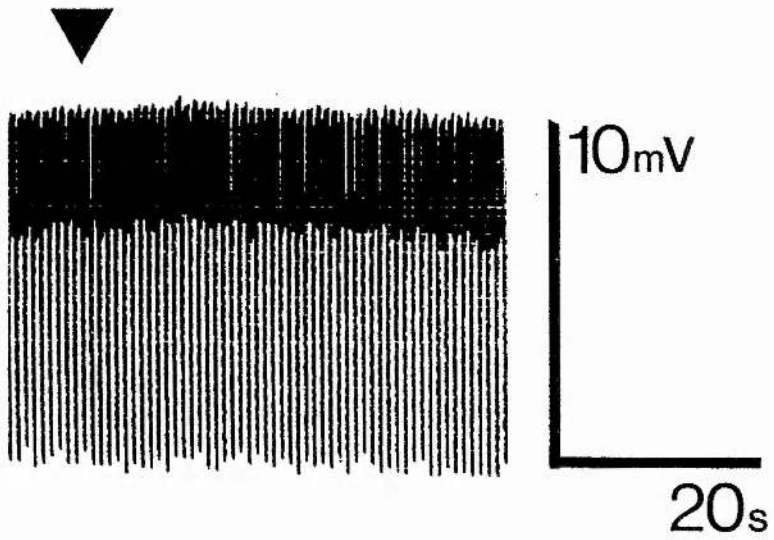


Figure 48 A typical current-clamp record indicating that pressure-applied SK&F 82526 has no effect on the membrane potential or input resistance of the cell. Current pulses were applied through a second microelectrode, and the resting potential was -56 mV.

each), at a concentration of 100 mM in the pressure-ejection micropipette, and 5 - 10 mM in the bath-application studies. Under current-clamp, the response of neurone D3 is similar to that elicited by dopamine, in that it is a depolarisation, associated with a modest fall in input resistance. The magnitude of the response, however, is not as great as that of a dopamine response.

Under voltage-clamp, the response to pressure-applied apomorphine is seen as an inward current at potentials near to the normal resting potential of the cell. At potentials both positive and negative to the normal resting potential of the cell, the magnitude of the current induced by apomorphine varies in a very similar manner to that of the current induced by dopamine. In a typical voltage-clamp experiment (Figure 49), the values of induced current were -45 nA at a command potential of -160 mV, -0.5 nA at -60 mV, and +8 nA at +30 mV.

Apomorphine was tested at concentrations as dilute as 1×10^{-3} M in the picospritzer pipette, but the response obtained was extremely small, and a concentration of 1×10^{-4} M failed to elicit any response.

In the present study, bromocryptine also proved to be a close mimic of the effects of dopamine. Under both current-clamp and voltage-clamp (7 experiments), the response to 100 mM bromocryptine was very similar to the response to dopamine. Here also, bromocryptine was pressure-applied at concentrations down to 1×10^{-3} M, and the

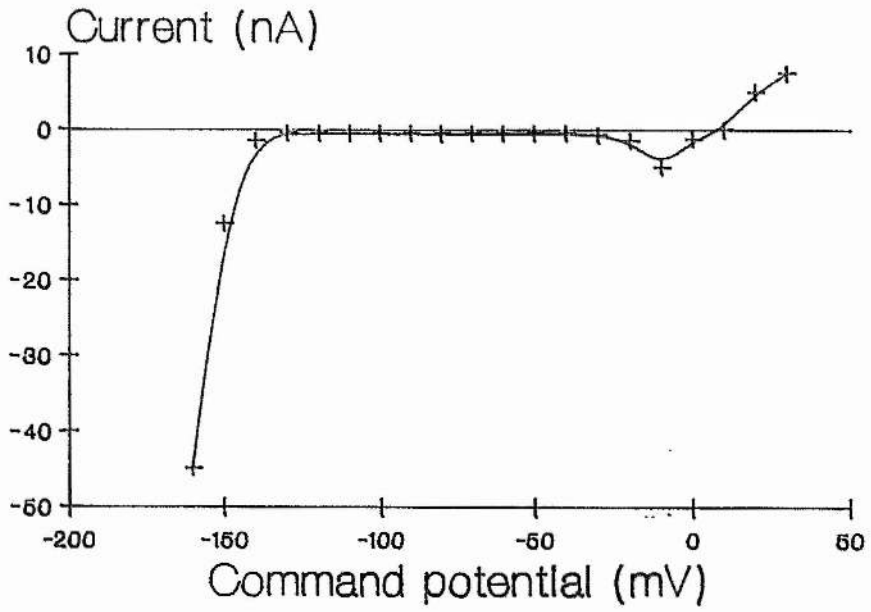


Figure 49 Difference curve indicating the voltage-dependence of the voltage-clamp response to pressure-applied apomorphine. Holding potential -60 mV.

response was slightly smaller than that to apomorphine at the same concentration.

Under current-clamp conditions, the response was a depolarisation, associated with a small fall in the input resistance of the cell. A typical voltage-clamp record (Figure 50) shows that the induced current had a very similar voltage-dependency to that of the response to dopamine. At a command potential of -160 mV, the induced current magnitude was -43 nA. At -60 mV, it was -0.5 nA, and at +30 mV, +12.5 nA.

In these experiments ergometrine proved to be a mimic of the effects of dopamine on the preparation. Applied at a concentration of 100 mM (in the ejection pipette), the response was easily repeatable, but again, at the lower concentration of 1×10^{-3} M, the response was much reduced by comparison with apomorphine.

Under current-clamp (6 experiments), the depolarising response was associated with a modest fall in input resistance. The magnitude of the response was slightly smaller than that elicited by dopamine. Under voltage-clamp (6 experiments), the response, seen as an inward current at potentials around the normal resting potential of the cell, showed a very similar voltage-dependency to the response to dopamine. In a representative experiment (Figure 51), the current magnitude at a command potential of -140 mV was -75 nA. At -60 mV, it was -0.5 nA, and at +30 mV, +12 nA.

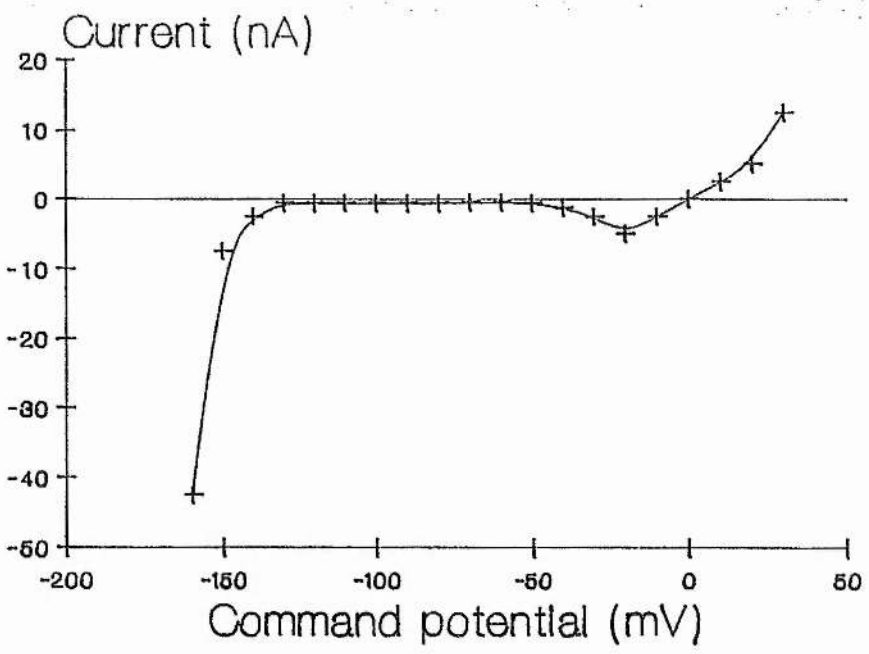


Figure 50 Difference curve indicating the voltage-dependence of the voltage-clamp response to pressure-applied bromocryptine. Holding potential was -60 mV.

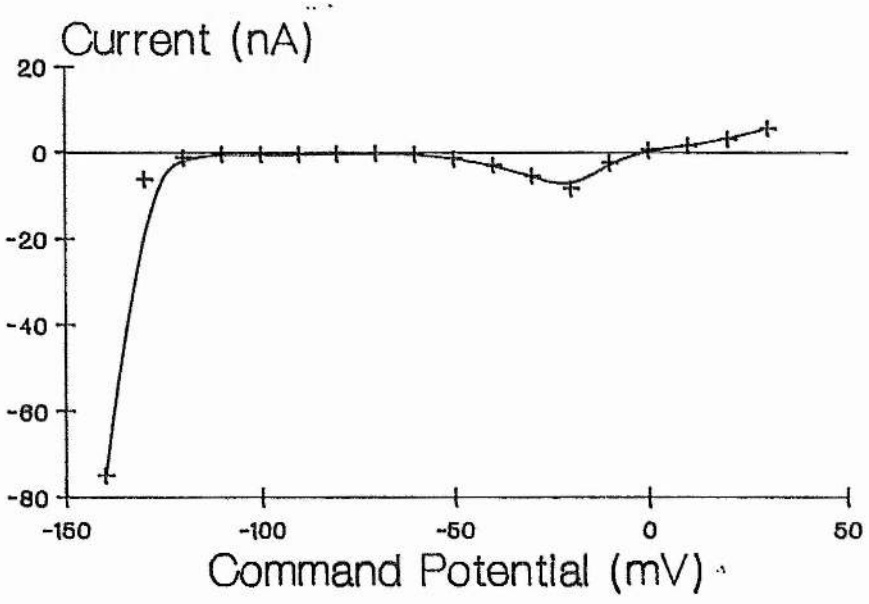


Figure 51 Difference curve indicating the voltage-dependence of the voltage-clamp response to pressure-applied ergometrine. Holding potential -60 mV.

The solubility of LY 171555 was as low as that of SK&F 82526, and consequently a concentration of 1 x 10^{-4} M (obtained by dissolving in 0.1 M HCl, and diluting with saline) was used.

In no case, under either current-clamp or voltage-clamp, did pressure-application of a 1 x 10^{-1}M solution produce any detectable effect. Bath-application of LY 171555 equally had no effect (10 experiments in all).

3.3.2.3 Antagonists Active at Both D_1 & D_2 Receptors

Under both current- and voltage-clamp conditions, fluphenazine reversibly suppressed the response to dopamine (9 experiments each). Under current-clamp, the size and duration of the depolarising response to dopamine was suppressed. In a representative voltage-clamp experiment (Figure 52), the magnitude of the dopamine-induced currents seen over the full range of membrane command potentials was reduced. The values obtained for the control run were -25 nA at a command potential of -140 mV, -0.5 nA at -60 mV, and +10 nA at +30 mV. Exposure to 1 x 10^{-6} M fluphenazine for 10 minutes reduced the induced current magnitudes to -10 nA at -180 mV (-0.75 nA at -150 mV), ≃0 nA at -60 mV, and +1 nA at +30 mV. Twenty-five minutes wash returned the response to full magnitude. No effect was observed on the resting current-voltage relationship of the cell.

Under current-clamp conditions, exposure to 1 x 10^{-7} M butaclamol reversibly suppressed the response to dopamine (5 experiments, Figure 53). In a typical voltage-clamp experiment (1 of 7, Figure 54), the

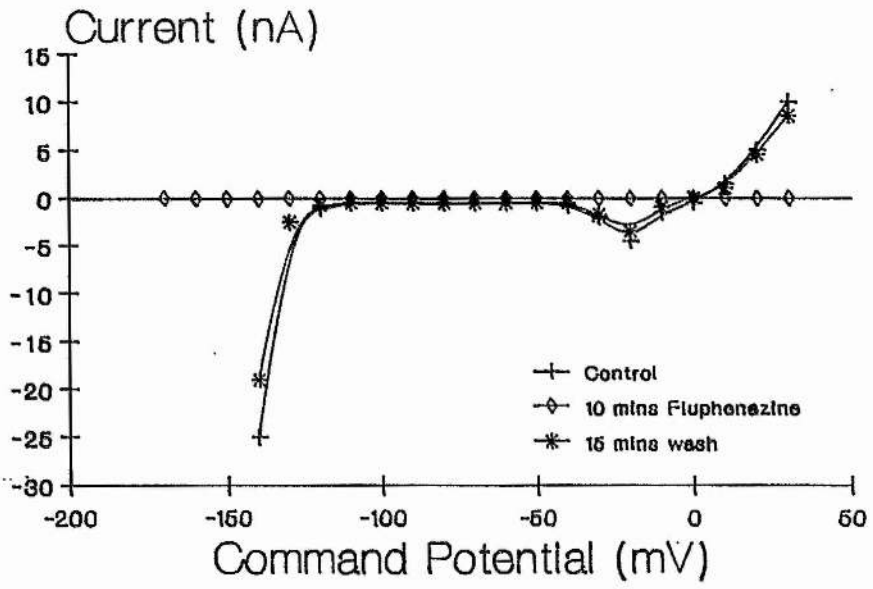


Figure 52 The reversible suppressive effect of 1 x 10^{-6} M fluphenazine on the voltage-clamp response to dopamine at a range of command potentials. Holding potential -60 mV.

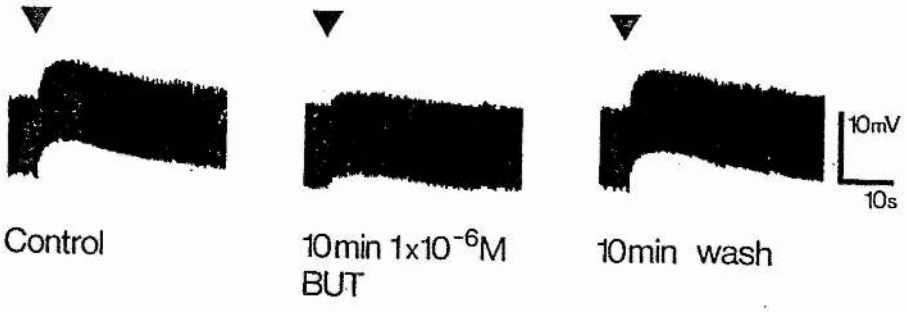


Figure 53 The suppressive effect of 1 x 10^{-6} M butaclamol on the current-clamp response to dopamine. Resting potential was -59 mV.

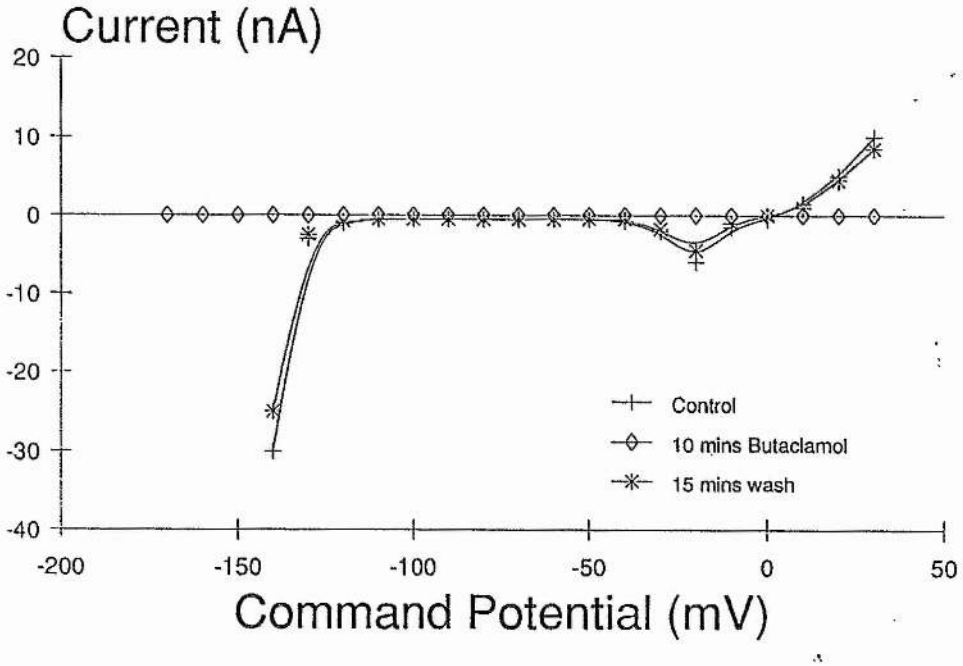


Figure 54 The suppressive effect of 1 x 10^{-6} M butaclamol on the voltage-clamp response to dopamine at at range of command potentials. Holding potential -60 mV.

control current magnitudes of -30 nA at a command potential of -140 mV, -0.5 nA at -60 mV, and +10 nA at +30 mV, were reduced to 0 nA across the full range of membrane command potentials after 10 minutes exposure to 1 x 10^{-7} M butaclamol. Following 15 minutes wash, the response was restored to values of -28 nA at -140 mV, -0.5 nA at -60 mV, and +8 nA at +30 mV. No effect was observed on the resting current-voltage relationship of the cell.

Flupenthixol was found to be active, under both current- and voltage-clamp conditions (6 experiments each under voltage- and current-clamp, and 4 additional tests for each of the lower concentrations down to 1 x 10^{-9}), at concentrations as dilute as 1 x 10^{-9} M. After incubation for 20 minutes, at a concentration of 1 x 10^{-6} M, it reversibly suppressed the response under current-clamp (Figure 55). In a typical voltage-clamp experiment (Figure 56), before exposure to 1 x 10^{-6} M flupenthixol, the current magnitudes were -35 nA at a command potential of -150 mV, -0.5 nA at -60 mV, and +11 nA at +30 mV. Following 10 minutes exposure to 1 x 10^{-6} M flupenthixol, these values were reduced to 0 nA at all command potentials. After 15 minutes wash, the response current magnitudes returned to normal, with values of -33 nA at -150 mV, -0.5 nA at -60 mV, and +10 nA at +30 mV. No effect was seen on the resting current-voltage relationship of the cell.

3.3.2.4 Antagonists Active at D_1 Receptors

As indicated in Section 3.2.3.1, SCH 23390 proved to be a potent and selective dopaminergic antagonist. Under current-clamp conditions,

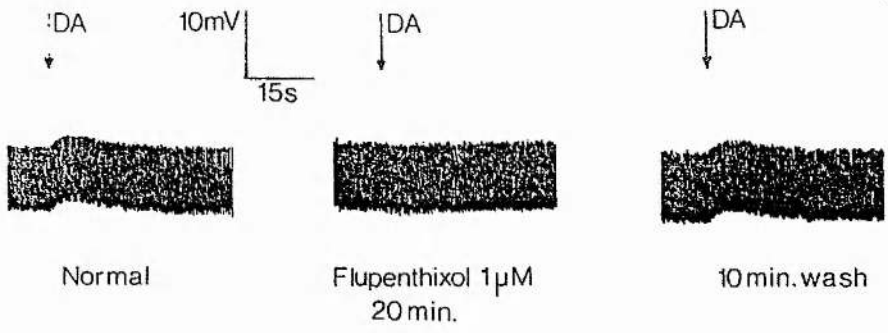


Figure 55 The suppressive effect of 1 x 10^{-6} M flupenthixol on the current-clamp response to dopamine. Current pulses were applied through a secon d microelectrode, and resting potential was -57 mV.

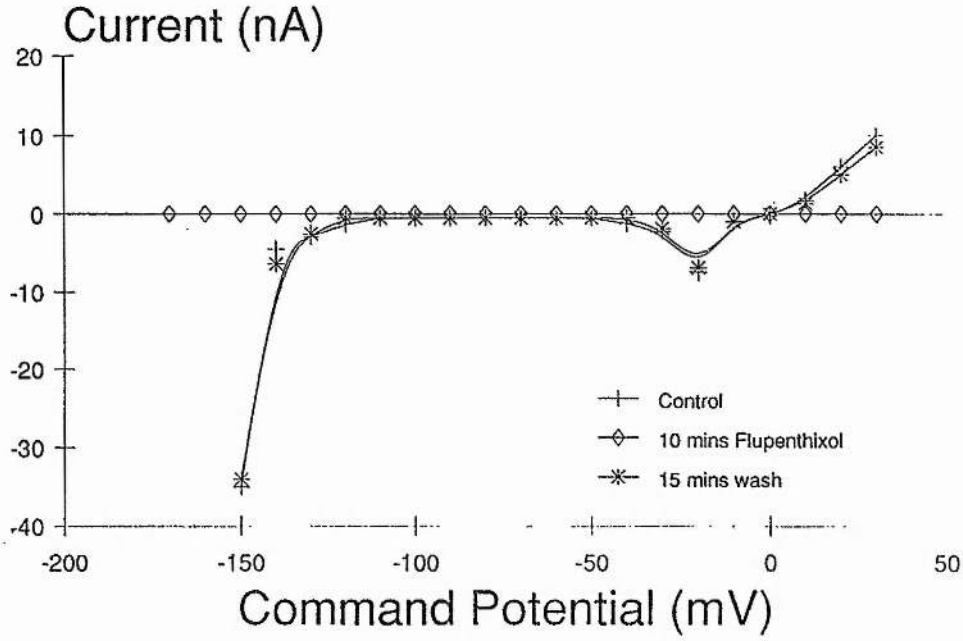


Figure 56 The suppressive effect of 1 x 10^{-6} M flupenthixol on the voltage-clamp response to dopamine at range of command potentials. Holding potential -60 mV.

at a concentration of 1 x 10^{-6} M, SCH 23390 reversibly suppressed the response to dopamine, and washed off completely after 10 to 20 minutes (Figure 34). At this concentration, the response was reliably and repeatably abolished. At concentrations progressively more dilute than this, the effect diminished, until at 1 x 10^{-9} M, it was virtually undetectable.

Under voltage-clamp conditions, the normal IV curve due to dopamine was flattened out completely at a concentration of 1 x 10^{-6} M, indicating a total blockade of the response even at relatively negative command potentials, where the response current would be expected to be at its largest. In a typical experiment (Figure 35), before the application of SCH 23390, at a command potential of -160 mV, the magnitude of the induced current was -13 nA, -0.5 nA at -60 mV, and +15 nA at +30 mV. After 5 minutes exposure to 1 x 10^{-6} M SCH 23390, these values were reduced to 0 nA at all potentials. After 10 minutes wash, the response had been restored, giving response current magnitudes of -10 nA at -160 mV, -0.5 nA at -60 mV, and +10 nA at +30 mV. No effect was seen on the resting current-voltage relationship of the cell.

SCH 23390 was tested, in one experiment only, on the response of neurone D3 to apomorphine, under voltage-clamp. In this experiment, a concentration of 1 x 10^{-6} M SCH 23390, after incubation for 7 minutes, significantly reduced the response currents under voltage-clamp over the complete standard range of command potentials. Although this result is unsubstantiated since it is from a single

experiment, it is indicative that the receptor upon which apomorphine is having its effect is being blocked by SCH 23390.

3.3.2.5 Antagonists Active at D_2 Receptors

Haloperidol suppressed the response of neurone D3 to dopamine under both current- and voltage-clamp conditions (6 experiments each). The effect was observed at concentrations as low as 1 x 10^{-9} M. In a typical voltage-clamp experiment (Figure 57), the control values of induced current were -26 nA at a command potential of -160 mV, -0.5 nA at -60 mV, and +15 nA at +30 mV. Following 15 minutes exposure to 1 x 10^{-9} M haloperidol, the response current was reduced to 0 nA at all command potentials. After 20 minutes wash, the response was restored, giving values of -17 nA at -160 mV, -0.5 nA at -60 mV, and +10 nA at + 30 mV. Under current-clamp, the magnitude of the depolarisation was completely suppressed by exposure to 1 x 10^{-9} M haloperidol, and recovered completely on wash-out (Figure 58). No effect was seen on the resting current-voltage relationship of the cell.

Under both current-clamp and voltage-clamp conditions, spiroperidol produced an irreversible blockade of the response (7 experiments each). In a representative voltage-clamp experiment (Figure 59), the control run produced values for the induced current magnitudes of -25 nA at a command potential of -150 mV, -0.5 nA at -60 mV, and +14 nA at +30 mV. After 15 minutes exposure to 1 x 10^{-5} M spiroperidol, the response current magnitudes were reduced to 0 nA at all command potentials. Following 45 minutes wash, the response was still 0 nA,

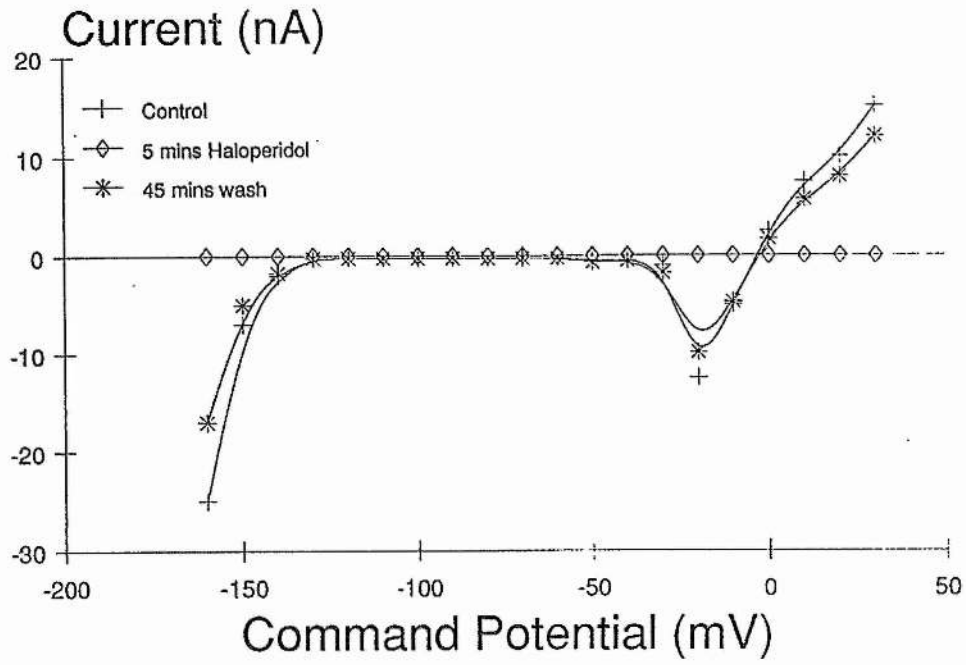


Figure 57 The suppressive effect of 1 x 10^{-9} M haloperidol on the voltage-clamp response to dopamine at range of command potentials. Holding potential -60 mV.

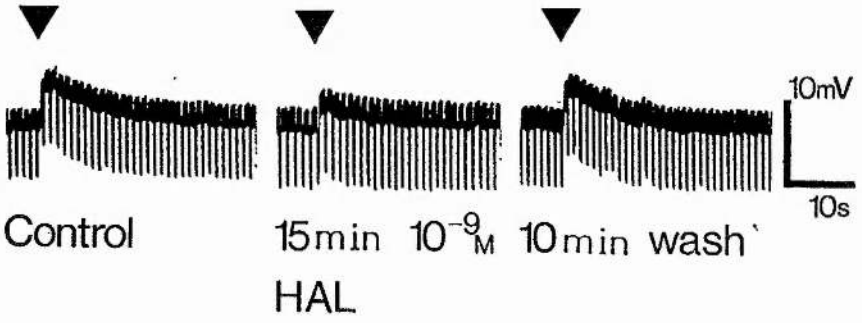


Figure 58 The suppressive effect of 1 x 10^{-9} M haloperidol on the current-clamp response to dopamine. Current pulses were applied through a second microelectrode, and resting potential was -56 mV.

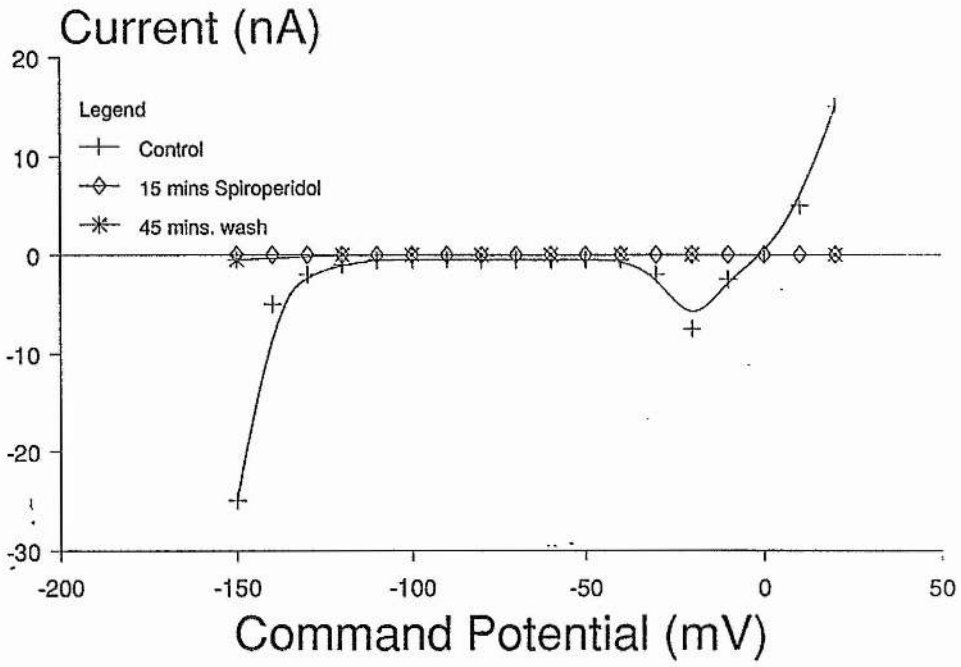


Figure 59 The suppressive effect of 1×10^{-6} M spiroperidol on the voltage-clamp response to dopamine at range of command potentials. The effect was not reversible. Holding potential -60 mV.

and showed no significant sign of recovery. This effect was repeated under current-clamp conditions. No effect was seen on the resting current-voltage relationship of the cell.

Metoclopramide, at concentrations in excess of 10^{-4} M, and incubation times in excess of 40 minutes, had no detectable effect on either the depolarising response to dopamine under current-clamp, or the dopamine-induced currents seen under voltage-clamp across the full range of membrane command potentials (9 experiments each). A typical voltage-clamp experiment (Figure 41), shows that the magnitude of the induced currents was not affected by exposure for up to 25 minutes to 1 x 10^{-4} M metoclopramide. No effect was seen on the resting current-voltage relationship of the cell.

Under both current- and voltage-clamp conditions YM 09151-2 reversibly suppressed the response to dopamine (8 experiments each). Under current-clamp, the size and duration of the response was reduced (Figure 60). In a representative voltage-clamp experiment (Figure 61), the magnitude of the dopamine-induced currents was suppressed over the complete range of command potentials. Prior to the exposure of the preparation to YM 09151-2, the current magnitudes were -12.5 nA at a command potential of -160 mV, -0.5 nA at -60 mV, and +15 nA at +30 mV. Following 5 minutes exposure to 1 x 10^{-6} M YM 09151-2, the values were reduced to 0 nA at all command potentials. After 10 minutes wash, the response returned, and the values were -10 nA at -160 mV, -0.5 nA at -60 mV, and +10 nA at +30 mV. No effect was seen on the resting current-voltage relationship of the cell.

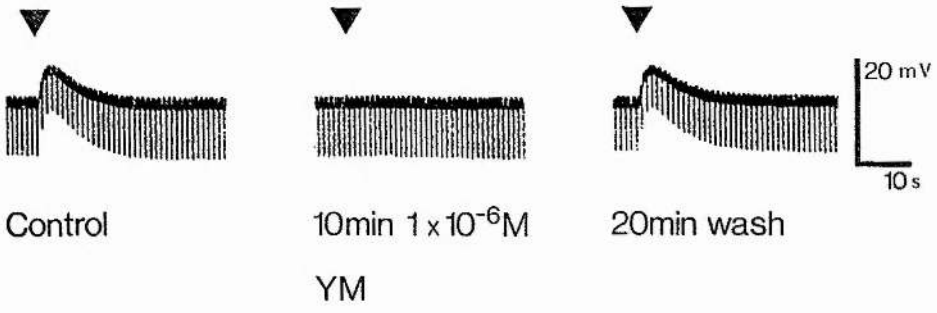


Figure 60 The suppressive effect of 1 x 10^{-6} M YM 09151-2 on the current-clamp response to dopamine. Current pulses were applied to the cell through a second microelectrode, resting potential -58 mV.

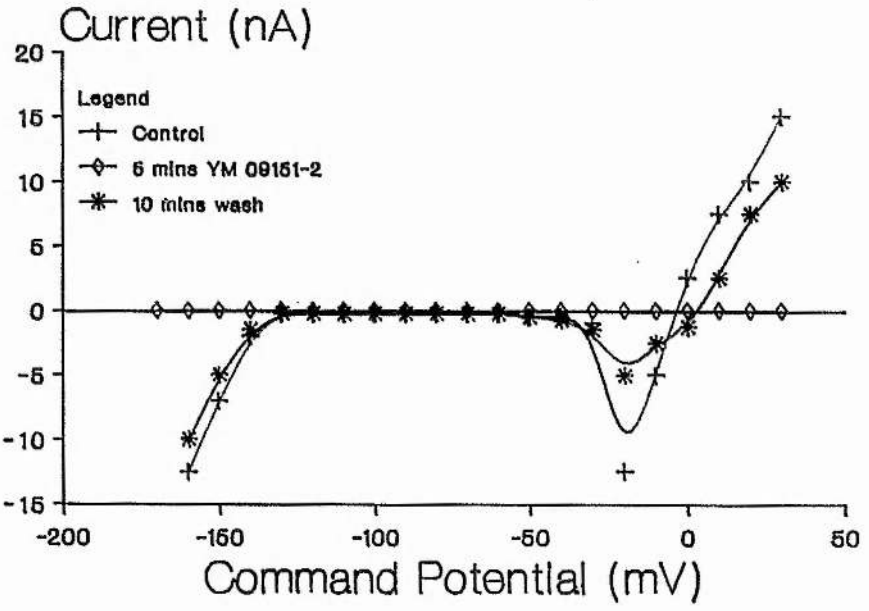


Figure 61 The suppressive effect of 1 x 10^{-6} M YM 09151-2 on the voltage-clamp response to dopamine at range of command potentials. Holding potential -60 mV.

Sulpiride and chlorpromazine have presented something of a problem in this study since it did not prove possible to achieve stable recordings for long periods of time from cells in the presence of either agent. In some experiments, the cell depolarised shortly after the introduction of sulpiride to the circulation. In others, the cell hyperpolarised, and at the same time a very marked fall in input resistance was seen, associated with a suppression of the response. The cell did not normally recover from these effects, even after prolonged washing (for example, see Figure 62). This was true of a wide range of concentrations, from 1 x 10^{-9} M to 1 x 10^{-4} M. At no point was a reliable, reversible blockade of the response achieved without dramatic, and usually irreversible, side-effects.

If sulpiride and chlorpromazine were having an antagonist effect on the response to dopamine, then they may well be achieving this end by effectively short-circuiting the membrane resistance, by virtue of indiscriminate effects on the cell membrane. This result must be seen as unusual, since nowhere in the literature is there any mention of either sulpiride or chlorpromazine having any non-specific effects on the resting properties of experimental systems in which they have been applied.

It is to be concluded that both sulpiride and chlorpromazine were having non-specific effects whose nature is not entirely clear. They were unpredictable, and often appeared to irreversibly damage the preparation as a whole (other cells impaled after wash-off had very poor resting potentials approximately -10 to -20 mV; cf normal values of -50 to -60 mV). Previous experiments had shown that these

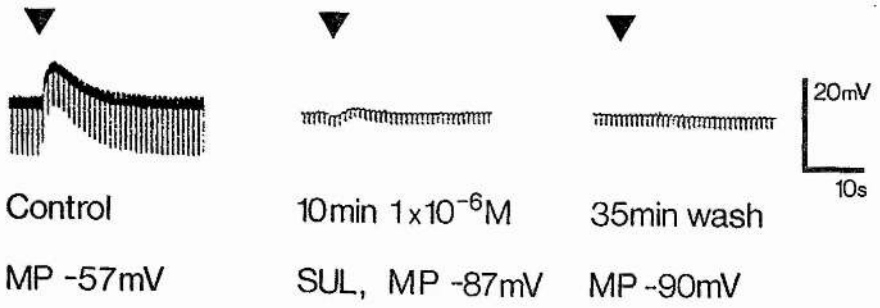


Figure 62 The effect of 1 x 10^{-6} M sulpiride on the current-clamp response to dopamine and the input resistance of the cell. The effect was irreversible. Current pulses were applied to the cell through a second microelectrode. Resting potential before the application of sulpiride was -56 mV, and after 35 minutes wash it was -18 mV.

effects were not the result of the actions of the vehicle in which the drug was dissolved, and sulpiride, therefore, did not prove to be a useful tool in the present investigation.

3.3.3 Discussion

The experiments detailed in section 3.2 indicated that the actions of dopamine in this preparation were mediated via receptors pharmacologically distinct from those mediating responses to noradrenaline, octopamine and acetylcholine. This, in addition to the evidence provided by the differences between the current voltage curves for the neurone in the presence of these agents strongly suggested that specific - dopamine receptors were present on this neurone.

The aim of the experiments detailed in this section was to determine a pharmacological profile for this putative dopamine receptor, and to ascertain whether or not it corresponded to either of the two classes (D_1 and D_2) of the mammalian central receptor classification scheme of Kebabian & Calne (1979).

In addition to pharmacological differences, there are distinctions between the ways in which the receptor is linked to adenylate cyclase in these two types. In D_1 receptors, activation causes stimulation of adenylate cyclase, and hence an increase in the level of intracellular cyclic AMP. Activation of D_2 receptors either has no effect or depresses adenylate cyclase activity, causing cyclic AMP levels to fall.

Agents which affect intracellular cyclic AMP metabolism have previously been shown to be ineffective in altering either the response to dopamine or the resting properties of the cell membrane. Pitman & Baker (1989) have shown that neither dibutyryl cyclic AMP, the phosphodiesterase inhibitor IBMX, nor the adenylate cyclase activator forskolin had any effect on the neurone. However, Nathanson & Greengard (1973; 1974) demonstrated that intracellular levels of cyclic AMP were elevated by octopamine, and, to a lesser extent, by dopamine in cockroach thoracic ganglion cells.

These results indicate that dopamine receptors in different preparations may differ substantially. In this case, electrophysiological evidence suggests that there is no linkage to adenylate cyclase, while biochemical studies indicate the presence of a dopamine-sensitive adenylate cyclase. It is unlikely, therefore, that the receptor present here is a 'classical' D_1 receptor, and it appears to share at least some characteristics with the mammalian D_2 receptor. The pharmacological profile determined here indicates that these receptors do not conform to either type.

The effects of dopamine were mimicked by the morphine derivatives apomorphine and bromocryptine, the dopaminergic ergot ergometrine, and the naphthalene A-6,7,DTN. Under current-clamp, all depolarised the neurone. Under voltage-clamp, the voltage-dependency of the response in all cases was similar to that of the dopamine response. The same region of negative-slope resistance was observed at potentials around -20 mV, as was the dramatic increase in inward current magnitude at potentials more negative than -140 mV. The

relative potencies of these four agonists appears to be ADTN > ergometrine > apomorphine > bromocryptine. ADTN produced a response which under identical conditions was experimentally indistinguishable from the response to dopamine.

Conversely the synthetic agonists SK&F 82526 (D_1) and LY 171555 (D_2) could not be made to produce a response at any concentration tested. However, these two compounds were only soluble in saline to a limited extent and therefore higher concentrations could not be tested. It appears therefore that neurone D3 is depolarised by relatively non-specific dopaminergic agonists, but unaffected, at least at the concentrations tested, by specific agents such as SK&F 82526 and LY 171555. This evidence suggests that the receptor present here does not conform precisely to either type D_1 or D_2.

Although specific agonists had no effect on the insect preparation, responses to dopamine could be blocked by antagonists specific to D_1 receptors (SCH 23390), agents specific to D_2 receptors (haloperidol, spiroperidol, YM 09151-2), and agents showing a lower degree of selectivity (flupenthixol, butaclamol and fluphenazine).

In some respects, the receptor present here corresponds to the D_2 receptor, in that the morphines, ergometrine and ADTN, all act as full agonists, and cyclic AMP does not appear to be involved. At D_1 sites they act as partial agonists or antagonists. In addition, D_2 antagonists block the response to dopamine. However, since D_1 agents also do this, the receptor cannot be said to conform entirely to either type, but perhaps to represent a third subdivision.

At this point it is important to discuss the observed effects of metoclopramide, sulpiride, and chlorpromazine. All of these agents are accepted in the literature as D_2 antagonists. Sulpiride is one of the benchmark antagonists used by Kebabian & Calne (1979).

In this preparation, however, metoclopramide had no detectable effect on the response to dopamine. As has been shown in section 3.3, metoclopramide was successfully used as an agent distinguishing between responses to dopamine and octopamine. Since the literature suggests that metoclopramide is both a D_2 antagonist, and an antagonist of octopamine receptors (Evans, 1981; 1984a), this provides further supporting evidence that the receptor present on neurone D3 does not correspond to the D_2 receptor.

The pharmacological evidence presented here might allow two conclusions to be drawn. Firstly that a single class of dopamine receptor exists, but with pharmacological properties intermediate between mammalian D_1 and D_2 types; secondly that two populations of receptors exist together on neurone D3.

Two pieces of evidence oppose the second conclusion. In the first place, responses to dopamine are abolished completely by antagonists specific for both D_1 and D_2 receptors. If both types were present, only a proportion of the response would be expected to be suppressed. In addition, agents which alter intracellular cyclic AMP metabolism have been shown to be ineffective in changing any of the characteristics of either the response or the resting properties of the cell (Pitman & Baker, 1989).

The conclusion that dopamine receptors are present in this preparation which do not conform to types D_1 or D_2 has only recently received indirect support from experimental evidence obtained from other phyla. Previously, most authors had concluded that dopamine receptors present in their preparations conformed to one of these types.

For example, the receptors found to mediate both excitatory and inhibitory responses in growth hormone producing cells of *Lymnaea* have been shown to conform to the mammalian classification scheme (Stoof et al, 1985; 1986). However, Gospe (1983) was more cautious, suggesting that although similarities between molluscan and mammalian receptors did exist, insufficient evidence had been collected at that time to conclude whether or not mulluscan receptors conformed precisely to the mammalian system.

More recently, Werkman, Lodder, de Vlieger & Stoof (1987) have shown that the D_2 receptors present on growth hormone producing cells in *Lymnaea* show some differences in pharmacology from mammalian D_2 receptors. Bokisch & Walker (1988) have concluded that receptors mediating hyperpolarising responses in *Helix* neurones could not be placed into the mammalian classification scheme. Likewise, Audesirk (1989) has shown that receptors on *Lymnaea* neurones which mediate both hyperpolarising the biphasic responses also cannot be included.

It was not possible to assess the actions of sulpiride and chlorpromazine on the response to dopamine, sonce both produced non-specific effects on the resting properties of the cell. In both

cases, massive depolarisation or hyperpolarisation, accompanied by a substantial fall in input resistance occurred. This suggested an overall short-circuiting of membrane conductance. These effects do not appear to have been reported in the literature.

The manner in which these agents produce their effects is unclear, although several possibilities exist. They could be directly affecting the membrane itself, altering the lipid phase and thus changing membrane resistance. They could be altering the selectivity of ionophores in the cell membrane, or affecting one of the membrane-based ion exchange or ion transport systems. However, there is no evidence to suggest which if any of these is the case.

There is now evidence to suggest, therefore, that molluscan dopamine receptors are not as similar to mammalian receptors as has been previously thought. If this is the case in molluscs, it might equally well be true in insects. The dopamine receptors characterised here clearly do not conform to either type D_1 or D_2. It will be interesting to see whether further types are identified in the insect CNS in the future.

3.3.4 Summary

A range of agonist agents, specific to D_1 or D_2 receptors was tested on neurone D3. Of these, the D_1 agonist ADTN was a potent mimic of the effects of dopamine, while the D_1 agent SK&F 82526 had no apparent effect. The D_2 agonists apomorphine, bromocryptine and

ergometrine all produced excitatory effects similar to dopamine, but LY 171555 had no effect.

Antagonists were also tested, on the response to dopamine, which either had no reported specificity for either class of receptor, such as fluphenazine, butaclamol and flupenthixol; specificity for D_1 receptors, such as SCH 23390; or for D_2 receptors, such as haloperidol, spiroperidol, metoclopramide, YM 09151-2, sulpiride and chlorpromazine. There was no consistency in the effects shown by these agents.

The results of these experiments are summarised in Tables 3 and 4.

TABLE 3

Summary of Pharmacological Profile - Agonists

DRUG		TARGET RECEPTOR	PRESENT STUDY
DOPAMINE	(10^{-2}M)*	D_1/D_2	++
SK & F 82526	(10^{-3}M)*	D_1	--
ADTN	(10^{-2}M)*	D_1	++
APOMORPHINE	(10^{-2}M)*	D_2	++
BROMOCRYPTINE	(10^{-2}M)*	D_2	++
ERGOMETRINE	(10^{-2}M)*	D_2	++
LY 171555	(10^{-3}M)*	D_2	--

++ = FULL AGONIST -- = NO EFFECT
* = USED FOR PRESSURE-APPLICATION

TABLE 4

Summary of Pharmacological Profile - Antagonists

DRUG		TARGET RECEPTOR	PRESENT STUDY
FLUPHENAZINE	(10^{-6}M)	D_1/D_2	+
(+)BUTACLAMOL	(10^{-7}M)	D_1/D_2	+
FLUPENTHIXOL	(10^{-9}M)	D_1/D_2	+
SCH 23390	(10^{-6}M)	D_1	+
HALOPERIDOL	(10^{-9}M)	D_2	+
SPIROPERIDOL	(10^{-6}M)	D_2	++
METOCLOPRAMIDE	(10^{-4}M)	D_2	-
YM 09151-2	(10^{-6}M)	D_2	+
s-SULPIRIDE	(Variable)	D_2	+?
CHLORPROMAZINE	(Variable)	D_2	+?

+ = REVERSIBLE BLOCK ++ = IRREVERSIBLE BLOCK
-- = NO EFFECT +? = UNCERTAIN EFFECT

SECTION 4 - CONCLUSIONS

The response of the neurone D_{α} to dopamine (Fleming, 1986; Pitman & Baker, 1988) has been characterised in terms of its pharmacology. The response is a depolarisation, and there may be an involvement of sodium, potassium, or chloride ions in the mediation of the response, althought this is not clear. Calcium plays an important role, but whether as a charge carrier or as a trigger for other membrane-based events is unclear.

The results presented here show that dopamine depolarises a motoneurone in the insect CNS, but at the same time it does not appear to be acting as a primary excitatory transmitter. The voltage-dependency of the response, causing an increase in the magnitude of the response current at potentials positive to the resting potential of the cell, might allow dopamine to exert a primarily modulatory role in the cockroach CNS.

The pharmacological profile, taken in conjunction with the difference in current voltage curves for dopamine, noradrenaline, octopamine, and acetylcholine indicates that dopamine is acting at specific receptors and not at receptors for these other transmitters. The dopamine receptor present here does not however conform to the classification scheme for vertebrate central dopamine receptors of Kebabian & Calne (1979). This may reflect evolutionary changes in the dopamine receptor, since hyperpolarising and biphasic responses

to dopamine in some molluscs also seem to be mediated by receptors which do not conform to this scheme (Bokisch & Walker, 1988; Audesirk, 1989).

There is still doubt, however, as to whether the dopamine responses recorded in neurone D_3 are mediated by a single class of receptor or through a mixed receptor population. Unitary curent recordings may demonstrate conclusively whether or not a single class of receptor is involved.

Further research is still necessary in order to identify conclusively the ionic currents underlying the response to dopamine, and to ascertain whether dopamine does indeed modulate the activity of the common inhibitory motoneurone, D_3.

APPENDIX 1

SALINE SOLUTIONS

Saline constitutions were based upon those used by Yamasaki & Narahashi (1959), and modified by Pitman (1975).

Normal saline solution had the following composition:

NaCl	1M	53.5	ml
KCl	1M	3.1	ml
$CaCl_2$	1M	9.0	ml
pH		7.2	

This solution was continuously circulated by bubbling with oxygen, which allowed for prolonged survival of the preparation in the bath.

Saline solutions for ion-substitution experiments were made up as follows:

Sodium-free saline

by equimolar substitution with TRIS-HCl

TRIS-HCl	1M	248.8	ml
KCl	1M	3.1	ml
$CaCl_2$	1M	9.0	ml
pH		7.2	

The concentration of TRIS-HCl was increased to 248.8 mM to compensate for the lower degree of dissociation of TRIS compared to NaCl.

31mM K^+ saline

by equimolar substitution of Na^+ with K^+

NaCl	4M	46.6	ml
KCl	1M	31.0	ml
$CaCl_2$	1M	9.0	ml
TES buffer		2.292	gl^{-1}
pH		7.2	

Calcium-free saline

The proportion of NaCl was increased to compensate for the loss of ionic strength resulting from the removal of Ca^{2+}

NaCl	4M	55.5	ml
KCl	1M	3.1	ml
TRIS-HCl	1M	1.0	ml
	or		
TES buffer	1M	10.0	ml
pH		7.2	

47mM chloride saline

NaCl was replaced with Na-isethionate, in an equimolar fashion

NaCl	4M	6.5	ml
KCl	1M	3.1	ml
$CaCl_2$	1M	9.0	ml
Na-isethionate		27.8	g
TES buffer		2.292	g
pH		7.2	

36mM calcium saline

NaCl was replaced by $CaCl_2$

NaCl	4M	49.7	ml
KCl	1M	3.1	ml
$CaCl_2$	1M	36.0	ml
TES buffer		2.292	g
pH		7.2	

Chloride-free saline

The chloride anion was replaced by acetate in all salts

NaAc	1M	214.0	ml
KAc	1M	3.1	ml
$Ca(Ac)_2$	1M	9.0	ml
TRIS-Ac	1M	10.0	ml
pH		7.2	

APPENDIX 2

NAME	CHEMICAL NAME
$^{+\ 1/2}$ A-6,7-DTN	6,7-dihydroxy-1,2,3,4-tetrahydro-naphthalene
$^{+\ 1/2}$ DOPAMINE	3-hydroxy Tyramine
$^{+\ 1}$ SK&F 38393	2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine
$^{+\ 1}$ SK&F 82526	6-chloro-2,3,4,5-tetrahydro7,8-dihydroxy-1-(4-hydroxy phenyl) -1H-3-benzazepine
$^{+\ 2}$ APOMORPHINE	apomorphine
$^{+\ 2}$ BROMOCRYPTINE	2-bromo-α-ergocryptine
$^{+\ 2}$ LY 171555	(-) 4,4a,5,6,7,8,8a,9-octahydro-5-n-propyl-2H-pyrazolo-3,4-g-quinoline
$^{-\ 1/2}$ CHLORPROMAZINE	2-chloro-10-(3-dimethylaminopropyl)-phenothiazine
$^{-\ 1/2}$ FLUPHENAZINE	1-(2-hydroxyethyl)-4-[3-(trifluoromethyl-10-phenothiazinyl) -propyl]-piperazine
$^{-\ 1/2}$ (+)BUTACLAMOL	1H-benzo-[6,7,1-cyclohepta-[1,2,3-de]-pyrido-[2,1-a]-isoquinolin-3-ol,3-(1,1-dimethylethyl)-2,3-4,4a,8,9,13b, 14-octahydro,HCl(3-α; 4a-α; 13b-β)
$^{-\ 1}$ cis-(Z)-FLUPENTHIXOL	cis-(Z)-2-trifluoromethyl-9-(3-[4-(hydroxyethyl)-1-piperazinyl]-propylidene)-thioxanthene
$^{-\ 1}$ SCH 23390	7-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine
$^{-\ 2}$ HALOPERIDOL	4'-fluoro-4-(4-hydroxy-4-p-chlorophenyl piperidino)-butyrophenone
$^{-\ 2}$ METOCLOPRAMIDE	4-amino-5-chloro-N-(2-diethylaminoethyl)-2-methoxybenzamide
$^{-\ 2}$ SPIPERONE	≡ SPIROPERIDOL 8-[3-(p-fluorobenzoyl)propyl]-1-phenyl-1,3,8-triazaspiro-[4,5]decan-4-one
$^{-\ 2}$ SULPIRIDE	N-1-[ethylpyrrolidin-2-yl-methyl]-2-methoxy-5-sulphamoyl benzamide
$^{-\ 2}$ YM-09151-2	N-[1-benzyl-2-methylpyrrolidin-3-yl]-5-chloro-2-methoxy-4-methyl-amino-benzamide

$^{+\ 1/2}$ = D_1/D_2 agonists, $^{+\ 1}$ = D_1 agonists, $^{+\ 2}$ = D_2 agonists

$^{-\ 1/2}$ = D_1/D_2 antagonists, $^{-\ 1}$ = D_1 antagonists, $^{-\ 2}$ = D_2 antagonists

APPENDIX 3

Dopamine

Freely soluble in aqueous media. At physiological pH (7.2), it begins to decompose within a few minutes, darkening the solution. This may be overcome by dissolving in a 1% solution (in either saline or water, depending on application) of sodium metabisulphite or ascorbate. This lengthens the usable life to several hours.

Octopamine

See **Dopamine**

Acetylcholine

Very soluble. Hygroscopic in solid form. Stability good, keeps in solution for several hours.

γ-Amino Butyric Acid (GABA)

Freely soluble. Needs no stabilization. Keeps well for several hours.

Apomorphine

Very insoluble. Will dissolve sparingly (max. conc. $\simeq 10^{-5}$M) in saline. Dissolves better in HCl, but if too concentrated, will come out of solution when pH is altered to physiological levels. Very unstable. Solution turns green after a few minutes as Apomorphine oxidizes. Must be used immediately. May need to be sonicated.

Bromocryptine

Similar to Apomorphine, though slightly more soluble. Same process and precautions apply. May need to be sonicated.

SK&F 82526

sparingly soluble at low pH, will remain in solution at relatively low concentrations ($\simeq 10^{-5}$M) at pH 7.2.

LY 171555

Sparingly soluble in saline at pH 7.2. Max. conc. $\simeq 10^{-4}$M

Chlorpromazine

Freely soluble in saline at pH 7.2.

Fluphenazine

Freely soluble.

Spiroperidol (= Spiperone)

Sparingly soluble, needs long spell on the stirrer!

(+)-Butaclamol

Soluble at max. conc. $\simeq 10^{-4}$M.

cis-Z-Flupenthixol

Freely soluble.

Haloperidol

Virtually insoluble in saline at pH 7.2. Dissolve in HCl first, Sonicate if possible. Neutralize before use. (note added after prolonged use:- dissolve in HCl, mix/grind with glass rod, add saline, spin for up to 1 hr until dissolved. Works for a stock concentration of 5×10^{-5} M.)

Metoclopramide

Freely soluble.

s-Sulpiride

Soluble. Max. Conc. ≃ 10^{-4}M

YM 09151-2

Soluble. Max. Conc. ≃ 10^{-4}M.

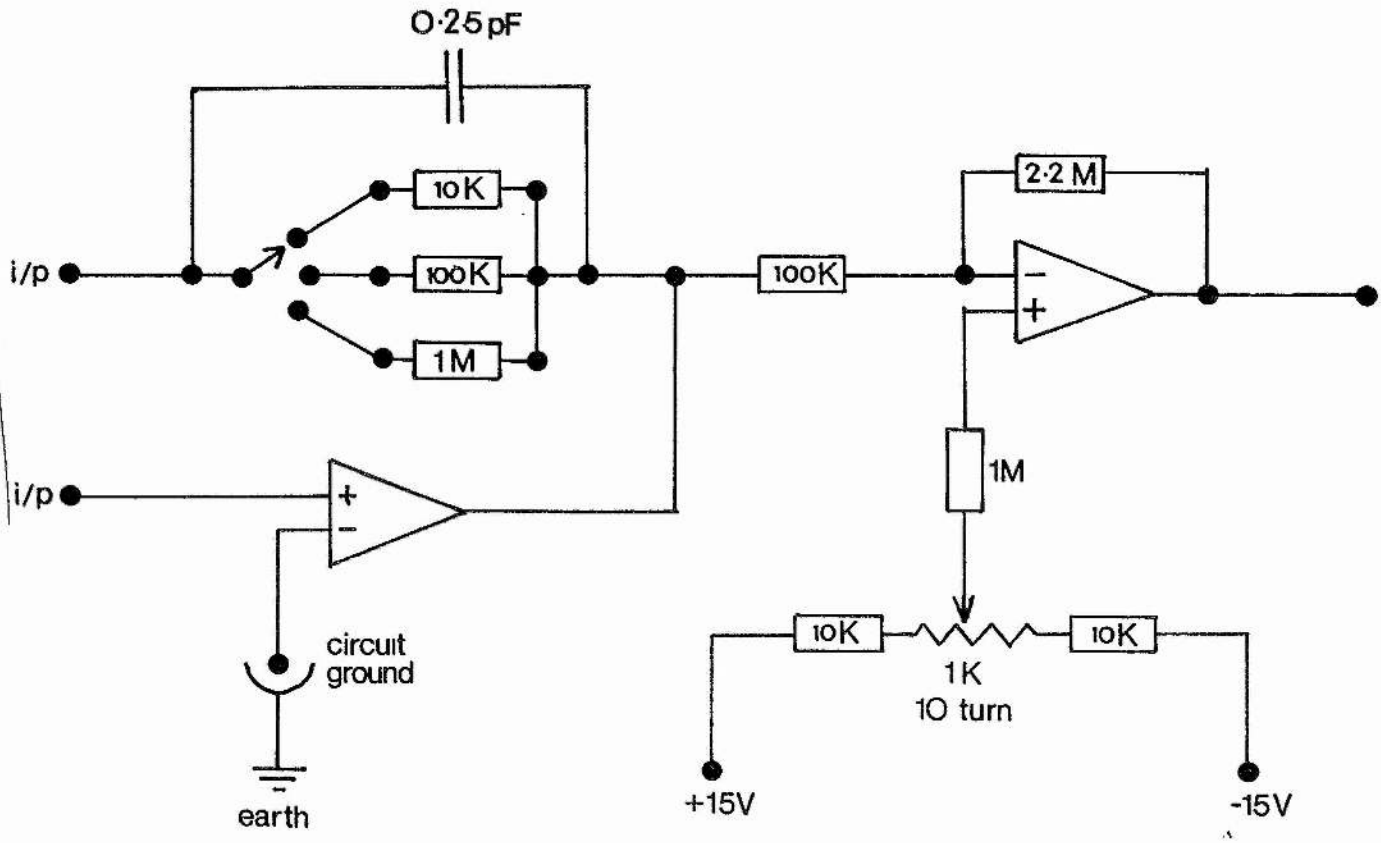
Ergotamine

Soluble in saline at conc. of 10^{-4} M.

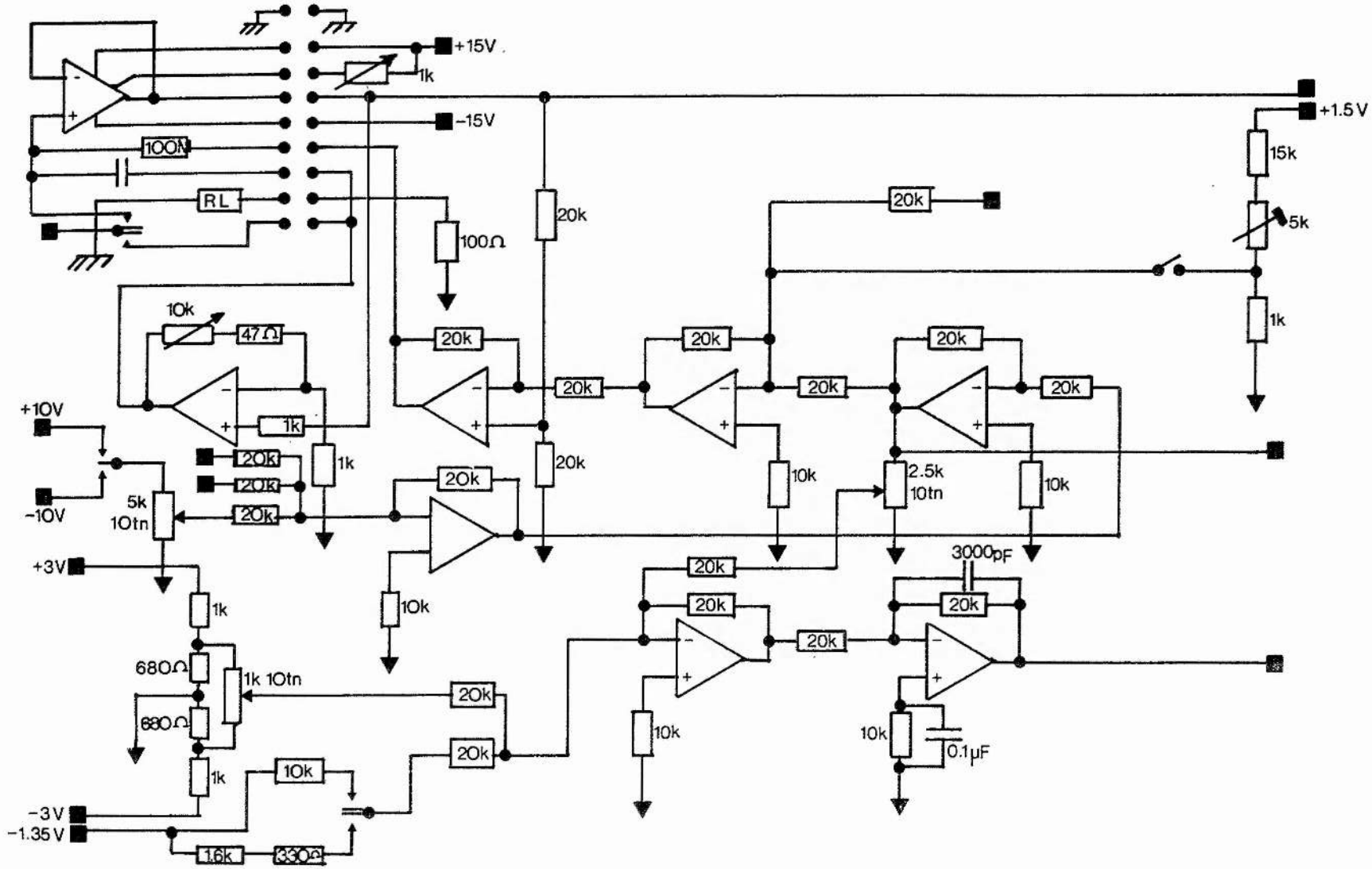
All other agents were easily soluble in normal saline at pH 7.2.

APPENDIX 4

Circuit diagrams of recording apparatus.



Current-voltage convertor (Current Monitor).

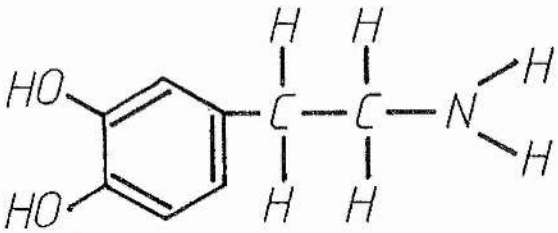


Intracellular recording amplifier.

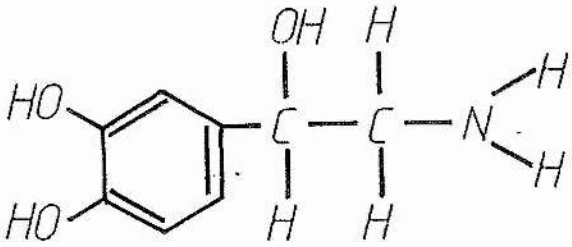
APPENDIX 5

Chemical structures of dopaminergic agents, and neurotransmitters.

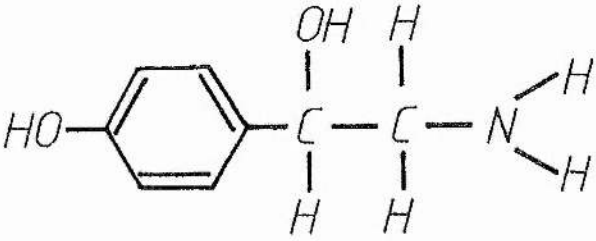
DOPAMINE



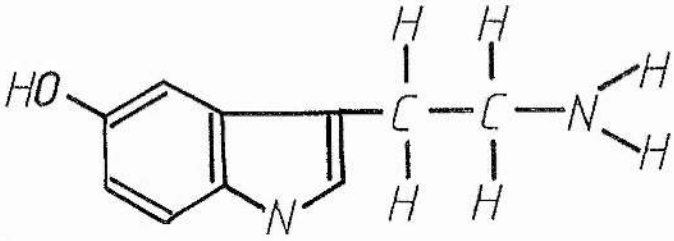
NORADRENALINE



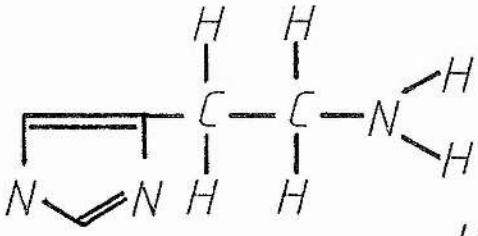
OCTOPAMINE



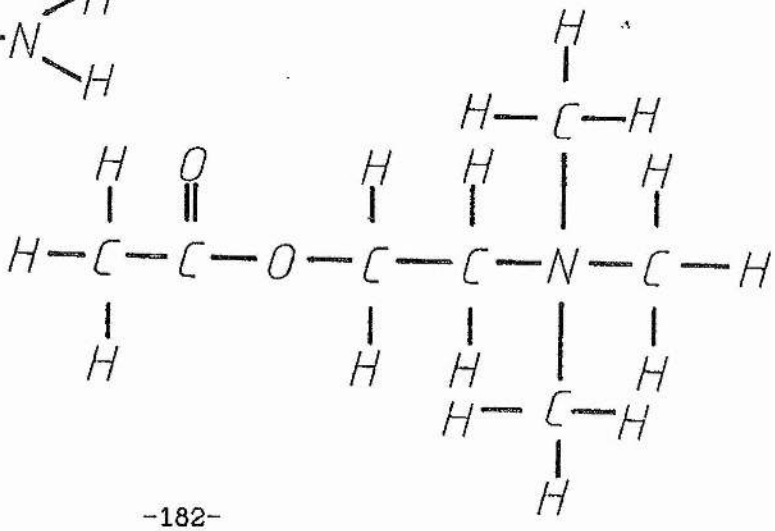
SEROTONIN



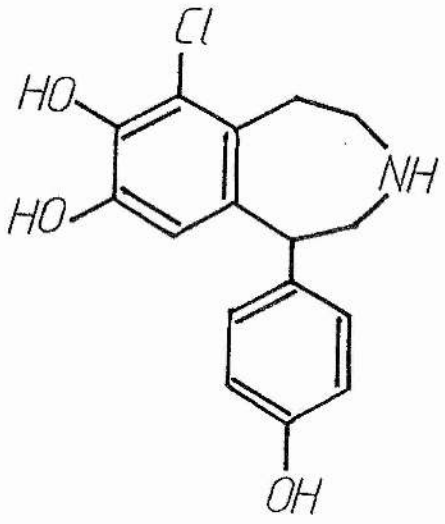
HISTAMINE



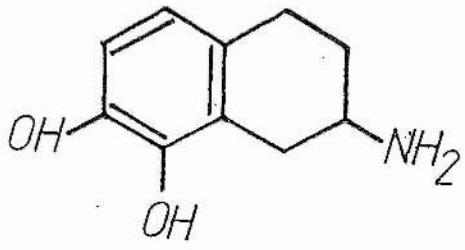
ACETYLCHOLINE



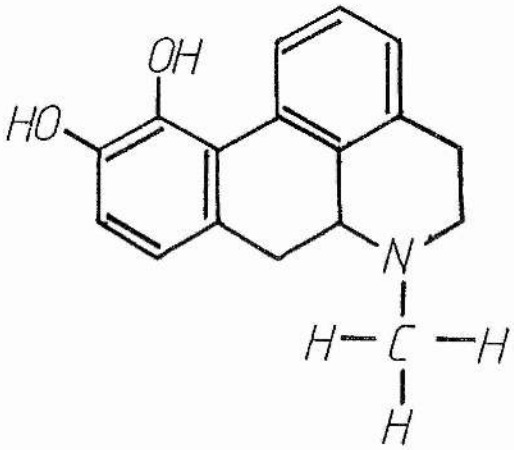
SK&F 82526



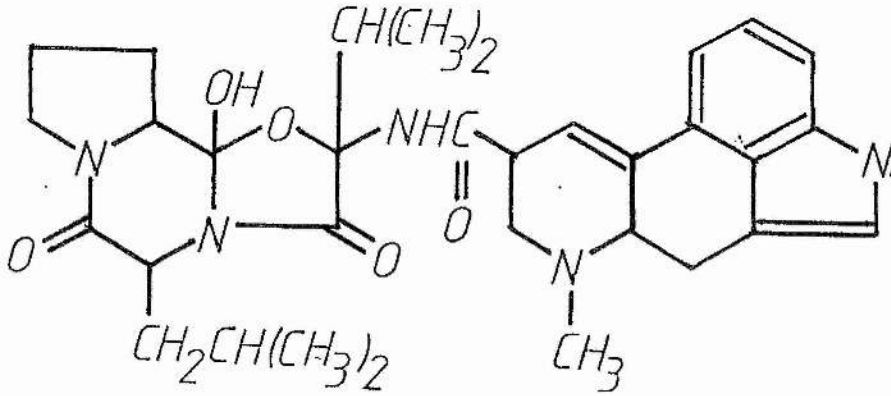
A-6,7-DTN



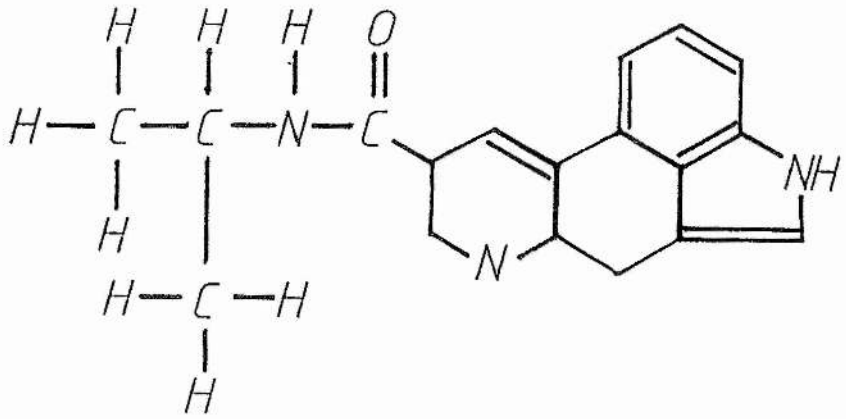
APOMORPHINE



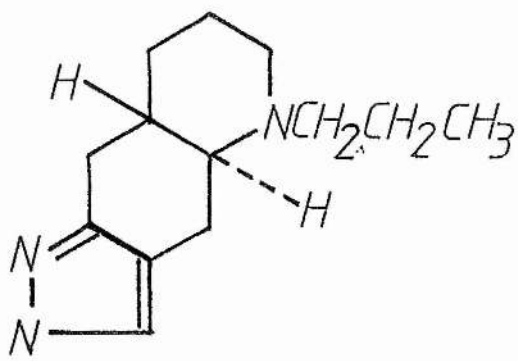
BROMOCRYPTINE

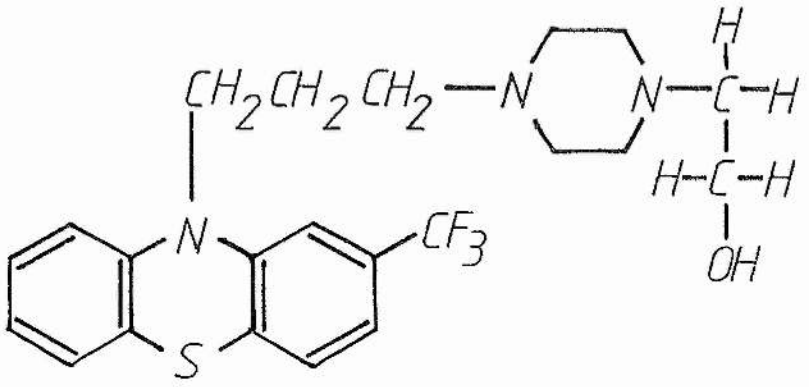


ERGOMETRINE



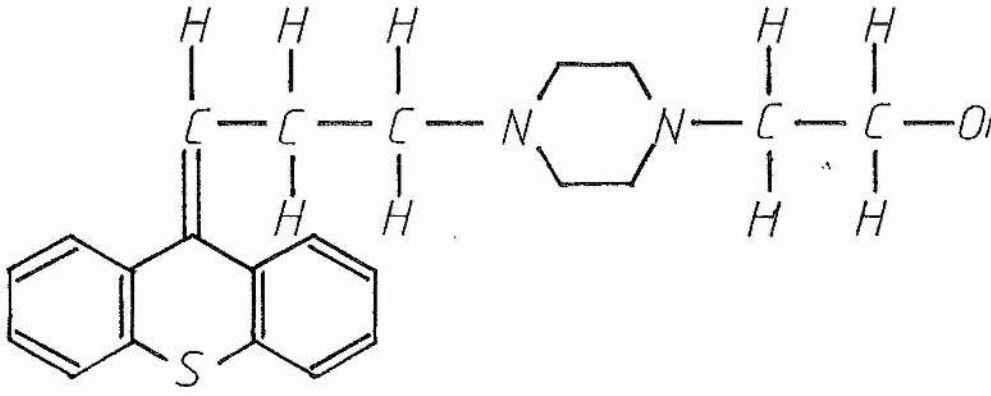
LY171555



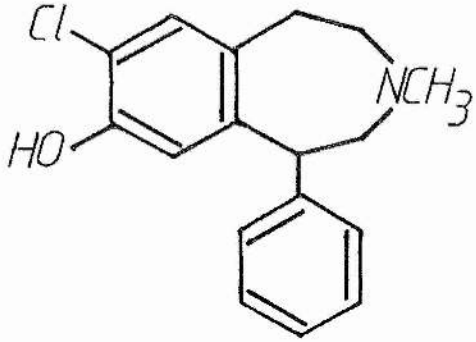


FLUPHENAZINE

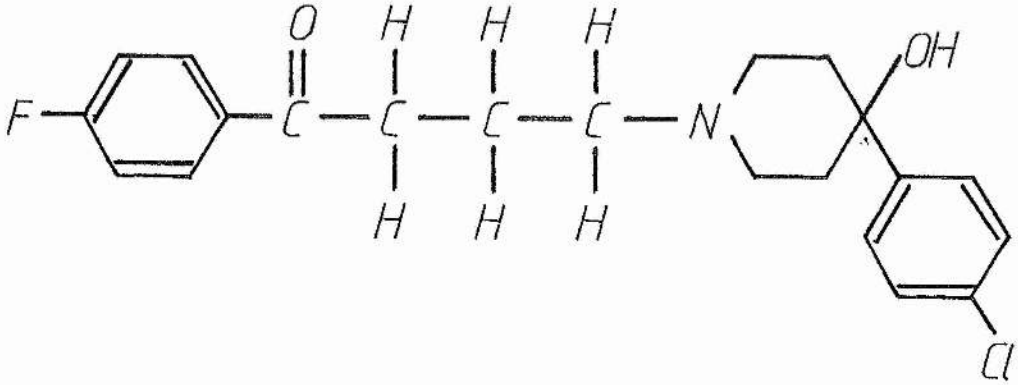
FLUPENTHIXOL



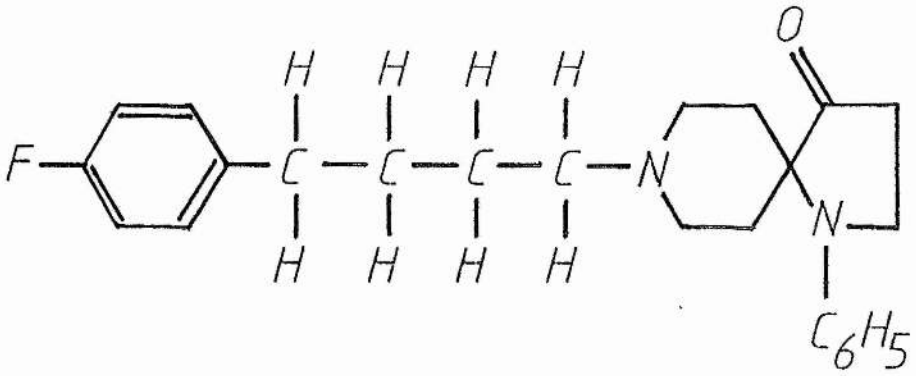
SCH 23390



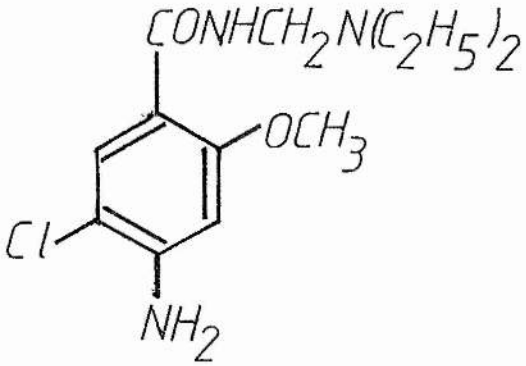
HALOPERIDOL



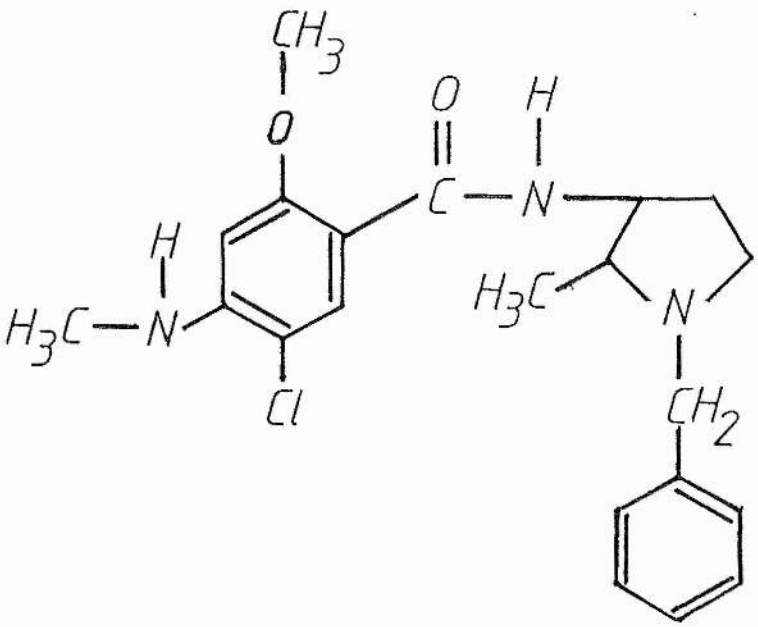
SPIROPERIDOL



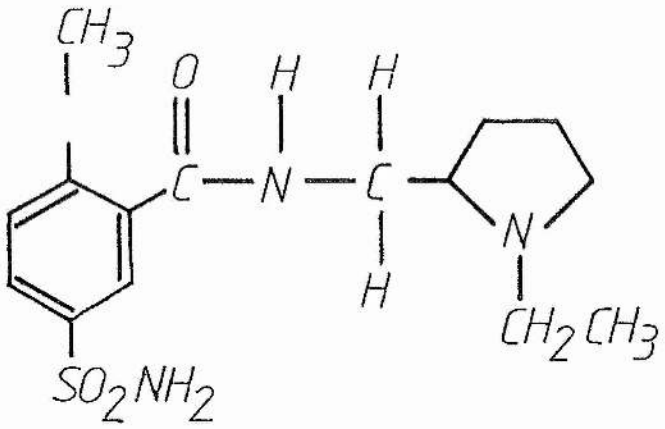
METOCLOPRAMIDE



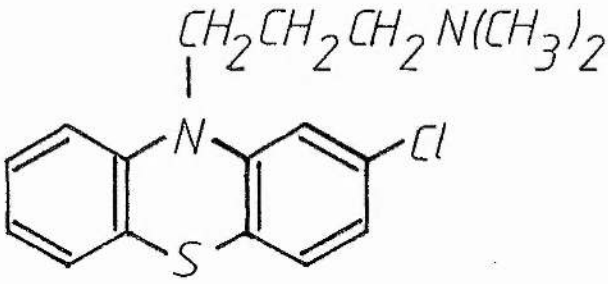
YM 09151-2



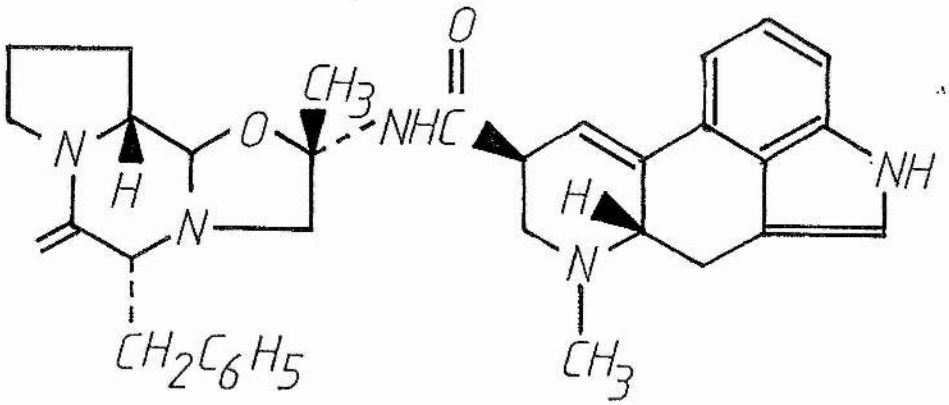
SULPIRIDE



CHLORPROMAZINE



ERGOTAMINE



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