THE EFFECTS OF DRUGS ON NEUROTRANSMISSION IN THE VAS DEFERENS

A thesis presented for the Degree of Doctor of Philosophy

by

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CONTENTS

PUBLICATIONS

Some of the work carried out during the course of this study has been published and reprints of the following articles may be found inside the back cover. The references are:-

FORSYTH, K.M., GALLAGHER, A.P. & POLLOCK, D. (1986).

Effects of reserpine or 6-hydroxydopamine on responses of mouse and rat vas deferens to field stimulation. Br. J. Pharmac., 87, 94P.

FORSYTH, K.M., LESLIE, C.A. & POLLOCK, D. (1986).

Thyroxine alters pre- and post-synaptic sensitivity in the mouse vas deferens. Br. J. Pharmac., 89, 828P.

FORSYTH, K.M. & POLLOCK, D. (1987).

Morphine and clonidine potentiate field stimulation-induced (^{3}H)-noradrenaline in the mouse vas deferens. J. Physiol., 381, 109P.

FORSYTH, K.M. & POLLOCK, D. (1987).

The effects of drugs on field stimulation-induced (^{3}H)-NA overflow in the mouse vas deferens. Abstract No. P946, IUPHAR 10th International Congress of Pharmacology, Sydney, 1987.

FORSYTH, K.M. & POLLOCK, D. (1987).

Clonidine and morphine increase (^{3}H)-noradrenaline overflow in mouse vas deferens. Br. J. Pharmac. (In Press).

FORSYTH, K.M., POLLOCK, D. & SHAW, A.M. (1987).

Clonidine and morphine enhance field stimulation-induced inositol phospholipid (PI) hydrolysis in the mouse vas deferens. Br. J. Pharmac. (Submitted).

SUMMARY

1) The aim of this study was to investigate the effects of field stimulation and drugs on neurotransmission in the vas deferens.

2) Field stimulation of mouse isolated vasa resulted in biphasic motor responses that were abolished by tetrodotoxin (TTX). The initial component of this response was selectively inhibited by morphine and clonidine. The second component was inhibited by phentolamine.

3) After 6-hydroxydopamine (6-OHDA) pretreatment, field stimulation produced a small, monophasic contraction, which was unaffected by phentolamine and by TTX. Vasa from 6-OHDA pretreated mice were supersensitive to noradrenaline (NA).

4) In control anaesthetised rats, motor responses of the in situ vas to field stimulation or to spinal nerve stimulation were biphasic and were abolished by TTX. After 6-OHDA pretreatment, in situ field stimulation produced monophasic contractions that were resistant to TTX, suggesting that these responses may be non-neuronal, resulting from direct stimulation of supersensitive smooth muscle.

5) Morphine and clonidine potentiated the second noradrenergic component of the biphasic motor response to field stimulation at a time when the overflow of radioactivity from vasa preincubated with (^{3}H)-NA was increased.

6) The ability of morphine and clonidine to potentiate the field stimulation-induced overflow of radioactivity occurred in the presence of a combination of drugs which block the removal mechanisms for NA.

7) NA and electrical field stimulation increased phosphoinositide hydrolysis, reflected in [^{32}P]-phosphatidic acid (PA) formation in the mouse vas deferens and these responses were blocked by prazosin and by TTX.

8) Neither morphine nor clonidine affected basal levels of [^{32}P]-PA formation or the ability of NA to enhance [^{32}P]-PA formation but both drugs potentiated the ability of field stimulation to increase the formation of [^{32}P]-PA. This suggests that these drugs enhance the field stimulation-induced release of NA in the mouse vas deferens. A possible explanation for the findings that morphine and clonidine apparently inhibit release of one co-transmitter whilst potentiating the release of another is discussed.

9) Part of the study investigated the effects of chronically treating mice with thyroxine (T_4). The level of T_4 in the serum was increased in mice pretreated with T_4, and this was accompanied by changes in pre- and post-synaptic receptor sensitivity.

10) Pretreatment with T_4 produced a presynaptic subsensitivity to morphine and clonidine and a postsynaptic subsensitivity to NA and carbachol. In these animals, the ability of morphine and clonidine to potentiate the overflow of (^{3}H)-NA was diminished. A possible explanation for these observations is considered.

INTRODUCTION

The existence of what is now known as the autonomic nervous system has been recognised for several centuries. The term 'sympathetic' applied to the peripheral nerves was introduced in 1732 by the Danish anatomist Winslow (Bowman & Rand, 1981), who believed that this system of nerves was responsible for regulating the 'sympathies' or interrelationships of organs in the body. The modern concept of the autonomic nervous system is based to a large extent on the anatomical and physiological studies of Gaskell and Langley. Gaskell (1886) demonstrated the functional connection through the white rami communicantes between the central nervous system and the sympathetic chains, and the connections of the grey rami to spinal nerves. He also recognised that the prevertebral ganglia were part of the sympathetic division. Gaskell grouped the cranial, sacral and thoracolumbar outflows from the spinal cord together with the sympathetic trunks and the prevertebral and other ganglia as the 'involuntary nervous system' (1916). Langley (1898) first proposed the designation 'autonomic nervous system', which included both the sympathetic and parasympathetic divisions, and this term has now been generally adopted.

NEUROCHEMICAL TRANSMISSION

The development of the concept that the nervous impulse is transmitted across synapses and neuroeffector junctions by a chemical mediator arose largely from studies of autonomic nervous function (Elliott, 1904; Loewi, 1921). Initially, there were two theories of transmission: the electrical theory and the humoral theory (Du Bois Reymond, 1877; Kühne, 1888; Elliott, 1904; Loewi, 1921; Cannon & Bacq, 1931). The electrical theory held that transmission was brought about by the spread of nerve action currents to the tissue. However, the nerve impulse must in some way achieve amplification in order to

excite the larger muscle mass. The humoral theory, which proposes that chemicals with specific actions are released from nerves, provides a more satisfactory explanation for amplification of impulses. This theory also provides an explanation for the observation that both excitation and inhibition can be produced in one tissue by stimulation of both sympathetic and parasympathetic nerves (Loewi, 1921).

Throughout the history of physiology, drugs have had an important role in analysing the operation of biological systems (Bernard, 1856; Langley, 1905; Dale, 1914). The effects of drugs have been examined in poorly understood systems, providing useful information about drug action and biological function. Had physiologists relied solely on the use of drugs whose mechanisms of action were fully understood, it is likely that little advance in our knowledge would have been made.

The development of the theory of chemical transmission and the evidence implicating acetylcholine (ACh) and noradrenaline (NA) as the chemical mediators of the autonomic nervous system has been reviewed (Gershon, 1970). A remarkable similarity between the effects produced by muscarine and the effects of peripheral nerve stimulation had been observed, and the blocking action of atropine noted (Dixon, 1907; Dixon & Brodie, 1903). However, it was later established that ACh rather than muscarine was likely to be the parasympathetic transmitter substance (Dale, 1914).

A similar pattern had been noted in the action of suprarenal extracts, sympathetic nerve stimulation and administration of adrenaline (Langley, 1901; Elliott, 1904). On stimulation of nerves at the lower end of the sympathetic chain in adrenalectomised animals, a substance passed into the blood producing the effects of sympathetic stimulation on other organs (Cannon & Bacq, 1931). This transmitter of sympathetic effects was referred to as 'sympathin' and was suggested to be either adrenaline or a very closely-related substance (Bacq,

1933). However, the effects of the transmitter carried by the blood from sympathetic nerve endings and the effects of injected adrenaline were not identical. This observation led to the theory that during sympathetic nerve stimulation, adrenaline is indeed released, but acts solely as a mediator, combining with an excitatory or an inhibitory substance localised in the receptors to form two complexes which would be the active substances (Cannon & Rosenbleuth, 1933). This theory received little support, and evidence was later presented that noradrenaline (NA) is the predominant catecholamine released on stimulation of the sympathetic nerves. There was no evidence that it was modified in any way before reaching the effector cells (Von Euler, 1946). Several studies have now confirmed and extended these observations, and all the evidence indicates that in mammals, the main transmitters liberated on stimulation of the post-ganglionic sympathetic and parasympathetic nerves are, respectively, NA and ACh.

POSTSYNAPTIC RECEPTORS

In the physiology of nerve transmission, the idea of a specialised tissue element on which the nerve impulse acted, which intervened between nerve and muscle and which was the site of action of such poisons as curare, long preceded the theory of chemical neuro-transmission (Bernard, 1857). On the basis of a famous experiment in which Bernard showed that nicotine applied to a particular part of the surface of a muscle elicited twitching and that this effect was prevented by curare, Langley (1905) postulated the existence of a 'receptive substance' located on the muscle rather than on the nerve endings. He suggested that this 'receptive substance' existed in more than one form in autonomic effector cells.

At an early stage it was realised that ACh had two main types of action which could be reproduced by the drugs muscarine and nicotine.

Muscarinic receptors are present in heart, smooth muscle and salivary glands, and are excited by muscarine and allied synthetic substances and blocked by atropine and atropine-like drugs. Nicotinic receptors, present in skeletal muscle, parasympathetic and sympathetic ganglia and the adrenal medulla, are activated by many substances structurally-related to nicotine but are blocked by high concentrations of nicotine (Dale, 1914). When the muscarinic receptors are blocked by atropine, the effects of ACh acting on nicotinic receptors in sympathetic ganglion cells are uncovered (Dale, 1914). It was postulated, therefore, that there were two types of receptor for ACh, and these were designated muscarinic and nicotinic. The distinction between the two types of cholinoceptor isoreceptors was clarified because of the historical accident that many of the potent alkaloids that attracted the attention of early pharmacologists acted specifically on either muscarinic or nicotinic sites. Relatively few studies on isoreceptors of cholinoceptors have been carried out. However, there have been several studies on isoreceptors of adrenoceptors, starting with Ahlquist's differentiation in 1948 between α- and β-adrenoceptors and extended by Furchgott in 1972, based on the relative potencies of various adrenergic agonists and on the susceptibility of blockade by specific drugs. However, it was not until the introduction of specific β-adrenoceptor antagonist drugs that the classification of α- and β-adrenoceptors was accepted (Powell & Slater, 1958). Thereafter, β-adrenoceptors were further subdivided into β_1- and β_2-adrenoceptors on the basis of the differential sensitivity of various tissues to a series of β-adrenoceptor agonists and antagonists (Lands, 1967).

PRESYNAPTIC RECEPTORS

In the peripheral nervous system, the magnitude of a tissue's response to nerve stimulation is related to the rate of transmitter release and this in turn is controlled by the frequency of impulse traffic, which is centrally determined (Brazier, 1960). However, from evidence accumulated over the last 3 decades, it is clear that transmitter release can also be controlled peripherally. Various observations suggest that neurally-released, hormonal and locally-formed substances, such as the prostaglandins, can influence transmitter release (see reviews, Starke, 1977; Westfall, 1977). This form of peripheral control appears to be mediated by receptors located on nerve varicosities - the sites of transmitter release from nerves. However, for a long time it was generally accepted that nerve endings specialised in the production, storage and release of chemical transmitter but were devoid of receptors, which were thought to be confined to the postsynaptic membranes of the cells upon which the neurotransmitters acted (Dale, 1952). Contrary observations had been intermittently reported but had received little attention. In tracing the development of pharmacology, examples can be found where advances in understanding were followed by periods of tunnel vision. For example, theories such as electrical neurotransmission (Kühne, 1888) and, later, autonomic nerves releasing only ACh or NA, took years to be supplanted by new ideas. Eventually the weight of evidence refuting a long-accepted principle renders a change in our view inevitable. However, recent years have seen a remarkable change. Nerve terminals have been found to be invested with a multitude of receptor-types (Stjärne, 1975b; Langer, 1977; Starke, 1977; 1981).

Much of the evidence which has established the hypothesis of local regulation of transmitter release has been derived from experiments in which the effects of agonist and antagonist drugs have been examined on

the overflow of transmitter released by nerve stimulation. The overflow of transmitter is the amount that escapes recapture by nerves ($Uptake_1$), extraneuronal cells ($Uptake_2$) or enzymatic inactivation, and is recovered in organ perfusates or tissue bathing fluid.

The first demonstration that drugs could increase sympathetic transmitter overflow was provided by Brown and Gillespie (1956, 1957) using the perfused cat spleen, which is a suitable organ for overflow studies because of its 'open type' of circulation. The amount of NA appearing in the venous blood following adrenergic nerve stimulation was related to the frequency of splenic nerve stimulation, with a maximum overflow at a frequency of 30 Hz (Brown & Gillespie, 1957). The overflow of transmitter was not altered by inhibitors of monoamine oxidase (MAO) (Brown & Gillespie, 1957; Brown et al., 1959) which, at that time, was thought to be the main enzyme responsible for the inactivation of NA. In the cholinergic system, it was known that ACh was destroyed by a cholinesterase, and this somewhat simpler mechanism of transmitter removal had been projected to the adrenergic system in order to simplify it. However, when the α-adrenoceptor antagonists dibenamine and phenoxybenzamine (PBA) were added to the blood-perfused spleen, there was a large increase in NA overflow, which was greater with stimulation at a frequency of 10 Hz than at 30 Hz. Knowing that both drugs blocked α-adrenoceptors, Brown and Gillespie concluded that NA released by nerve endings was taken up by receptor sites in the smooth muscle before being destroyed. When these receptor sites were blocked by PBA or dibenamine, most of the released amine overflowed into the venous blood.

This conclusion was later challenged by Paton (1960) on the basis of similar experiments carried out on the adrenal gland. The nerves innervating adrenal medullary cells release ACh which acts on the gland to release adrenaline and NA. At this site, as at the nerve endings,

the amount of amine appearing in the blood leaving the gland was dependent on the frequency of stimulation, with very little overflow at low frequencies. Brown and Gillespie had attributed this effect at nerve endings to a combination of amine with receptors, yet in this situation there are no local receptors. Paton offered another explanation. He suggested that some of the amine liberated by the medullary cell was taken back into the cell between stimuli, and the greater amount in the venous blood at high frequencies of stimulation followed from the reduction in time available for this uptake. The existence of a neuronal uptake mechanism ($Uptake_1$) was soon demonstrated and its characteristics established (Iversen, 1967). Drugs such as PBA were found to have the ability to inhibit $Uptake_1$ (Hertting et al., 1961; Gillespie, 1966; Iversen, 1967), thereby adding weight to Paton's hypothesis. An extraneuronal uptake process ($Uptake_2$) was also characterised (Avakian & Gillespie, 1968; Kalsner & Nickerson, 1969). It was suggested that PBA increased transmitter release by blocking neuronal uptake (Thoenen et al., 1964). However, PBA and other α-adrenoceptor antagonists can increase NA overflow in concentrations which do not inhibit neuronal or extraneuronal uptake (Starke, 1977; Westfall, 1977). Indeed, it is doubtful whether blockade of the uptake mechanisms alone can significantly increase NA overflow (Kalsner, 1981). In particular, it is paradoxical that in the vas deferens, which possesses a dense adrenergic innervation, inhibition of the uptake mechanisms with highly selective drugs should result in such a small increase in NA overflow (Farnebo & Malmfors, 1971).

In the early 1970's, four groups of workers independently proposed the hypothesis that NA regulates its own release by activating α-adrenoceptors located on the nerve terminal (Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Langer et al., 1971; Starke, 1971). Several

lines of evidence supported this view. PBA and other α-adrenoceptor antagonists, used in concentrations too low to inhibit neuronal or extraneuronal uptake, were found to increase NA overflow (Langer, 1970; Starke et al., 1971; Cripps & Dearnaley, 1972). Drugs which blocked neuronal uptake caused only a small increase in the overflow of NA following nerve stimulation. In contrast, α-adrenoceptor antagonists produced a much larger increase in transmitter overflow. Haggendal (1970) reported that PBA enhanced the amount of NA in the overflow from the nerve stimulated tissue even after inhibition of local re-uptake of liberated NA.

The idea that presynaptic α-adrenoceptors may regulate the nerve stimulation-evoked release of transmitter has been substantiated by observations that PBA and phentolamine increase the overflow of dopamine-β-hydroxylase, an enzyme present in adrenergic storage vesicles. This enzyme is not affected by uptake or degradative enzymes, and is released by exocytosis during nerve stimulation along with NA. Since dopamine-β-hydroxylase is, therefore, a good marker of NA release from nerves, the increased overflow of this granular component probably results from an increased release from the amine granules in the nerve terminals (Johnson et al., 1971; De Potter et al., 1971). Further evidence for the existence of a negative feedback mechanism was provided by α-adrenoceptor agonists, which inhibit NA overflow evoked by nerve stimulation in many tissues including the rabbit heart (Starke et al., 1971), rabbit pulmonary artery (Starke et al., 1975), cat spleen (Kirpekar et al., 1973), rabbit ear artery (Rand et al., 1973) and guinea-pig vas deferens (Stjärne, 1974). A characteristic feature of the ability of α-adrenoceptor agonists and antagonists to affect NA release is their ineffectiveness at high frequencies of stimulation (Brown & Gillespe, 1957; Langer, 1977). Difficulties arise in attempting to relate the ineffectiveness of

exogenous α-adrenoceptor agonists and antagonists using such stimulation parameters to the presynaptic receptor hypothesis. If the concentration of neurally-released NA was sufficiently high to fully activate the presynaptic α receptors and exert a maximum inhibition, then this could not be further enhanced by exogenous agonists (Langer, 1977; Starke, 1977). However, this explanation fails to accommodate the observation that α-adrenoceptor antagonists have little effect at high frequencies of stimulation. If NA was indeed exerting a maximum inhibitory effect then, under such circumstances, α-adrenoceptor antagonists would be expected to have their greatest effect to potentiate the release of transmitter. Such inconsistencies have led to the validity of the presynaptic receptor hypothesis being challenged (Kalsner, 1982; 1983a;b; Kalsner & Quillan, 1984) and an alternative explanation being put forward to account for the effects of antagonists on stimulation-evoked efflux of transmitter (Kalsner, 1983a). α-Adrenoceptor antagonist-induced increases in field stimulation-induced transmitter overflow should follow a predictable pattern if due to the interruption of a negative feedback system. The increases in overflow should be proportional to the level of ongoing feedback in the absence of antagonists and should be explicable in terms of the amount of NA present in the synapse to activate presynaptic receptors during nerve stimulation. However, in a variety of tissues from several species this was not found (Kalsner & Quillan, 1984). α-Adrenoceptor antagonists had their most pronounced effects to enhance overflow when the concentration of transmitter in the synapse was low, contrary to expectations for a negative feedback system.

The process of transmitter release from sympathetic nerve terminals is believed to be related to the duration of the action potential. Prolongation of the stimulation period allows the calcium

channels to stay open longer, permitting a greater entry of the ion and an increase in the amount of transmitter released. It has been proposed that the presynaptic action of α-adrenoceptor antagonists is linked to a prolongation of the duration of depolarisation and, consequently, is most likely to be seen with short pulse durations (Kalsner, 1983a). This possible explanation is substantiated by the finding that tetraethylammonium, a selective blocker of outward potassium channels (Szurszewski, 1978) known to prolong the duration of the action potential, acts to increase transmitter efflux (Kalsner & Quillan, 1984).

The development of a variety of new α-adrenoceptor agonists provided evidence that, although the inhibitory effect of NA was mediated through α-adrenoceptors, these were not identical with the familiar postjunctional α-adrenoceptors and differed in their sensitivity to drugs (Cambridge et al., 1977; Doxey et al., 1977). On the basis of this evidence, adrenoceptors were classified as either α_1-(postjunctional) or α_2-(prejunctional) subtypes (Langer, 1977; Starke, 1977; 1981). It was assumed that the entire population of postjunctional α-adrenoceptors was of the α_1 subtype. However, a sub-population of α-adrenoceptors, which were resistant to the selective α-adrenoceptor antagonist prazosin was later identified in vascular smooth muscle of rat and cat (Bentley et al., 1977). This was supported by a similar finding in human arteries (Moulds & Jauernig, 1977) and led to the breakdown of the original anatomical classification and the emergence of a new pharmacological classification of non-neuronal or postjunctional α_2-adrenoceptors (Docherty et al., 1979; Timmermans et al., 1979). These α_2-adrenoceptors have been reported in many organ systems, for example, platelets, pancreatic islets, adipocytes and kidneys. Thus, autonomic neurotransmission is brought about by chemical transmitters which are

released in response to nerve impulses. These transmitters interact with specific receptors on the postsynaptic effector cell to trigger a response, and their release is subject to regulation by receptors situated presynaptically on nerve endings.

SENSITIVITY CHANGES IN AUTONOMIC EFFECTORS

A characteristic of intrinsic importance for the survival of an organism is its ability to adapt to the external environment. These adaptations, which are prompted by environmental stimuli, vary in time-course, ranging from evolutionary changes which take many years to develop, to rapid adaptations required to protect the animal from a sudden hostile environment (Gibson, 1981). Many of the rapid alterations are initiated by the release of chemical mediators which act in concert, providing a physiological means of adapting tissue sensitivity to meet the particular requirements of the animal (Guyton, 1971). Two systems which are of prime importance in maintaining the conditions necessary for the continued existence of an organism are the autonomic nerves and the endocrine glands (Bernard, 1878; Canon, 1929). Both of these systems are involved in influencing smooth muscle sensitivity, which is not fixed, but is subject to change. Under certain conditions the amount of stimulant required to produce a response is less than normal, and this phenomenon is known as super-sensitivity.

i) Supersensitivity

Supersensitivity (Cannon & Rosenbleuth, 1949; Trendelenburg, 1972; Fleming et al., 1973; Thesleff, 1974; Westfall, 1981) generally occurs as a result of the removal of a tonic stimulus, and early observations of changes in the sensitivity of effector organs involved surgical denervation (Budge, 1855). Two types of super-sensitivity could be demonstrated as a result of denervation of the

cat nictitating membrane (Langer & Trendelenburg, 1966; Trendelenburg, 1966), one of which was specific for NA, the other of which was non-specific. The specific supersensitivity produced by NA could be attributed to the destruction of the adrenergic neurones, which are normally sites of loss for exogenously administered NA. A similar type of supersensitivity occurs when the block of inactivation is at a postjunctional site (Graefe & Trendelenburg, 1974; Trendelenburg & Graefe, 1975). This type of supersensitivity has been described as 'deviation' supersensitivity on account of its occurrence after removal of the sites of loss of the agents concerned (Fleming, 1975). As a consequence of this loss, the concentration of agonist which reaches the receptors of the effector organ increases, resulting in an enhanced response.

Non-specific or 'non-deviation' supersensitivity has been attributed to an increase in receptor number (Langer & Trendelenburg, 1968; Bito & Dawson, 1970). Sensitivity changes occur at several receptors, and the phenomenon is slow in onset, often occurring several days after surgical denervation of a tissue. It is likely that there is also a change in the effector cells at a level beyond the agonist-receptor interaction, for example, at sites where the excitation of several receptors converge on a common biochemical pathway (Hudgins & Fleming, 1968; Fleming, 1968).

Supersensitivity of a tissue to a receptor ligand is reflected in a leftwards displacement of the dose-response curve for one or more agonists (Cannon & Rosenbleuth, 1949). This occurs when the responses to all submaximal doses of agonist are increased, but the maximum response is unchanged. This is the type of supersensitivity most commonly seen in smooth muscle (Fleming *et al.*, 1973). However, an increase in the maximum reponse can also be interpreted as

supersensitivity and has been observed in several tissues (Pollock et al., 1972; Gardiner et al., 1974; Gibson & Pollock, 1975).

ii) Subsensitivity

Subsensitivity, or desensitisation of a tissue to a receptor ligand is reflected in a rightwards displacement of the dose-response curve for that compound (Rang & Ritter, 1969). However, a reduction in the maximum response can also be interpreted as a subsensitivity, together with, or in the absence of, a rightwards displacement of a dose-response curve (Waud, 1975).

The term homologous desensitisation is used to describe the specific desensitisation which occurs when cells are re-challenged with the same agonist used in the initial exposure (Su et al, 1976a;b). In the case of the β-adrenoceptors, this type of desensitisation is thought to involve the sequestration of the receptors away from their effector in the plasma membrane. The sequestered receptors may then become down-regulated (Harden, 1983; Perkins & Hertel, 1987).

The term heterologous desensitisation describes the non-specific reduction in sensitivity that occurs when cells are incubated with one agonist and subsequently found to the subsensitive not only to that agonist, but to several others (Su et al., 1976a;b). Heterologous desensitisation involves functional uncoupling of receptors in the absence of sequestration or down-regulation (Harden, 1983).

The analysis of drug-receptor interactions using classical pharmacological techniques in isolated tissues or in situ with perfused organs can be prone to limitations. The use of direct receptor labelling techniques has several advantages over more traditional pharmacological techniques, such as minimising the problem of drug-receptor equilibrium and eliminating the effects of drugs distal to the receptor (Minneman & Molinoff, 1980; Nahorski, 1981). However, although the measurement of specific binding of radiolabelled high

affinity agonists and antagonists has demonstrated that alterations in the number of receptors may contribute to at least some forms of drug-induced desensitisation (Creese & Sibley, 1981; Harden, 1983), it is difficult to draw conclusions from experiments employing only this technique. The lack of a final biological response can be a distinct disadvantage, particularly in experiments utilising high affinity radiolabelled antagonists which, unlike agonists, have little or no intrinsic activity. Such ligands are, therefore, of limited value in detecting any alterations in the mechanisms linking receptor activation to a change in cellular responsiveness.

FACTORS AFFECTING SENSITIVITY CHANGES

The endocrine glands release hormones into the bloodstream to exert regulatory influences on distant tissues. Two important groups of hormones which exert an influence on autonomic function are the corticosteroids and the thyroid hormones.

i) Corticosteroids

The adrenocorticosteroids have been implicated in sensitivity changes in various smooth muscles. In general, corticosteroid administration enhances the response to catecholamines in smooth muscle (Gibson & Pollock, 1975), liver (Exton *et al.*, 1972), adipose tissue (Brodie *et al.*, 1966) and salivary gland (Almagren & Jonason, 1973). Acute administration of cortocosteroids inhibits the extraneuronal uptake of catecholamines (Iversen & Salt, 1970) and, therefore, produces a deviation supersensitivity in some tissues (Kaumann, 1972). This is characterised by a leftward shift in the dose-response curve for NA, with no effect on the maximum response. Chronic administration of corticosteroids enhanced the maximum contractile response of the anococcygeus muscle to both NA and ACh (Gibson & Pollock, 1975). Blockade of extraneuronal uptake produced no

alteration in the sensitivity of this tissue, suggesting that the mechanism of action of chronic corticosteroid administration is not due to the blockade of extraneuronal uptake, but may be due to altered ion balance (Gibson, 1981). Corticosteroids can alter ionic distribution between the intracellular and extracellular space (Beck & McGarry, 1962). Two of the most important ions involved in smooth muscle contraction are Na^+ and Ca^{2+} (Bohr, 1964; Godfraind, 1975). Altering the Na^+ concentration had a greater effect on muscle contractility, the maximum contractile response varying directly with the external Na^+ concentration. Exposure of tissues to the Na^+ pump inhibitor ouabain resulted in a supersensitivity similar to that produced by corticosteroid administration, suggesting that if Na^+ is involved in the production of corticosteroid-induced supersensitivity then an enhanced intracellular Na^+ concentration may be responsible (Gibson & Pollock, 1976). Another possible explanation of the enhanced neuro-effector responses other than changes in ion concentrations is that corticosteroids may affect the prostaglandin synthesis pathway (Rascher et al., 1980).

ii) **Thyroid Hormones**

These hormones, triiodothyronine (T_3) and thyroxine (T_4), released from the thyroid gland under the control of a pituitary hormone thyrotrophin, have multiple effects throughout the body on growth and metabolism. They also influence the sensitivity of sympathetically-innervated tissues to agonists (Gibson, 1981).

The possibility of a link between the thyroid gland and the sympathetic nervous system has been recognised for many years because of the striking similarity between the symptoms of hyperthyroidism and increased activity of the sympathetic nervous system (Reith, 1865). Most of the early scientific evidence supported this suggestion, neuro-effector responses to catecholamines being enhanced in

hyperthyroidism and diminished in hypothryoidism (Waldstein, 1966). However, the effects of the thyroid hormones on the functioning of autonomic neuro-effectors are complex and often contradictory, with not all effector organs displaying an increased sensitivity to catecholamines in the hyperthyroid state (Macmillan & Rand, 1962).

Most studies on the effects of thyroid hormones on tissue sensitivity have involved the use of cardiac tissue. Hyperthyroidism increases and hypothyroidism decreases the sensitivity of the heart to β-adrenoceptor agonists. Ligand binding studies support these results and indicate that hyperthyroidism increases the number of β-adrenoceptors and decreases the number of α-adrenoceptors, whilst hypothyroidism has the opposite effects (Kunos, 1977). Indeed, in cardiac tissue, thyroid hormones have complex effects and may alter more than the receptor number (Tse et al., 1980).

The apparent parallel changes in sensitivity and receptor number led to the suggestion that there might be a single adrenoceptor that can exist in either the α or β state according to the circulating levels of T_4 (Kunos, 1977). Here there is a problem. In the heart, although both types of adrenoceptor can be found on nerve terminals, most of the α-adrenoceptors lie presynaptically on adrenergic and cholinergic nerve terminals, while β-adrenoceptors exist mainly on postsynaptic non-neuronal tissues. Generally, ligand binding studies fail to take account of this fact, which makes it difficult to accept the hypothesis of an allosteric change in a single receptor. Nevertheless, the ligand binding studies draw attention to the fact that some of the alterations in receptor number due to altered thyroid status may involve changes in pre- as well as post-synaptic receptors.

Little attention has been focussed on effects of thyroid hormones on presynaptic receptors despite the important role of α_2-adrenoceptors in regulating transmitter release at the sympathetic neuroeffector

junction (Gillespie, 1980). Part of this study, therefore, investigated the effects of altered T_4 levels on the sensitivity of both pre- and post-synaptic receptors in the mouse deferens.

NON-ADRENERGIC, NON-CHOLINERGIC TRANSMISSION

Hints of the existence of autonomic nerves that release transmitters other than ACh or NA can be found in the early literature (Langley, 1898; McSwiney & Robson, 1929; Ambache, 1951). Responses resistant to blocking drugs were first recognised in the cholinergic parasympathetic nervous system because of the availability of the powerful muscarinic receptor blocking drug, atropine. As early as 1898, Langley noted that stimulation of the vagus nerves could produce relaxation of the stomach. This relaxation was best revealed after blockade of the excitatory action of cholinergic fibres with atropine (McSwiney & Robson, 1929). Inhibitory nerves mimicking sympathetic nerve stimulation were discovered much later because of the lack of a selective potent adrenergic blocking drug. This changed with the introduction of adrenergic neurone blocking agents, which were first used to analyse the response of the stomach to nerve stimulation. It was shown that neither the inhibitory response to vagal stimulation in the guinea-pig stomach (Martinson & Muren, 1963) nor the inhibitory response to transmural stimulation in the guinea-pig, kitten or mouse stomach (Paton & Vane, 1963) was blocked. The first suggestion that these responses were produced by non-adrenergic inhibitory neurones arose from observations that when the responses to both adrenergic and cholinergic nerve stimulation had been blocked in the guinea-pig taenia coli, large transient hyperpolarisations and relaxations were produced, even with single pulses. The possibility that these were due to direct stimulation of muscle was excluded since they were abolished by tetrodotoxin. They were thus established as inhibitory junction

potentials (i.j.ps) resulting from stimulation of non-adrenergic, non-cholinergic (NANC) neurones (Burnstock et al., 1963; 1964). The presence of NANC nerves has been reported throughout the gastrointestinal tract of many vertebrate species (Burnstock et al., 1966), as well as in other organs, including lung, urinary bladder, trachea, oesophagus, anococcygeus, seminal vesicles and parts of the cardiovascular system (Burnstock, 1969; Furness & Costa, 1973; reviews by Burnstock, 1972; 1979).

The next step was to try to determine the principal transmitter in these nerves. Many substances were examined, including catecholamines, 5'-hydroxytryptamine (5-HT), adenosine 3'-5' monophosphate (cAMP), histamine, prostaglandins, various amino acids and some polypeptides. Most were rejected either because they did not mimic the nerve-mediated response, or their action was produced via stimulation of nerves and not by direct action on smooth muscle. A purine nucleotide, probably adenosine triphosphate (ATP), emerged as the most likely contender (Burnstock et al., 1970; 1972). This finding was followed by a systematic series of experiments to investigate whether or not ATP satisfied the criteria necessary for establishing a substance as a neurotransmitter, namely:

synthesis and storage of transmitter in nerve terminals,

release of transmitter during nerve stimulation,

postjunctional responses to exogenous transmitter that mimic responses to nerve stimulation,

enzymes that inactivate the transmitter and/or an uptake system for the transmitter or its breakdown products, and

drugs that produce parallel blocking or potentiating effects on the responses to both exogenous transmitter and nerve stimulation.

On the basis of the evidence which was obtained from these studies (see reviews by Burnstock, 1972; 1979; 1981), the purinergic nerve hypothesis was formulated. In 1971, nerves, utilising ATP as the principal transmitter, were termed 'purinergic' (Burnstock, 1971) and a tentative model for the storage, release and inactivation of ATP for purinergic nerves was proposed (Burnstock, 1972). It was suggested that following release, that fraction of ATP not bound to receptors is rapidly broken down, possibly by extracellular enzymes. The penultimate product, adenosine, is taken back into the nerve endings for resynthesis to ATP. Any adenosine not taken up in this way is broken down to inosine, which is pharmacologically inactive, cannot be taken up by nerves and leaks into the circulation. This system is comparable to that for ACh, where the extracellular enzyme cholinesterase rapidly breaks down the released transmitter, and the end product choline is reincorporated into the nerve and resynthesised to ACh, ready for release.

Since the purinergic nerve hypothesis was postulated there has been much debate, with evidence presented both for and against (Burnstock, 1979; Stone, 1981; Gillespie, 1982). Although ATP mimics well in most tissues the response to non-adrenergic inhibitory nerves, there are discrepancies, mainly in the evidence concerning the stomach and oesophagus (Gillespie, 1972; Ohga & Taneika, 1977; Daniel et al., 1979). However, systematic studies by electronmicroscope suggested that it was unlikely that all NANC nerves were a single population with only one transmitter. Up to nine morphologically distinguishable types of neurones were revealed in the enteric plexuses, including some nerve profiles containing a complex mixture of vesicles, suggesting that they may contain more than one transmitter (Gabella, 1972; Cook & Burnstock, 1976). These findings were followed by several investigations based on the new histochemical

approach which followed the advances in peptide chemistry. With pure, identified peptides, highly selective immune sera were produced, and these in turn were used for fluorescence histochemistry. Autonomic nerves containing many polypeptides have been described (Hokfelt et al., 1980). (For a list of the substances currently considered to be transmitters or putative transmitters in autonomic nerve fibres see Burnstock, 1986).

CO-TRANSMISSION

A widely held belief about the organisation of the nervous system was that each nerve cell makes and releases only one transmitter. This has been described as 'Dale's Law'. Dale, in fact, never suggested that neurones contain only one neurotransmitter, but proposed that each neurone releases the same neurotransmitter at all its synapses (Dale, 1935). 'Dale's Law' was probably widely accepted because it fitted in with the belief prevalent for many years that efficient transmission required only a single stimulatory or inhibitory transmitter.

The suggestion that some nerve cells store and release more than one transmitter was based largely on the early studies of Burn and Rand (1965). According to their hypothesis, ACh is present together with NA in adrenergic axons and forms an intermediate link between nerve impulses and the release of NA from the nerve terminals (Burn & Rand, 1965). There is now considerable experimental support for the co-existence of peptides or purine nucleotides together with classical neurotransmitters (Chan-Palay & Palay, 1984). It is clear that 'Dale's Law' has been invalidated by the discovery of numerous examples of co-existence of putative neurotrans-mitters (see O'Donohue et al., 1985).

Much of the evidence for co-transmission in sympathetic nerves has come from years of experimental study of the vas deferens.

THE VAS DEFERENS

The vas deferens is the duct which carries spermatozoa from the testes to the urethra. It is composed of three layers of smooth muscle, an outer and inner longitudinal layer surrounding an inner circular layer. As in the muscle layers of most visceral tissues, muscle cells in the vas deferens are arranged in branching bundles of irregular cross-section surrounded by connective tissue sheaths. The muscle bundles rather than the individual muscle cells are the effector units (Bennett & Burnstock, 1968). In most mammals the vas deferens is probably the most densely innervated muscle in the body. It is supplied with an autonomic ground plexus in which postganglionic axons branch to form preterminal axons from which transmitter is released (Sjöstrand, 1965; Burnstock, 1970). Preterminal axons are predominantly non-myelinated, surrounded by a Schwann cell sheath and beaded. The beaded appearance is due to the varicosities of functional nerve endings which are the anatomical sites of release. In the varicose regions of these axons (0.5-1 μm in diameter and 1-3 μm long), which are packed with vesicles and mitochondria, the Schwann cell sheath is often incomplete, leaving them naked (Burnstock, 1970).

The vas deferens has the highest NA content of any tissue, varying between 5 and 20 μg/g of tissue in several different species (Sjöstrand, 1965; Blakeley *et al.*, 1970). A dense adrenergic innervation has been demonstrated by the use of histochemistry (Falck *et al.*, 1965). It has, however, been known for some time that the motor response to nerve stimulation is resistant to α-adrenoceptor antagonists (Boyd *et al.*, 1960).

In early experiments on adrenergic neurotransmission, the isolated vas deferens was stimulated for short periods at regular intervals. It was generally believed that the motor transmitter, NA, elicited a simple monophasic mechanical response. However, using longer periods

of stimulation, Swedin (1971) uncovered a secondary component of the mechanical response in rat and guinea-pig vasa. The motor response to field stimulation was now seen to be more complex than previously considered, consisting of an initial rapid contraction, or 'twitch', which declined, to be replaced by a slower, better-maintained, 'secondary' response. The twitch response, unlike the secondary component, was shown to be resistant to α- and β-adrenoceptor blocking drugs and reserpine pretreatment. Only the neurone blocking drugs guanethidine and bretylium were capable of abolishing both phases of the contractile response. Swedin (1971) proposed that NA, on release from adrenergic nerves, rapidly reaches high concentrations in the narrow neuromuscular gap and this effectively stimulates the local junctional receptors. On sustained stimulation, the continuously released surplus of NA diffuses out of the narrow neuromuscular cleft to stimulate extrajunctional receptors, thereby mediating the prolonged secondary phase of the mechanical response.

Ambache and Zar (1971) reported a very low sensitivity of the longitudinal muscle to the motor action of NA, and an inhibitory effect of both sympathomimetic drugs and NA on the nerve-stimulated twitch response. The twitch height failed to be depressed either by combined block of α- and β-adrenoceptors, or by irreversible antagonism with PBA which blocked more than 1000 twitch-matching doses of NA. In preparations from animals pretreated with reserpine, the motor response to field stimulation was unaffected, and exogenous NA inhibited the twitch responses of the field stimulated vas deferens. From their evidence, Ambache and Zar concluded that motor transmission was 'non-adrenergic'. The inability of α- and β-adrenoceptor blocking drugs and the ability of NA to inhibit the twitch response in the guinea-pig vas deferens also led von Euler and Hedqvist (1975) to support the conclusion reached by Ambache and Zar (1971). The physiological

function of the dense adrenergic innervation was postulated to be a modulator for the non-adrenergic motor response, the NA acting prejunctionally on the non-adrenergic neurones to inhibit the release of the unknown transmitter (Ambache et al., 1972).

Although sixteen years have passed since the first conflicting reports (Ambache & Zar, 1971; Swedin, 1971), the nature of motor transmission in the vas is still under debate. Whilst the use of drugs has greatly enhanced our understanding of transmitter mechanisms in the vas, many observations remain difficult to explain.

Several experimental observations suggest that the initial phase of the contractile response to field stimulation in vasa from various species is mediated by ATP acting on P_2-purinoceptors and may be termed 'purinergic'. First, the time course of the first component is mimicked by exogenous ATP, not NA (Sneddon & Westfall, 1984). Secondly, it is blocked by the P_2-purinoceptor antagonist arylazido aminopropionyl ATP ($ANAPP_3$) (Fedan et al, 1981; Sneddon et al, 1982; Sneddon & Westfall, 1984). Thirdly, it is abolished in tissues desensitised to ATP by exposure to the stable analogue α,β-methylene ATP (α,β-MeATP) (Meldrum & Burnstock, 1983). The underlying electrical event, the excitatory junction potential (e.j.p.), also exhibits these purinergic characteristics. First, local application of ATP but not NA can exactly mimic the time course of the e.j.p. (Sneddon & Westfall, 1984). Secondly, e.j.ps are blocked by $ANAPP_3$ (Sneddon et al., 1982; Sneddon & Westfall, 1984) and by α,β-MeATP (Sneddon & Burnstock, 1984) but not by α-adrenoceptor blockers. Thirdly, e.j.ps are not reduced by pretreatment with reserpine (Sneddon & Westfall, 1984).

The second component of the response to field stimulation has been shown to be adrenergic by all the pharmacological tests employed. It is blocked by α-adrenoceptor antagonists (McGrath, 1978; Sneddon &

Westfall, 1984; Sneddon et al., 1984), inhibition of NA release by stimulation of prejunctional α-adrenoceptors with lysergic acid diethylamide (LSD) (Booth et al., 1978) and depletion of NA stores with reserpine (Booth et al., 1978; Sneddon & Westfall, 1984). Further evidence for the existence of two transmitters comes from experiments utilising the calcium channel antagonist nifedipine, which eliminates the initial twitch response leaving the second component intact (French & Scott, 1981). Since, on examination of the electrical events underlying the mechanical response, nifedipine was found to abolish the smooth muscle action potential, leaving the second component intact, there appears to be a fundamental difference between the two types of response to nerve stimulation in the vas deferens (Blakeley et al., 1981). Whether or not the two transmitters are contained in a single or in separate nerves is still doubtful, since selective destruction of sympathetic nerves by 6-hydroxydopamine (6-OHDA) apparently inhibits only the adrenergic component in some studies (Booth et al., 1978; Brown et al., 1983) but inhibits both components in others (Fedan et al., 1981; French & Scott, 1983).

However, it has been suggested that NA may be the sole transmitter, producing its contractile effects through two processes, the first involving e.j.ps which summate to initiate propogated action potentials but does not involve classical α-adrenoceptors. These γ receptors may be present in a number of sympathetically-innervated tissues (Hirst & Neild, 1980; 1984). The second process does not involve e.j.ps or propogated action potentials but is mediated by α_1-adrenoceptors.

Most of the experimental evidence suggests that the response to field stimulation in the vas deferens can be accounted for largely in terms of the combined effects of NA and ATP, although a third factor appears to participate in transmission. After the 'removal' of both

NA- and ATP-mediated components, a small residual contraction remains. It has been postulated that the recently discovered 36-amino acid gut-brain peptide neuropeptide Y (NPY) (Tatemoto, 1982) may mediate this response (Stjärne & Åstrand, 1985; Stjärne et al., 1986). A high level of NPY-like immunoreactivity (NPY-LI) has been shown to exist in vasa deferentia of several species (Lundberg et al., 1982; 1983; 1984). Although NPY has only weak contractile effects on the smooth muscle of the vas, it greatly potentiates the contractile effects of exogenous NA and α,β-MeATP, thus NPY may have additional contractile effects expressed through its co-transmitters. Exogenous NPY depressed the twitch phase of the field stimulation-evoked contractile response of mouse and rat vasa, but not the contraction evoked by exogenous NA (Allen et al., 1982; Lundberg et al., 1982). NPY also depressed the secretion of (^{3}H)-NA and both phases of the contractile response to field stimulation in the rat vas deferens, suggesting that NPY inhibits the secretion of both NA and the putative transmitter ATP (Lundberg & Stjärne, 1984).

While the mechanism by which nerve stimuli evoke secretion of quanta of ATP and NA from individual varicosities of the guinea-pig and mouse vasa deferentia is beginning to be understood (Cunnane & Stjärne, 1982; 1984; Stjärne, 1985; Stjärne & Åstrand, 1984; 1985), the characteristic features of the secretion of endogenous NPY from these sympathetic nerves are not yet known. Experiments in the pig spleen, however, have shown that splenic nerve stimulation at a low continuous frequency releases mainly NA, while intermittent activation with high frequency bursts preferentially releases NPY-LI (Lundberg et al., 1986). The nerve stimulation-evoked release of NPY-LI was enhanced following either α-blockade with phentolamine or NA depletion after resperine pretreatment. This was especially evident at low frequencies of

stimulation suggesting that NA exerts an inhibitory prejunctional influence via α-adrenoceptors on the release of NPY-LI (Lundberg et al., 1986; Stjärne et al., 1986).

The dense adrenergic innervation of their smooth muscle layers has led to the widespread use of rodent vasa deferentia in the study of adrenergic mechanisms. In particular, nerve-induced contractions have proved useful in assaying compounds which act at prejunctional receptors to inhibit transmitter release, for example, opioid receptors in the mouse vas deferens (Henderson et al., 1972). Such studies are possible despite incomplete understanding of the neuro-transmission process. However, the wealth of information obtained over the past sixteen years on neurotransmission in the vas deferens indicates that, much as we would like to simplify the biological systems we study, inconsistencies cannot be ignored in order to force observations into generally accepted hypotheses.

A tissue possessing such complexities as the vas deferens requires a combination of techniques to be integrated in providing a clearer understanding of its activity. This study set out to determine, using a combination of well-established and some novel techniques :-

1) If, indeed, there is evidence for the existence of more than one transmitter, are they contained in a single nerve or in separate nerves?
2) How does altering the stimulation parameters affect the release of the co-transmitters?
3) Are the co-transmitters involved in mutual regulation of release by a presynaptic action?
4) What effect does altering the circulating levels of T_4 have on the sensitivities of pre- and post-synaptic receptors?

MATERIALS AND METHODS

INVESTIGATION INTO THE INFLUENCE OF THYROID HORMONES ON PRE- AND POST-SYNAPTIC SENSITIVITY IN THE MOUSE VAS DEFERENS

i) Preparation of Tissues

Adult, male, T.O. mice (25-30 g) were anaesthetised with halothane and killed by decapitation. The blood was collected, allowed to clot and then centrifuged to obtain the serum, which was frozen until required for radioimmune assay. The vasa deferentia were excised, placed in a petri-dish containing Krebs-bicarbonate solution of the following composition (mM): NaCl (118.1), KCl (4.7), $MgSO_4$ (1.0), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0), glucose (11.1), and the adjoining blood vessels and connective tissues were removed. Each tissue was mounted vertically (epididymal end uppermost) with an initial resting tension of 500 mg, in a 25 ml double-jacketed organ bath containing Krebs solution. This was maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2. Changes in tension of the isolated tissues were recorded isometrically with a Grass FT03 force-displacement transducer and displayed on a Grass Model 7 polygraph.

ii) Investigation of Presynaptic Changes

To examine any thyroid-induced changes in presynaptic receptor sensitivity, the vasa deferentia from treated and untreated mice were stimulated electrically by field stimulation through silver ring electrodes with a Grass S88 stimulator. Trains of stimuli were achieved using the following parameters: 20 Hz; 500 μs pulse width; 500 msec. duration; every 100 sec., supramaximal voltage. Field stimulation causes the release of transmitter from nerve endings. In the mouse vas deferens, the presynaptic α_2-adrenoceptors and opiate receptors can inhibit this release and can, therefore, inhibit the tissue response to field stimulation. The selective α_2-adrenoceptor agonist, clonidine, and opiate receptor agonist, morphine, were used to determine the sensitivity of these presynaptic receptors. Increasing

concentrations of agonist were added to the organ bath, each dose being washed out after maximum inhibition of the tissue response to a single dose was achieved. The tissue was allowed to recover before the addition of a subsequent dose.

iii) Investigation of Postsynaptic Changes

The responses of isolated vasa deferentia from treated and untreated animals to the α_1-adrenoceptor agonist, NA, and the muscarinic cholinoceptor agonist, carbachol, were examined to determine any thyroid-induced changes in postsynaptic sensitivity in smooth muscle. Increasing concentrations of agonist were added to the bath, each concentration remaining in contact with the tissue for 30 sec. before being washed out and 5 min. being allowed to elapse between drug additions. Responses to each concentration were measured as grammes tension developed by the tissue and as a percentage of the maximum response.

iv) Radioimmune Assay of Free Serum Thyroxine Levels

Radioimmune assay of serum thyroxine (T_4) allowed the effectiveness of the pretreatment to be monitored. An Amersham Amerlex T_4 RIA kit (IM3050) was used for the immune assay of free T_4 in the serum obtained from treated and untreated mice. This method depended upon the competition between ^{125}I-T_4 and free T_4 in the serum for a limited number of binding sites on a T_4-specific antibody. The proportion of the ^{125}I-T_4 bound to the antibody was inversely related to the concentration of free T_4 present in the serum. Standards (containing known amounts of T_4) and the serum samples were mixed with both ^{125}I-T_4 and T_4-specific antibody suspension and then allowed to stand for 45 min. Centrifugation (2000 x g, 10 min., room temperature) produced a pellet of antibody with its bound ^{125}I-T_4 and T_4 beneath a liquid supernatant. The supernatant was discarded and the pellet counted in a gamma counter. A standard curve was

constructed showing the concentration of free T_4 versus ^{125}I counts, from which the free T_4 content of the serum samples could be obtained.

v) **Measurements and Statistics**

Dose-response curves, using both absolute responses in grammes tension developed by the muscles and with responses expressed as a percentage of the maximum response, were constructed for each agonist. All tissues, whether treated or untreated, were exposed to the four agonists in a random order. Each response was expressed as a mean with a standard error of the mean ($\bar{x} \pm$ S.E. mean). To compare results, Student's t-test was used. Levels of significance: * $0.05>P>0.01$; ** $0.01>P>0.001$; *** $P<0.001$.

(^3H)-NORADRENALINE OVERFLOW EXPERIMENTS

Adult, male, T.O. mice (25-30 g) (unless specified), were stunned and killed by exsanguination. Vasa deferentia were dissected, freed from connective tissue and transferred to 0.5 ml of Krebs bicarbonate buffer containing 12 µCi of (^3H)-noradrenaline ((^3H)-NA), previously gassed with a mixture of O_2, 95%/CO_2, 5%. Vasa were incubated in the radioactive solution (37°C, 30 min.), inserted into silver ring electrodes and transferred to organ baths (2 ml capacity), where they were attached to Stratham force displacement transducers (resting tension 0.5 g) to record motor responses isometrically. Vasa were continuously superfused with Krebs solution (37°C, 4 ml/min., 1.5 hr.) to remove loosely bound (^3H)-NA not taken up into nerves.

At the end of this preliminary washing period, the superfusion was stopped and, with the organ baths filled with Krebs solution, vasa were stimulated with a Grass stimulator (model S88) at a frequency of 20 Hz for a period of 5 sec., pulse width 0.5 msec., supramaximal voltage. Motor responses were displayed on a Grass polygraph (model 7). At the end of each period of stimulation and at intervals between

stimulations, the contents of the organ baths were collected in liquid scintillation vials containing 10 ml of Ecoscint (National Diagnostics). The radioactivity in each vial was counted in a Packard Tricarb liquid scintillation counter and the fractional release obtained with each stimulation was calculated.

All counts were corrected for quenching and the results expressed as d.p.m. The (^{3}H)-NA released into the organ baths during stimulation was separated from its metabolites by column chromatography.

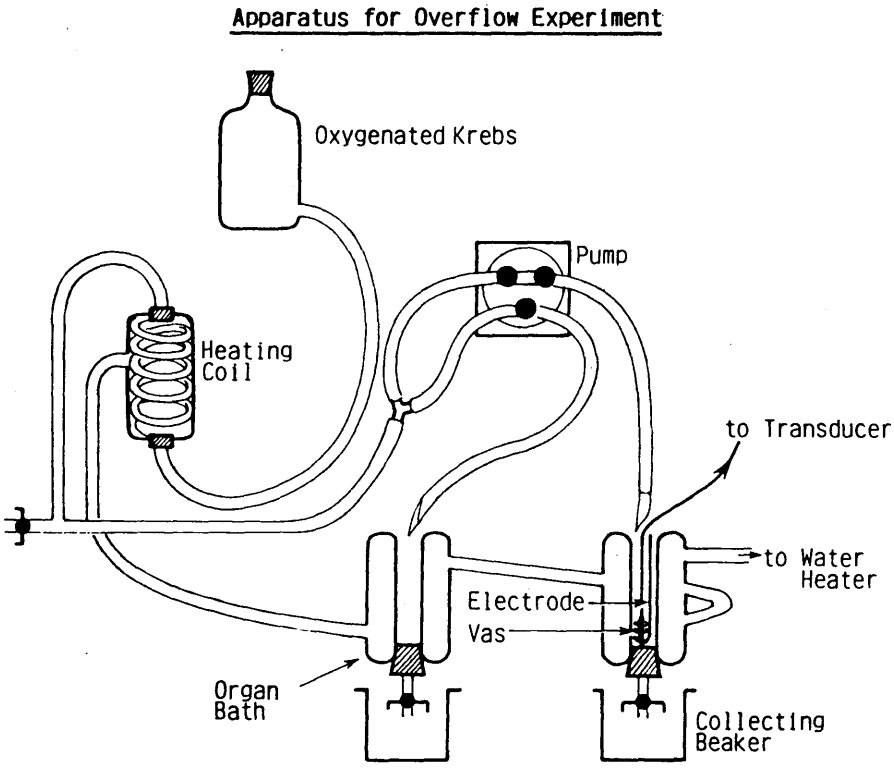


FIGURE 1. Diagram of apparatus used for overflow experiments.

METABOLISM STUDIES

Alumina was treated according to the method of Crout (1961) and washed with 0.2 M sodium acetate before drying. Dowex 50W x 4 (200-400 mesh) was washed several times with 2 M NaOH (containing 1% Na-EDTA) at 50°C until the supernatant was clear, washed with H_2O, 2 M HCl, H_2O and, finally, equilibrated with 0.01 M HCl. Columns used for chromatography were 0.5 cm in diameter and stoppered with cotton wool. 200 mg of activated alumina was used and washed before use with 5 ml 0.2 M sodium acetate (pH 8.2), Dowex columns were filled to a height of 1.5 cm (0.5 ml) with resin and washed with 2 ml H_2O.

10 µg of unlabelled NA and of each NA metabolite were added as carrier to each sample. The pH of the samples was adjusted to 8.2-8.4 with 0.5 M Tris buffer (pH 8.2) then immediately added to the column. After addition, the column was washed through with a further 1 ml 0.2 M sodium acetate and 2 ml H_2O. The effluent and washing contained the O-methylated metabolites 3-methoxy-4-hydroxyphenylglycol (MOPEG), 3 methoxy-4-hydroxymandelic acid (VMA) and normetanephrine (NMN).

The (^{3}H)-NA and (^{3}H)-DOPEG fraction was then eluted with 3.5 ml 0.2 M acetic acid. After washing the column with 4 ml 0.5 M acetic acid, which elutes approximately 5% of both NA and DOPEG, the deaminated acid, DOMA, was eluted with 3 ml 0.2 M HCl.

(^{3}H)-NA was separated from the (^{3}H)-DOPEG by means of the strong cation exchange resin Dowex-50W x 4. 2 ml of the (^{3}H)-NA/(^{3}H)-DOPEG fraction was poured onto the column and washed through with 2 ml H_2O. The effluent and washings contained DOPEG. After washing with a further 2 ml H_2O, which was discarded, the NA was eluted with 3 ml 3 M HCl.

The O-methylated fraction was separated using Dowex-50W x 4 columns. The O-methylated deaminated metabolites (VMA and MOPEG) passed through the column and were collected in the effluent. NMN was

retained by the Dowex and subsequently eluted with 2 ml 6 M HCl/ethanol (1:1 v/v).

1 ml of each separated fraction was added to 10 ml of Ecoscint in a counting vial and radioactivity was measured in a Packard Tricarb liquid scintillation counter.

The method of separation was essentially that of Graefe et al. (1973) and Nicol (1975), with the following modifications. Volumes of eluates were increased so that aliquots could be taken for counting before column separation. This allowed corrections for recovery to be made in each sample. The volume of 0.2 M acetic acid used to elute the NA/DOPEG fraction from the alumina column was increased from 2 ml to 3.5 ml and the 0.2 M HCl used for elution of (^{3}H)-DOMA was increased from 2.5 to 3 ml. The concentration of HCl required to elute (^{3}H)-NA was increased from 2 M to 3 M and the volume from 2 to 3 ml. These alterations were made to ensure complete recovery of (^{3}H)-NA.

Calculation of metabolism results

The amount of radioactivity appearing in each of the metabolite samples compared to that in the sample before separation gave an indication of the recovery and efficiency of each column. The recovery for the alumina column was 87.9 $\pm$ 1.7 % (n = 8) and for the Dowex columns 98.6 $\pm$ 4.5% and 91.9 $\pm$ 1.8 % (n = 8) respectively.

The amount of (^{3}H)-NA and (^{3}H)-metabolites present in the pre-stimulation sample were subtracted from the amount in each sample obtained after nerve stimulation.

(^{3}H)-NA and its metabolites were expressed as a percentage of the total ^{3}H present in the samples.

Results were expressed as mean $\pm$ standard error of the mean. Significance was assessed by Student's t test.

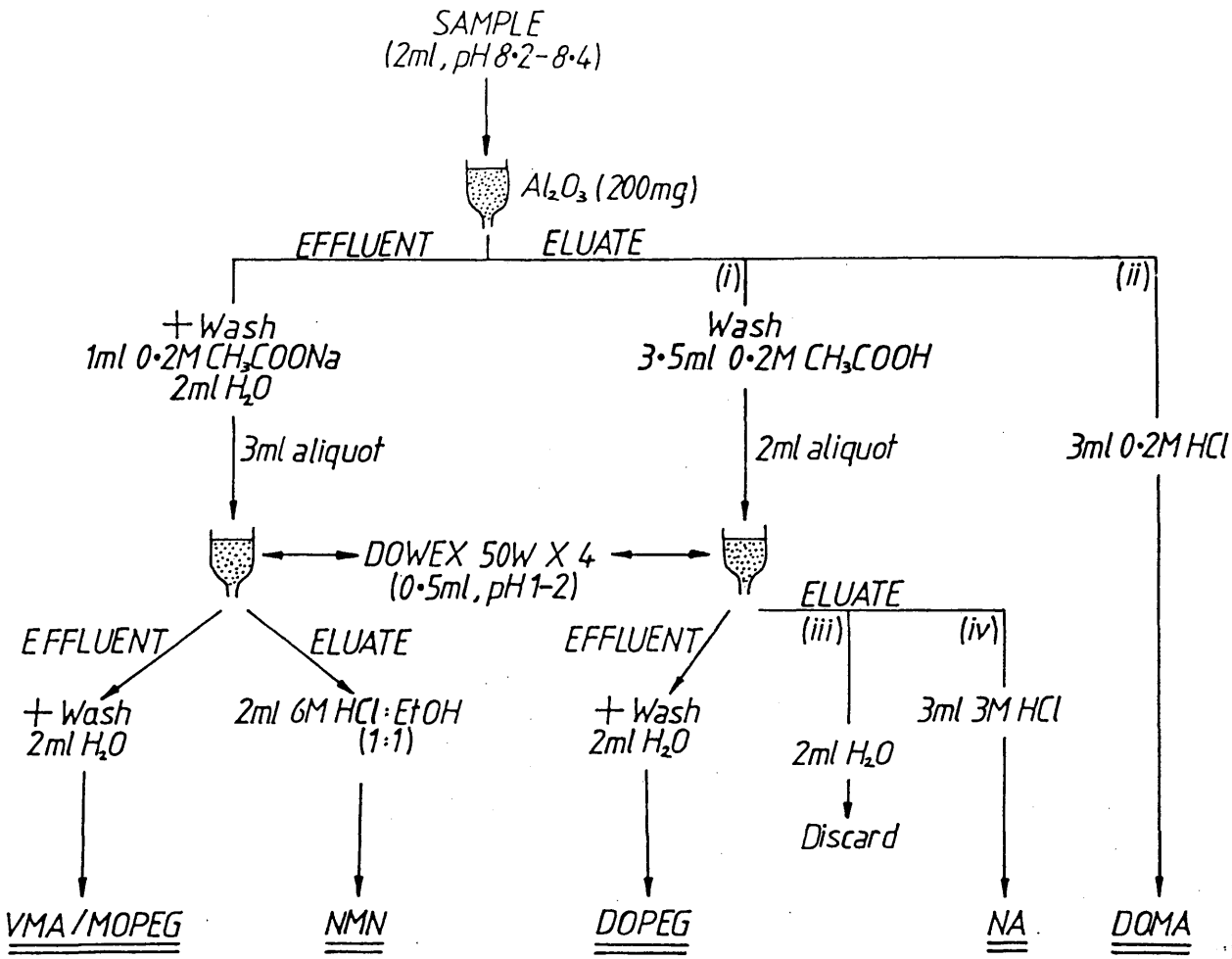


FIGURE 2. Flow diagram of the chromatographic separation of (^{3}H)-NA from its metabolites. 2 ml samples were applied to the alumina colums. The effluent and washings contained COMT metabolites. The acetic acid eluate contained the NA and DOPEG. The HCl eluate from the alumina contained DOMA. The COMT metabolites were separated by a DOWEX-50W x 4 column into VMA/MOPEG and NMN. The NA-DOPEG fraction was also separated by a DOWEX-50W x 4 column into DOPEG and NA.

IN VIVO EXPERIMENTS

Male Wistar rats (200-250 g) were anaesthetised with pentobarbitone (60 mg/kg, i.p.). In each animal the trachea was cannulated to allow respiration to be maintained artifically with a Palmer pump. In addition, the carotid artery and the femoral vein were cannulated to permit blood pressure (B.P.) to be recorded and drugs to be administered intravenously. An electrode consisting of a stainless steel tube within a Teflon tube (Gillespie *et al.*, 1970) was inserted between thoracic vertebrae T_8 and T_9 and pushed down the spinal canal to the point of furthest penetration in the sacrum. The length of the stimulating electrode extruded from the shielding Teflon tube could be varied but was usually 1-2 cm, so that the spinal nerves leaving the column from L_5 down to the sacrum were stimulated. A brass rod, used as an indifferent electrode, was inserted under the skin, dorsal to and along the length of the vertebral column. Tubocurarine was administered (1 mg/kg, i.v.) to block neuromuscular transmission and prevent contractions of voluntary muscle interfering with responses of the vas deferens.

One vas deferens was exposed and attached by means of a thread and sewn through the muscle to a Grass FT03 force displacement transducer. The resting tension was adjusted to 1 g and 2 platinum electrodes were placed around the vas to permit electrical field stimulation of the vas *in situ*.

With the spinal electrode exposed from L_5 down to the sacrum, supramaximal stimulation with 0.5 ms pulses at various frequencies caused the vas to contract, with either no effect or, occasionally, a small depressor effect on B.P. The motor response of the vas to stimulation of the spinal nerves was similar to that produced by field stimulation and was biphasic when the chart recorder speed was fast. If the length of electrode exposed within the vertebral canal was

increased by withdrawing the Teflon shielding, stimulation produced large pressor responses, which developed more slowly and outlasted the contractile response of the vas. In these experiments, the motor response of the vas was poorly maintained and the decay of this response coincided with the onset of the pressor responses, which was caused by stimulation of the spinal outflows to the adrenals. This pressor response was blocked by phentolamine (1 mg/kg, i.v.), which simultaneously prolonged the motor response of the vas. Throughout these studies, the position of the electrode within the spinal canal was monitored radiographically.

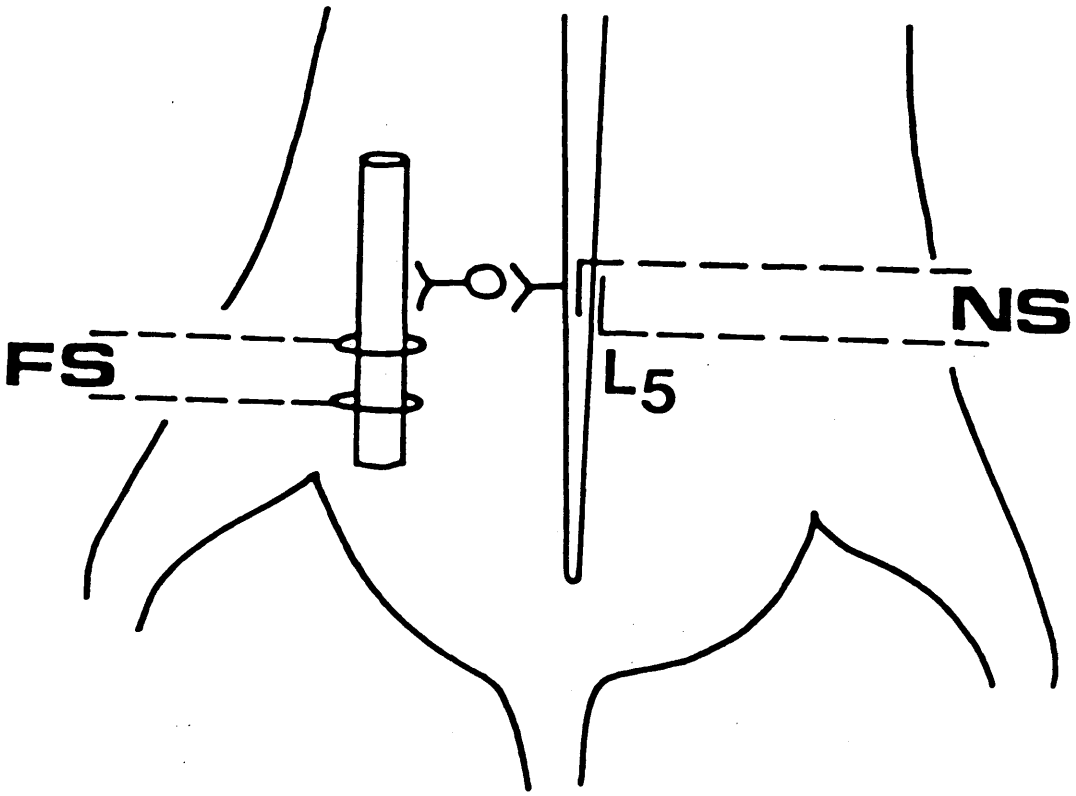


FIGURE 3. Schematic representation of the *in situ* anaesthetised rat preparation. FS: Vasa were field stimulated by means of 2 platinum electrodes placed around the vas. NS: Nerve stimulation was obtained by an electrode inserted into the spinal canal and the stimulation of the spinal motor outflows to the vas.

PHOSPHOLIPID EXTRACTION AND SEPARATION OF PHOSPHATIDIC ACID (PA) BY THIN LAYER CHROMATOGRAPHY

Male Porton mice (25-30 g) were stunned and killed by exsanguination. Vasa deferentia were dissected, freed from connective tissue, dried on filter paper and weighed. A numbered thread was attached to each of the vasa, which were transferred to test-tubes containing 2 μCi of [^{32}P]-orthophosphate per vasa in 3 ml of Hepes buffer (mM:NaCl 11.4, KCl 4.7, $MgCl_2$ 1.2, glucose 11.1, Hepes 10.1, pH adjusted with NaOH to 7.2). Vasa were incubated in this radioactive solution (37°C, 2 hr.), removed and washed 3times in 250 ml of Krebs buffer, previously gassed with a mixture of O_2, 95%/CO_2, 5% to remove loosely bound [^{32}P]-orthophosphate.

Experiments were carried out in two ways. In some experiments vasa were inserted into silver ring electrodes, transferred to organ baths each containing 2 ml of Krebs buffer (37°C) and attached, with a resting tension of 0.5 g, to a Stratham force displacement transducer. These vasa were stimulated (20 Hz, 5 sec., pulse width 0.5 msec., supramaximal voltage) with a Grass model S88 stimulator. At the peak of each field stimulation-induced motor response, which was displayed on a Grass model 7 polygraph, each vas was removed and plunged into liquid nitrogen. In other experiments vasa were not subjected to field stimulation but were exposed to agonists. In these studies vasa were incubated in Krebs solution containing agonists (37°C, 5 min.) and then plunged into liquid nitrogen.

Each frozen vas was transferred to 2 ml of a mixture of chloroform/methanol/10 M-HCl (25:50:4, v/v/v) in a glass Potter-Elvehjem homogeniser, in which the vas was homogenised. To each homogenate was added 1.25 ml of chloroform and 2.05 ml of water to partition the aqueous and organic phases. The contents of each tube were vortexed and centrifuged (1000 x g, 15 min.) to separate the

aqueous and organic layers. The lower (chloroform) layer, which contained the extracted phospholipids from each sample, was carefully transferred using a Pasteur pipette to a test-tube and reduced to dryness at 40°C under oxygen-free nitrogen gas.

The extracted lipids from each sample were redissolved in 75 µl of a mixture of chloroform and methanol (9:1, v/v) and spotted on to silica gel thin layer chromatography (t.l.c.) plates (10 cm x 10 cm) for two dimensional separation of phospholipids according to the procedure described by Yavin and Zutra (1977). Briefly, this t.l.c. system employs a basic solvent (chloroform/methanol/aqueous methylamine (40%) 130:60:150 v/v/v), neutralisation with HCl fumes, an acid ether wash in the second dimension with diethylether/glacial acetic acid 190:10, v/v, and an acidic solvent (chloroform/acetone/methanol/glacial acetic acid/water 100:40:20:30:10, v/v/v/v/v) also in the second dimension. In order to facilitate identification of the [^{32}P]-PA, unlabelled carrier PA (10 µg/ml) was added to each plate. Individual spots were detected by exposing the plates to iodine vapour and the spot corresponding to PA was scraped into liquid scintillation vials each containing 10 ml of Ecoscint (National Diagnostics). The [^{32}P] radioactivity was quantified by liquid scintillation spectroscopy. Results were expressed as % change in [^{32}P]-PA formation over controls. Means were compared using Student's t test.

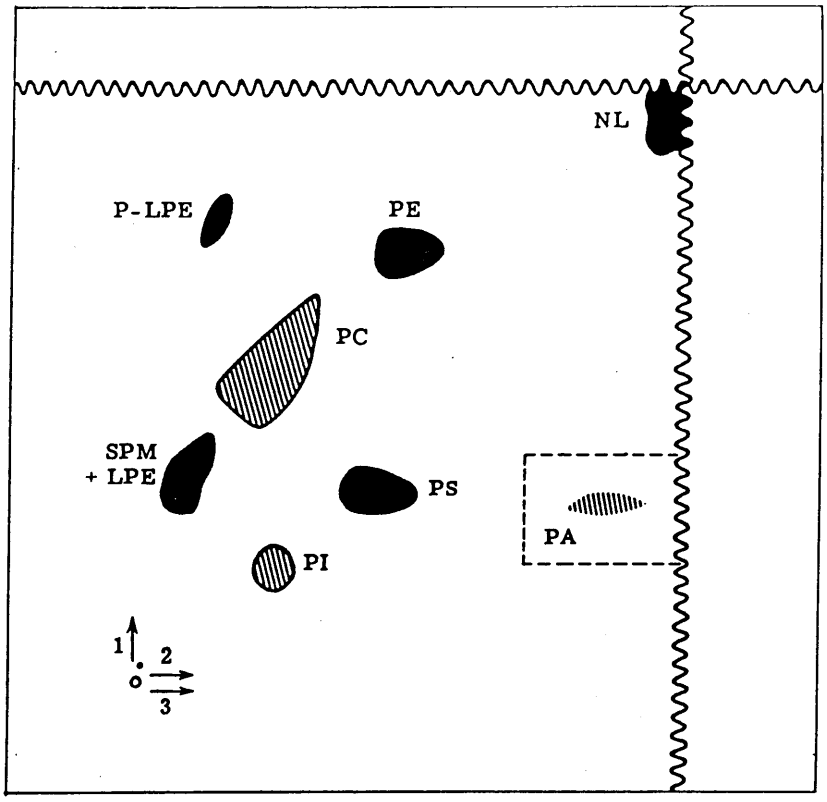


FIGURE 4. Schematic representation of phospholipid separation by two-dimensional thin layer chromatography. Phospholipids identified by iodine staining are indicated. O - Origin; PI - phosphatidyl inositol; PC - phosphatidyl choline; PA - phosphatidic acid; PS - phosphatidyl serine; PE - phosphatidyl ethanolamine; P-LPE - plasmalogen lyso-phosphatidyl ethanolamine; SPM - sphyngomyelin; LPE - lyso-phosphatidyl ethanolamine; NL - neutral lipids.

1) chloroform methanol methylamine (40%) (130:60:15 v/v/v)

2) diethyl ether acetic acid (190:10 v/v)

3) chloroform acetone methanol acetic acid water (100:40:20:30:10 v/v/v/v/v)

Drugs Used

The drugs used during the project were adenosine 5' triphosphate (Sigma), α,β-methylene ATP (Sigma), L-ascorbic acid (Sigma), atropine sulphate (Sigma), carbamoylcholine chloride (Sigma), clonidine hydrochloride (Sigma), desipramine hydrochloride (Sigma), dL-3,4-dihydroxymandelic acid (Sigma), dL-3,4-dihydroxyphenylglycol (Sigma), Tyr-D-Ala-Gly-n-Me-Phe-Gly-O/enkephalin (Cambridge Research Biochemicals), 6-hydroxydopamine hydrobromide (Sigma), dL-4-hydroxy-3-methoxymandelic acid (Sigma), bis-4-hydroxy-3-methoxyphenylglycol (Sigma), morphine hydrochloride (MacFarlan Smith), naloxone hydrochloride (Sigma), L-(7,8-^{3}H) noradrenaline (Amersham), L-noradrenaline bitartrate (Koch-Light), dl-normetanephrine hydrochloride (Sigma), 17,β-oestradiol (Sigma), pentobarbitone sodium (May and Baker), phenoxybenzamine hydrochloride (Smith, Kline and French), trans-2-phenylcyclopropylamine hydrochloride (Sigma), phentolamine mesylate (Ciba), prazosin hydrochloride (Pfizer), reserpine (Sigma), tetrodotoxin (Boehringer), L-thyroxine (Sigma), d-tubocurarine (Wellcome), tyramine hydrochloride (Sigma), yohimbine hydrochloride (Sigma).

The drugs were prepared in 0.9% NaCl, except when otherwise stated.

L-(7,8-^{3}H)-noradrenaline (8-14 Ci mmol^{-1}) supplied in 0.02 M acetic acid:ethanol (9:1 v/v), was resuspended in distilled water containing ascorbic acid (5.7 x 10^{-3} M) to prevent breakdown of the catecholamine.

Reserpine was dissolved in a 4% solution of L-ascorbic acid.

Phenoxybenzamine and 17,β-oestradiol were made up in absolute alcohol, but were never added in volumes greater than 0.25 µl/ml of Krebs solution.

ANIMAL PRETREATMENTS

I L-Thyroxine (L-T_4)

L-T_4 was administered orally in the drinking water. Preliminary studies indicated that a 25 g mouse drinks 5 ml of water per day, so L-T_4 was added to the water to give a solution of 100 mg/l. This gave each mouse a dose of 20 mg/kg/day. The mice were treated for ∮ 2 weeks before being examined.

II 6-Hydroxydopamine (6-OHDA)

6-OHDA was administered to mice and rats as follows:-

i) 2 x 50 mg/kg (i.p.) on day 1

ii) 2 x 100 mg/kg (i.p.) on day 3

Experiments were carried out on days 4 and 5.

III L-T_4 + 6-OHDA

L-T_4 was administered orally in the drinking water to give mice a daily dose of 20 mg/kg. 6-OHDA was administered during the period of treatment with L-T_4 on day 1 (2 x 50 mg/kg i.p.), day 4 (2 x 100 mg/kg i.p.) and on the last day of treatment with L-T_4 i.e. ∮ 2 weeks (2 x 100 mg/kg i.p.).

The mean volume of water drunk by mice was determined and this volume was used to calculate the concentration of drug that needed to be added to the drinking water to provide the mice with the required drug dosage. Since the volume of drug solution drunk and, therefore, the amount of drug administered varied from day to day, the doses administered were correct only to within ± 8% of the stated doses.

IV Reserpine.

Reserpine was administered to mice as follows:-

i) 2 x 1mg/kg (i.p.) on day 1
ii) 2 x 1mg/kg (i.p.) on day 2
iii) 2 x 1mg/kg (i.p.) on day 3

Experiments were carried out on day 4.

RESULTS

PART I

RESPONSES OF MOUSE ISOLATED VASA DEFERENTIA TO ELECTRICAL FIELD STIMULATION, AND THE EFFECTS OF ACUTE AND CHRONIC DRUG ADMINISTRATION ON THESE RESPONSES

Field stimulation of the mouse vas deferens produced a biphasic response, consisting of an initial fast component followed by a more prolonged contraction (Fig. 5a). The first component was enhanced and the second component inhibited by phentolamine (10^{-6} M) (Fig. 5b).

Morphine (10^{-6} M, 10^{-5} M) inhibited the first component and potentiated the second component of the biphasic contractile response evoked by field stimulation (Figs. 6, 7). Both the inhibitory effect of morphine on the first component and the enhancing effect of morphine on the second component were antagonised by naloxone (10^{-6} M) (Fig. 7).

Clonidine (10^{-9} M-10^{-7} M) also inhibited the initial component and potentiated the second component of the motor response to field stimulation (Fig. 8). TTX (3×10^{-6} M) abolished both components of the biphasic response to field stimulation (Fig. 8).

ATP (10^{-3} M) inhibited both components of the biphasic motor response to field stimulation. Although the ability of NA (3×10^{-5} M) to induce a contraction was unaffected, ATP (10^{-3} M) could no longer elicit a contractile response at a time when the field stimulation-evoked motor response was inhibited by ATP (10^{-3} M) (Fig. 9).

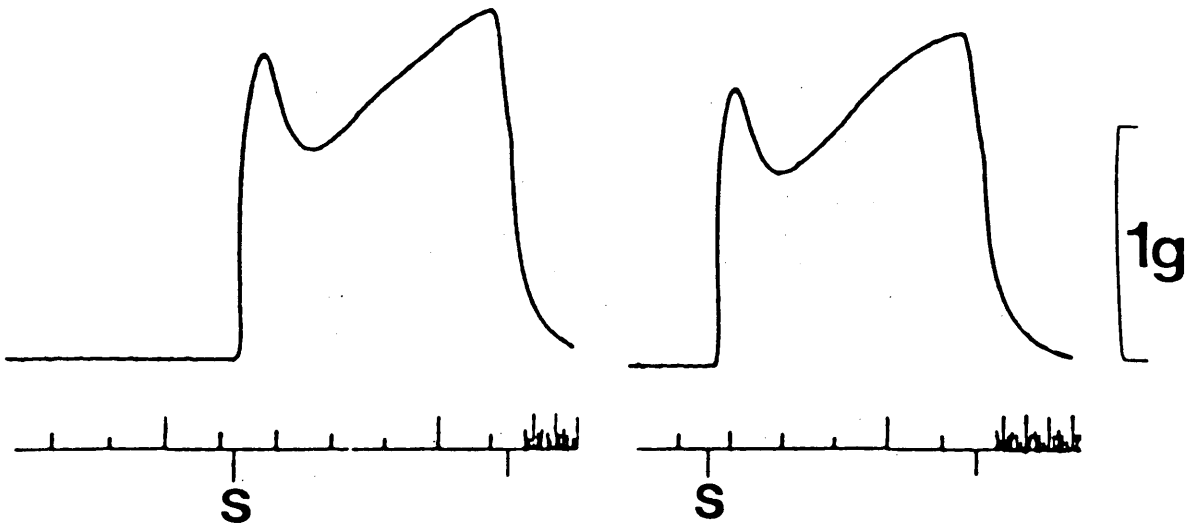


FIGURE 5a. Typical biphasic contractions of the mouse vas deferens to field stimulation at 20 Hz starting at S and lasting for 5 sec.

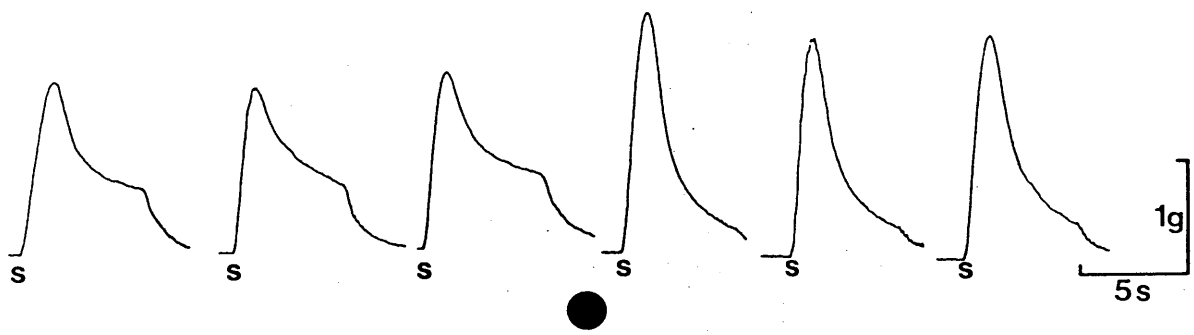


FIGURE 5b. Contractions of the mouse vas deferens to field stimulation (20 Hz, starting at S and lasting for 5 sec). Phentolamine (0, 10^{-6} M) enhanced the initial component and inhibited the second component.

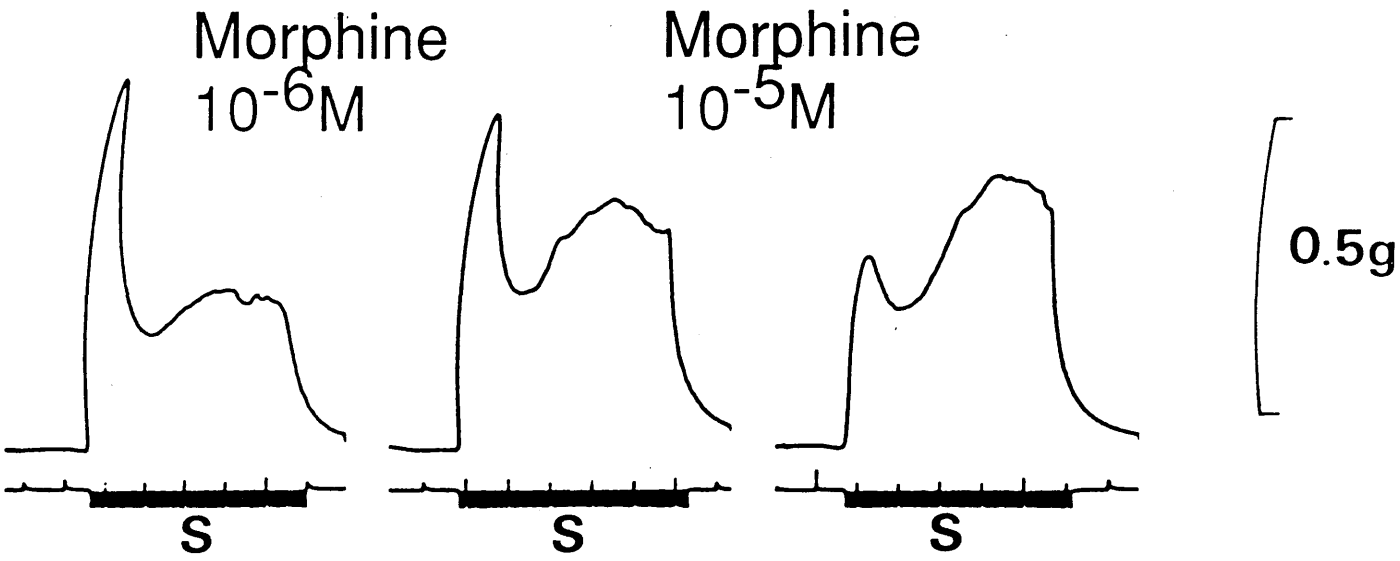


FIGURE 6. Biphasic contractions of the mouse vas deferens to electrical field stimulation at 20 Hz, starting at S and lasting for 5 sec. Morphine (10^{-6} M) added between the first and second responses slightly reduced the initial component and potentiated the second component. When the concentration of morphine was increased from 10^{-6} M to 10^{-5} M, between the second and third responses, the initial component was further reduced and the second component further potentiated.

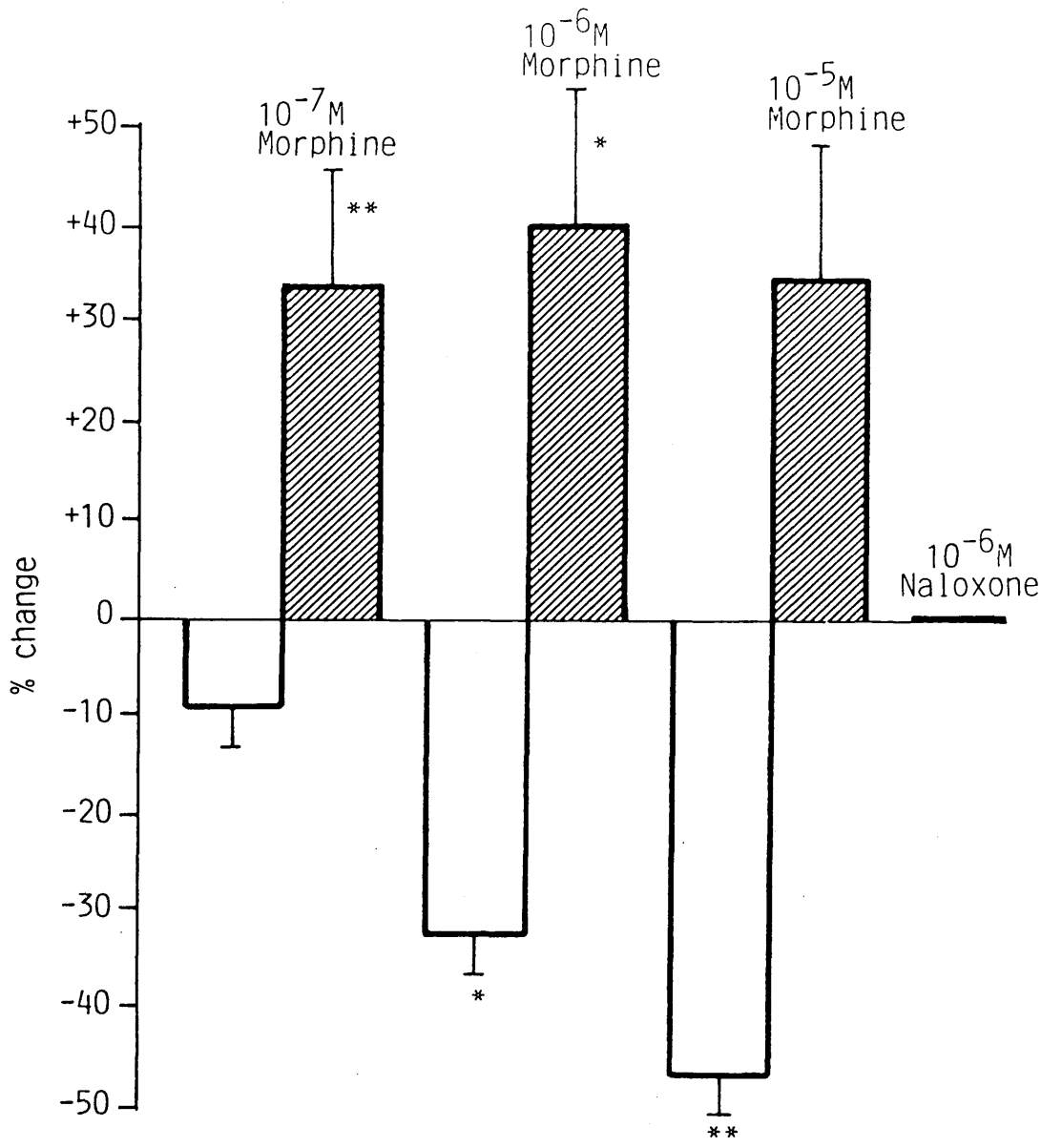


FIGURE 7. Effects of morphine (10^{-7} M-10^{-5} M) on biphasic motor responses of the vas deferens to field stimulation. The results are expressed as percentage changes from controls. The lower, open histograms show the dose-related inhibitory effects of morphine on the initial NANC component of the motor response. The upper, shaded histograms show the potentiating effect of morphine on the second noradrenergic component of the motor response. Naloxone (10^{-6} M) antagonised the effects of morphine (10^{-5} M) on both phases of the motor response to field stimulation. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* $0.05>P>0.01$, ** $0.01>P>0.001$ for comparison with corresponding controls.

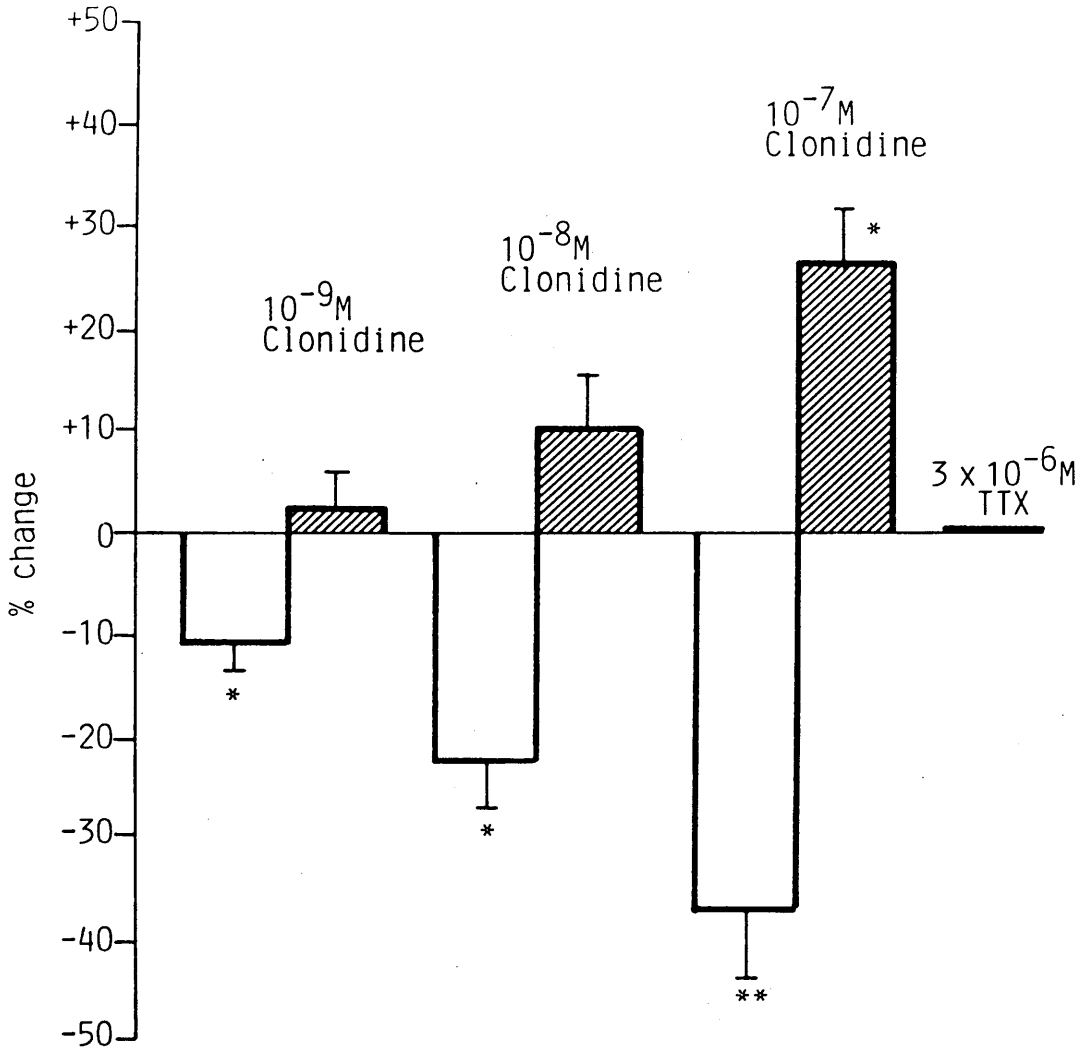
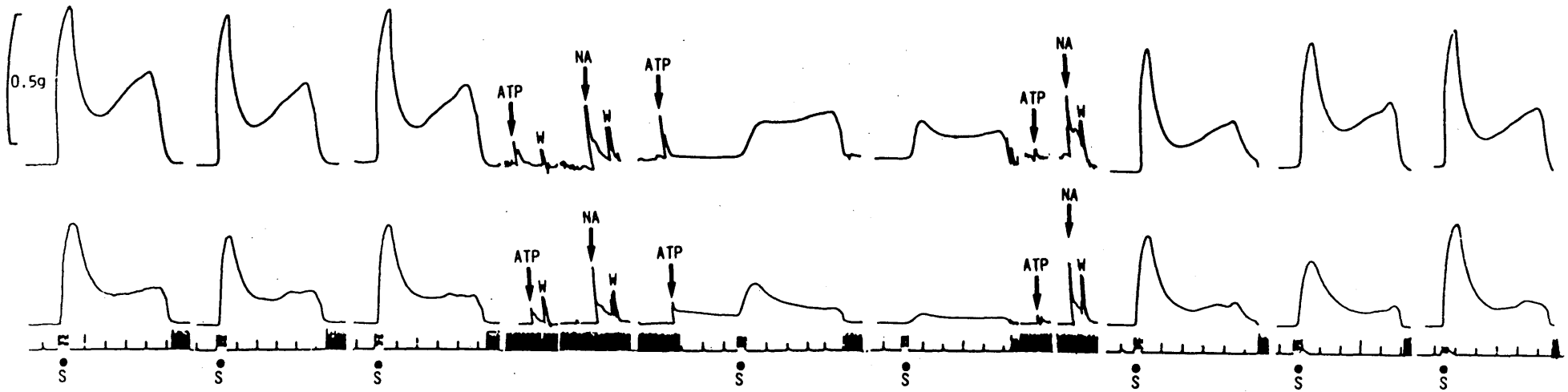


FIGURE 8. Effects of clonidine (10^{-9} M-10^{-7} M) on biphasic motor responses of the vas deferens to field stimulation. The results are expressed as percentage changes from controls. The lower, open histograms show the dose-related inhibitory effects of clonidine on the initial NANC component of the motor response. The upper, shaded histograms show the potentiating effect of clonidine on the second noradrenergic component of the motor response. Tetrodotoxin (TTX, 3 x 10^{-6} M) blocked both phases of the motor response to field stimulation. Each column represents the mean ($\pm$ S.E. mean) of 7 observations. * $0.05>P>0.01$, ** $0.01>P>0.001$ for comparison with corresponding controls.

FIGURE 9. Contractions of the mouse vas deferens to field stimulation (20 Hz, starting at S and lasting for 5 sec.). Exogenous ATP (10^{-3} M) produced a small, short-lived contraction and NA (3×10^{-5} M) produced a more sustained contaction which was reversed by washing. ATP (10^{-3} M) inhibited the contractile response to field stimulation (20 Hz, starting at S and lasting for 5 sec.). In the absence of washing, ATP (10^{-3} M) produced a negligible response, but the contractile response to NA (3×10^{-5} M) was maintained. Following washing, field stimulation-induced responses were of similar magnitude to those obtained prior to drug addition.


0.5g
ATP
W
NA
W
ATP
ATP
W
NA
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Reserpine pretreatment changed the nature of the contraction evoked by field stimulation, producing an inhibition of the second component of the biphasic motor response (Fig. 10).

6-OHDA pretreatment also changed the nature of the motor response to field stimulation. The typical biphasic response was converted to a monophasic contraction which was unaffected by phentolamine (10^{-6} M) (Fig. 11).

Contractile responses to field stimulation in control vasa were abolished by TTX (10^{-6} M, 3×10^{-6} M), which had no effect on responses of tissues from 6-OHDA pretreated animals (Fig. 12).

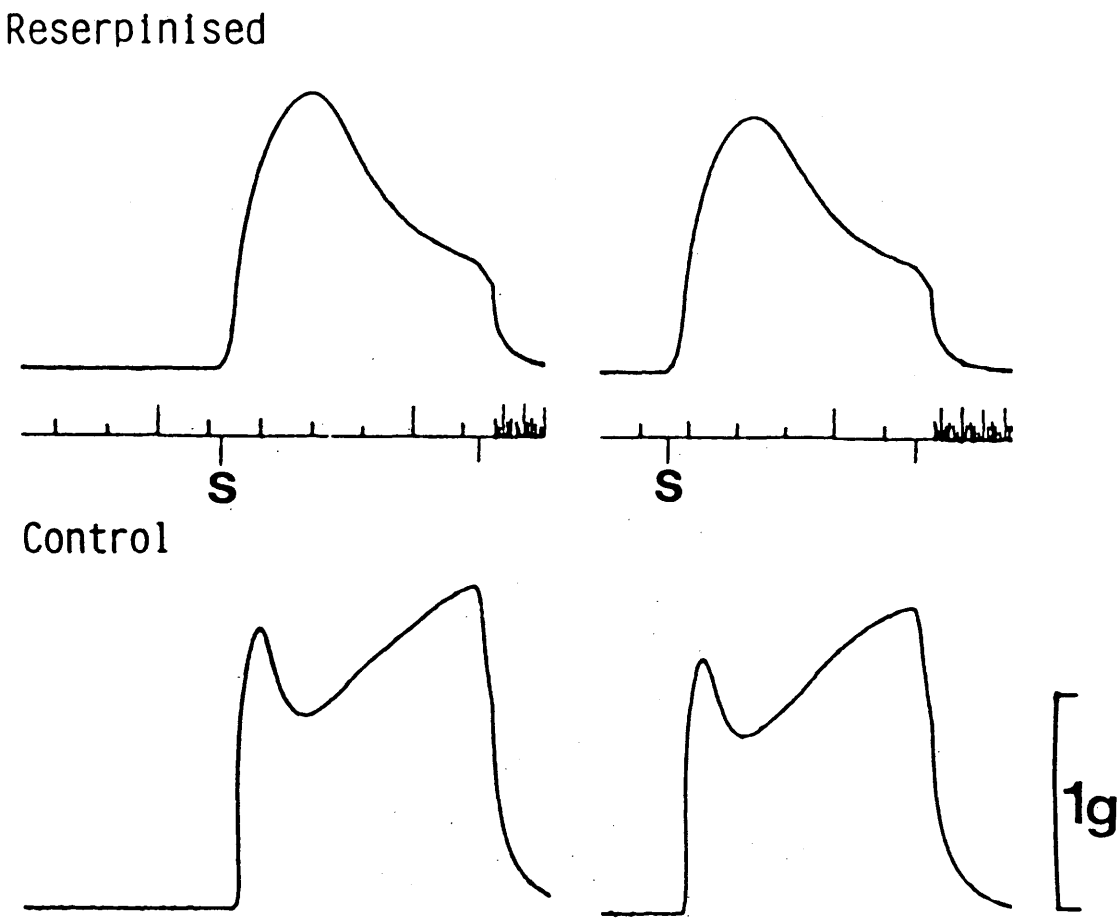

FIGURE 10. Contractions of the mouse vas deferens to field stimulation at 20 Hz, starting at S and lasting for 5 sec. Upper panel shows the inhibitory effect of reserpine pretreatment on the second component of the biphasic motor response to field stimulation. Lower panel shows typical biphasic contractile responses to field stimulation.

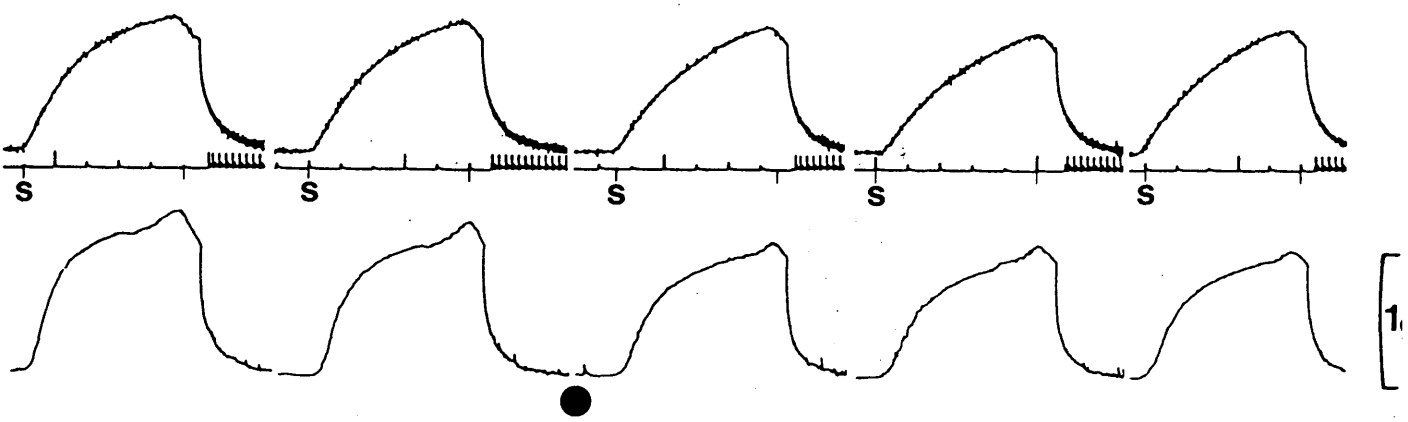

FIGURE 11. Monophasic contractions to field stimulation (20 Hz, starting at S and lasting for 5 sec.) in vasa from 6-OHDA pretreated mice, showing the lack of effect of phentolamine (●), (10^{-6} M).

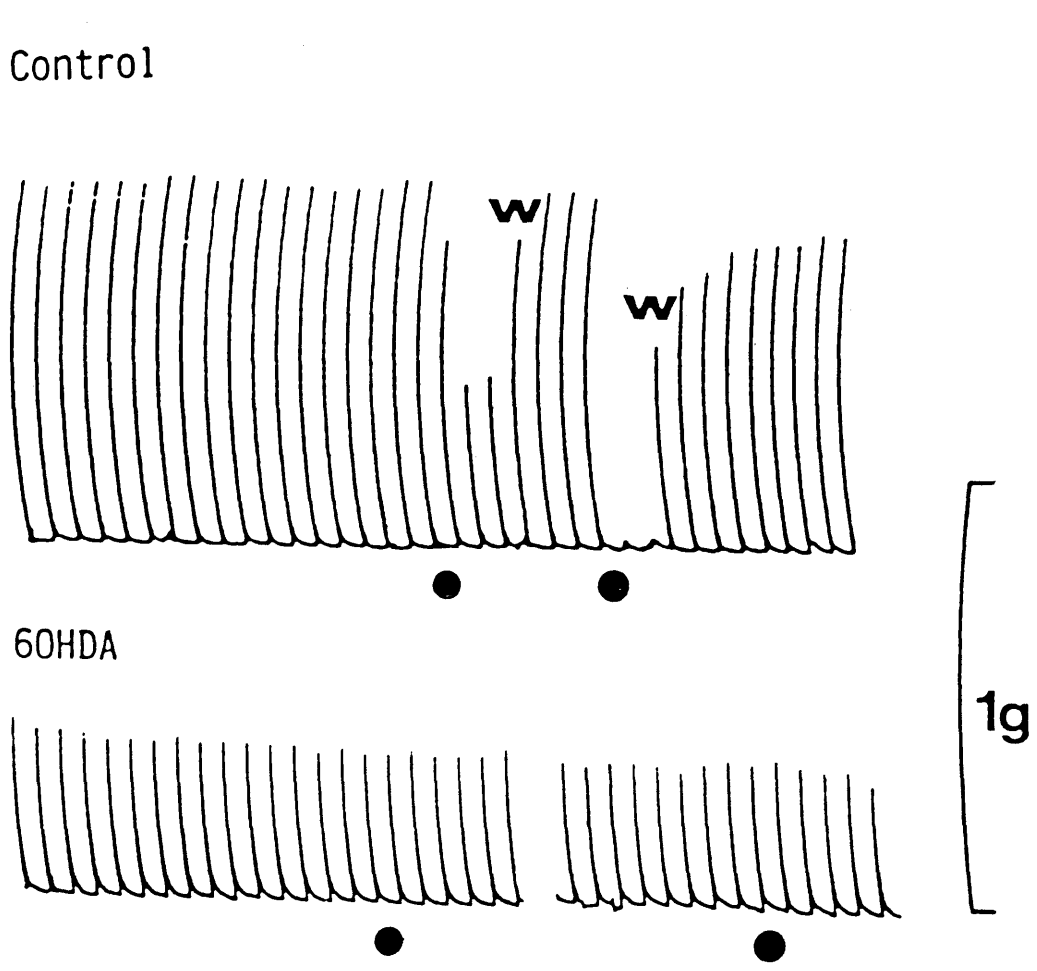

FIGURE 12. Traces showing the inhibitory effects of TTX on responses of isolated mouse vasa to field stimulation and the lack of effect of TTX in vasa from 6-OHDA pretreated animals. Upper trace from a control vas to trains of 100 pulses, delivered at 20 Hz and 100 sec. intervals. TTX (●, 10^{-6} M and ●, 3 x 10^{-6} M) produced dose-dependent inhibitions that were reversed with washing. Lower trace shows responses of a vas from a 6-OHDA pretreated mouse to trains of 100 pulses at 20 Hz every 100 sec., on which TTX (●, 10^{-6} M and ●, 3 x 10^{-6} M) had no effect.

PART II

EFFECTS OF ACUTE AND CHRONIC DRUG ADMINISTRATION ON RESPONSES OF MOUSE ISOLATED VASA

Pretreatment with 6-OHDA increased the sensitivity of isolated vasa to NA. The supersensitivity was characterised by a large increase in the maximum response to NA (Fig. 13).

High concentrations of ATP, added exogenously to the bath, elicited contractile responses (Figs. 14, 15).

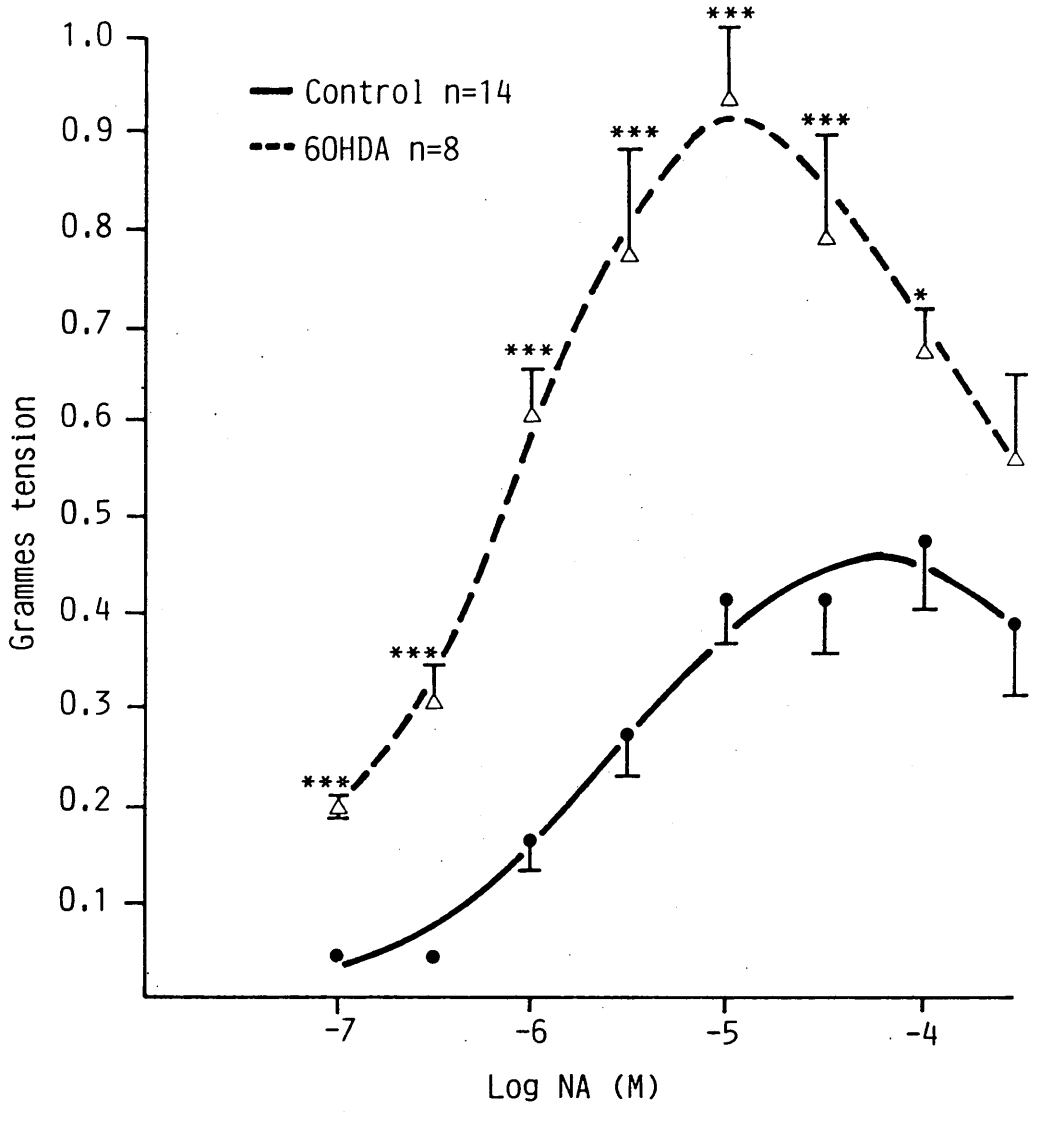

FIGURE 13. Dose-response curves to NA in isolated vasa from control (●——●) and 6-OHDA pretreated (△——△) mice. Each point is the mean (± S.E. mean) of ≤ 8 observations.

* 0.05>P>0.01, *** P<0.001.

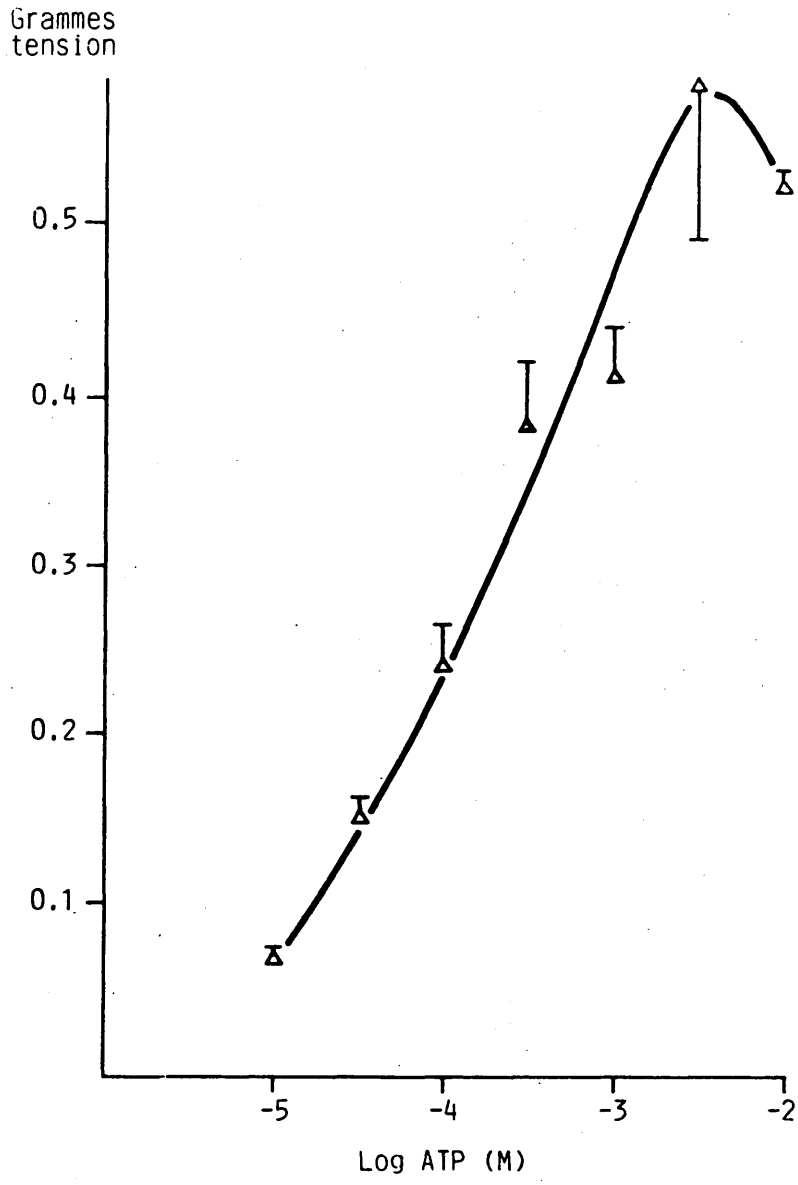

FIGURE 14. Dose-response curve showing the effect of ATP in vasa. Each point is the mean (± S.E. mean) of 4 observations.

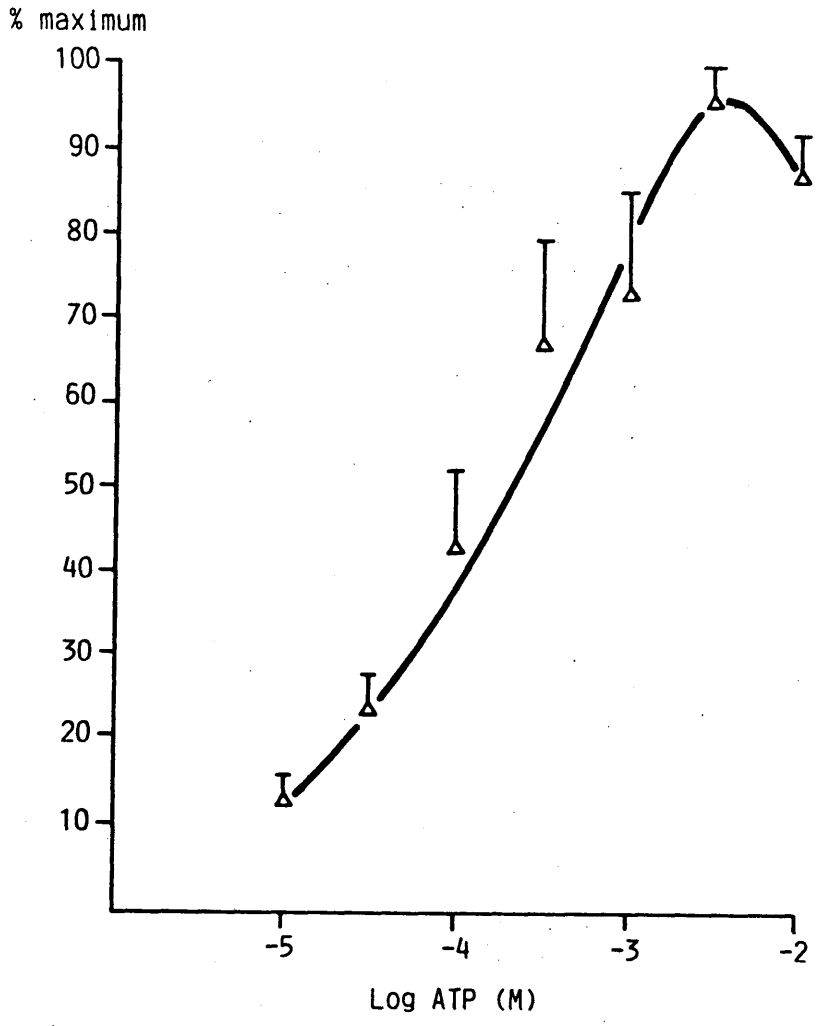

FIGURE 15. Dose %-response curve showing the effects of ATP in vasa. Each point is the mean (± S.E. mean) of 4 observations.

PART III

EFFECTS OF 6-OHDA PRETREATMENT AND ACUTELY ADMINISTERED DRUGS ON RESPONSES OF THE IN SITU VAS DEFERENS OF THE ANAESTHETISED RAT TO FIELD STIMULATION AND STIMULATION OF THE SPINAL MOTOR NERVES

Stimulation of the vas *in situ*, either via an electrode inserted into the spinal canal, or by field stimulation with local electrodes placed around the vas, produced biphasic responses similar to those produced by field stimulation in isolated vasa (Fig. 16).

In anaesthetised control rats, the shape of the response to *in situ* field stimulation was altered and the response to stimulation of the spinal outflows was inhibited by clonidine (500 μg/kg). These effects were antagonised by yohimbine (1 mg/kg) (Fig. 16). Control responses were abolished by TTX (T, 2 mg/kg) (Fig. 17).

6-OHDA pretreatment changed the nature of the motor response evoked either by nerve stimulation or by field stimulation. The small residual monophasic contraction, evoked by stimulation of the spinal motor outflows, was abolished by TTX (T, 2 mg/kg). The monophasic residual response evoked by *in situ* field stimulation was reduced, but not abolished, by TTX (T, 2 mg/kg) (Fig. 17).

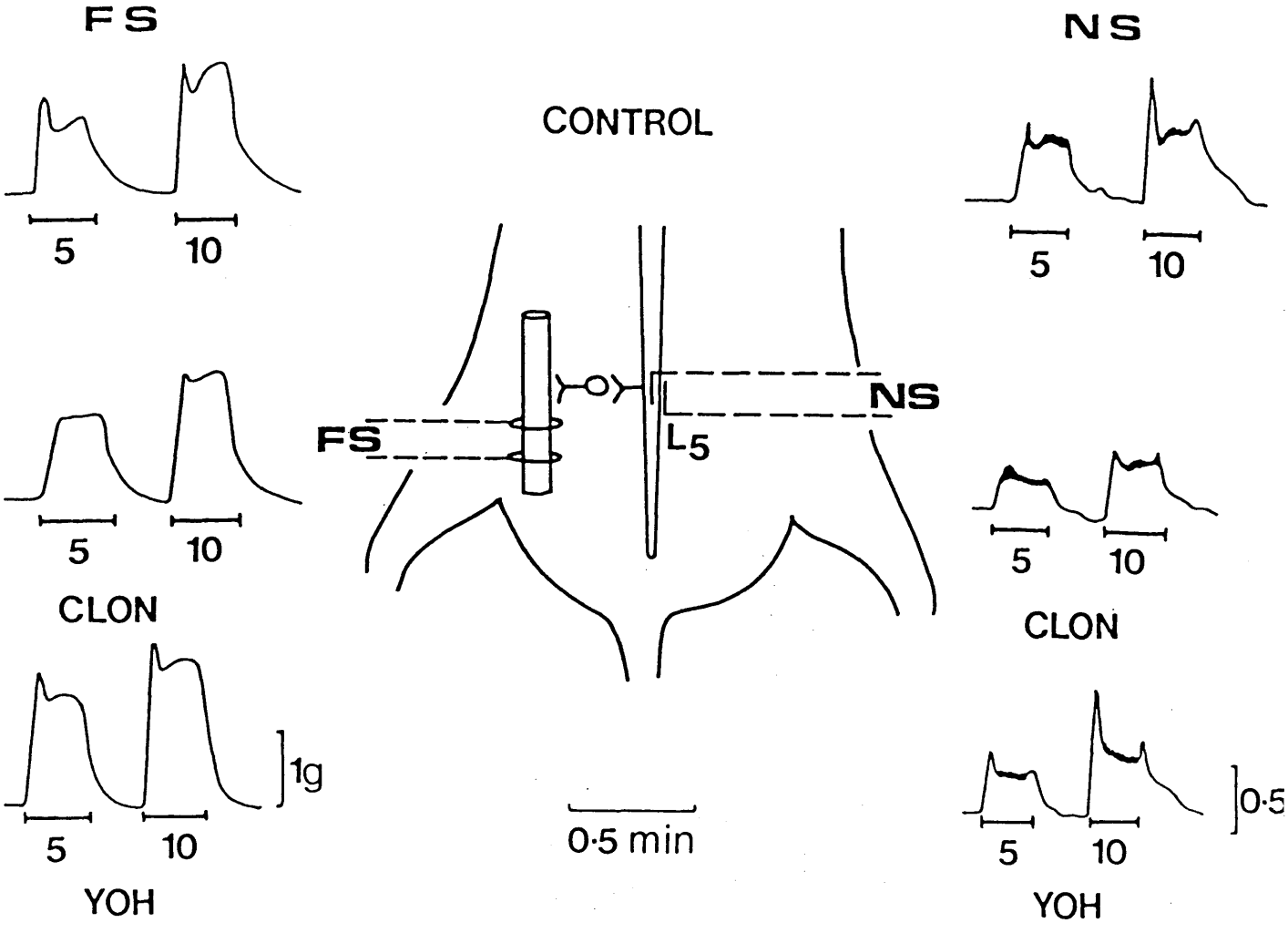

FIGURE 16. Contractile responses of the <u>in situ</u> vas deferens to electrical field stimulation and stimulation of spinal motor nerves in the anaesthetised cat.

FS: Field stimulation at different frequencies for the periods indicated by the bars produced characteristic biphasic responses. Clonidine (500 μg/kg) altered the shape of the contractile response, slowing the rate of rise of the response and altering its biphasic nature, resulting in a more monophasic contraction. Yohimbine (1 mg/kg), antagonised the ability of clonidine to slow the rate of rise and the shape of the response and, in addition, enhanced both phases of the contraction above the size of the control response.

NS: In the same animal, the biphasic contractile response to stimulation of the spinal motor nerves to the vas was inhibited by clonidine (500 μg/kg) and this inhibition was also antagonised by yohimbine (1 mg/kg).

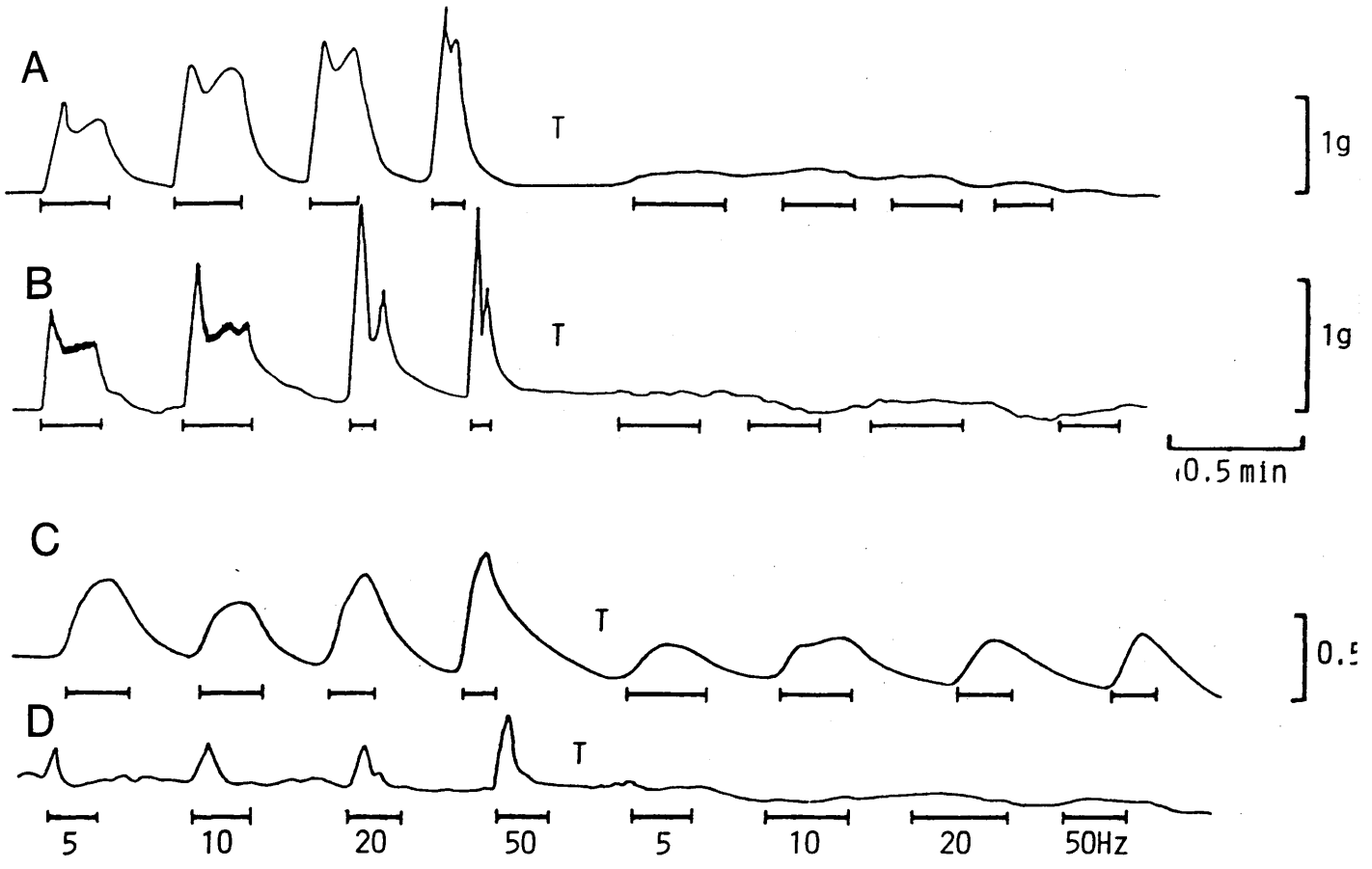

FIGURE 17. Contractile responses of the in situ vas deferens to electrical field stimulation and to stimulation of the spinal motor nerves in an anaesthetised intact rat and in an anaesthetised, 6-OHDA pretreated rat. Electrical stimulation was applied at the frequencies shown and for the periods indicated by the bars.

The upper panel shows biphasic motor responses to in situ field stimulation (A) and stimulation of the spinal motor nerves (B) in the same control rat, abolished by 2 mg/kg TTX (T).

The lower panel shows small, monophasic responses to in situ field stimulation in a 6-OHDA pretreated rat (C). These responses were reduced but not abolished by 2 mg/kg TTX (T). In the same rat, spinal motor nerve stimulation produced a very small motor response that was abolished by 2 mg/kg TTX (T).

PART IV

EFFECTS OF THYROXINE PRETREATMENT ON PRE- AND POST-SYNAPTIC RECEPTOR SENSITIVITY

Thyroxine (T_4)-pretreatment increased serum free T_4 levels (Fig. 18). This change was accompanied by alterations in the sensitivity of the vas to agonists acting at both pre- and post-synaptic sites.

T_4-pretreatment reduced the inhibition produced by morphine and clonidine, but had little effect on the maximum percentage inhibitions produced by these drugs (Figs. 19, 20, 21, 22).

T_4-pretreatment also reduced the maximum contractile response to NA with no displacement of the NA dose-response curve (Figs. 23, 24).

Pretreatment with T_4 reduced the maximum contractile response to carbachol and produced a rightward displacement of the carbachol dose-response curve (Figs. 25, 26).

Pretreatment with a combination of T_4 + 6-OHDA similarly increased the maximum contractile response to carbachol in comparison to pretreatment with 6-OHDA alone (Fig. 27).

Combined pretreatment of T_4 + 6-OHDA increased the maximum contractile response to NA to a greater extent than pretreatment with 6-OHDA alone (Fig. 28).

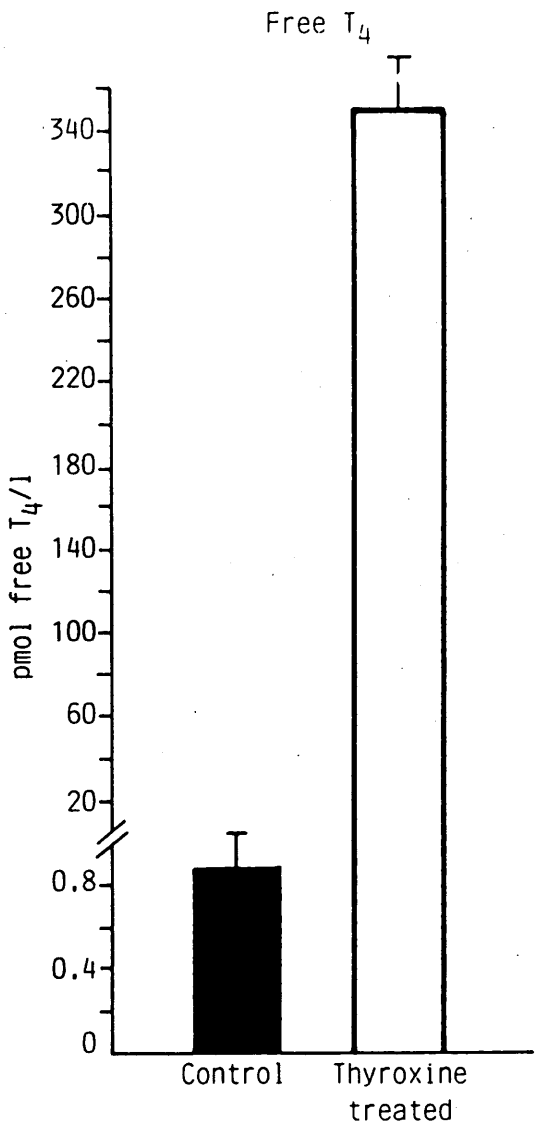

FIGURE 18. The unbound T_4 levels in plasma, measured in pmol free T_4/l, in both control and T_4-pretreated mice. Each column represents the mean ($\pm$ S.E. mean) of $\leq$ 8 observations. *** P<0.001.

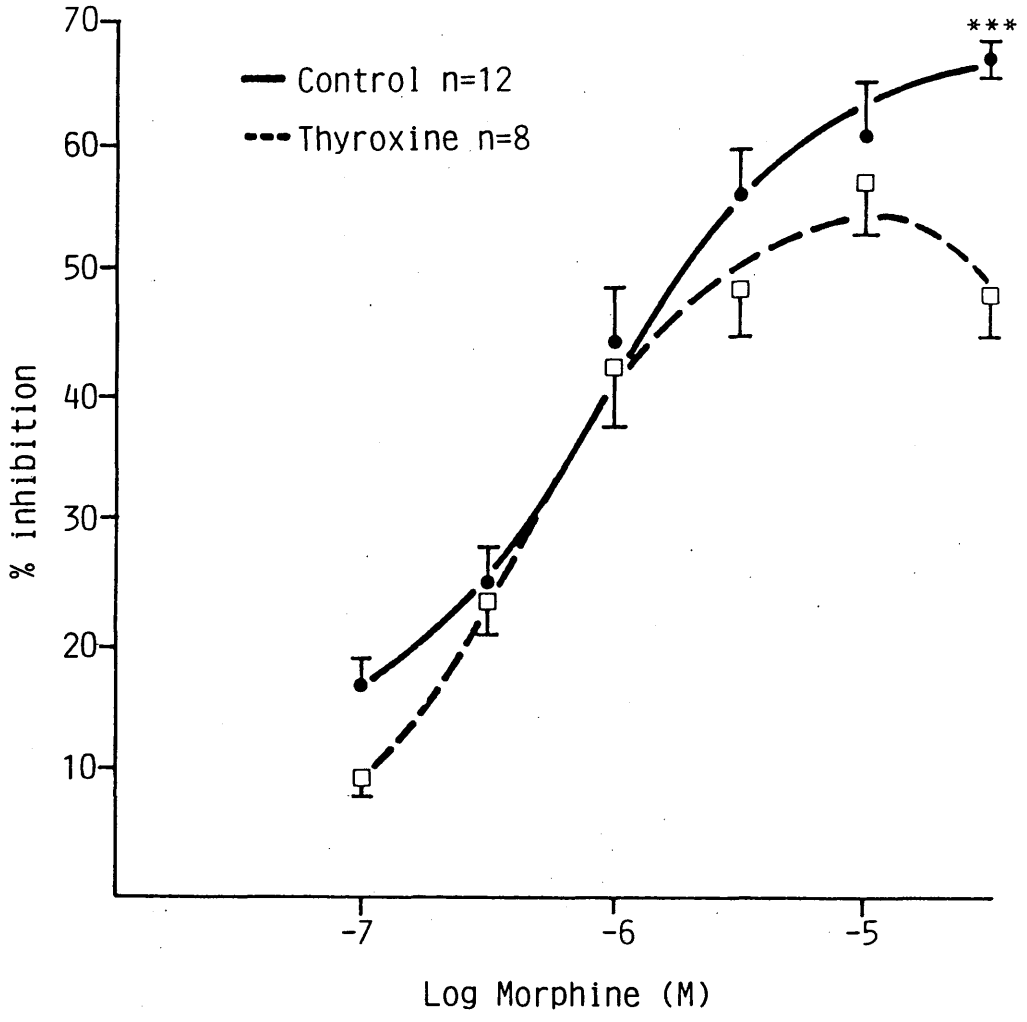

FIGURE 19. Dose-response curves showing inhibitory effects of morphine in vasa from control (●——●) and T_4-pretreated (□——□) mice, stimulated at 20 Hz for 0.5 msec. Each point is the mean (± S.E. mean) of ≤ 8 observations. *** P<0.001.

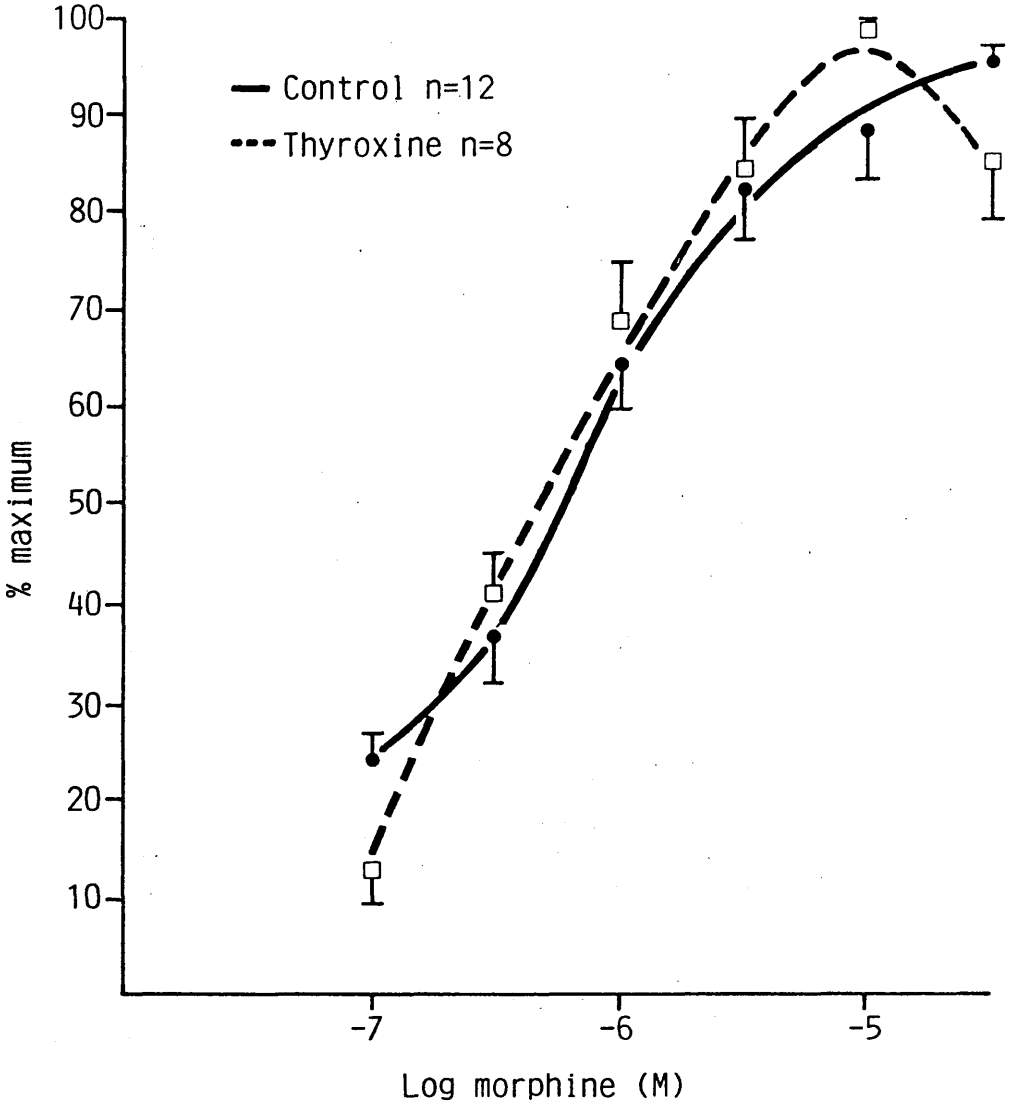

FIGURE 20. Dose %-response curves showing effects of morphine in vasa from control (●——●) and T_4-pretreated (□——□) mice, stimulated at 20 Hz for 0.5 msec. Each point is the mean (± S.E. mean) of ≤ 8 observations.

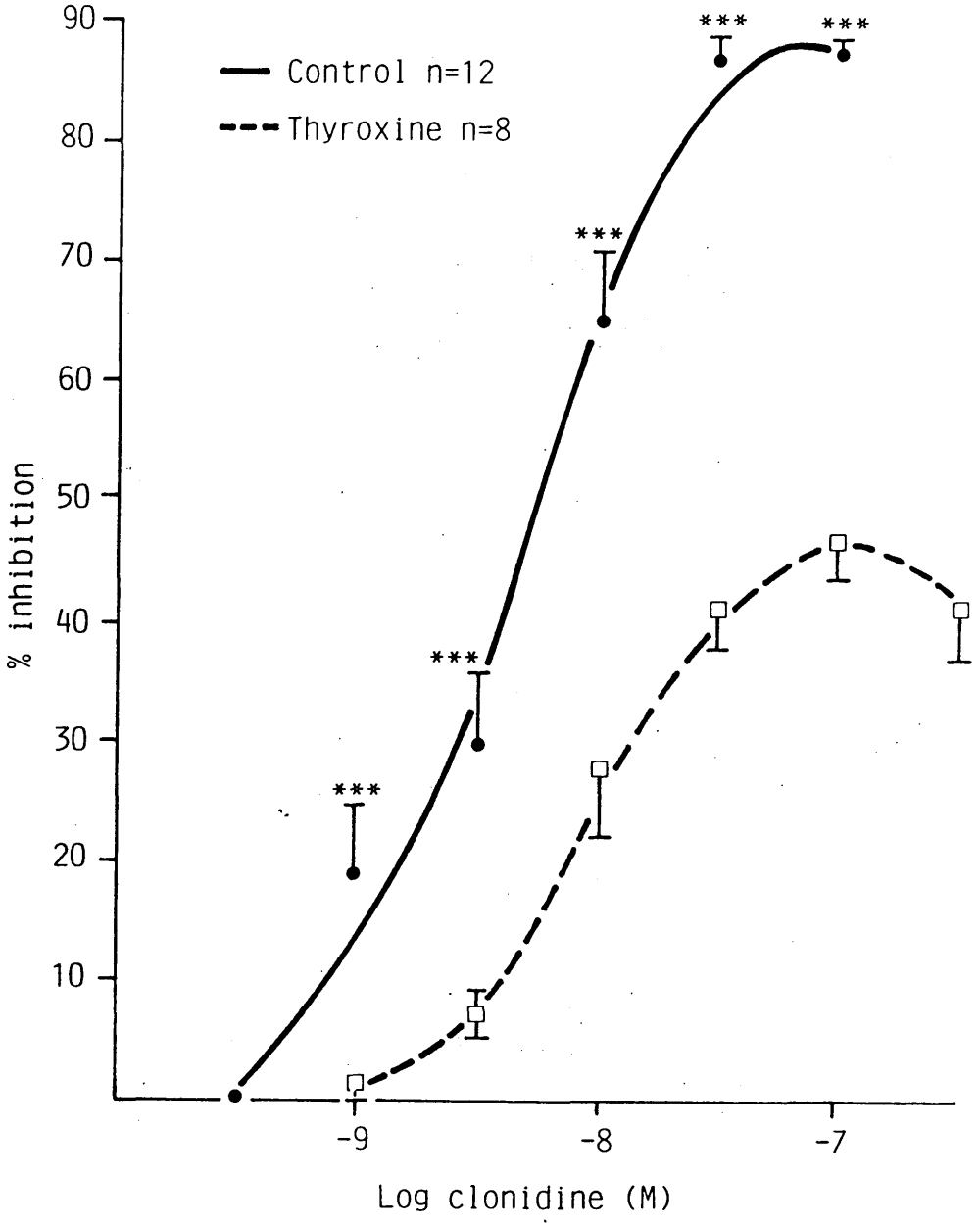

FIGURE 21. Dose-response curves showing inhibitory effects of clonidine in vasa from control (●——●) and T_4-pretreated (□——□) mice, stimulated at 20 Hz for 0.5 msec. Each point is the mean (± S.E. mean) of 8 observations. *** P<0.001.

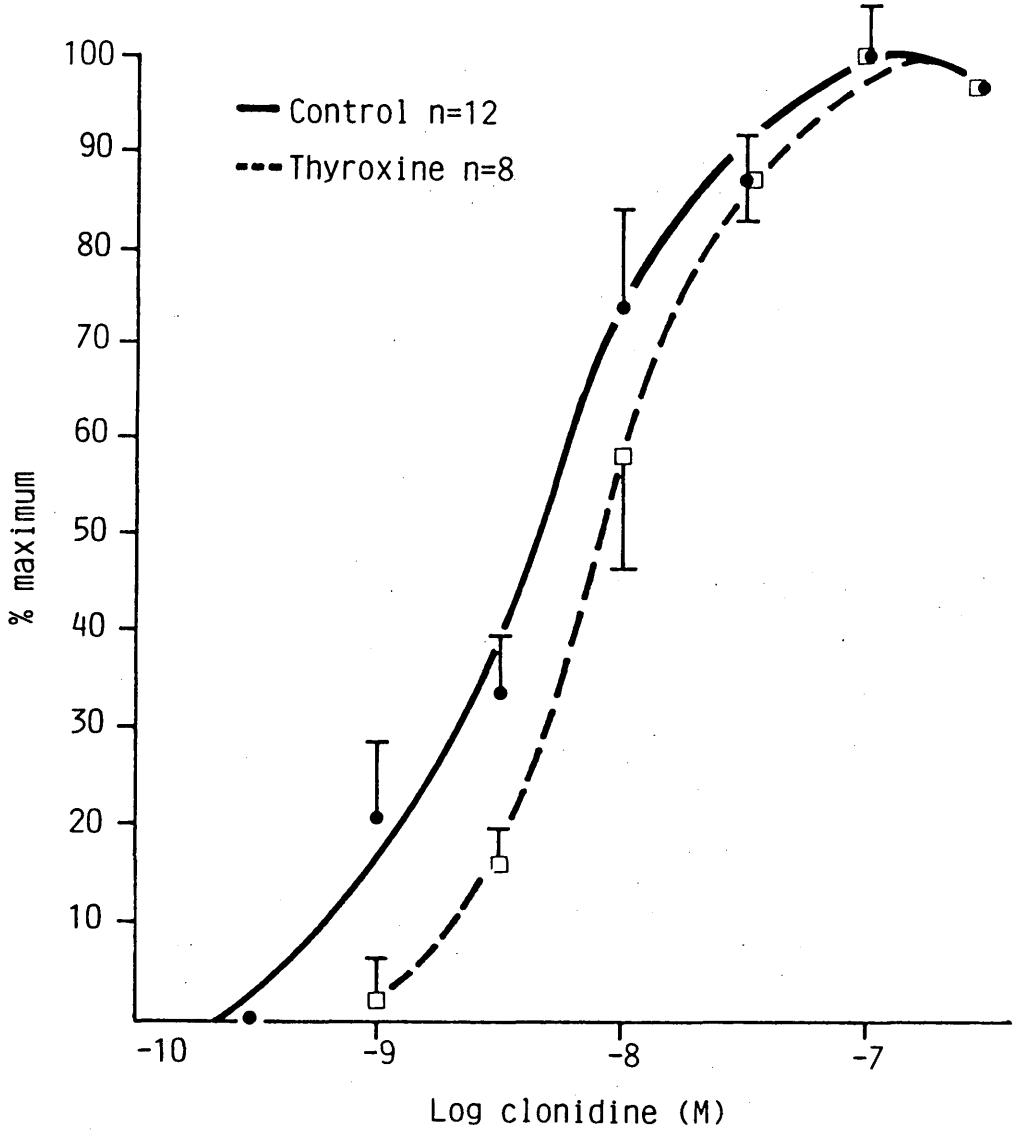

FIGURE 22. Dose %-response curves showing effects of clonidine in vasa from control (●——●) and T_4-pretreated (□——□) mice, stimulated at 20 Hz for 0.5 msec. Each point is the mean (± S.E. mean) of ≤ 8 observations.

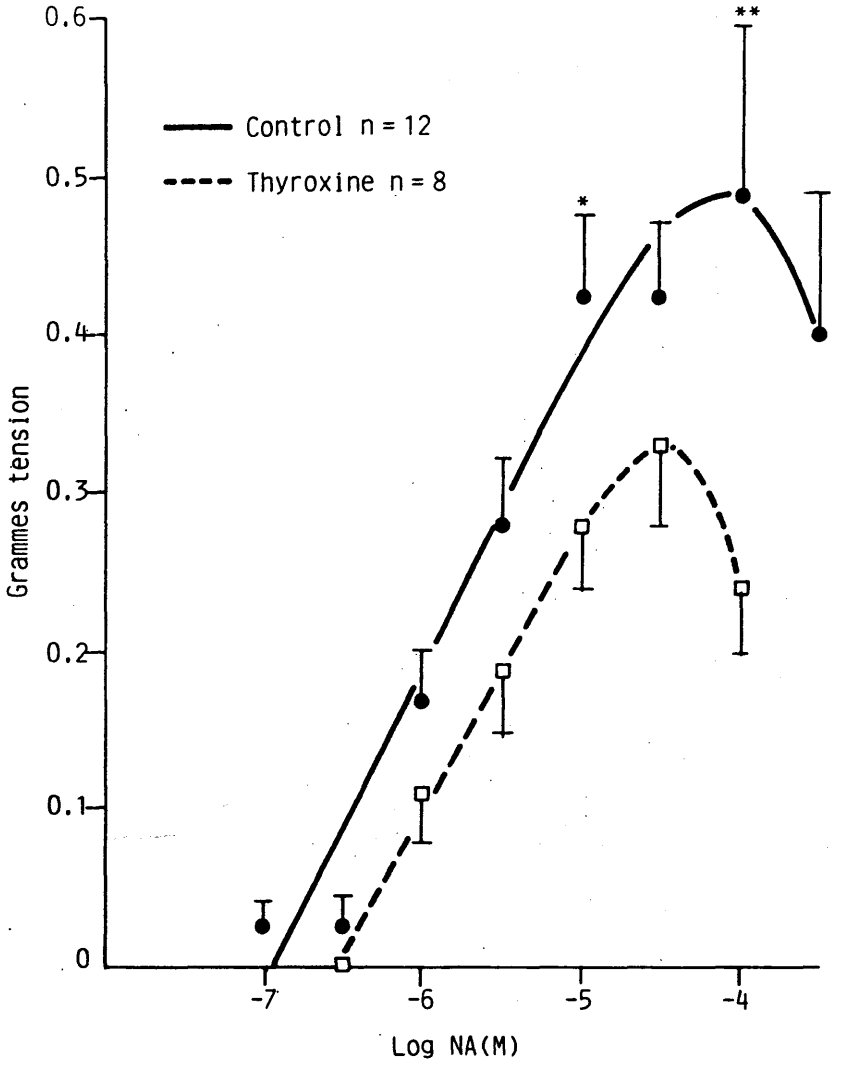

FIGURE 23. Dose-response curves showing effects of NA in vasa from control (●——●) and T_4-pretreated (□——□) mice. Each point is the mean (± S.E. mean) of ≤ 8 observations.

* 0.05>P>0.01; ** 0.01>P>0.001.

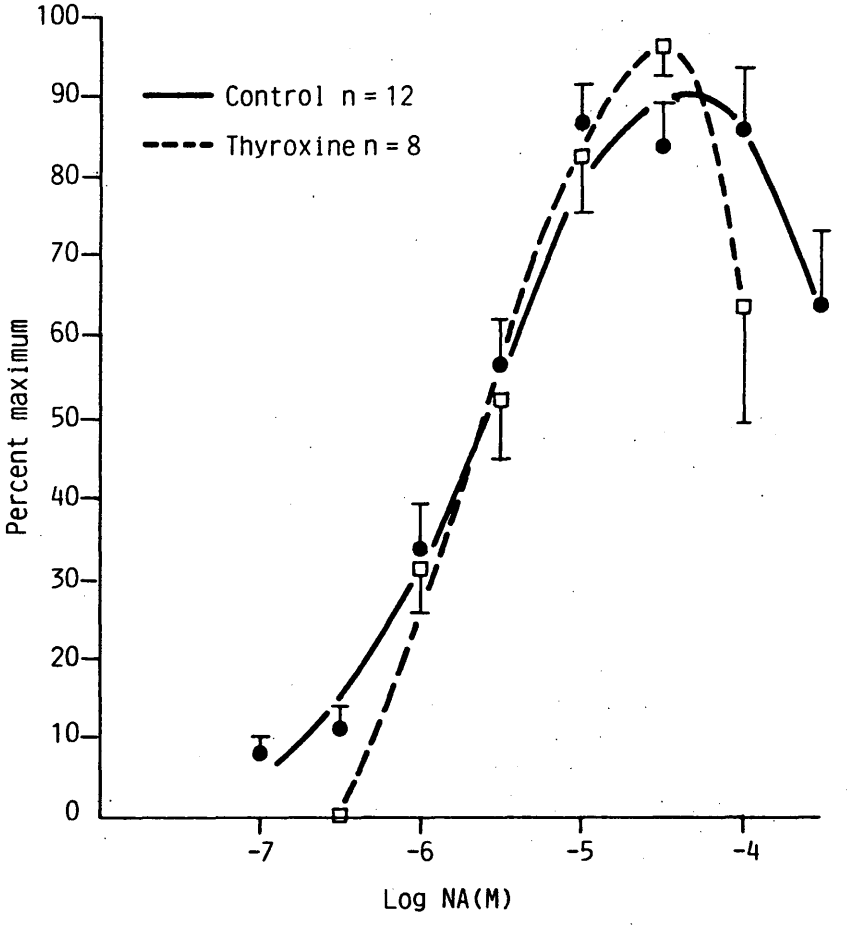

FIGURE 24. Dose %-response curves showing effects of NA in vasa from control (●——●) and T_4-pretreated (□——□) mice. Each point is the mean (± S.E. mean) of ≤ 8 observations.

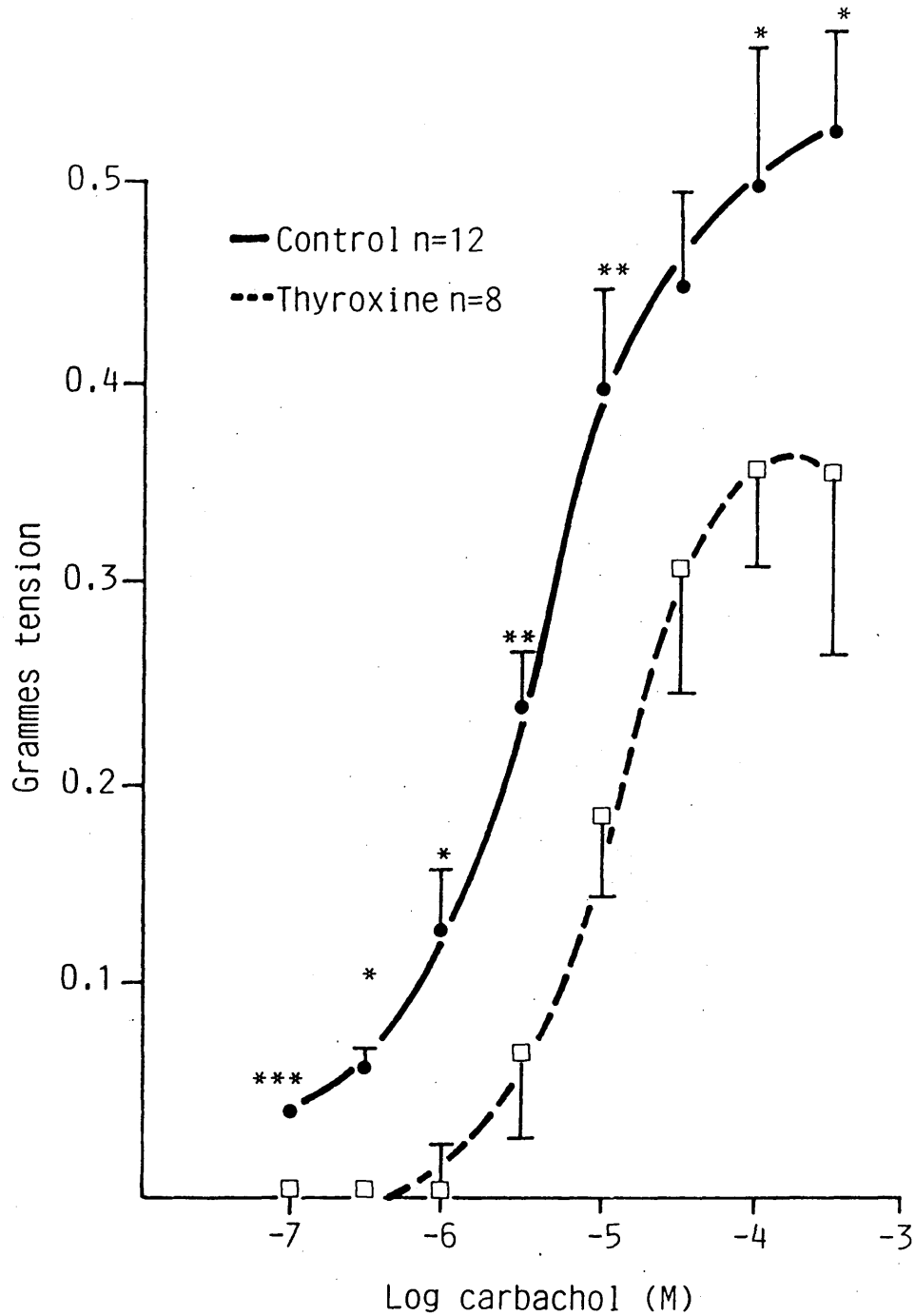

FIGURE 25. Dose-response curves showing effects of carbachol in vasa from control (●——●) and T_4-pretreated (□——□) mice. Each point is the mean (± S.E. mean) of ≥ 8 observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001.

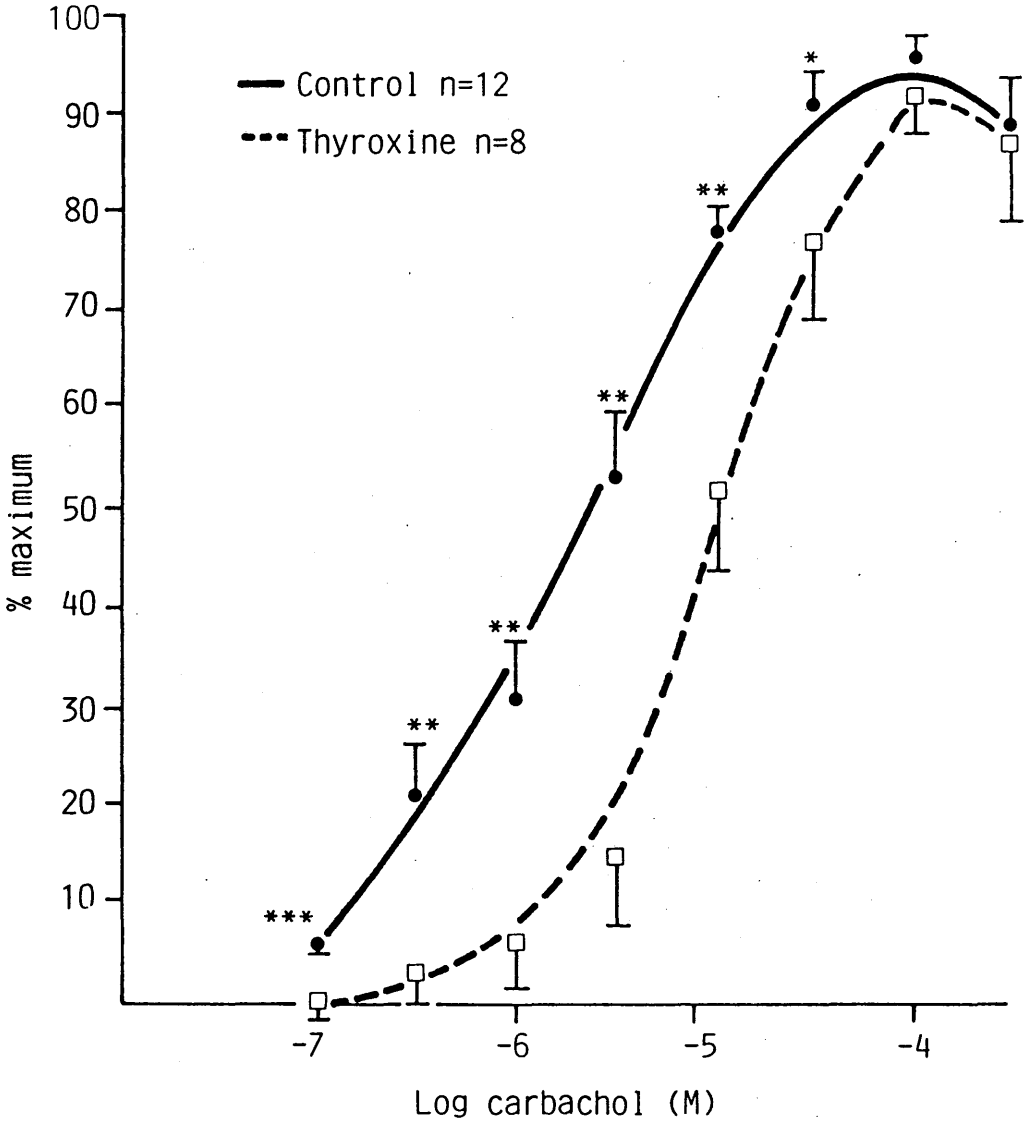

FIGURE 26. Dose %-response curves showing effects of carbachol in vasa from control (●——●) and T_4-pretreated (□——□) mice. Each point is the mean (± S.E. mean) of ≤ 8 observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001.

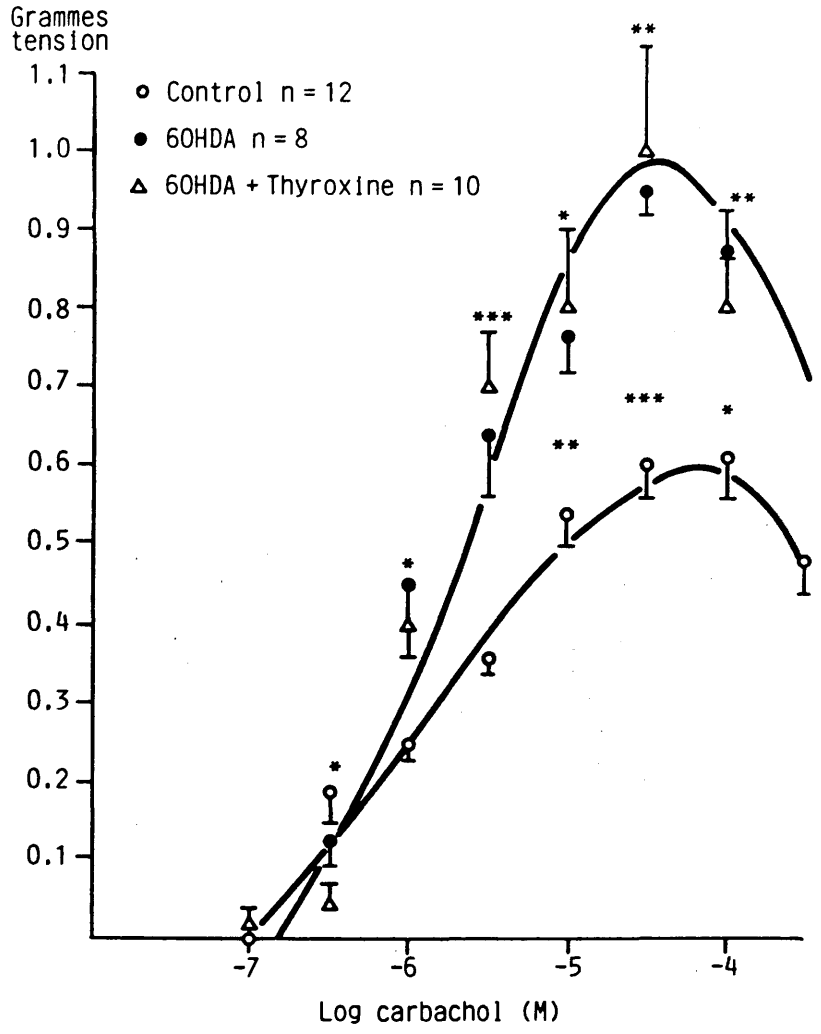

FIGURE 27. Dose-response curves to carbachol in isolated vasa from control (O——O), 6-OHDA pretreated (●——●) and 6-OHDA + T_4 pretreated (△——△) mice. Responses to carbachol were potentiated in vasa pretreated with 6-OHDA and a combination of 6-OHDA + T_4. The uppermost asterisks at 3 x 10^{-5} M, 10^{-6} M and 3 x 10^{-6} M indicate significant changes resulting from 6-OHDA and 6-OHDA + T_4 pretreatments in comparison with controls. The uppermost asterisks at 10^{-4} M, 3 x 10^{-4} M and 10^{-5} M indicate significant changes resulting from a combination of 6-OHDA + T_4 pretreatment in comparison with controls. At these same concentrations, the lower asterisks indicate significant changes resulting from 6-OHDA pretreatment in comparison with controls. Each point is the mean (± S.E. mean) of ≮ 8 observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001.

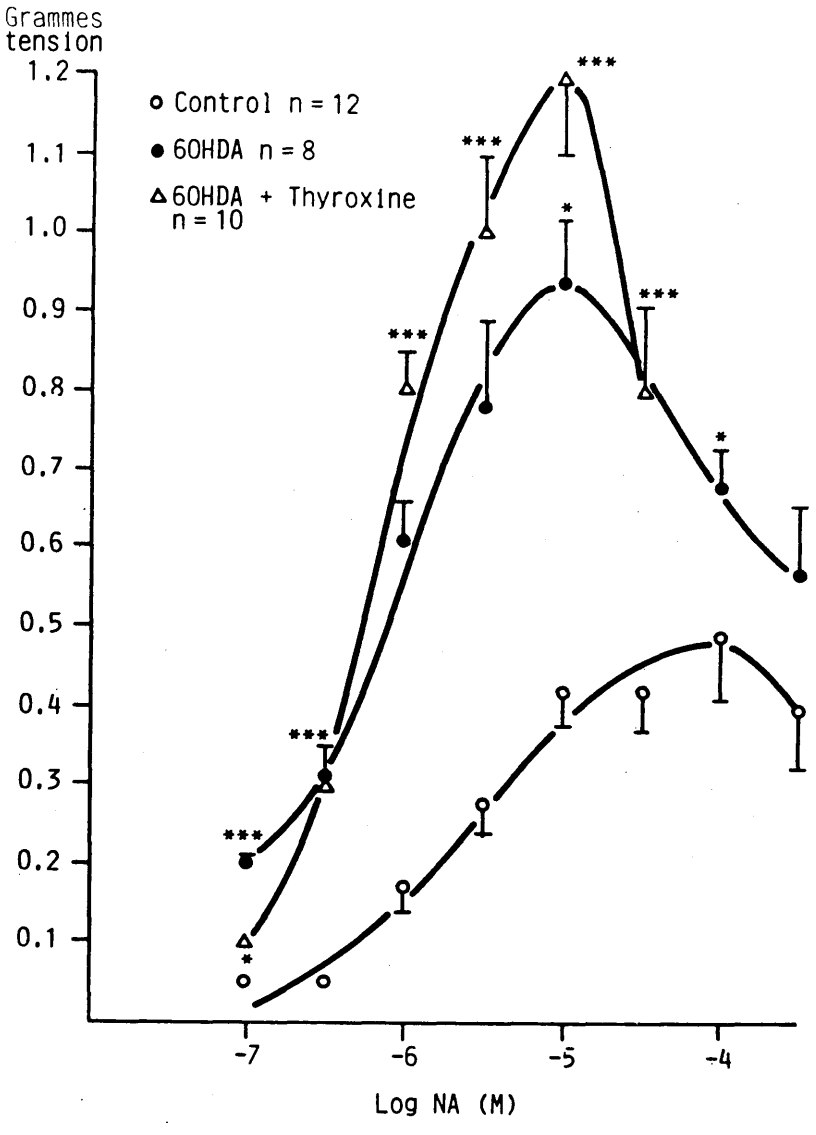

FIGURE 28. Dose-response curves to NA in isolated vasa from control (O——O), 6-OHDA pretreated (●——●) and 6-OHDA + T_4 pretreated (△——△) mice. Responses to NA were enhanced in vasa from 6-OHDA pretreated mice and were further potentiated in vasa from mice pretreated with a combination of 6-OHDA + T_4. Asterisks indicate significant changes resulting from 6-OHDA and 6-OHDA + T_4 pretreatments in comparison with controls. The asterisk at 10^{-7} M indicates a significant difference between vasa from controls and from mice pretreated with a combination of 6-OHDA + T_4. The asterisk at 10^{-5} M indicates a significant difference between vasa from 6-OHDA pretreated mice and mice pretreated with a combination of 6-OHDA + T_4. Each point is the mean ($\pm$ S.E. mean) of $\leq$ 8 observations.

* $0.05>P>0.01$, *** $P<0.001$.

PART V

EFFECTS OF FIELD STIMULATION ON THE OVERFLOW OF ^{3}H AND THE EFFECTS OF DRUGS ON THIS OVERFLOW

Electrical field stimulation of the vas increased the amount of radioactivity released into the Krebs solution in the organ bath. Such increases coincided with the field stimulation-evoked contractions, after which further collection of Krebs solution between stimulations contained only low levels of radioactivity. Chromatographic separation of (^{3}H)-NA from its metabolites showed that field stimulation increased the overflow of (^{3}H)-NA. Whereas only 34% of the spontaneous ^{3}H overflow was recovered as (^{3}H)-NA, almost 80% of the ^{3}H radioactivity released into the bathing solution during field stimulation was (^{3}H)-NA (Table I).

Table I. Percentage of (^{3}H)-NA and its (^{3}H)-metabolites in Krebs solution bathing isolated, field stimulated vasa deferentia.

Time	(^{3}H)-NA (%)	(^{3}H)-NMN (%)	(^{3}H)-deaminated metabolites (%)
Before stimulation	34 ± 8 (5)	33 ± 6 (5)	30 ± 10 (5)
During stimulation	77 ± 8 (5)	16 ± 5 (5)	7 ± 3.5 (5)

mean percentages ± S.E. mean (n)

deaminated metabolites : DOMA, DOPEG, MOPEG, VMA

^{3}H overflow was unaffected by treatment with a combination of tranylcypromine 10^{-5} M, desmethylimipramine 10^{-5} M and 17,β-oestradiol 10^{-5} M, which block respectively MAO and the neuronal and extraneuronal uptake of NA (Fig. 29). PBA (4×10^{-5} M), which, in addition to blocking these disposal mechanisms, also blocks presynaptic α_2-adrenoceptors, potentiated ^{3}H overflow (Fig. 30).

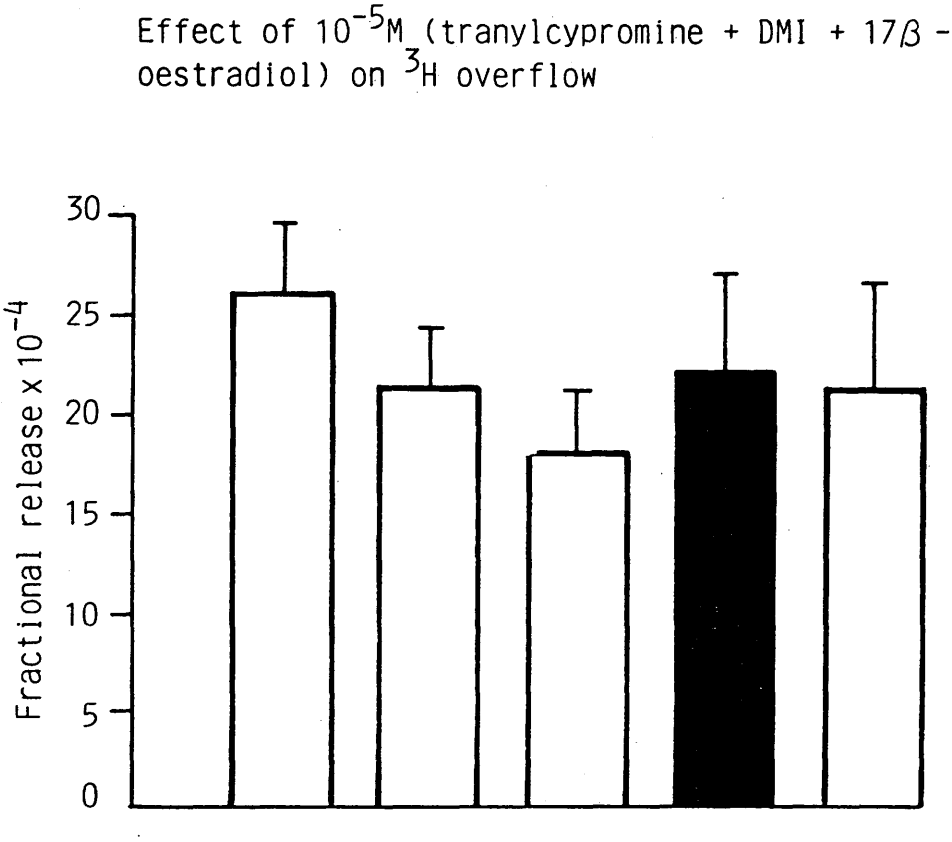

FIGURE 29. Effect of tranylcypromine 10^{-5} M, + desmethylimipramine 10^{-5} M + 17,β-oestradiol 10^{-5} M, on field stimulation-evoked overflow of ^{3}H in the vas deferens. The open histograms show successive control responses prior to drug addition. The shaded histogram shows the lack of effect of the combination of drugs to block monoamine oxidase (MAO) and the neuronal and extraneuronal uptake of NA in the same experiments. Each column represents the mean (± S.E. mean) of 6 observations.

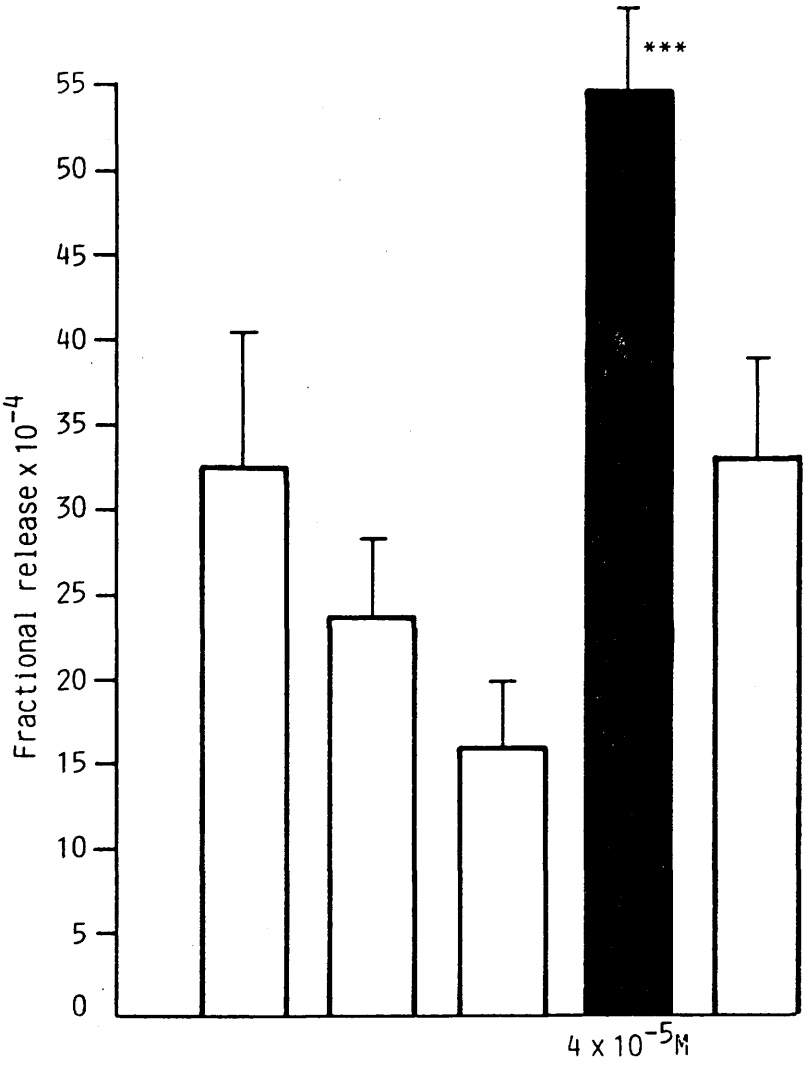

FIGURE 30. Effect of phenoxybenzamine (4 x 10^{-5} M) on field stimulation-evoked overflow of ^{3}H in the vas deferens. The open histograms show successive control responses prior to drug addition. The shaded histogram shows the potentiating effect of phenoxybenzamine (4 x 10^{-5} M) in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

*** $P<0.001$ for comparison with control prior to drug addition.

Morphine (10^{-7} M-10^{-5} M) increased the overflow of radioactivity from vasa of both T.O. (Fig. 31) and Porton (Fig. 32) mice and this effect was antagonised by naloxone (10^{-6} M) (Fig. 33). The ability of morphine to enhance ^{3}H overflow occurred in the presence of a combination of drugs which block MAO and the neuronal and extraneuronal uptake of NA (Fig. 34).

Clonidine (10^{-9} M-10^{-7} M) also increased the overflow of radioactivity from vasa of both T.O. (Fig. 35) and Porton (Fig. 36) mice. The ability of clonidine to increase ^{3}H overflow occurred in the presence of blocking drugs (Fig. 37).

Chromatographic separation of (^{3}H)-NA from its metabolites showed that there was little change in the percentage of (^{3}H)-NA released into the bathing solution during field stimulation in the presence of morphine or clonidine (Table II).

Effect of morphine on ^{3}H overflow.

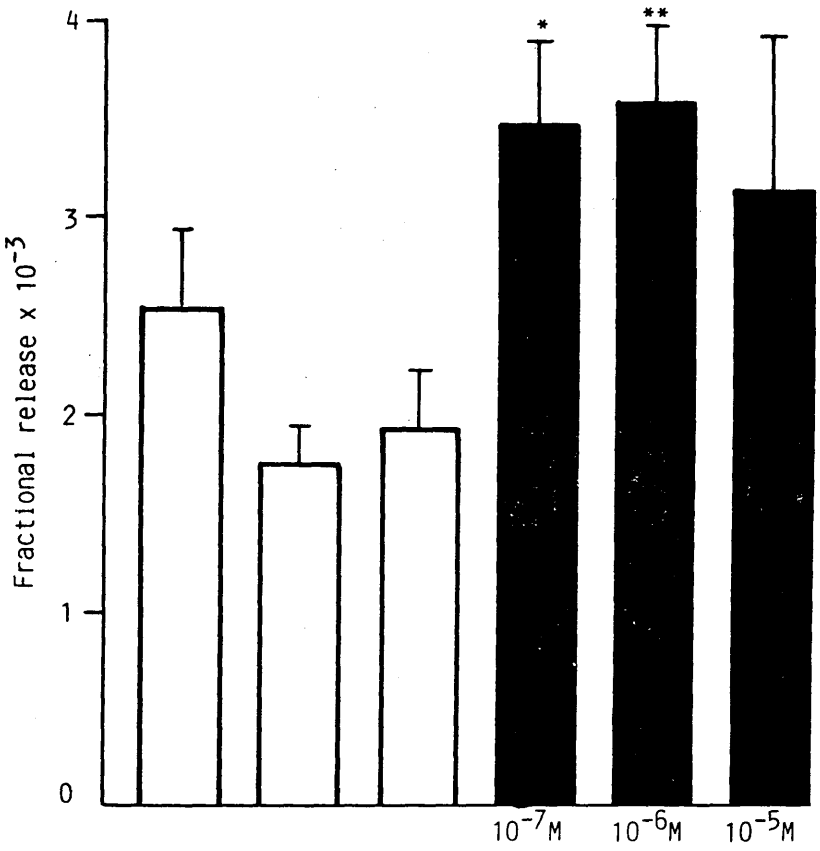

FIGURE 31. Effect of morphine (10^{-7} M-10^{-5} M) on field stimulation-evoked overflow of ^{3}H in vasa from T.O. mice. The open histograms shown successive control responses prior to drug addition. The succeeding 3 shaded histograms show the potentiating effect of morphine added in increasing concentrations (10^{-7} M-10^{-5} M) in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* 0.05>P>0.01, ** 0.01>P>0.001 for comparison with control prior to drug addition.

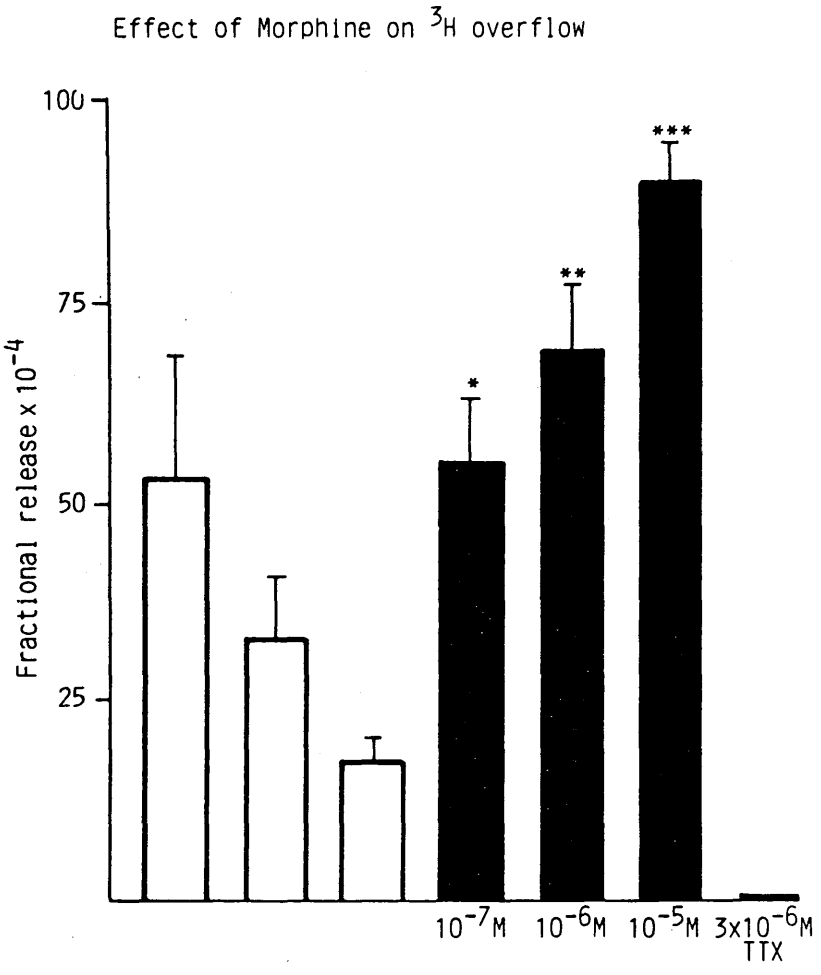

FIGURE 32. Effect of morphine (10^{-7} M-10^{-5} M) on field stimulation-evoked overflow of ^{3}H in vasa from Porton mice. The open histograms show successive control responses prior to drug addition. The succeeding 3 shaded histograms show the potentiating effect of morphine added in increasing concentrations (10^{-7} M-10^{-5} M) in the same experiments. Tetrodotoxin blocked field stimulation-evoked ^{3}H overflow. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001 for comparison with control prior to drug addition.

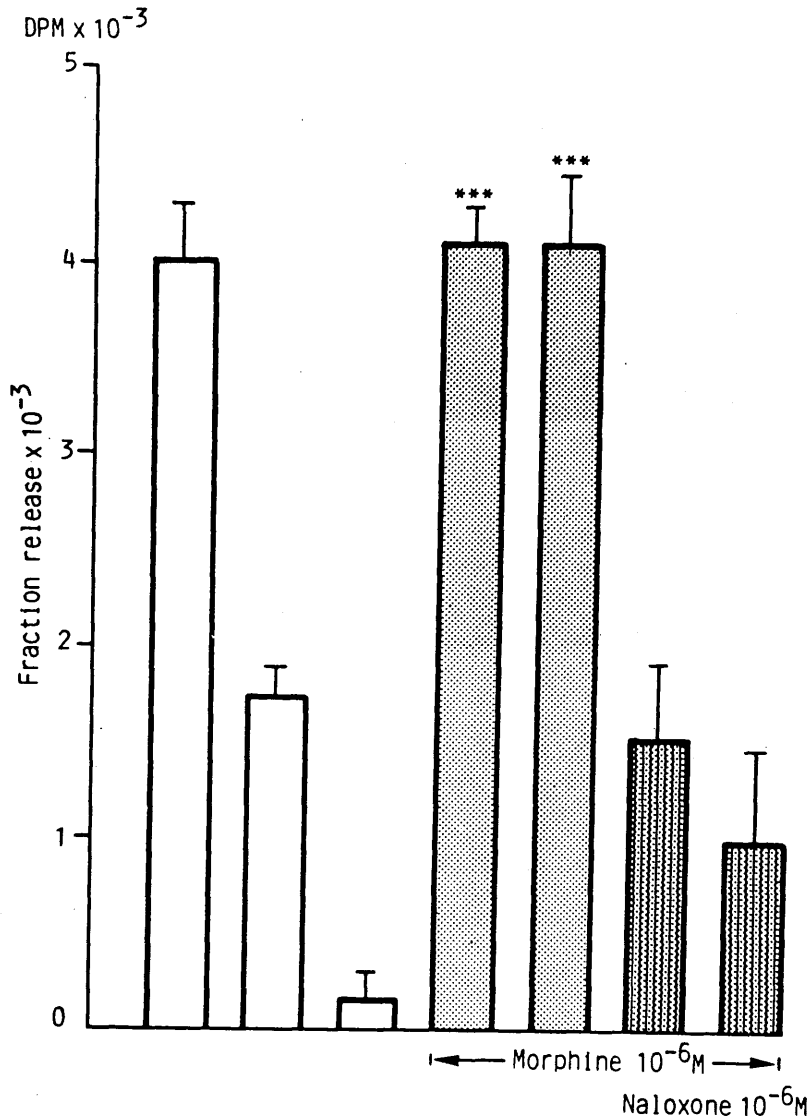

FIGURE 33. Effect of morphine (10^{-6} M) on field stimulation-evoked overflow of ^{3}H in the vas deferens. The open histograms show successive control responses prior to drug addition. The succeeding 2 stippled histograms show the potentiating effect of morphine (10^{-6} M) in the same experiments. Thereafter, the addition of naloxone (10^{-6} M) (stippled/striped histograms) antagonised the effect of morphine (10^{-6} M). Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

*** $P<0.001$ for comparison with control prior to drug addition.

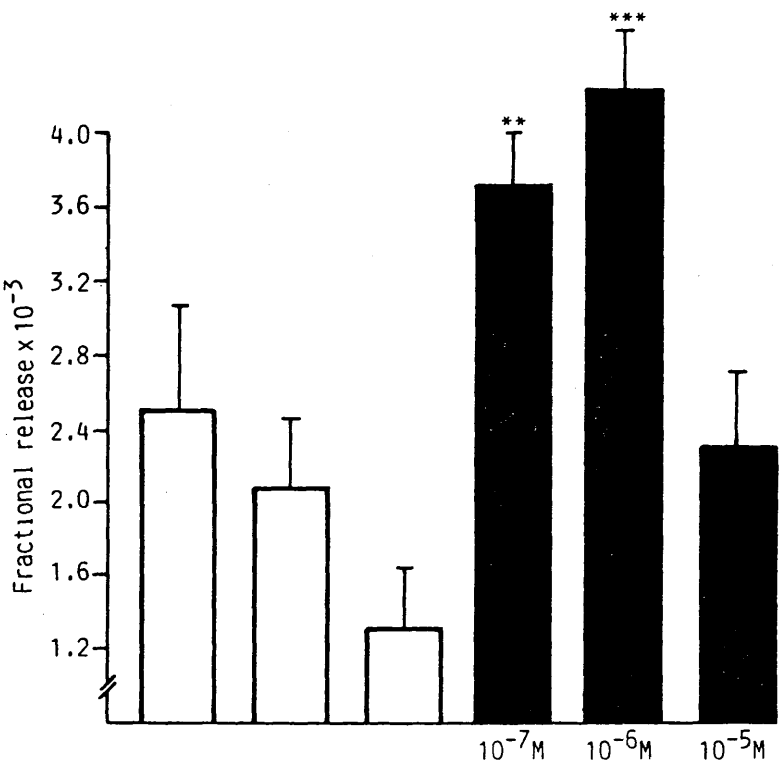

FIGURE 34. Effect of morphine (10^{-7} M-10^{-5} M) on field stimulation-induced overflow of ^{3}H in the vas deferens, in the presence of a combination of drugs (tranylcypromine 10^{-5} M, desmethylimipramine 10^{-5} M, 17-β-oestradiol 10^{-5} M) to block MAO and the neuronal and extraneuronal uptake of NA. The open histograms show successive control responses prior to drug addition. The succeeding 3 shaded histograms show the potentiating effect of morphine added in increasing concentrations (10^{-7} M-10^{-5} M) in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 8 observations.

** $0.01>P>0.001$, *** $P<0.001$ for comparison with control prior to drug addition.

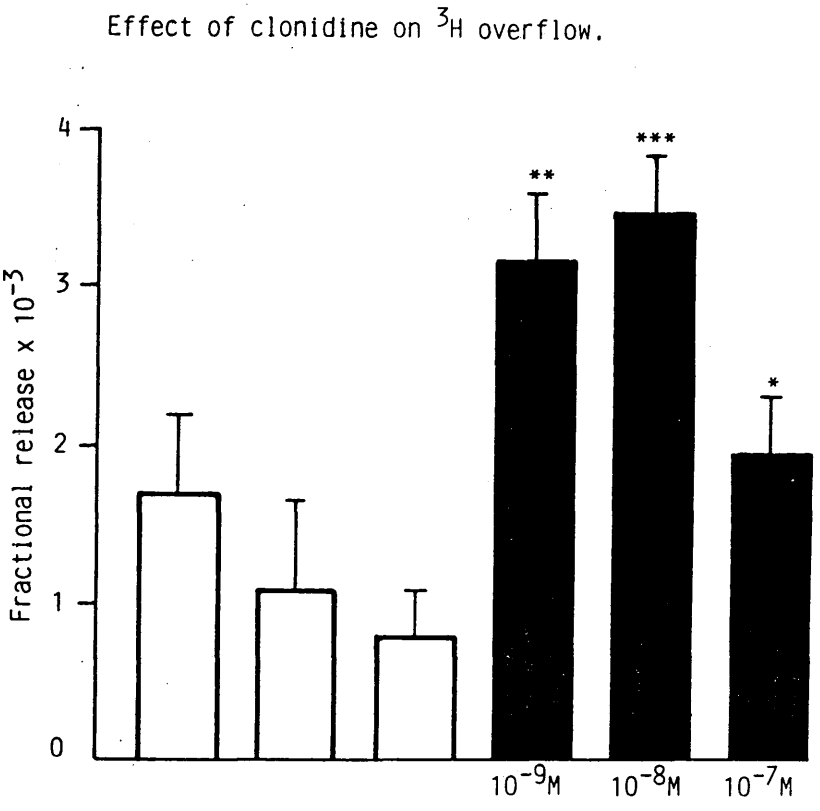

FIGURE 35. Effect of clonidine (10^{-9} M-10^{-7} M) on field stimulation-evoked overflow of ^{3}H in vasa from T.O.. mice. The open histograms show succesive control responses prior to drug addition. The succeeding 3 shaded histograms show the potentiating effect of clonidine added in increasing concentrations (10^{-9} M-10^{-7} M) in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001 for comparison with control prior to drug addition.

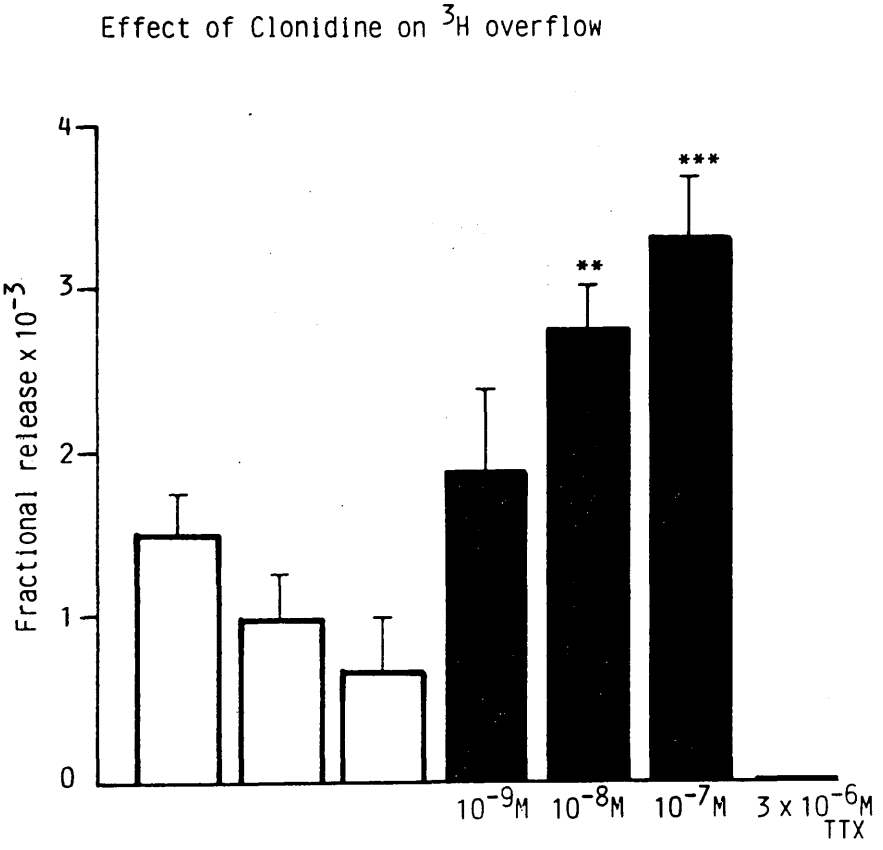

FIGURE 36. Effect of clonidine (10^{-9} M-10^{-7} M) on field stimulation-evoked overflow of ^{3}H in vasa from Porton mice. The open histograms show successive control responses prior to drug addition. The succeeding 3 shaded histograms show the potentiating effect of clonidine added in increasing concentrations (10^{-9} M-10^{-7} M) in the same experiments. Tetrodotoxin (TTX, 3 x 10^{-6} M) blocked field stimulation-evoked ^{3}H overflow. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

** 0.01>P>0.001, *** P<0.001 for comparison with control prior to drug addition.

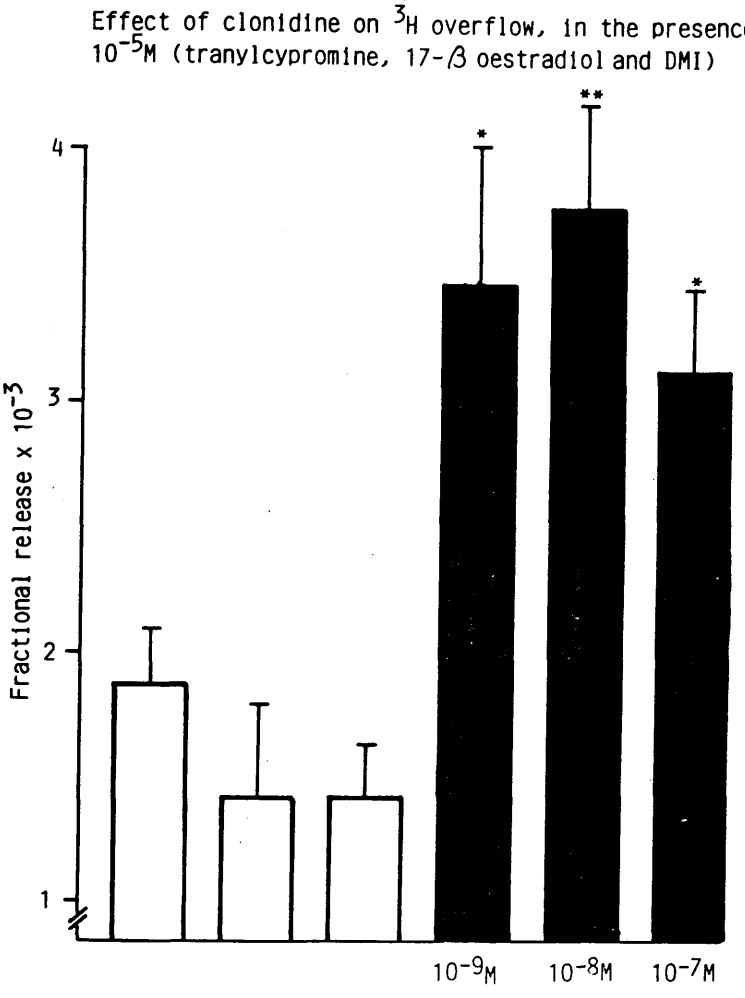

FIGURE 37. Effect of clonidine (10^{-9} M-10^{-7} M) on field stimulation-induced overflow of ^{3}H in the vas deferens, in the presence of a combination of drugs (tranylcypromine 10^{-5} M, desmethylimipramine 10^{-5} M, 17-β-oestradiol 10^{-5} M) to block monomaine oxidase (MAO) and the neuronal and extraneuronal uptake of NA. The open histograms show successive control responses prior to drug addition. The succeeding 3 shaded histograms show the potentiating effect of clonidine added in increasing concentrations (10^{-9} M-10^{-7} M) in the same experiments. Each column represents the mean (± S.E. mean) of 6 observations. * 0.05>P>0.01, ** 0.01>P>0.001 for comparison with control prior to drug addition.

Table II. Percentage of (^{3}H)-NA and its (^{3}H)-metabolites in Krebs solution bathing isolated, field stimulated vasa deferentia.

Time	(^{3}H)-NA (%)	(^{3}H)-NMN (%)	(^{3}H)-deaminated metabolites (%)
Before stimulation	34 ± 8 (5)	33 ± 6 (5)	30 ± 10 (5)
During stimulation	77 ± 8 (5)	16 ± 5 (5)	7 ± 3.5 (5)
During stimulation in the presence of morphine (10^{-5} M)	72 ± 4 (4)	18 ± 3 (4)	10 ± 3 (4)
During stimulation in the presence of clonidine (10^{-7} M)	70 ± 8.5 (4)	18 ± 5.5 (4)	12 ± 6.5 (4)

mean percentages ± S.E. mean (n)

deaminated metabolites : DOMA, DOPEG, MOPEG, VMA

Like clonidine and morphine, yohimbine (10^{-8} M, 10^{-7} M) enhanced the overflow of ^{3}H and the potentiation was similar in the absence and presence of tranylcypromine (10^{-5} M), desmethylimipramine (10^{-5} M) and 17,β-oestradiol (10^{-5} M) (Fig. 38).

TTX (3×10^{-6} M) reduced field stimulation-induced increases in radioactive overflow to basal levels (Figs. 32, 36).

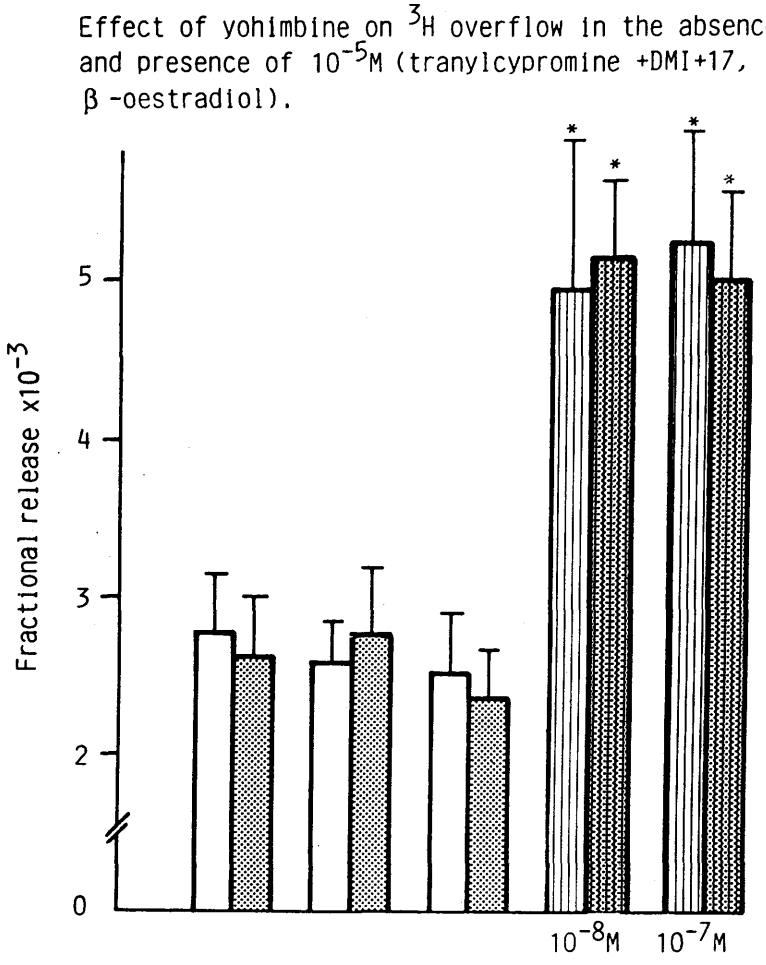

FIGURE 38. Effect of yohimbine (10^{-8} M, 10^{-7} M) in the absence and presence of tranylcypromine 10^{-5} M, + desmethylimipramine 10^{-5} M + 17,β-oestradiol 10^{-5} M, on field stimulation-evoked overflow of ^{3}H in the vas deferens. The 3 open histograms show successive control responses prior to drug addition. The successive 2 vertical striped histograms show the significant potentiating effect of yohimbine (10^{-8} M, 10^{-7} M) in the same experiments. The 3 stippled histograms show successive control responses prior to drug addition. The successive 2 vertical striped/stippled histograms show the significant potentiating effect of a combination of yohimbine (10^{-8} M, 10^{-7} M) + tranylcypromine 10^{-5} M, + desmethylimipramine 10^{-5} M + 17,β-oestradiol 10^{-5} M in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

* $0.05 > P > 0.01$ for comparison with control prior to drug addition.

Neither morphine (10^{-7} M-10^{-5} M) nor clonidine (10^{-9} M-10^{-7} M) produced any significant enhancement of ^{3}H overflow in T_4-pretreated mice (Figs. 39, 40).

In the presence of PBA (4×10^{-5} M), ^{3}H overflow was increased significantly in mice pretreated with T_4 (Fig. 41).

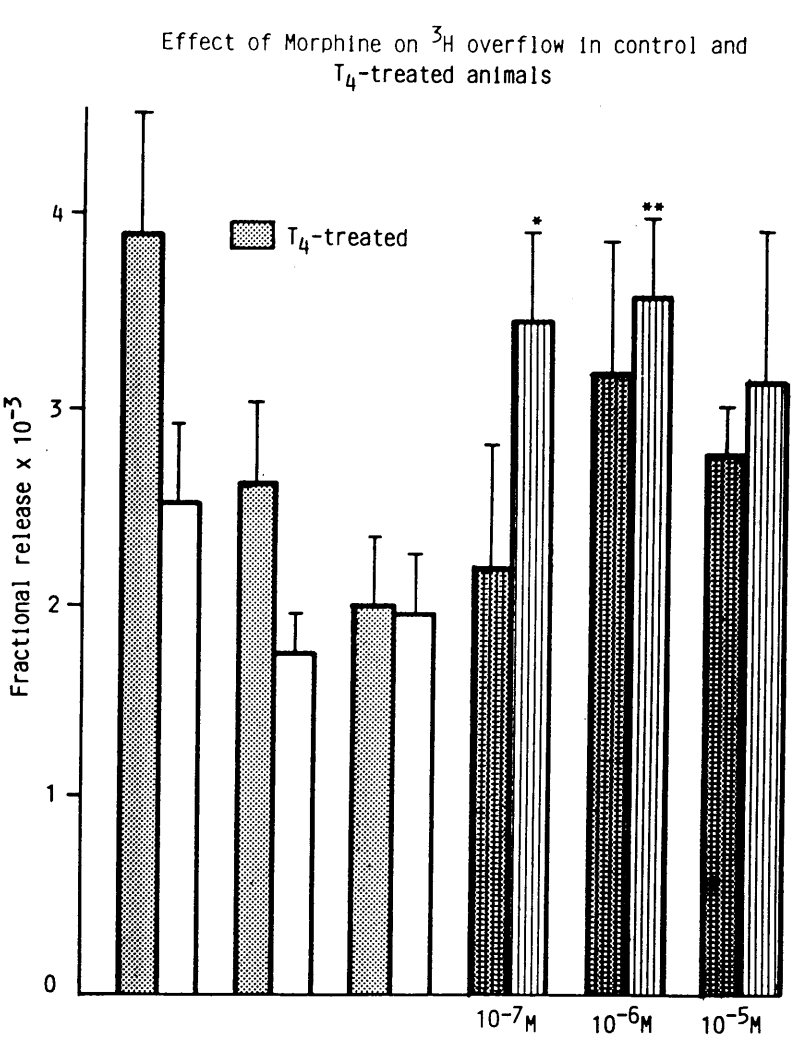

FIGURE 39. Effect of morphine (10^{-7} M-10^{-5} M) on field stimulation-evoked overflow of ^{3}H in vasa from control and T_4-pretreated animals. The 3 open histograms show successive control responses prior to drug addition. The 3 stippled histograms show successive responses in tissues from T_4-pretreated animal prior to drug addition. The succeeding vertical striped and vertical striped/stippled histograms show the effect of morphine added in increasing concentrations (10^{-7} M-10^{-5} M) in, respectively, tissues from control and T_4-pretreated animals, in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* 0.05>P>0.01, ** 0.01>P>0.001 for comparison with control prior to drug addition.

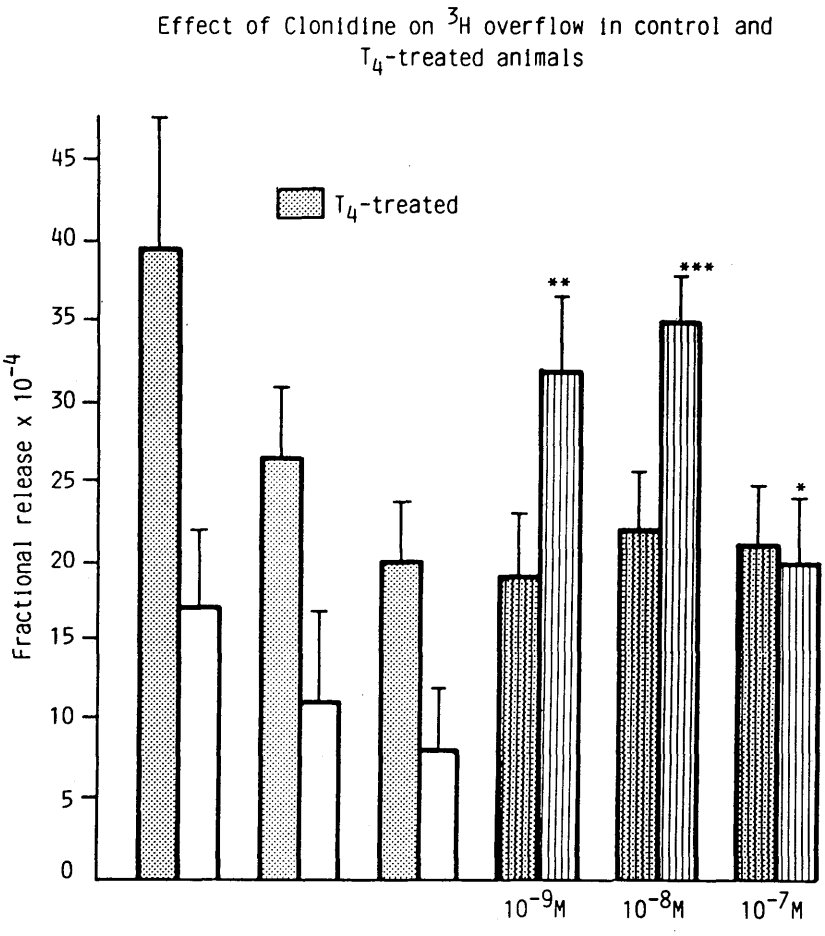

FIGURE 40. Effect of clonidine (10^{-9} M-10^{-7} M) on field stimulation-evoked overflow of ^3H in vasa from control and T_4-pretreated animals. The 3 open histograms show succesive control responses prior to drug addition. The 3 stippled histograms show successive responses in tissues from T_4-pretreated animals prior to drug addition. The succeeding vertical striped and vertical striped/stippled histograms show the effect of clonidine added in increasing concentrations (10^{-9} M-10^{-7} M) in, respectively, tissues from control and T_4-pretreated animals in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001 for comparison with control prior to drug addition.

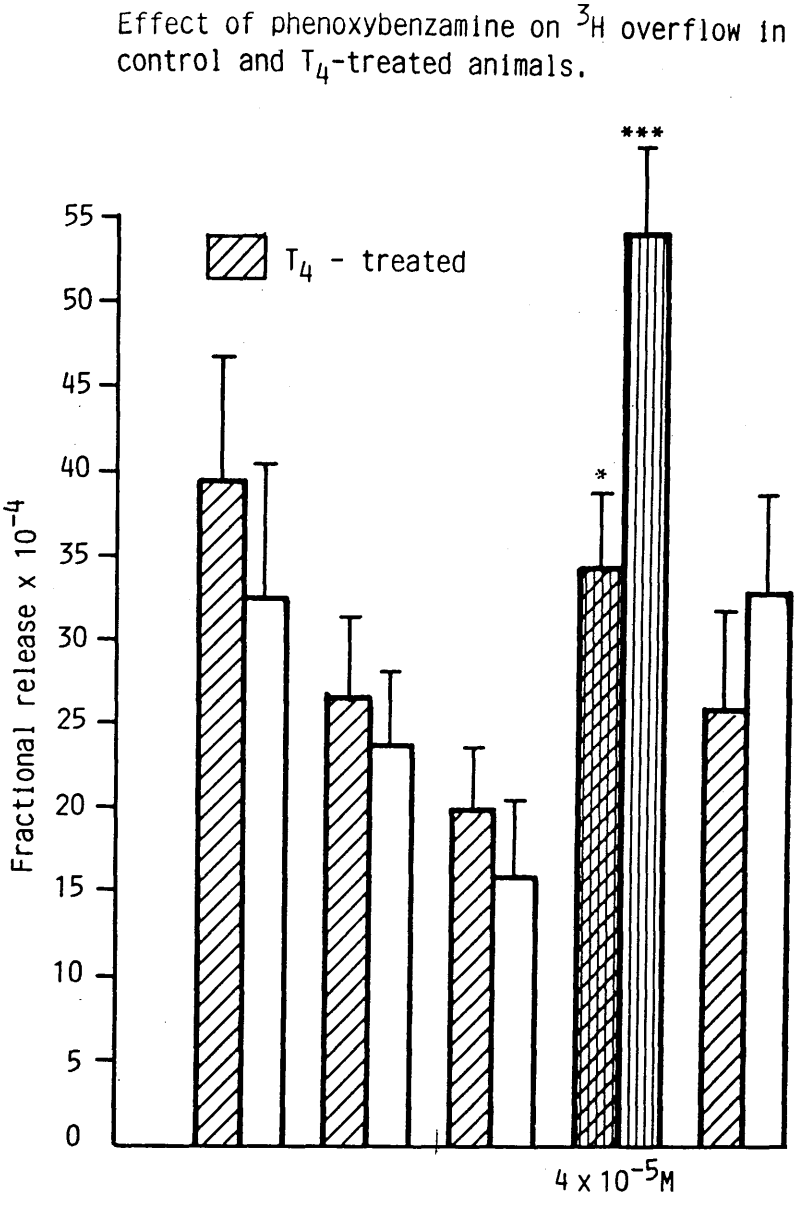

FIGURE 41. Effect of phenoxybenzamine (4 x 10^{-5} M) on field stimulation-evoked overflow of ^{3}H in vasa from control and T_4-pretreated animals. The 3 open histograms show successive control responses prior to drug addition. The diagonal striped histograms show successive responses in tissues from T_4-pretreated animals prior to drug addition. The succeeding vertical striped and vertical/diagonal striped histograms show the potentiating effect of phenoxybenzamine (4 x 10^{-5} M) in, respectively, tissues from control and T_4-pretreated animals. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

* 0.05>P>0.01, *** P<0.001 for comparison with control prior to drug addition.

PART VI

EFFECT OF VARYING THE STIMULATION PARAMETERS ON THE OVERFLOW OF ^{3}H

The effect of altering the stimulation parameters was investigated on the overflow of ^{3}H.

Successive stimulations at a constant frequency (20 Hz) for a fixed number of pulses (100) caused a reduction in the overflow of ^{3}H, which levelled off after the 4th stimulation (Fig. 42).

Keeping the number of pulses constant at 100, but increasing the frequency of stimulation throughout the experiment, commencing with stimulation at 1 Hz for a period of 100 sec. and ending with stimulation at 20 Hz for a period of 5 sec., caused a reduction in ^{3}H overflow throughout the course of the experiment (Fig. 43).
If the experiment was carried out in the reverse order, that is, if the frequency of stimulation was decreased throughout the experiment, commencing with stimulation at a frequency of 20 Hz for a period of 5 sec. and ending with stimulation at a frequency of 1 Hz for a period of 100 sec., the amount of radioactivity released was enhanced (Fig. 44).

A similar pattern was obtained if vasa were stimulated 3 times at each frequency and the mean ^{3}H overflow was calculated (Figs. 45, 46).

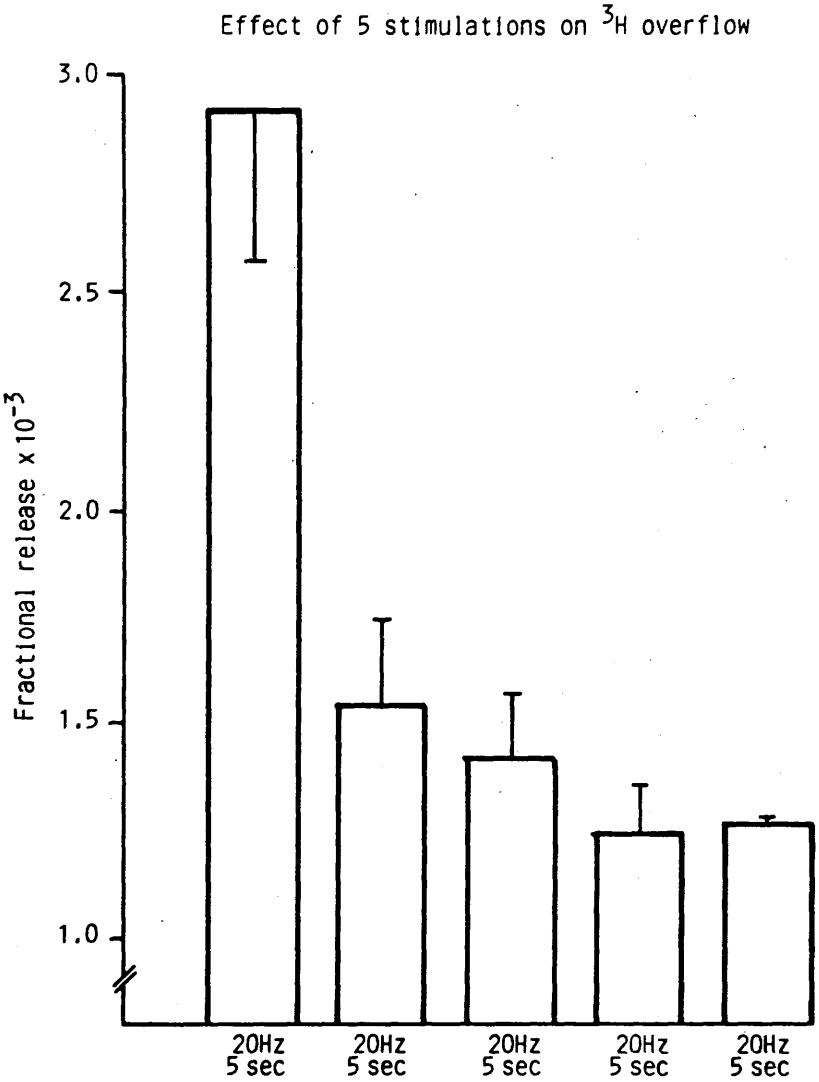

FIGURE 42. Effect of 5 consecutive stimulations at a frequency of 20 Hz for a period of 5 sec. Each column represents the mean (± S.E. mean) of 24 stimulations.

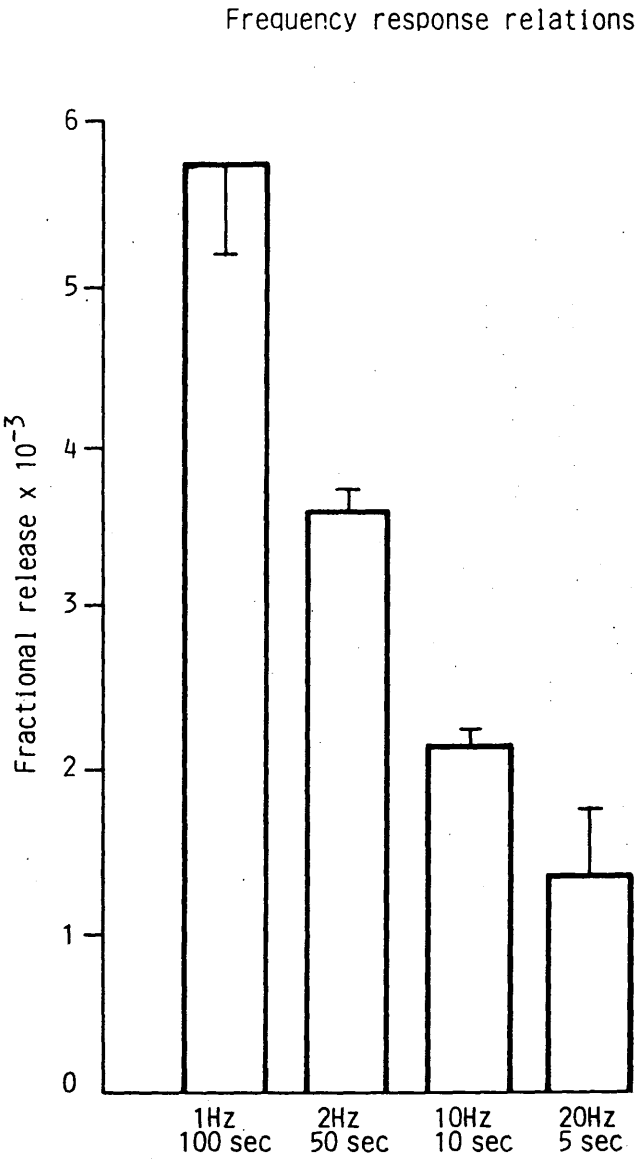

FIGURE 43. Frequency response relationship for a constant number of pulses (100) at increasing frequencies of stimulation from 1 Hz to 20 Hz. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

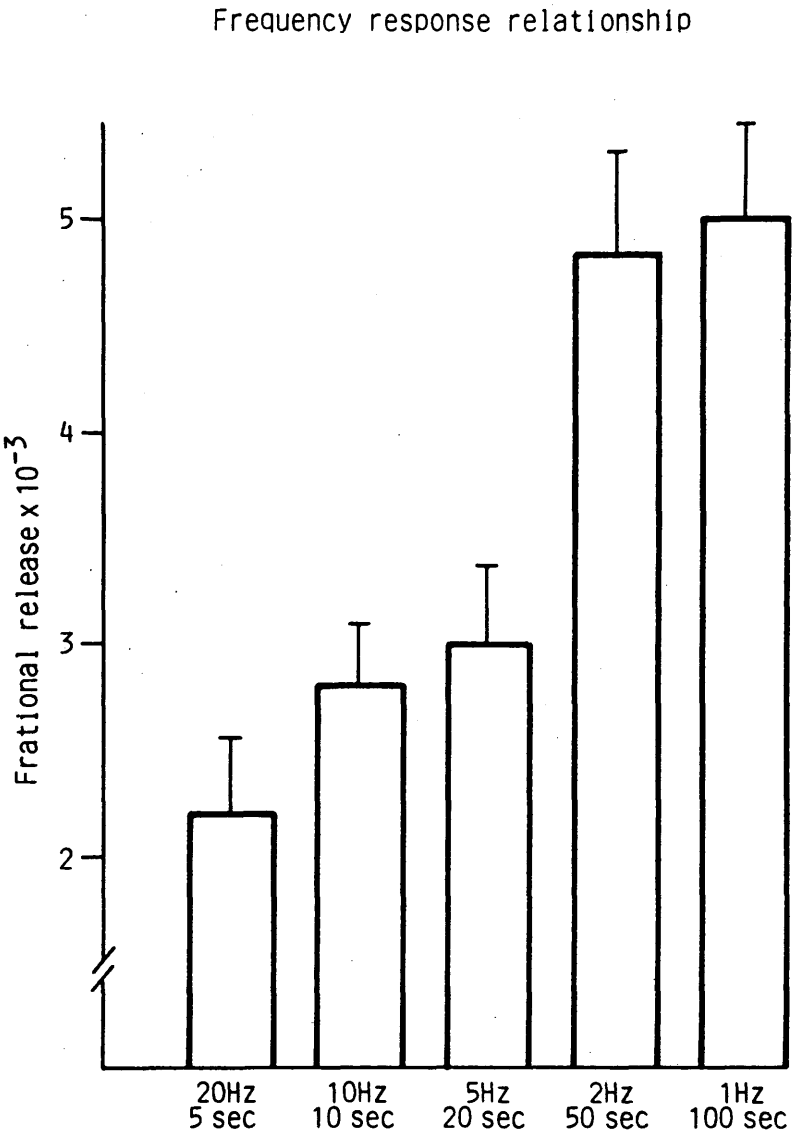

FIGURE 44. Frequency response relationship for a constant number of pulses (100) at decreasing frequencies of stimulation from 20 Hz to 1 Hz. Each column represents the mean (± S.E. mean) of 6 observations.

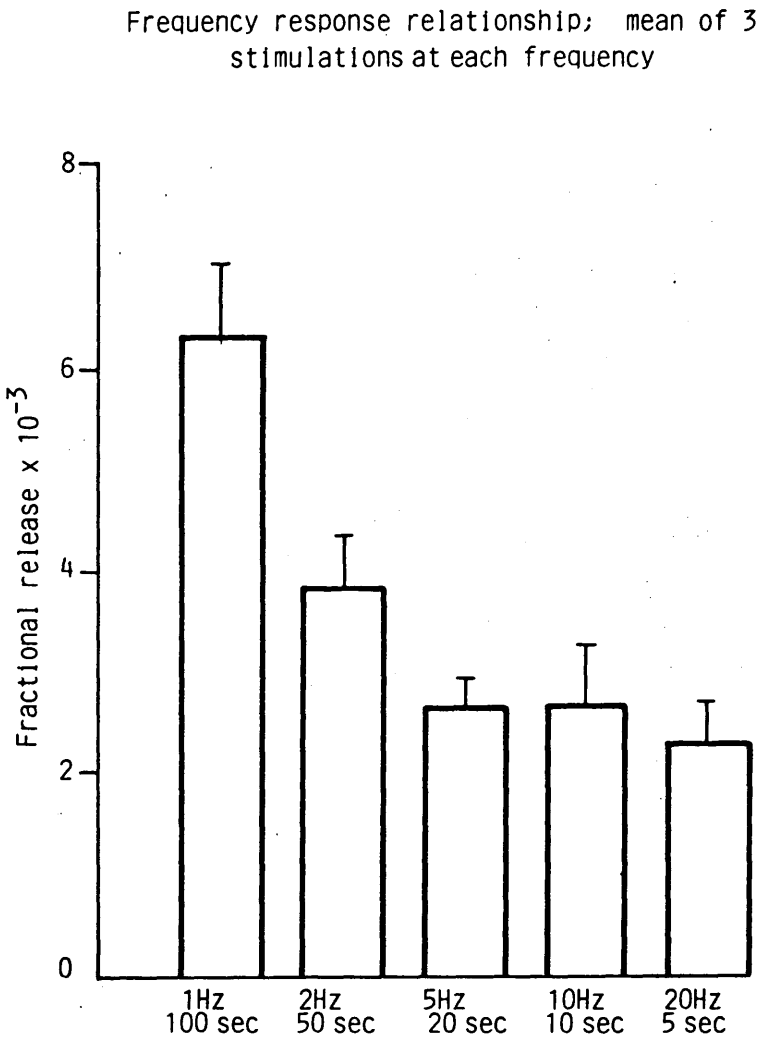

FIGURE 45. Frequency response relationship for a constant number of pulses (100) at increasing frequencies of stimulation from 1 Hz to 20 Hz. In each experiment tissues were stimulated 3 times and the mean response was calculated. Each column represents the mean ($\pm$ S.E. mean) of 4 separate experiments.

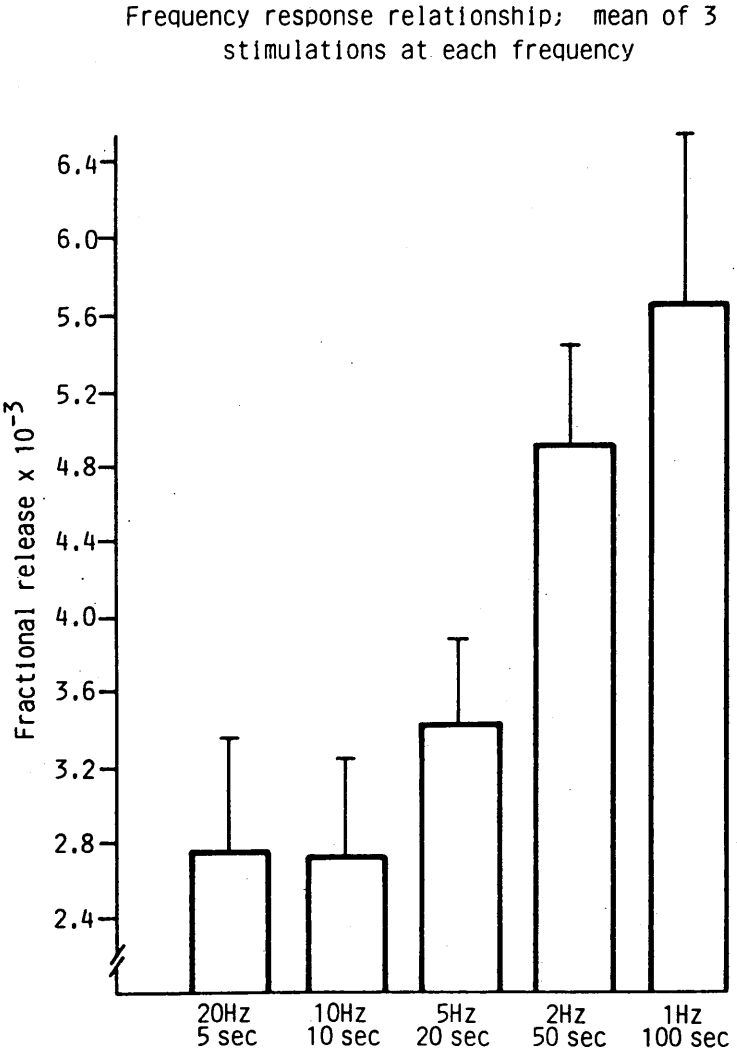

FIGURE 46. Frequency response relationship for a constant number of pulses (100) at decreasing frequencies of stimulation from 20 Hz to 1 Hz. In each experiment, tissues were stimulated 3 times and the mean response was calculated. Each column represents the mean (± S.E. mean) of 4 separate experiments.

Maintaining a constant time of 100 sec. but increasing the frequency of stimulation throughout the experiment from 1 to 20 Hz, resulted in an enhanced overflow of ^{3}H (Fig. 47). If the experiment commenced with stimulation at a frequency of 20 Hz and ended with stimulation at a frequency of 1 Hz, the overflow of ^{3}H was seen to diminish throughout the experiment (Fig. 48).

In the presence of PBA (4×10^{-5} M), the overflow of ^{3}H at decreasing frequencies of stimulation, starting at 20 Hz for a period of 5 sec. and ending with stimulation at 1 Hz for 100 sec., was enhanced (Fig. 49).

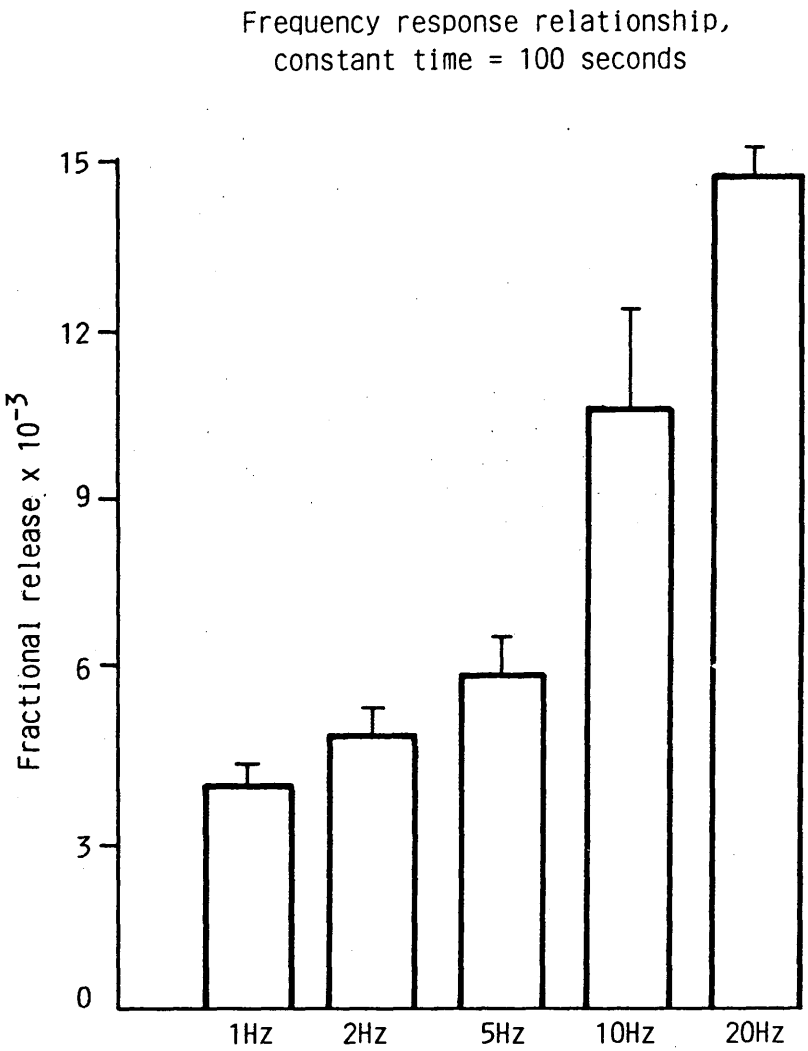

FIGURE 47. Frequency response relationship at increasing frequencies of stimulation from 1 Hz to 20 Hz for a constant time of 100 sec. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

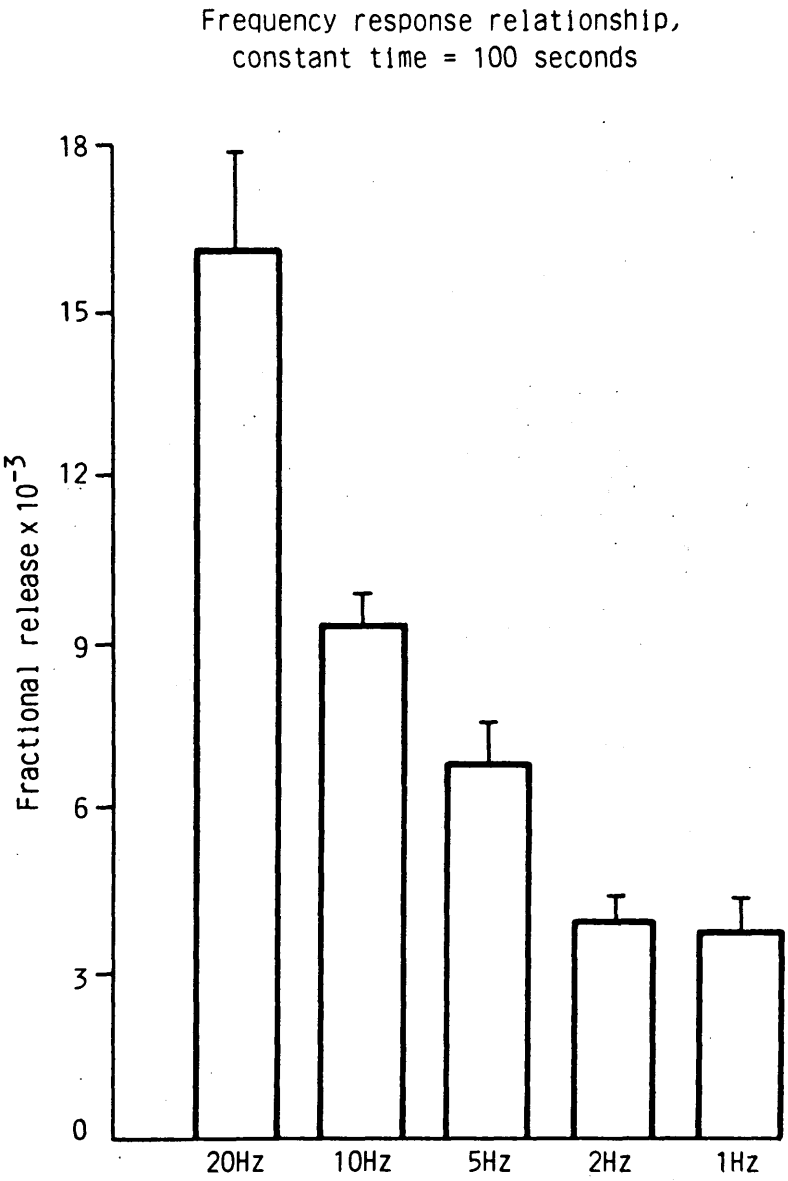

FIGURE 48. Frequency response relationship at decreasing frequencies of stimulation from 20 Hz to 1 HZ for a constant time of 100 sec. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

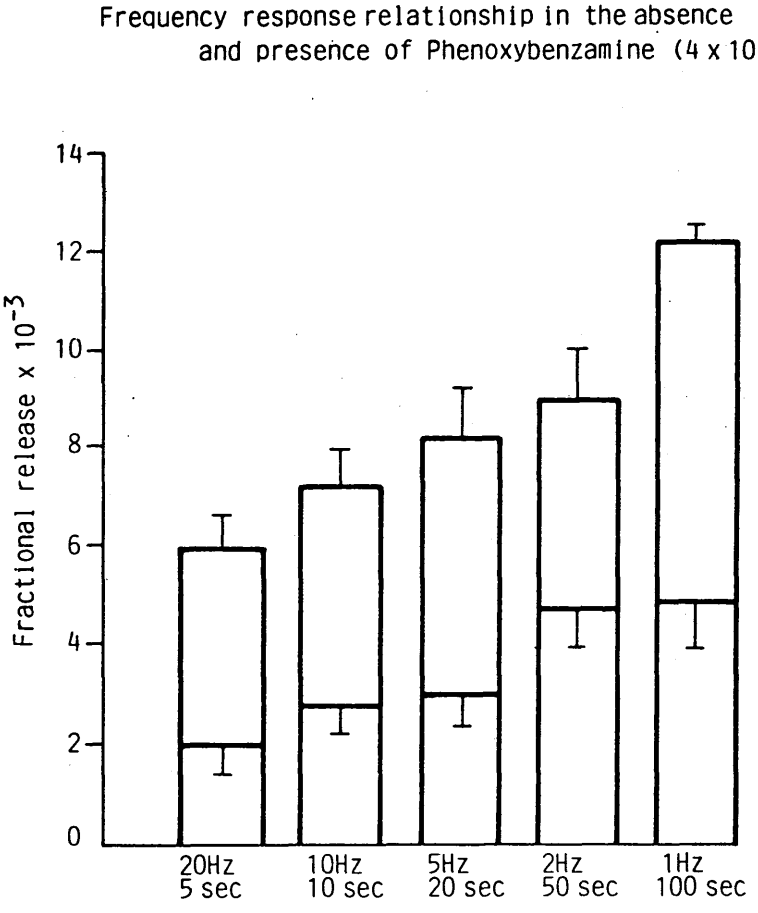

FIGURE 49. Frequency response relationship for a constant number of pulses (100) at decreasing frequencies of stimulation from 20 Hz to 1 Hz in the absence (lower section of column) and presence (whole column) of phenoxybenzamine (4 x 10^{-5} M). Each column represents the mean ($\pm$ S.E. mean) of 6 observations.

PART VII

EFFECT OF FIELD STIMULATION AND AGONISTS ON THE FORMATION OF [^{32}P]-PA

PI hydrolysis in the mouse vas deferens was monitored by the increased formation of [^{32}P]-PA in vasa preincubated with [^{32}P]-orthophosphate (Fig. 50).

NA (10^{-5} M) increased the formation of [^{32}P]-PA in vasa prelabelled with [^{32}P]-orthophosphate. This effect of NA was antagonised by prazosin (10^{-6} M) (Fig. 51). Neither ATP (10^{-3} M), nor α,β-MeATP (10^{-6} M), altered the synthesis of [^{32}P]-PA (Fig. 51).

Electrical field stimulation increased the formation of [^{32}P]-PA and this response was antagonised by prazosin (10^{-6} M) and by TTX (10^{-6}M) (Fig. 52).

Neither morphine (10^{-6}M) nor clonidine (10^{-8}M) had any effect on the basal levels of [^{32}P]-PA formation nor on the ability of NA to enhance [^{32}P]-PA formation (Fig. 53).

Morphine (10^{-5} M, 10^{-4} M) potentiated the field stimulation-induced increase in [^{32}P]-PA formation and this effect was antagonised by naloxone (10^{-6}M) (Fig. 54).

Clonidine (10^{-8} M, 10^{-7} M) potentiated the field stimulation-induced increase in [^{32}P]-PA formation in a dose-dependent manner (Fig. 55).

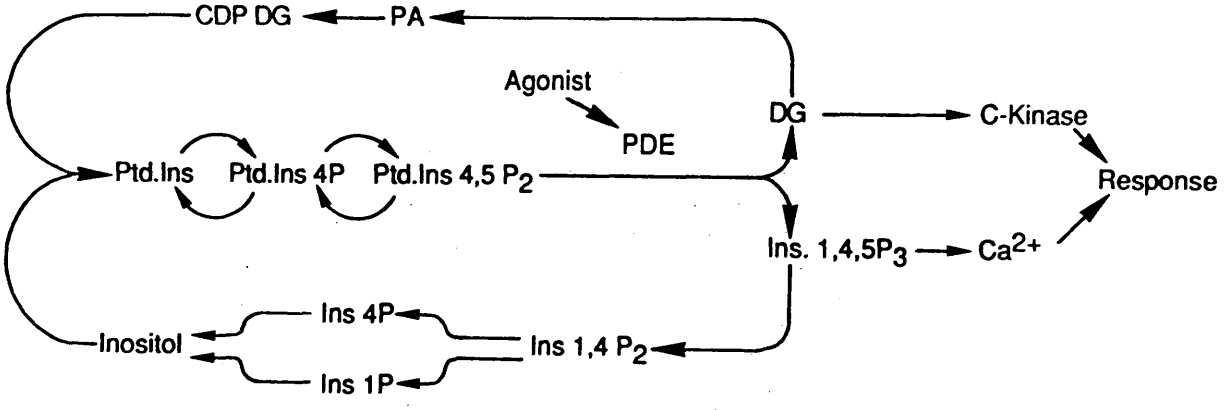

FIGURE 50. Outline of inositol lipid metabolism. The key event is the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Ptd.Ins 4,5 P_2) to give diacylglycerol (DG) and inositol 1,4,5-trisphosphate (Ins 1,4,5 P_3), both of which function as second messengers. A DG kinase converts DG to phosphatidic acid (PA) which is increased when the PI cycle is activated.

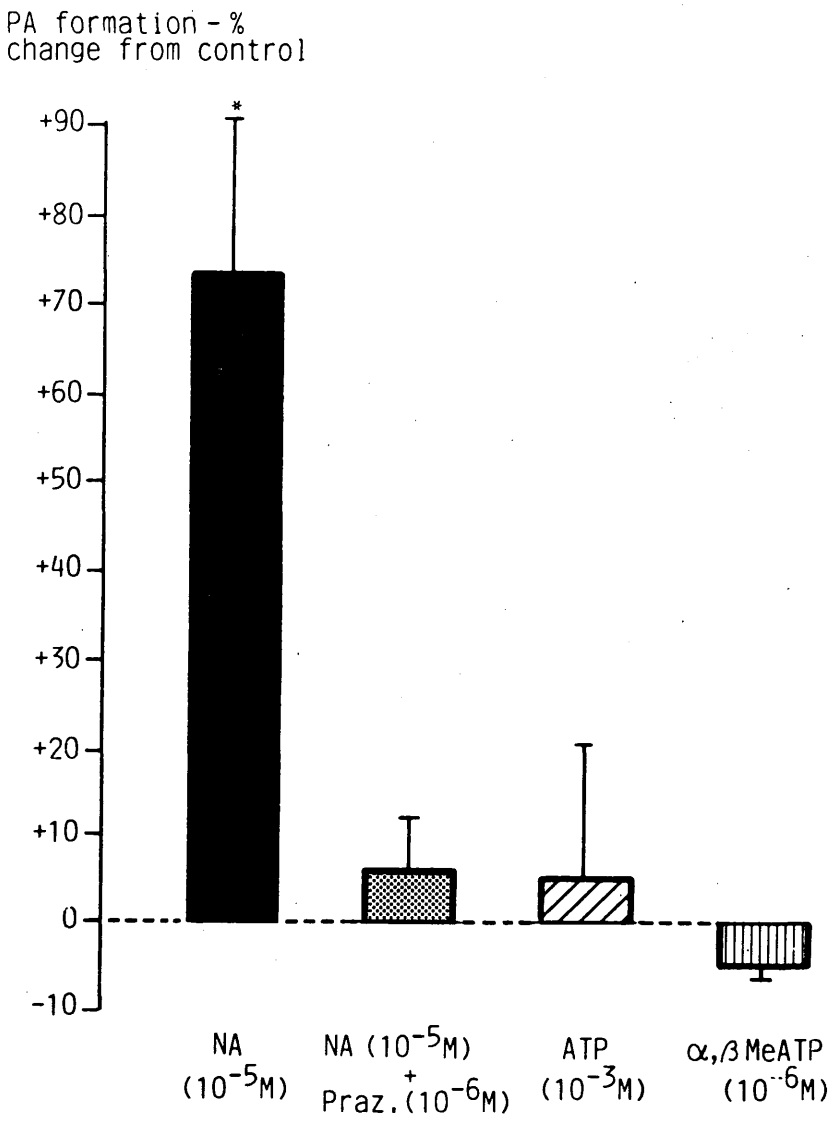

FIGURE 51. Effect of NA, NA + prazosin, ATP and α,β-MeATP on the formation of [^{32}P]-PA in the vas deferens. NA (10^{-5} M, shaded histogram) increased PA formation and this effect was antagonised by prazosin (10^{-6} M, stippled histogram). Neither ATP (10^{-3} M, diagonal striped histogram) nor α,β-MeATP (10^{-6} M, vertical striped histogram) had any effect on the formation of PA. Each column represents the mean (± S.E. mean) of 4 observations.

* 0.05>P>0.01.

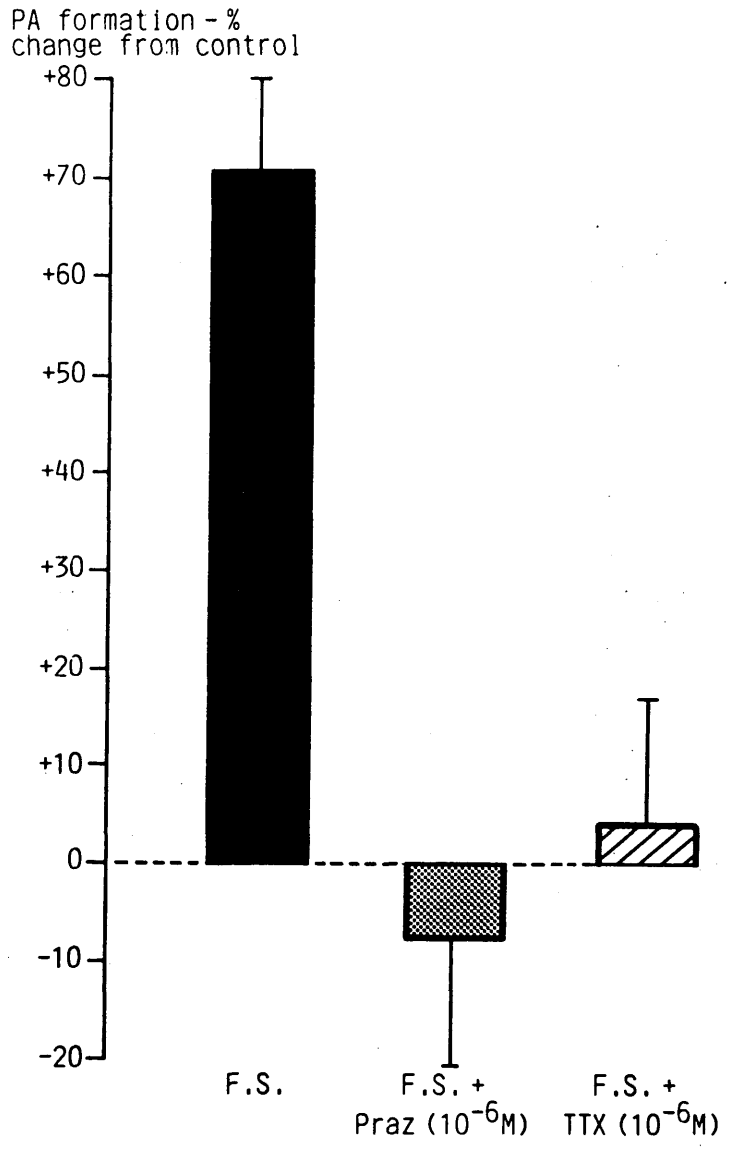

FIGURE 52. Effect of field stimulation, field stimulation + prazosin and field stimulation + TTX on the formation of [^{32}P]-PA in the vas deferens. Field stimulation (shaded histogram) increased PA formation and this effect was antagonised by prazosin (10^{-6} M, stippled histogram), and by TTX (10^{-6} M, diagonal striped histogram). The 1st column represents the mean ($\pm$ S.E. mean) of 12 observations, the 2nd and 3rd columns represent the mean ($\pm$ S.E. mean) of 4 observations.

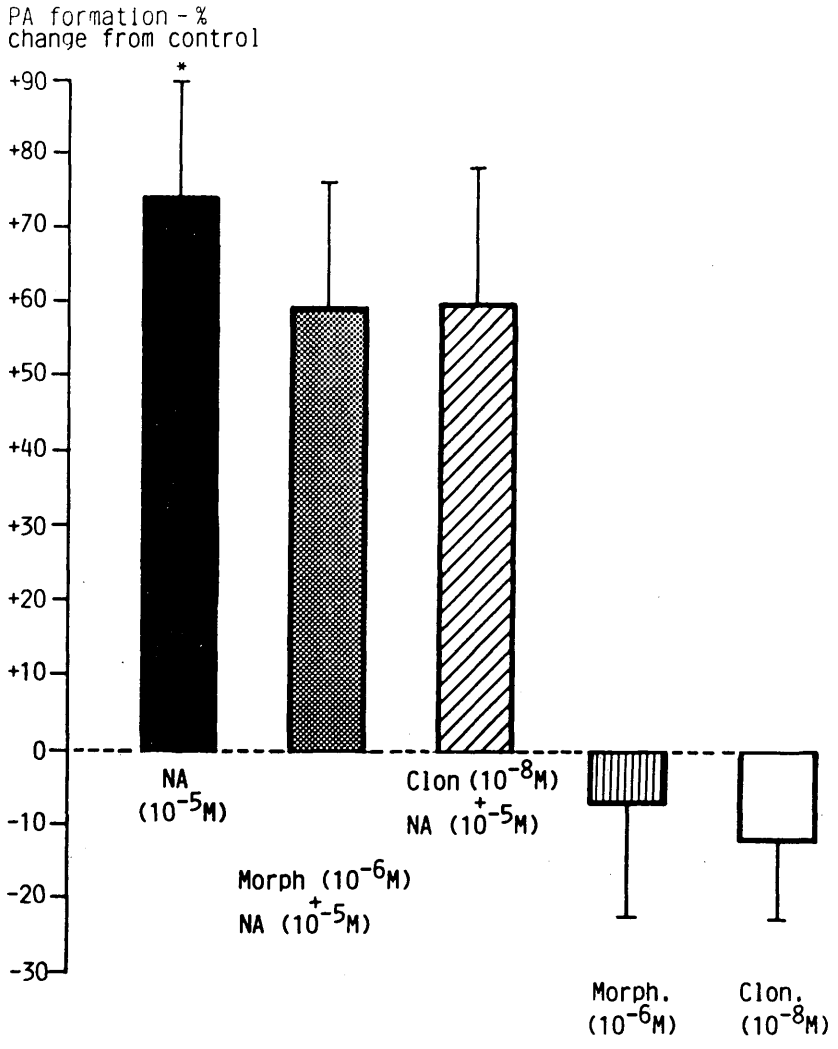

FIGURE 53. Effect of NA, morphine + NA, clonidine + NA, morphine and clonidine on the formation of [^{32}P]-PA in the vas deferens. NA (10^{-5} M, shaded histogram) increased PA formation and this enhancement was not potentiated any further by morphine (10^{-6} M, stippled histogram) or clonidine (10^{-6} M, diagonal striped histogram). Neither morphine (10^{-6} M, vertical striped histogram) nor clonidine (10^{-8} M, open histogram) had any effect directly on PA formation. Each column represents the mean (± S.E. mean) of 4 observations.

* $0.05>P>0.01$.

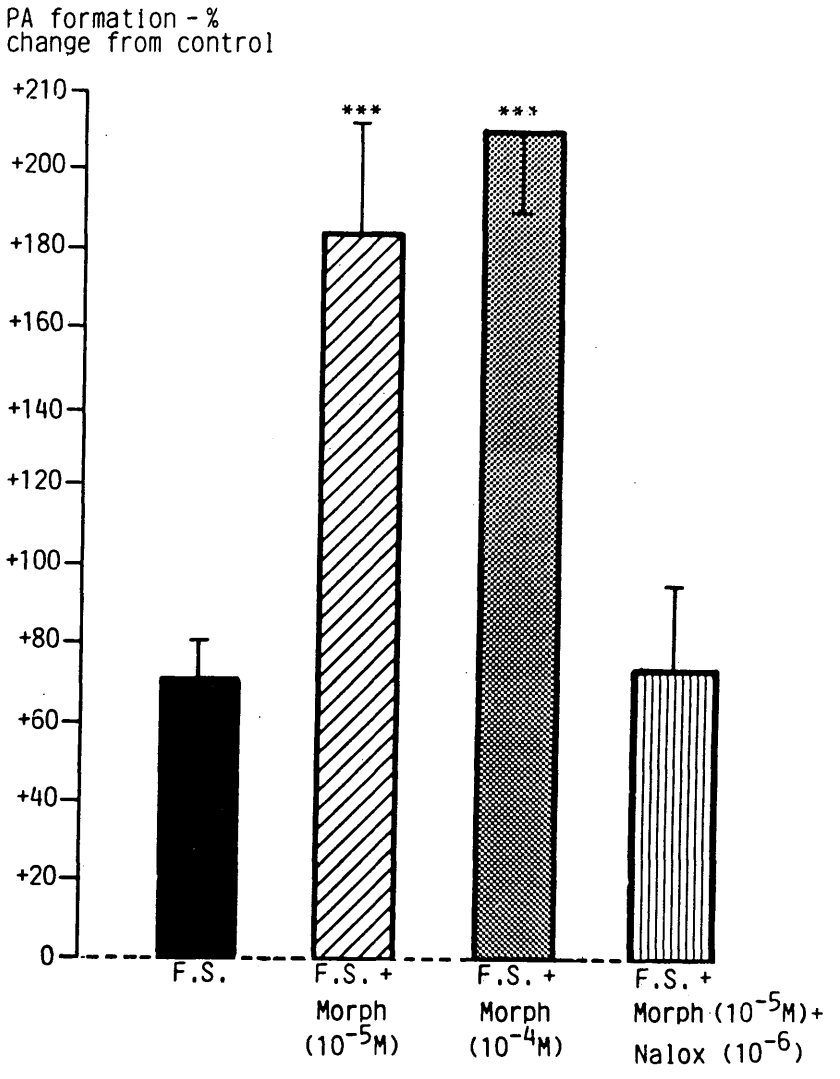

FIGURE 54. Effect of field stimulation, field stimulation + morphine and field stimulation + morphine + naloxone on the formation of [^{32}P]-PA in the vas deferens. Field stimulation (shaded histogram) increased PA formation. This increase was potentiated by morphine (10^{-5} M, diagonal striped histogram; 10^{-4} M, stippled histogram). In the presence of naloxone (10^{-6} M, vertical striped histogram) the addition of morphine did not result in any enhancement of the field stimulation-induced increase in PA formation. The 1st column represents the mean ($\pm$ S.E. mean) of 12 observations, the remaining 3 columns represent the mean ($\pm$ S.E. mean) of 4 observations.
*** $P<0.001$.

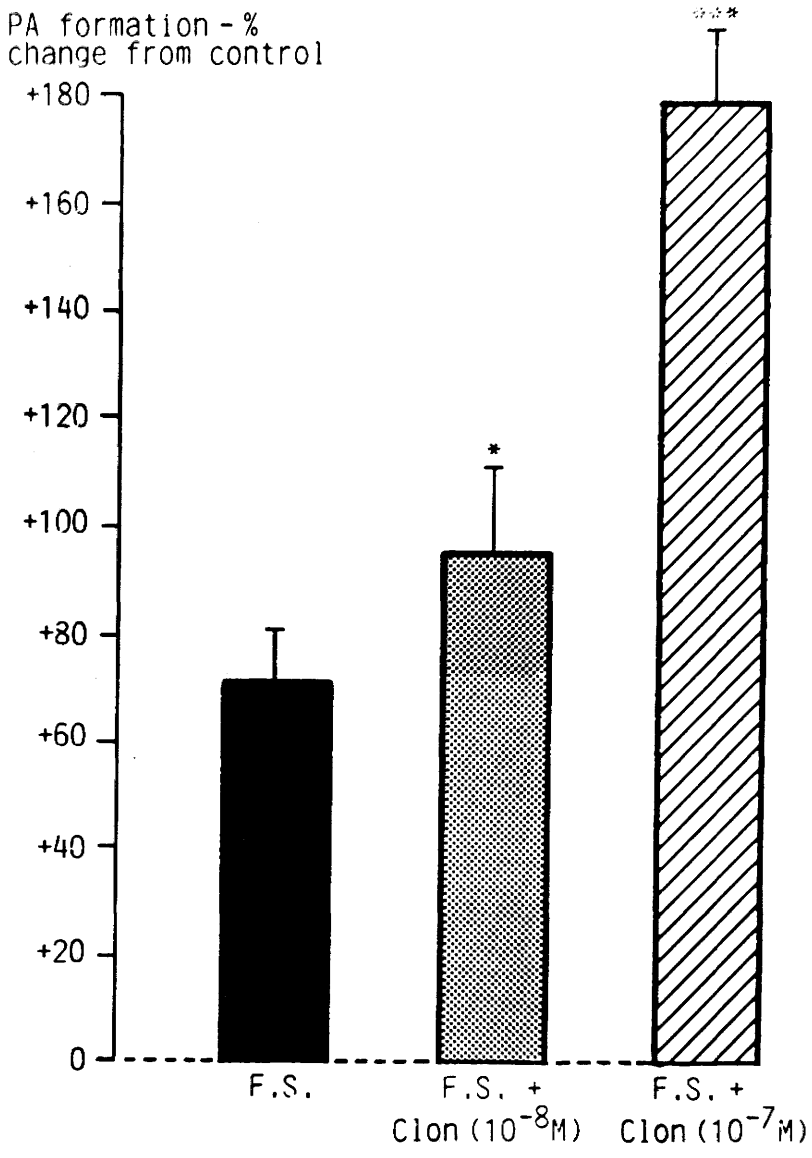

FIGURE 55. Effect of field stimulation and field stimulation + clonidine on the formation of [^{32}]-PA in the vas deferens. Field stimulation (shaded histogram) increased PA formation. This increase was potentiated in a dose-related manner by clonidine (10^{-8} M, stippled histogram; 10^{-7} M, diagonal striped histogram). The 1st column represents the mean (± S.E. mean) of 12 observations, the 2nd and 3rd columns represent the mean (± S.E. mean) of 4 observations.
* $0.05>P>0.01$, *** $P<0.001$.

DISCUSSION

This study has answered some of the questions posed in the introduction, but has raised others that may be more difficult to answer. It is clear that field stimulation-induced responses of the mouse vas deferens are biphasic, confirming observations obtained in vasa from other species (Ambache et al., 1972; Blakeley et al., 1981; Fedan et al., 1981; Brown et al., 1983; Sneddon et al., 1984). Evidence obtained in this and other studies indicates that, whereas the second component of the biphasic response to field stimulation is adrenergic, the initial component is neither adrenergic nor cholinergic (NANC). Despite the general acceptance of the existence of an NANC transmitter that mediates the initial component of the biphasic response in vasa from several species, there remains a possibility that this response could arise from neurally-released NA acting on adrenoceptors that differ from the more familiar adrenoceptors.

Results from experiments carried out on vascular smooth muscle have led to the suggestion that two types of excitatory receptor exist for NA (Hirst & Neild, 1980; 1984). When activated, one type produced a response that was not associated with any change in the membrane potential and could be abolished by the α_1-adrenoceptor antagonist prazosin. The other type of receptor was associated with a depolarisation and was unaffected by prazosin. It was proposed that this response resulted from the interaction of NA with an adrenoceptor which differed from the classical α-adrenoceptor and it was named the γ receptor (Hirst & Neild, 1980; 1984). These observations obtained from arteriolar smooth muscle were thought to provide a possible explanation for the paradoxical failure of α-adrenoceptor antagonists to block transmission in the vas deferens. One experimental obstacle which prevents acceptance of NA as the sole transmitter in this tissue is the survival of the initial component after reserpine pretreatment

(Ambache & Zar, 1971; Swedin, 1971; Booth et al., 1978; Sneddon & Burnstock, 1984; Stjärne & Åstrand, 1985; Kirkpatrick & Burnstock, 1987). This study confirmed that reserpine pretreatment inhibited the second component but did not affect the initial component of the motor response to field stimulation in isolated vasa.

Other workers have presented evidence which supports an alternative hypothesis, namely that ATP, released as a co-transmitter with NA from sympathetic nerves in the vas deferens, mediates the initial contractile response (French & Scott, 1983; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984). Despite the extensive experimental results in support of co-release of ATP and NA, this explanation of transmission in the vas deferens has not been accepted without some reservations (Allcorn et al., 1986). However, the bulk of evidence favours the motor response being explained by the co-transmission hypothesis.

One question that has remained in dispute is whether or not the co-transmitters are contained in a single nerve or in separate nerves. Proponents of the separate nerve hypothesis claim that a residual response surviving 6-OHDA pretreatment is due to separate NANC nerves (Booth et al., 1978). However, the effects of drugs which are known to preferentially inhibit the initial component of the motor response were not investigated in these studies, nor were the effects of tetrodotoxin (TTX) examined in order to confirm that the residual contraction was neurally-mediated.

This study re-examined the effect of pretreating animals with 6-OHDA to determine whether or not any response survived this treatment and, if such a response remained, to investigate if it was abolished by TTX and affected by drugs which preferentially inhibited the initial component of the biphasic response of control vasa to field

stimulation. In addition to examining the effects of drugs on responses of vasa from 6-OHDA pretreated animals *in vitro*, a novel preparation was introduced, where the inherent problem associated with field stimulation, namely the possibility of direct stimulation of the smooth muscle, would be absent. The effects of stimulating the spinal motor outflows to the vas deferens of anaesthetised control and 6-OHDA pretreated rats were examined. By this means, stimulation of the nerves at their point of origin in the spinal canal permitted the effects of 6-OHDA pretreatment to be examined in a situation where direct stimulation of the smooth muscle could not occur. In addition, the responses of vasa to *in situ* field stimulation were investigated in these animals. Thus, it was possible to examine the effects of drugs on responses resulting from stimulation of the spinal motor nerves to the vas deferens and responses to *in situ* field stimulation of vasa in the same preparation.

Results obtained in this study confirmed that in isolated tissues a residual response survived pretreatment with 6-OHDA. However, this response was unaffected by clonidine or opiates, which preferentially inhibited the initial component of the biphasic response to field stimulation in control vasa. TTX, which normally abolished all responses to field stimulation in vasa from control animals, did not affect the residual response that survived pretreatment with 6-OHDA. This observation indicated that the residual response was not neurally-mediated but possibly resulted from direct stimulation of the smooth muscle, which was found to be supersensitive to NA. Thus, 6-OHDA pretreatment may not only have made the vas supersensitive to NA, but may also have lowered the threshold for direct stimulation of the smooth muscle. To distinguish a situation where the smooth muscle could be directly stimulated from one where this would not be possible,

the effects of clonidine, opiates and TTX were examined in anaesthetised animals. In controls, stimulation of the vasa, both by *in situ* field stimulation and *via* stimulation of the spinal motor nerves, produced biphasic responses, similar to those resulting from field stimulation *in vitro*. The initial component of the biphasic responses was inhibited by opiates and clonidine and the entire response was abolished by TTX. In 6-OHDA-pretreated animals, a residual response to *in situ* field stimulation persisted and, as in the isolated vas, TTX did not abolish this response. Thus, this residual contraction may have resulted from direct stimulation of the smooth muscle during *in situ* field stimulation, where the electrodes were closely applied to the vas. It could be argued that the residual response to field stimulation in 6-OHDA-pretreated animals was not due to direct stimulation of the smooth muscle but to a resistance to TTX and there is evidence that high intensity electrical stimulation of the vas deferens releases NA even in the presence of TTX (Illes *et al.*, 1984). Such an explanation seems unlikely, since it would require that responses to field stimulation in vasa from 6-OHDA-pretreated animals were TTX resistant but those from controls were not. When the spinal motor nerves to the vas were stimulated in these 6-OHDA-pretreated animals, a small contraction was obtained. However, this residual response, unlike the responses occurring as a result of field stimulation both *in vitro* and *in situ*, was abolished by TTX, suggesting that it was perhaps mediated by a few adrenergic fibres that survived pretreatment with 6-OHDA.

This study demonstrated that the field stimulation-induced contractile response surviving 6-OHDA-pretreatment resulted from direct stimulation of the smooth muscle which was supersensitive. The results are, therefore, in agreement with Fedan *et al.* (1981) and

French & Scott (1983), in favour of the existence of the transmitters in the vas deferens being in a single nerve, which is destroyed by 6-OHDA, resulting in the removal of any nerve-mediated response.

A biphasic mechanical response is indicative of more than one transmitter being released by field stimulation. However, this study confirmed that the two components of the biphasic response to field stimulation in the mouse vas deferens could be preferentially affected by drugs, providing further evidence for the release of more than one transmitter. α-Adrenoceptor antagonists such as phentolamine inhibited the second component of the motor response and both morphine and clonidine inhibited the initial component. However, surprisingly, at a time when morphine and clonidine were inhibiting the first component of the biphasic motor response, the second component was enhanced. This observation suggested that, not only was more than one transmitter released on field stimulation, but that the transmitters may be involved in a presynaptic feedback regulation. The results from the experiments designed to ascertain whether or not the transmitters in the vas are contained in a single nerve or in separate nerves, suggested that they are contained within a single nerve. Obviously this would require a more complex explanation for a possible cross-regulation than had the transmitters been released from separate nerves. However, it is unsatisfactory to examine the effects of drugs such as morphine and clonidine, which are generally thought to act presynaptically to inhibit the release of transmitter (Hughes et al., 1975a; Gillan et al., 1979), by monitoring postsynaptic events. Although the mouse vas deferens has been used to study adrenergic mechanisms by assaying the effects of compounds which act prejunctionally to inhibit transmitter release, a more direct method of examining the sensitivity of presynaptic receptors would be to measure

the output of transmitter. This eliminates the involvement of postsynaptic events in the analysis of the results.

Since NA is widely believed to be one of the transmitters in the vas deferens, part of this study investigated the effects of a variety of drugs on the release of NA from vasa preincubated with (^{3}H)-NA. Field stimulation of the vas increased the amounts of radioactivity released into the bathing solution, and these increases coincided with the field stimulation-evoked contractions. TTX abolished (^{3}H)-NA overflow, indicating that it was neurally mediated.

The inactivation of neurally-released NA is carried out by neuronal uptake, which reduces the concentration of released transmitter in the synaptic cleft (Langer, 1977). Thus, neuronal uptake of NA can regulate the amount of transmitter which is available to activate the presynaptic α-adrenoceptors (Langer, 1974). This study has shown that a combination of tranylcypromine, desmethylimipramine and 17,β-oestradiol which block, respectively, monoamine oxidase (MAO) and the neuronal and extraneuronal uptake of NA, did not produce a significant increase in the overflow of (^{3}H)-NA. This supports the view that blockade of neuronal uptake enhances the amount of transmitter available to activate presynaptic α-adrenoceptors, leading to enhanced feedback inhibition (Langer, 1977). However, combination of the drugs which block MAO, Uptake$_1$ and Uptake$_2$ with the α-adrenoceptor antagonist yohimbine, increased (^{3}H)-NA overflow to the same extent as yohimbine alone. If tranylcypromine, desmethylimipramine and 17,β-oestradiol enhanced the amount of NA in the synaptic cleft leading to a greater feedback inhibition *via* α-adrenoceptors, the combination of these drugs with yohimbine would be expected to have resulted in a greater overflow of (^{3}H)-NA when compared to the overflow obtained in the presence of yohimbine alone. This result was not obtained, thus the explanation

of the increased transmitter in the synaptic cleft arising from blockade of uptake, leading to enhanced activation of presynaptic α-adrenceptors, is inadequate for the mouse vas deferens. Considering the dense adrenergic innervation of this tissue, these paradoxical results are in agreement with other workers, who found that inhibition of the uptake mechanisms with highly-selective drugs produced little enhancement of NA overflow (Farnebo & Malmfors, 1971).

Phenoxybenzamine (PBA), which, in addition to blocking MAO and the neuronal and extraneuronal uptake of NA, also blocks presynaptic -adrenoceptors (Gillespie, 1980), significantly potentiated (^{3}H)-NA overflow, confirming that these receptors are involved in the regulation of NA release through a negative feedback mechanism (Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Langer et al., 1971; Starke, 1971).

The surprising observation that morphine and clonidine inhibited the initial NANC component of the motor response to field stimulation but potentiated the second component, was corroborated in the overflow experiments, where both of these drugs were found to increase the overflow of (^{3}H)-NA. It seems likely that the ability of morphine and clonidine to potentiate (^{3}H)-NA overflow was receptor-mediated, since it occurred at low concentrations and was dose-related. The ability of morphine to increase (^{3}H)-NA overflow was antagonised by naloxone. It appears that the ability of these drugs to increase (^{3}H)-NA overflow is due to a presynaptic action on release rather than on the removal mechanisms for NA, since the ability of both clonidine and morphine to enhance (^{3}H)-NA overflow persisted in the presence of tranylcypromine, desmethylimipramine and 17,β-oestradiol.

Although many studies have shown that the two phases of the field stimulation-induced motor response of the vas can be differentially

affected by drugs (e.g. Sneddon et al., 1984), the observation that morphine and clonidine, which are generally believed to act presynaptically to inhibit the release of transmitter (Hughes et al., 1975a; Gillan et al., 1979), actually enhanced the overflow of transmitter in this study was surprising.

Several studies have used inhibition of field stimulation-induced motor responses of the mouse vas deferens to assay opiate activity (Henderson et al., 1972; Hughes et al., 1975a; Henderson & Hughes, 1976). However, there are several ways in which the experiments of Henderson and Hughes differed from the present investigation; for example, many pairs of vasa were stimulated in order to obtain a measurable output of NA and experiments wherein the stimulated output declined by more than 10% per stimulus train were rejected, so that perhaps an unrepresentative sample was chosen. The opiate receptor antagonist, naloxone, was present for 20 minutes but the results were apparently not corrected for the background release of radioactivity over this period, perhaps giving a false impression of a reversal of inhibition due to a large basal level. Moreover, the stimulation parameters used to investigate the inhibitory effects of morphine on contractions elicited by field stimulation differed from those used to release and measure NA. Contractile responses were evoked by short trains of pulses at 0.1-1.0 Hz and these were readily blocked by opiates. However, much longer trains of pulses at 0.5, 1.5 or 15 Hz were used in studies to measure NA output (Henderson & Hughes, 1976). In one study the same stimulation parameters were used to evoke both contractions and NA output (Henderson et al., 1972) although results from only one experiment were shown. It is interesting that morphine was shown to have no effect on field stimulation-induced contractions at a frequency of 10 Hz, since morphine can inhibit contractile

responses by up to 75% at this, or a higher frequency. Such frequencies of stimulation are routinely used to assess the sensitivity of opiate receptors in tissues from both untreated and drug-pretreated animals (McCulloch & Pollock, 1985; Forsyth et al., 1987).

The results of this study are in agreement with those of Stjärne (1975a), who reported that clonidine "surprisingly" potentiated motor responses and (^{3}H)-NA overflow in the field stimulated isolated guinea-pig vas deferens. Before considering a possible explanation of the results, it is worth noting that although the adrenergic innervation of the vas deferens had been questioned (Ambache & Zar, 1971), Hughes et al. considered that "the motor innervation of the vas deferens is adrenergic and that morphine acts by inhibiting the release of NA from the motor nerve terminals". Since morphine inhibited contractions, and these contractions were considered to be adrenergic, it is difficult to envisage any explanation of the results other than morphine acting presynaptically to inhibit the release of the motor transmitter NA.

An important question arising from the results obtained in this study is, how could morphine and clonidine inhibit the release of the NANC transmitter, as indicated by the reduced size of the initial component, whilst potentiating the release of NA?

Several experimental observations suggest that there are at least 2 motor transmitters in the vas deferens, NA and an NANC transmitter that may be ATP (Fedan et al., 1981; Meldrum & Burnstock, 1983; Sneddon & Westfall, 1984). It has been proposed that ATP, released as a co-transmitter together with NA, activates postjunctional purinoceptors which are of the P_2 sub-type (Burnstock, 1986). Neurally-released ATP is thought to act on postjunctional P_2-purinoceptors to produce excitatory junction potentials (ejps),

which are the underlying mechanism producing the initial, phasic component of the field stimulation-induced mechanical response (Sneddon et al., 1982; Burnstock & Sneddon, 1984; Sneddon & Westfall, 1984). After its release and activation of postsynaptic receptors, ATP is rapidly broken down to adenosine which can act prejunctionally on P_1-purinoceptors (De Mey et al., 1979; Moody & Burnstock, 1982; Burnstock, 1986) to regulate transmitter release (Sneddon et al., 1984; Burnstock, 1986).

Yohimbine enhanced the amplitude of ejps (Illes & Starke, 1983) and potentiated both phases of the contractile response to field stimulation (Stjärne & Åstrand, 1985). These effects of yohimbine were absent when the NA stores were depleted with reserpine, which reduced the concentration of NA below the threshold necessary for activation of prejunctional α_2-adrenoceptors (Illes & Starke, 1983; Stjärne & Åstrand, 1985). These observations suggest that yohimbine interrupts an endogenous feedback mechanism on transmitter release. This study confirmed that yohimbine enhanced field stimulation-induced biphasic motor responses and (^{3}H)-NA overflow in the mouse vas deferens, providing direct evidence that neurally-released NA acts on prejunctional α_2-adrenoceptors to inhibit further NA release. This study also provided indirect evidence that NA may also inhibit release of the transmitter mediating the initial component of the contractile response, since yohimbine enhanced the amplitude of the initial component of the motor response.

It is possible that activation of prejunctional P_1-purinoceptors by a metabolite of the putative transmitter, ATP, leads to a parallel autoinhibition and neuromodulation. Interruption of such a feedback system would provide one possible explanation of the results obtained with morphine and clonidine, since introducing these drugs into such a

complicated system would be expected to produce complex effects. It is possible that morphine and clonidine inhibit the release of ATP. This action would lead to less feedback inhibition *via* prejunctional P_1-purinoceptors and, consequently, result in an enhanced release of NA.

This study set out to examine this hypothesis in two ways. Firstly, it sought to determine indirectly if, indeed, ATP or its metabolites were involved in a feedback modulation of transmitter release, by examining the effects of ATP on field stimulation-induced motor responses. Secondly, it examined the effects of morphine and clonidine directly on the release of ^3H in vasa preincubated with (^3H)-adenosine.

The results from experiments investigating the effects of ATP on field stimulation-induced biphasic motor responses were consistent with the hypothesis that a metabolite of ATP, possibly adenosine, acts on prejunctional P_1-purinoceptors to inhibit transmitter release. Both components of the motor response were inhibited in the presence of ATP, suggesting that activation of prejunctional P_1-purinoceptors leads to inhibition of the release of both ATP and NA. Although the response to exogenous NA was not inhibited in the presence of ATP, at a time when the field stimulation-induced motor response was reduced, the ability of the tissue to respond to exogenous ATP was inhibited. This suggests that the ability of postjunctional α_1-adrenoceptors to respond to exogenous NA is unaffected by adenine nucleotides, and provides further evidence that the reduction in amplitude of the second, noradrenergic component of the motor response to field stimulation is due to a reduction in neurally-released NA. It was not possible from such indirect experiments to determine whether or not the amount of ATP released by field stimulation was reduced in the presence of ATP, as

the postjunctional receptors were less able to respond to exogenous ATP. However, it seems unlikely that activation of P_1-purinoceptors would inhibit the release of NA, with no effect on ATP release. Thus, the results obtained from these studies are consistent with the hypothesis that morphine and clonidine inhibit release of the NANC transmitter, leading to the removal of a negative feedback inhibition on NA release. This explanation would seem plausible if NA and the NANC transmitter are stored in separate vesicles. Differential regulation of release of co-existing neurotransmitters can be expected when the co-transmitters are stored in separate vesicles (Bartfai, 1985). If either or both transmitters participate in presynaptic autoinhibition or mutual cross-regulation of co-transmitter release, then drugs that affect such mechanisms can be expected to have complex effects.

Since limited information can be obtained from experiments attempting to study the effects of drugs which act on presynaptic receptors by examining postsynaptic responses, this study investigated a second method of testing the proposed explanation of the actions of morphine and clonidine. The effects of these drugs were examined on the overflow of ^3H in vasa preincubated with (^3H)-adenosine. This procedure results in the incorporation of label into (^3H)-adenine nucleotides. There is evidence of ^3H release following transmural stimulation of guinea-pig vasa deferentia preincubated with (^3H)-adenosine (Westfall et al., 1978). However, it still remains unclear whether ATP is released from presynaptic (Silinsky, 1973; Burnstock et al., 1978) or postsynaptic (Kuchii et al., 1975; Meunier, 1975) sites. The results obtained from such studies attempting to clarify the origin of ATP release are very contradictory. One approach that has been adopted in the vas deferens in an attempt to locate the source

of ATP released by nerve stimulation, is to use a hypertonic bathing solution to prevent muscle contraction. Hypertonic solutions have been employed for electrophysiological investigations of smooth muscle (Tomita, 1970).

The results of this study, which examined ^{3}H overflow from vasa bathed in Krebs solution made hypertonic by the addition of sucrose (12.5%), failed to support or refute the hypothesis that morphine and clonidine inhibit the release of the NANC transmitter. The amounts of ^{3}H released into the bathing solution were small and were unaffected by either morphine or clonidine. This contraction-independent release of ^{3}H from (^{3}H)-adenosine-treated tissues was insensitive to the action of TTX. Electrophysiological evidence indicates that depolarisation of nerve terminals may release transmitter, even in the presence of TTX, which blocks the inward Na^{+} current (Katz & Miledi, 1965). This may provide an explanation for the TTX-insensitive ^{3}H release evoked by field stimulation in this study. Alternatively, it may be due to the release of radioactivity as a result of direct electrical stimulation of the muscle.

Of importance to any discussion of transmitter release and function is the site of origin of the released putative transmitter and, in particular, whether release occurs from pre- or post-synaptic sites. This area is more confusing and contradictory than any other aspect of ATP metabolism. There is little doubt that ATP is released, together with catecholamines, from adrenal chromaffin cells (Geffen & Livett, 1971; Burnstock, 1976). However, in the case of neurones, the situation is far from clear. Evidence has been presented both for (Su et al., 1971) and against (Stjärne et al., 1970; Lagercrantz, 1976; Fredholm et al., 1981) a neuronal release of purines. Evidence concerning the vesicular distribution of ATP in the vas deferens is

also conflicting, in contrast to NA, which is contained in the large and small dense-cored vesicles, first observed by Grillo and Palay (1962) and now characterised both morphologically (Geffen & Livett, 1971) and biochemically (Nelson & Molinoff, 1976). The distribution of dopamine-β-hydroxylase in the rat vas deferens implies that NA synthesis occurs in large dense-cored vesicles (Fried et al., 1978). All of the current evidence indicates that enzymically active dopamine-β-hydroxylase occurs primarily, if not exclusively, in large dense-cored vesicles (Fried et al., 1978) implying that the large vesicles possess a high capacity for NA synthesis. The newly-synthesised NA is present as a fast release pool which saturates rapidly, resulting in the overflow of NA to the cytoplasm. From there, NA is taken up and stored in the small vesicles (Fried et al., 1978; Klein & Lagercrantz, 1982).

In contrast to NA distribution, about which there is general agreement across various pharmacological disciplines, the distribution of ATP remains inconclusive. Early studies showed a NA:ATP ratio of 4:1 in noradrenergic vesicle preparations of splenic nerves and 1.3-3.7 in preparations from vas deferens (Geffen & Livett, 1971), therefore, NA was assumed to be stored with ATP in both small and large vesicles in the ratio of 4:1 (Geffen & Livett, 1971), as in adrenal chromaffin granules (Winkler, 1976). This fitted in well with the hypothesis of catecholamine storage in a binding complex together with ATP. However, these early findings were shown to be in error due to contamination (De Potter et al., 1970) and later studies with large vesicles free from mitochondrial contamination gave values of 7.5-12.1 (Lagercrantz, 1976).

It is difficult to draw conclusions about the distribution of ATP in the vas deferens since, even in castrated preparations, there is

muscular tissue present (Fried et al., 1978). There is evidence that small vesicles can accumulate (^{3}H)-ATP (Geffen & Livett, 1971), although the amounts may be quite small (Lagercrantz & Stjärne, 1974).

The available morphological and biochemical evidence supports the hypothesis that both the large and the small vesicles release NA on nerve stimulation (Trifaro & Cubbedo, 1981). Under normal physiological conditions it is assumed that small vesicles are responsible for the primary release of NA from nerve terminals. Large vesicles, which contain approximately ten times more NA per vesicle than small vesicles (Fried et al., 1984), probably become more important during more intense stimulation, such as was used in this study.

The existence of a second NANC transmitter in the vas deferens has been shown, although its distribution and possible involvement in transmission remain to be clarified. Immunohistochemical studies have uncovered the presence of neuropeptide Y-like immunoreactivity (NPY-LI) in vasa of a variety of animals (Lundberg et al., 1982; 1983). Neuropeptides appear to be stored only in large vesicles, as shown by electronmicroscopic and subcellular studies (Hökfelt et al., 1980; Fried, 1982) and, in the rat vas deferens, NPY has been suggested to be contained in large vesicles (Fried et al., 1984). NPY may potentiate the effects of NA and ATP in the mouse vas deferens, as exogenous NPY rapidly increased the contractions induced by NA and ATP (Stjärne et al., 1986). Thus, the contractile response to nerve stimulation in this tissue may be due to the dynamic interplay of 3 transmitter substances with the relative importance of NPY increasing with greater frequencies of stimulation. This may provide an explanation for some of the experimental observations obtained in this study. A greater overflow of (^{3}H)-NA was obtained with trains of 100 pulses at a

frequency of 1 Hz than with trains of 100 pulses at a frequency of 20 Hz. This occurred irrespective of whether the experiment commenced with the highest or the lowest frequency. It is possible that, at the higher frequencies of stimulation, complex feedback processes come into operation in order to "spare" the main transmitter from being depleted (Stjärne et al., 1986). It has been postulated that the importance of NPY increases with increasing frequencies of stimulation (Lundberg et al., 1986) and that NPY may have a dual role, firstly to "amplify" the effects of the main motor transmitters and, subsequently, to "turn off" the secretory activity, i.e. a transmitter sparing effect (Stjärne et al., 1986). The parameters of stimulation used in this study may have been optimal for the release of NPY (in agreement with Lundberg et al., 1986) and NPY released at a frequency of 20 Hz may have inhibited the release of NA. Since the overflow of NA obtained at a frequency of 1 Hz was significantly greater than at higher frequencies of stimulation, it may be that NA is being released at this frequency of stimulation primarily from small vesicles and the main regulation of release is through presynaptic α_2-adrenoceptors. The addition of PBA produced approximately twice the overflow of (^{3}H)-NA in comparison with controls. However, the addition of PBA to vasa stimulated at a frequency of 20 Hz produced a comparable increase in (^{3}H)-NA to that obtained at the lower frequency, indicating that presynaptic α_2-adrenoceptors are equally involved in regulation at low and high frequencies of stimulation. Therefore, since the difference in the amount of transmitter released does not appear to be due to varying regulation through presynaptic α_2-adrenoceptors, it is possible that NPY is involved in a "sparing effect", as has been postulated (Stjärne et al., 1986).

This study showed that the amount of (^{3}H)-NA released over the initial 3 stimulations in the absence of any drugs decreased and then levelled off. It is noteworthy that such a phenomenon has previously been reported in the guinea-pig vas deferens (Macrae, 1983). In a tissue such as the atria, where there is no evidence for co-transmission, this reduction in overflow does not occur (Boyle, personal communication). It is possible that the stimulation parameters used in this study elicited transmitter release from all vesicular types and a complicated system of feedbacks came into operation with the ultimate outcome of preventing NA from being depleted. It is difficult to explain why there was a consistent reduction in (^{3}H)-NA overflow over the first 3 stimulations unless this system, involving a fine balance among 3 transmitters, requires an adjustment period before NA release becomes constant. Such an explanation is weak and merely serves to illustrate why understanding of transmission in this tissue has remained elusive for so many years. It may be that in the interval between the first and fourth stimulations, the system attains an equilibrium whereby the ability of NPY to regulate the release of NA increases and then stays constant.

Experiments examining the effects of drugs on ^{3}H overflow from tissues preincubated with (^{3}H)-adenosine or (^{3}H)-NA may provide limited information. It is possible that incubation of tissues in a radioactive solution results in pools of transmitter being non-uniformly labelled. The magnitude of the effect on (^{3}H)-NA overflow obtained with morphine and clonidine may be exaggerated and give a misleading impression of its importance due to the possible inadequacies of this technique in labelling all of the stores of transmitter equally. Because of the possible drawbacks of the overflow technique and the lack of information from experiments

designed to measure release of the putative transmitter ATP, another approach was adopted to examine further the unexpected results obtained with morphine and clonidine from experiments examining their effects on mechanical responses and on (^{3}H)-NA overflow. The effects of NA and the putative transmitter ATP were compared to the effects of field stimulation in their ability to stimulate postsynaptic coupling mechanisms in the vas deferens. The possibility that morphine and clonidine act postsynaptically was examined, together with their proposed action of enhancing the field stimulation-induced release of NA.

Activation of smooth muscle by transmitters and drugs involves, at least in part, the release of intracellular calcium ($[Ca^{2+}]_i$) from the sarcoplasmic reticulum. Until recently, the mechanism by which this occurred was unknown. A common observation is that all agonists which induce a phosphatidylinositol (PI) response evoke a cellular response that results in an elevation of $[Ca^{2+}]_i$). This observation led to the introduction of a hypothesis suggesting a causal relationship between phosphoinositide hydrolysis and Ca^{2+} mobilisation (Michell, 1975). PI turnover is stimulated by adrenergic and cholinergic agonists in several tissues and this effect is mediated through α_1-adrenoceptors and muscarinic cholinoceptors respectively (Carnessa de Scarnati & Lapetina, 1974; Lapetina et al., 1976; Villalobos-Molina et al., 1982; Fox et al., 1985). Many cellular processes such as contraction (Michell, 1975; Burgess et al., 1984) are regulated by Ca^{2+}-mobilising receptors, which stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), with the consequent generation of the two intracellular second messengers 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) (Berridge & Irvine, 1984; Downes & Michell, 1985). IP_3 diffuses into

the cytosol to release Ca^{2+} from intracellular stores (Berridge & Irvine, 1984). DG can be phosphorylated by a kinase to phosphatidic acid (PA), and the levels of PA are increased when the PI cycle is activated. This provides a method of monitoring the turnover of PI.

This study investigated the effects of exogenous agonists on [^{32}P]-PA formation in the vas deferens and compared their effects to those of field stimulation. The effects of morphine and clonidine were examined on this system in an attempt to confirm their unusual action in the vas deferens, revealed by experiments examining both mechanical responses and transmitter overflow. NA and electrical field stimulation increased the formation of [^{32}P]-PA and these effects were antagonised by the α_1-adrenoceptor antagonist prazosin. These results suggest that in the mouse vas deferens, as in other smooth muscle (Abdel-Latif, 1974; Lapetina et al., 1976; Campbell et al., 1985), inositol phospholipids are intimately involved in signal transduction of the response triggered by α_1-adrenoceptor occupation. The field stimulation-induced enhancement of [^{32}P]-PA formation was inhibited by TTX, indicating that it was neurally-mediated. The ability of prazosin to block the effects of field stimulation on [^{32}P]-PA formation indicated that only the NA released by field stimulation had any effect on PI metabolism. If the NANC co-transmitter(s) had influenced the hydrolysis of PI, prazosin would have been unlikely to completely block the enhanced [^{32}P]-PA formation resulting from field stimulation. Neither ATP nor its stable analogue, α,β-MeATP, affected [^{32}P]-PA formation indicating that the field stimulation-induced NANC response mediated by ATP does not involve the hydrolysis of PI.

Morphine and clonidine had no effect on the basal levels of [^{32}]-PA formation or the ability of NA to enhance the formation of [^{32}P]-PA,

indicating that these drugs do not act postsynaptically either to affect [^{32}P]-PA formation directly or to enhance the effects of NA on [^{32}P]-PA formation. However, both drugs potentiated the ability of electrical field stimulation to increase the formation of [^{32}P]-PA. These results indicated that the site of action of morphine and clonidine is presynaptic, resulting in a potentiation of the release of NA. These results confirmed the previous findings from studies employing more traditional pharmacological techniques and suggested that the complex actions of morphine and clonidine to increase both the adrenergic component of the biphasic motor response of the mouse vas deferens and the overflow of (^{3}H)-NA are real effects and cannot be attributed to unexplained properties peculiar to a species or, alternatively, to a "presynaptic protecting effect", such as has been postulated to explain the similar effects of clonidine in the guinea-pig vas deferens (Stjärne, 1975a). This highlights the quandary faced by many pharmacologists when their experimental observations do not fit into accepted dogma. Do they simply ignore or dismiss their data?

It is interesting that the proposed alteration in the relative importance of the co-transmitters in the vas deferens, depending on the parameters of stimulation, has a parallel in the signal pathway utilising the second messengers generated from IP_2. Release of Ca^{2+} from the endoplasmic reticulum by IP_3 is only one branch of a bifurcating signal pathway. The other component is DG, which actives protein kinase C (Nishizuka, 1984). One very important aspect of this bifurcation is that the 2 branches often act synergistically with each other (Kaibuchi et al., 1983; Nishizuka, 1984) and both second messengers are often required to produce a maximal response. Ca^{2+} ionophores have been used to bypass the actions of IP_3 in elevating $[Ca^{2+}]_i$ levels. Phorbol esters, which mimic DG (Nishizuka,

1984), have been shown to induce a slowly-developing tonic contraction of vascular smooth muscle (Banthuluri & Deth, 1984). When added together, phorbol esters and Ca^{2+} ionophores produced a sustained contraction, thus in many cellular processes both IP_3 and DG are required to produce a maximum response.

In the vas deferens, it would seem that all the co-transmitters are intimately involved in the process of transmission. It may be that the vas responds to alterations in stimulation by shifting the emphasis from one neurotransmitter to another and triggering several different and rather complex feedback interactions in order to sustain transmission. Stimulation at high frequencies will produce a contraction which is the resultant effect of a small number of chemical messengers, avoiding the sole utilisation of any one, with the possibility of exhaustion.

It is interesting to speculate that the greater the number of co-transmitters, the greater the number of potential feedback interactions to control the release of the "main" transmitter.

Thyroxine-induced changes in sensitivity

Thyroid hormones are known to influence the sensitivity of sympathetically-innervated tissues to agonists (Gibson, 1981). Whereas most studies investigating the effects of these hormones have examined their effects on postsynaptic receptor sensitivity, this study set out to determine whether or not chronic T_4 pretreatment affected the sensitivity of presynaptic receptors and if the changes in postsynaptic receptor sensitivity occurred as a result of presynaptic changes. T_4 administration raised the levels of free T_4 in the plasma and produced changes in the responsiveness of the mouse vas deferens to drugs acting both pre- and post-synaptically. Chronic treatment with T_4 reduced the sensitivity of smooth muscle to NA and ACh. This subsensitivity was characterised by a reduction in the maximum response to NA with no shift in the position of the dose-response curve. In addition to a reduced maximum response to carbachol, there was a rightwards displacement of the dose-response curve for this agonist. The subsensitivity to both NA and carbachol is one example of a widespread phenomenon in which the response to several agonists is impaired (Su *et al.*, 1976a; b). This phenomenon, described as heterologous desensitisation, occurs by more than one mechanism (Harden, 1983). Since the turnover of inositol phospholipids is stimulated by adrenergic and cholinergic agonists in several tissues (Carness de Scarnati & Lapetina, 1974; Lapetina *et al.*, 1976; Fox *et al.*, 1985), it is possible that T_4 pretreatment acts at a stage in this cycle, disrupting the second messenger pathway linking activation of muscarinic receptors and α_1-adrenoceptors to a change in the responsiveness of the cell.

The results obtained from the field stimulated tissues indicated that T_4 pretreatment may also have influenced the sensitivity of

presynaptic receptors, as there was a reduction in the maximum capacity of both morphine and clonidine to inhibit field stimulation-induced responses. The stimulation parameters used in this part of the study, that is, trains of 10 pulses at a frequency of 20 Hz, were most likely to have released the NANC transmitter(s), as no detectable overflow of (^{3}H)-NA was obtained using these parameters of stimulation. In light of this evidence, it is interesting that clonidine could produce up to 90% inhibition of the twitch responses, since clonidine's accepted action as a prototype α_2-adrenoceptor agonist, is not sufficient to explain this observation. Clonidine also appears to activate purinoceptors, blocking a presynaptic autoinhibitory mechanism (Katsuragi & Furakawa, 1985). However, the results obtained in this study would suggest that clonidine may, indeed, activate purinoceptors but in the capacity of an agonist and not as an antagonist as proposed by Katsuragi and Furakawa (1985). Clonidine may stimulate a presynaptic autoinhibitory mechanism leading to a reduction of ATP release. This mode of action of clonidine alone would be unlikely to produce a 90% inhibition of field stimulation-induced twitch responses. Thus, clonidine's ability to inhibit twitches in the vas may be the resultant effect of blocking a presynaptic P_1-purinoceptor-mediated autoinhibition of ATP release together with blockade of a presynaptic α_2-adrenoceptor-mediated modulation of ATP release. Although NA can not be detected at the stimulation parameters used in these studies, there may be a sufficient threshold concentration of neurally-released NA in the synaptic cleft to activate presynaptic α_2-adrenoceptors and inhibit the release of ATP. Since T_4 pretreatment produced a significant subsensitivity to clonidine at all concentrations, but a significant subsensitivity to morphine only at the highest concentration, it is possible that the effect of T_4 pretreatment was on

the mechanisms activated by clonidine and the change in sensitivity of opiate receptors was secondary to this change. Both α_2-adrenoceptors and opiate receptors, located on nerve terminals, may be linked to a common mechanism that inhibits transmitter release (McCulloch & Pollock, 1985). It has been clearly established that presynaptic α_2-adrenoceptors mediate inhibition of adenylate cyclase (Jacobs, 1985) and there is some evidence that opioids may also modulate the activity of this transduction mechanism (West & Miller, 1983). It has been suggested that presynaptic purinoceptors may also inhibit adenylate cyclase (Sneddon et al., 1984) and, therefore, any adaptation in this common pathway may produce a subsensitivity to the effects of drugs acting presynaptically. Although it is possible that T_4 pretreatment merely had a "toxic" effect on nerve terminals, removing the inhibitory capacity of presynaptic receptors, this is an unlikely explanation of the results, since the ability of clonidine to inhibit responses was affected to a greater extent than morphine. A non-specific effect on nerve endings would have been more likely to affect the inhibitory capacity of both drugs similarly.

Results from experiments investigating the effects of pretreatment with a combination of T_4 + 6-OHDA suggested that the changes in postsynaptic receptor sensitivity occurred as a result of changes presynaptically. If the observed subsensities both pre- and post-synaptically had occurred independently of each other, T_4 pretreatment of denervated animals would have resulted in less of a supersensitivity than was obtained in animals treated with 6-OHDA alone. However, this was not the experimental observation that was obtained. Pretreatment with a combination of 6-OHDA + T_4 resulted in an even greater postsynaptic supersensitivity than occurred as a result of 6-OHDA pretreatment alone.

Limited information can be obtained from studies where the recorder speed was running slowly and field stimulation released only the NANC transmitter. Studies monitoring the overflow of (^{3}H)-NA revealed that in the absence of any drugs, there was an enhanced overflow of (^{3}H)-NA, indicating that T_4 pretreatment inhibited the presynaptic mechanisms which normally regulate transmitter release. It is possible that these receptors became subsensitive because there was more NA in the vicinity of the receptors due to an impairment of the removal mechanisms for NA. This was tested by blocking MAO and the neuronal and extraneuronal uptake of NA. The results showed that T_4 pretreatment did not significantly alter these processes. This study provided evidence that T_4 pretreatment produced a subsensitivity of presynaptic α_2-adrenoceptors. PBA, which blocks the uptake processes and both pre- and post-synaptic -adrenoceptors, produced less of an enhancement of (^{3}H)-NA overflow in tissues from treated animals than from controls. It is possible that PBA may also block presynaptic P_1-purinoceptors and, in T_4 pretreated animals, the reduced overflow of (^{3}H)-NA resulted from a combined subsensitivity of both α_2-adrenoceptors and P_1-purinoceptors on nerve endings.

Morphine and clonidine did not produce a significant enhancement of (^{3}H)-NA overflow in vasa from T_4 pretreated animals unlike their effect in vasa from control animals. This observation is consistent with the explanation that T_4 pretreatment resulted in a change in the sensitivity of presynaptic receptors, inhibiting their ability to regulate transmitter release. This observation further supports the experimental results obtained with morphine and clonidine throughout the study since, if these drugs normally inhibit a presynaptic inhibitory regulatory mechanism on transmitter release, leading to the enhanced release of NA, preventing this feedback from operating

efficiently by pretreating animals with T_4 will prevent morphine and clonidine from enhancing NA release.

In conclusion, the effects of morphine and clonidine have confirmed the complexity of transmission in the vas deferens, first suggested by Ambache and Zar (1951). The evidence obtained in this study is consistent with the hypothesis that the vas possesses more than one transmitter. Drugs such as morphine and clonidine may preferentially inhibit the release of at least one of the co-transmitters which normally regulates the release of NA. Altering the hormonal environment of this tissue produced results consistent with this proposed explanation. This study provided answers for some of the unresolved questions in the vas, such as whether or not the transmitters are contained in a single nerve or in separate nerves, and if chronic T_4 pretreatment altered the sensitivity of presynaptic receptors. However, other questions have been raised in the course of the study:

(1) What is the transmitter responsible for the 'spike' component of the mechanical response? Is it ATP?

(2) If it is, indeed, ATP, what is the vesicular distribution of this putative transmitter?

(3) What is the relative importance of NPY in the vas deferens?

(4) How could drugs preferentially inhibit release of one transmitter at high frequencies of stimulation, even if the transmitters are stored in separate vesicles?

Of interest in this study was the ability of chronic T_4-pretreatment to abolish the effect of drugs on NA release. This may provide an important means of altering the ability of drugs to act presynaptically to interfere with complex feedback regulations on

transmitter release and provides further evidence that chronic hormone treatment produces multiple effects throughout the body, both pre- and post-synaptically.

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