A Study of the local modulatory effect of Nitric Oxide and histamine on neurotransmission in the mouse vas deferens

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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April 1998
ABSTRACT

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The effects of Nitric Oxide (NO) and histamine as modulatory agents of autonomic neurotransmission in the mouse vas deferens have been investigated. Electrophysiological, pharmacological, and histochemical approaches were adopted to investigate their various activities.

Histochemically, NO synthase was demonstrated through the reaction of its NADPH-diaphorase activity with NBT or BPST and their conversion into dark blue positive deposits. Under the electron microscope NADPH-diaphorase positive areas are associated with the outer mitochondrial membranes and some parts of the nuclear membrane.

Mechanical responses evoked by electrical field stimulation gave information about pre- and post-junctional effects. Whereas agonist evoked contractions gave information about post-junctional responses, L-arginine (3x10^{-4} M) reduced nerve evoked contractions, suggesting a NO sensitivity. L-arginine methyl ester (L-AME) 2.3x10^{-4} M (NO synthase inhibitor), increased nerve evoked contractions. This suggested a physiological depressive role for NO synthase. L-arginine shifted the dose-response curve to the right, whereas L-AME shifted the curve to the left, indicating a post-junctional modulatory mode of action. Extracellular focal recording techniques recorded excitatory junction currents (EJCs). EJCs reflect the neuronal release of ATP. Quantal analysis of EJCs amplitude permitted the determination of pre- and post-junctional effects. L-arginine (3.4x10^{-4} M) reduced EJCs amplitude and mean quantal content. Quantal analysis also revealed a pre-junctional mode of action. PTIO (3x10^{-8} M) increased EJCs amplitude, and reversed the inhibitory effect of L-arginine. L-arginine revealed a low-threshold current of a positive polarity, abolished by noradrenaline and increased by yohimbine and idazoxan, indicating a possible existence of autoinhibitory feedback mechanisms controlling the new current.

Exogenous histamine reduced EJCs amplitude. Quantal analysis revealed both pre- and post-junctional sites of action. Thioperamide (10^{-7} M) and (R)-α-methyl histamine (10^{-8} M) confirmed the presence of H_3 receptors pre-junctionally. Ranitidine (10^{-5} M) and dimaprit (10^{-6} M) confirmed the presence of H_2 receptors pre- and post-junctionally. The presence of H_2 and H_3 receptors indicate that endogenous histamine acts on those receptors to depress contraction.

The results showed that NO-mediated neuromodulatory mechanisms exist in this tissue, which modulate pre- and post-junctional activities. The results confirmed the presence of pre-junctional H_3 receptors, and pre- and post-junctional H_2 receptors. Both receptors modulate transmission in this tissue.
ACKNOWLEDGEMENT

I am extremely grateful for the assistance and the unending support that I have received over the last few years from my supervisors Prof A. G. H Blakeley.

I am thankful to Dr. John Scott for his technical help and support. In addition, I would like to thank the staff in the Department of Pre-clinical Sciences, at Leicester University, in particular, Mrs. Judy McWilliam from electron microscopy, and Mr. Symon Byrne. Not to forget friends and relatives both in Kuwait and in England for their prayers and support. And finally, I would like to thank my husband Ali for his support.
To my Parents
In the name of God, the most Compassionate, the most Merciful

On no soul God place a burden greater than it can bear. It gets every good that it earns, and it suffers every ill that it earns. Our Lord, condemn us not if we forget or fall into errors; Our Lord, Lay not on us a burden like lay on those before us; Our Lord, lay not on us a burden greater than we have strength to bear. Blot out our sins, and grant us forgiveness. Have mercy on us. You are our protector.

(al Baqarah, 286)
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1- INTRODUCTION

The aim of the work included in this thesis was to investigate the functional modulation of sympathetic neurotransmission using the mouse vas deferens as a model system. In particular the roles of Nitric Oxide (NO) and histamine were examined in this system. Results obtained in previous work from this laboratory showed that NO and histamine both modulate transmission in the mouse vas deferens (Ph. D. Thesis, Patterson R. V., University of Leicester, 1996). Those results showed that L-arginine (the natural precursor for NO synthase in the production of NO) depresses nerve evoked contraction, whereas, NO synthase inhibitors such as L-AME (L-arginine methyl ester), had the opposite effect. In addition, application of exogenous histamine was shown to depress nerve evoked contractions by a physiological mechanism. Dr. Patterson's results have been confirmed and extended in this thesis. In the next sections the functional and the structural aspects of the sympathetic system will be reviewed. Special reference will be made to the rodent vas deferens in terms of its structure, function and innervation.

1-1 Neurotransmission:

The process of neurotransmission involves the transfer of information from one neurone to another, or from a neurone to a muscle, gland or other effector cell. Sometimes the nerve cell membrane is fused with the post-synaptic membrane and
transmission occurs through a flow of ions between the two cells, this is termed electrical transmission. Electrical synapses are characterised by their rapid speed of transmission due to the fact that cells are connected structurally. In most cases, however, the transfer of information occurs across a narrow gap between the cells, by means of a chemical substance which is released from transmitting neurone. This is described as chemical transmission which mediate excitatory or inhibitory mechanisms. At the chemical synapses a synaptic cleft (20-40\mu m) separates the pre- and post-synaptic sites. The pre-synaptic terminals contain localised collections of vesicles, the synaptic vesicles, which are filled with chemical neurotransmitter. Chemical transmitters are released from synaptic vesicles in response to pre-synaptic action potentials. Following the release step, the transmitter binds to receptors sites located on the post-synaptic sites, resulting in an opening or closing of ion channels and consequently altering the membrane conductance and potentials of the post synaptic cell. (Kandel et al., 1989).

Chemical transmission is often subjected to modulation, if the site of modulation was pre-synaptic, this is termed pre-junctional modulation. On the other hand, if the site of the modulation is on the target cell, the modulation is described as post-junctional. Transmission in the autonomic nervous system is a process which has many modulatory influences. The work in this thesis will focus on the concept of local neuromodulation, where locally released agents can alter neurotransmission either by prejunctional modulation of the amount of transmitter released, or by post-junctional modulation of the time course or intensity of action of released transmitter. In this thesis the work will concentrate on the neuromodulatory role of histamine and Nitric Oxide (NO). The mouse vas deferens was chosen because it receives a dense sympathetic innervation, which makes it an ideal tissue for studies investigating some of the
local modulatory factors affecting sympathetic transmission. It is, therefore, useful at this point to review briefly the current knowledge of the neurotransmission and, in particular, the neurotransmitter release process.

1-2 *The quantal hypothesis:*

The current understanding of the mechanisms underlying the release of neurotransmitters originates from a series of studies in the early of 1950s. Katz and his colleagues in the early 1950s demonstrated and recorded small spontaneous random depolarisations from the end-plate region at the skeletal muscle penetrated by an intracellular recording electrode (Fatt & Katz, 1952). These random potentials were increased in size by the anticholinesterase prostigmine, reduced by curare, and absent in muscle that had been previously denervated. They suggested that these potentials, termed miniature end-plate potentials (MEPP), were the result of the release of acetylcholine from the nerve terminals. They also demonstrated that MEPP resulted from the release of a multimolecular packet of acetylcholine rather than a single molecule.

As a result of stimulation of the motor nerve innervating the muscle fibre, a large end-plate potential (EPP) was recorded, which had a similar time course to that of the MEPP. The relationship between MEPP and EPP was determined by reducing the Ca\(^{2+}\) content of the bathing solution. This resulted in reduction in the EPP amplitude with no corresponding effect on the amplitude of the MEPP. In low Ca\(^{2+}\) solutions individual EPP fluctuated in amplitude in a random step-wise manner (the size of the step unit as the EPP approximated to the size of the MEPP), in other words the EPPs amplitude became similar to MEPPs amplitude. In very low Ca\(^{2+}\) many stimuli
failed to evoke an EPP. On the basis of the above observation Fatt and Katz (1952) proposed the quantal hypothesis, where the EPP is made up of several hundreds multimolecular units (quanta), which are identical in size to the MEPP.

Boyd and Martin (1956) through their work on the skeletal neuromuscular junction of feline tenuissimus in low Ca\textsuperscript{2+} solutions, showed that EPP was made up of units (i.e. quanta) of the same size as the MEPP. They estimated the normal quantal content of the EPP to be about 200-300 units, when the probability of release was low and the amplitude of the events could be fitted to a Poisson distribution. In 1956 Liley reported that transmitter release at the skeletal neuromuscular junction of the rat diaphragm was also quantal in nature. Liley also reported that the amplitude distribution of the EPP when the probability of release was reduced could be fitted by Poisson distribution. These observations suggest that the release of a quanta is a stochastic process and the arrival of the action potential at the terminal result in a brief rise in the probability of release of all quanta present. The release of quanta in such a process would be independent on the simultaneous release of other quanta.

The precise use of quantal analysis was possible because at the skeletal neuromuscular junction, each muscle cell fibre receives only one synaptic input, and the muscle cells are electrically isolated from each other. The end plate potential is shown to be made up of quantal units, each unit (or multimolecular packet), corresponds in size to the spontaneous miniature end-plate potential (Boyd and Martin, 1956). On the other hand, due to the multiple innervation and electrical coupling between the cells at the autonomic neuroeffector junction, the application of quantal analysis was not thought to be possible. Indeed the time course of recorded spontaneous excitatory junction potential (SEJP) is much shorter than that of the excitatory junction potential.
(EJP) and the EJP is often bigger than the SEJP. However, Blakeley and Cunnane, in 1979, showed that the EJP could be broken down into elements (Discrete Events) when matched with the amplitude and time course of the spontaneous excitatory junction potentials in the same cell, were able to carry out limited quantal analysis of autonomic neuroeffector transmission.

The junction potentials recorded in individual smooth muscle cells are the result of two processes. First, due to the release of transmitter from a large and variable number of varicosities in the terminal regions of one or more nerves. Second, due to the spread of electrical activity from neighbouring smooth muscle cells across low-resistance pathways (see Bennett and Burnstock, 1968).

With the aid of the electron microscope and by histochemical studies the detailed relationship of individual nerves to single smooth muscle cells was made possible, when coupled with information inferred from electrophysiological studies of transmission. All of this allowed a proposal building of a model of the sympathetic neuromuscular junction (Burnstock and Iwayama, 1971). This model concluded that the transmitter must be released from large numbers of terminal varicosities. The effector is the muscle bundle rather than a single smooth muscle cell. In this smooth muscle bundle some, but not all cells are directly innervated. In addition, the cells are electrically coupled. And finally, an all or non action potential is initiated when a muscle cell becomes depolarised, and the potential is propagated through the tissue.

The importance of quantal analysis of transmission at chemical synapses lies in the ability to determine the nature of the junctional mechanisms that operate normally and during modulation of transmission. It is possible to work the site of modu-
lation by implementing quantal analysis. Presynaptic sites of modulation affect the probability of transmitter release and the number of quanta released. Whereas, quantal size is usually determined by post synaptic factors. One of the strengths of quantal analysis is that it allows one to separate the various junctional mechanisms.

1-3 Morphological correlate of the quantum

In the mid 1950s morphological studies showed the presence of membrane bound vesicles close to the prejunctional membrane in a number of different nerve terminals including those innervating skeletal muscle (Palade, 1954; Palay, 1954; De Robertis and Bennett, 1955). De Robertis and Bennett (1955) suggested that these vesicles might be involved in transmitter storage and transport. Del Castillo and Katz (1955), suggested that the vesicles may provide the morphological correlate of the quantum, which lead to the vesicle hypothesis (i.e. that the transmitter content of a synaptic vesicle represents the quantum). Supporting this suggestion De Robertis in (1967), was able to show that the synaptic vesicles prepared from various tissues by homogenisation and centrifugation contain neurotransmitters. It has been suggested that at the skeletal neuromuscular junction a major fraction of the acetylcholine released by nerve action potentials originates from the cytoplasm rather than the vesicular store (see Tauc, 1982). However, studies with vesamicol, an inhibitor of the vesicular acetylcholine carrier, support the view that released acetylcholine is vesicular in origin (Marshal and Parsons, 1987).
Early studies of neurotransmission at the autonomic neuroeffector junction

Intracellular recording techniques were developed by Burnstock and Holman (1961), to investigate electrical activity at the sympathetic neuroeffector junction. Intracellular recordings were made from smooth muscle cells in the outer longitudinal muscle layer of the guinea-pig vas deferens. When cells were penetrated with high resistance microelectrodes and the excitatory nerves innervating the preparation were electrically stimulated, a transient depolarisation was recorded, which has been called the excitatory junction potential (EJP). EJPs were graded with stimulus intensity and increased in amplitude response to the first few pulses in a train of stimuli. This characteristic increase in EJP amplitude is frequency dependent, and this process is termed facilitation. In the absence of nerve stimulation, spontaneous excitatory junction potentials (SEJPs) were recorded. SEJP is thought to be caused by the release of a multimolecular packet of neurotransmitter from a varicose nerve terminal (Burnstock and Holman, 1961, 1962a).

The presence of SEJPs might suggest that transmitter release at the sympathetic neuroeffector junction is packeted. However, unlike the MEPP and EPP at the skeletal neuromuscular junction, which have closely similar time course, the time courses of the EJP in the vas deferens lasts 4-5 times longer than that of SEJP. Bennet in 1972 thought that the explanation for this difference in the time course was a result of electrical coupling of smooth muscle. When a single packet of transmitter is released spontaneously, there is an increase in membrane conduction at the site of release. As the current generated at this site spreads rapidly, the time course of the potential change (i.e., the SEJP) as a result is brief. When the nerve is stimulated, transmitter is released at a number of different points throughout the muscle. When the
current spreads from the source point, it results in a depolarisation of the whole muscle, which in turn limits further current spread. In this case the time course of the potential change will be determined by the relatively slow process of charge dissipation across the muscle cell membrane (see Brock and Cunnane, 1992).

The quantal nature of the post junctional response to nerve stimulation has not been possible to be analysed statistically at the autonomic neuroeffector junction, for three major reasons. Firstly, the difference in time course of SEJP and EJP means that they are not directly comparable. Secondly, the amplitude distribution of the SEJPs is not unimodal and is positively skewed. This is because of small events often disappearing into the noise level of the recording system, making it impossible to determine the mean quantal size. Thirdly, the statistical tests are only valid if the activity of a single junction can be identified or if the innervation is limited to a specific muscle fibre. Smooth muscle cells receive multiple innervation with electrical coupling between adjacent cells, the electrical activity recorded in any one cell reflects the activity of transmitter released both locally and at sites remote from the recording electrode. These properties of smooth muscle in part explain why the SEJP amplitude distribution is skewed. Transmitter action close to the recording electrode produces a larger SEJP than that occurring at a distant release site, leading to attenuation of signal spreading electrically. However, variation in the number of transmitter molecules in the packets and/or variation in the degree of coupling between neighbouring smooth muscle cells may also contribute to the SEJP amplitude distribution (see Brock and Cunnane, 1993).

Using conventional electrophysiological techniques, the relationship between spontaneous and evoked transmitter release at the sympathetic neuroeffector junction
cannot be established with any degree of certainty. It was for this reason that much of our early understanding of the mechanisms involved in the storage and release of transmitter at the sympathetic neuroeffector junction came initially from biochemical studies of catecholamine secretion (see Brock and Cunnane, 1993).

1-5 The excitatory transmitters of the rodent vas deferens (co-transmission)

Electrical stimulation of the rodent vas deferens evokes a biphasic response (Swedin, 1971; Birmingham and Freeman, 1976; Fedan, 1981), consisting of a rapid contraction (phase I) which is associated with EJP (Burnstock and Holman, 1961; Stjarne and Astrand, 1985), followed by a second phase (phase II) of sustained contraction. The two phases are species dependent, more clearly seen in the rat than in the guinea pig vas deferens for example (Birmingham and Freeman, 1976).

Both phases of the contractile response are sensitive to the adrenergic neurone blocking agents, bretylium and guanithidine, suggesting sympathetic involvement (Kuriyama, 1963; Burnstock and Holman, 1964). The second phase of the contraction is inhibited by the α-adrenoceptor antagonist phentolamine and by pretreatment of the tissue with reserpine, suggesting that this phase is mediated by NA (Burnstock and Holman, 1964). The first phase of the contraction is resistant to all of these procedures (Wedin, 1971). Burnstock (1976) thought that certain nerves may release more than one transmitter. The initial phasic component of the contraction is predominantly purinergic, as it is abolished by P2x-receptor antagonists arylazideo aminopropinoyl-ATP (ANAPP3) (Sneddon et al., 1982; Sneddon and Weastfall,
1984). It is also abolished by α-β-meATP (Meldrum and Burnstock, 1983), suramine (Baily and Hourani, 1995) and PPDS (Mc Learn et al., 1994).

It is generally accepted that ATP is the primary mediator of the fast contraction (phase I), and noradrenaline is the primary mediator of the slow contraction (phase II). But some suggested that both noradrenaline and ATP contribute to both phases of contractions. This is based on the observation of that the adrenergic and purinergic antagonist reduced both phases of contraction (Fedan et al., 1981; Stjarne and Astrand, 1985). However, those observation are difficult to interpret, because noradrenaline and ATP can each increase the post-synaptic action of the other (Holck and Marks, 1978).

Although the EJPs are abolished by tetrodotoxine (TTX) and guanethidine, they are resistant to α-adrenoceptor blockade (see Von Kugelgen and Starke, 1991). In the 1980s, EJPs were first shown to be mediated by ATP in the guinea pig vas deferens, where they are inhibited by the P2 purinoceptor antagonist ANAPP3 (Sneddon et al., 1982; Sneddon and Westfall, 1984). In most tissue NA released from sympathetic nerves has no effect on the membrane potential of smooth muscle cells.

Most evidence to date suggests that both ATP and NA are stored in both large and small dense cored vesicles (Klein, 1982; Lagercrantz & Fried, 1982) and are co-released (Burnstck, 1990). Thus it follows that the SEJP and discrete events may also measure indirectly the quantal release of NA. 6-hydroxydopamine (6-OHDA) and guanethidine are sympatholytic, causing degradation of nerve terminals. In the vas deferens and blood vessels both drugs block the release of noradrenaline and ATP (Fedan et al., 1981; Cheung, 1982; Kirkpatric and Burnstock, 1987). However, some
work has raised the question as to whether NA and ATP, under certain conditions, are released and/or stored in the same way in sympathetic nerves (Trachte, 1988; Ellis and Burnstock, 1989, 1990). On the other hand, Mshgina, Mermet, Gouon and Stjärne (1992), from parallel studies of ATP and NA secretion using electrophysiological and electrochemical methods, concluded that their data was consistent with the traditional model, that nerve impulses evoked the release of mixed multimolecular packets of ATP and NA.

1-6 Biochemical studies of Noradrenaline (NA) secretion

Biochemical studies and chemical assays have played an important role in revealing a considerable amount of information regarding noradrenaline (NA) synthesis, storage, and secretion. Estimation of the NA content of single varicosities in different tissues are in good agreement: $4 \times 10^{-15}$ g for rat iris and vas deferens (Dahlstrom et al., 1966), $10^{-14}$ g for rabbit pulmonary artery (Bevan et al., 1969), and $3.4 \times 10^{-14}$ g for guinea pig uterine artery (Bell and Vocf, 1971).

It is generally agreed that most of the NA in terminal varicosities is contained in granular vesicles (Hokfelt, 1969; Blaschko and Smith, 1971). This store is heterogeneous. Two population of vesicles have been shown morphologically in the varicosities of postganglionic sympathetic nerves, namely small (25-60 μm) and large (70-160 μm) dense cored vesicles. NA is found in both light and heavy particulate fractions, which correspond to small and large dense cored vesicles respectively (Smith and Winkler, 1972).
Pharmacological evidence suggested that only NA stored in vesicles is released by nerve stimulation at the sympathetic neuroeffector junction. Reserpine is a compound which blocks the uptake of NA into storage vesicles, and consequently depletes the vesicular store. In the presence of monoamine oxidase inhibitors (drugs which prevent the metabolism of cytosolic noradrenaline), reserpinized tissues are able to sequester tritiated noradrenaline via the neuronal uptake mechanism. This indicates that the sequestered NA is free in the cytosole and is not taken up by vesicles (Iversen et al., 1965; Potter, 1967). In these tissues, the electrically evoked release of tritiated NA is markedly inhibited compared with control tissue (Potter, 1967), suggesting that only NA present in vesicles can be released by nerve impulses.

More support for the vesicular origin of the released NA comes from demonstrating that two vesicular proteins, dopamine β-hydroxylase and chromogranine A, present in the NA-containing particulate fraction isolated from dog and calf splenic nerve axon, were released in a calcium dependent manner in response to nerve stimulation (De Potter et al., 1969; Smith et al., 1970). Electron microscopic methods have revealed vesicles fusing with the plasma membrane in the varicosities of sympathetic nerve, providing further evidence for an exocytotic mechanism (Thureson-Klein, 1983; Thureson-Klein, 1990; see Fillenz, 1992)

There is evidence suggesting that both small and large dense vesicles are involved in transmitter release. The β-hydroxylase activity/NA ratio in tissue perfusate from electrically stimulated dog and calf spleen is lower than in the NA-containing particulate fraction isolated from splenic nerve (Smith et al., 1970). This particulate fraction corresponds to the heavy particulate fraction isolated from nerve terminal. Comparing the light and the heavy particulate fraction, the light particulate fraction
contains a very low dopamine β-hydroxylase content in relation to its noradrenaline content (Fried, 1980). As a result the probable explanation for the low dopamine β-hydroxylase content of the splenic tissue perfusate is that released NA comes from both small and large dense cored vesicles while dopamine β-hydroxylase is only secreted from the large dense vesicles (Thureson-Klein, 1983).

In conclusion, a number of evidence now supports the idea that neurotransmitters are stored in sympathetic vesicles and released by exocytosis. The membrane fusion and subsequent release of the catecholamine content of a single vesicle from cultured chromaffin cells has more recently been detected by a combination of patch clamp and fast cyclic voltametric recording techniques (Chow et al., 1992).

1-7 Electrophysiology of adrenergic transmission

The rodent vas deferens receives a dense innervation by sympathetic noradrenergic neurones (Sjostrand, 1965). As mentioned before (section 1-6) stimulation of those neurones produces a strong contraction of both longitudinal and circular smooth muscle layers of the vas deferens, and the result is often a biphasic contraction (Swedin, 1971). The biphasic contraction consists of a rapid phase of contraction which is associated with EJP (Burnstock and Holman, 1961), which is followed by a second slower phase of contraction, which is independent on membrane potential changes.

Degeneration of adrenergic neurones by 6-OHDA produces a great reduction or abolishes EJP and smooth muscle contraction (Wadsworth, 1973). In addition, all
excitatory responses of vas deferens to nerve stimulation are abolished by noradrenergic neurone blocking agents such as, guanethidine and bretylium (Burnstock and Holman, 1964). However, following the depletion of neuronal stores of noradrenaline with reserpine or after application of α-adrenergic antagonists only the second phase of contraction reduced. Whereas, the first phase of contraction and EJP are largely unaffected by these drug treatements (Burnstock and Holman, 1964; Swedin, 1971).

The above pharmacological analyses have led to the general agreement that both phases of contraction of vas deferens are mediated by transmitter release from adrenergic nerve terminals. On the other hand, the inability of exogenous noradrenaline to mimic the EJP and the fast contraction suggests that these responses are not due to noradrenaline acting on adrenoceptors (Meldrum and Burnstock, 1983). The resistance of the EJP and the fast contraction to α-adrenoceptors antagonists is likely to be mediated by substance other than noradrenaline, but released from adrenergic neurones (Ambache and Zar, 1971). Some studies demonstrated the co-existence of noradrenaline and NPY (neuropeptide Y) in sympathetic neurones supplying the vas deferens (Lundberg et al., 1982). The EJP and the fast contraction are mimicked by exogenous ATP or α-β-MeATP (Meldrum and Burnstock, 1983), but not by NPY (Stjarne et al., 1986).

Many neurotransmitters have both pre-synaptic and post-synaptic modulator action. For example, noradrenaline (NA) and acetylcholine (Ach) act on receptors located on autonomic nerve terminals to modify the release of transmitters, including themselves (Starke, 1987). Starke proposed that adrenergic nerve terminals are endowed with α-adrenoceptors related to structures on the effector tissues, which on reaction with α-stimuli (e.g. NA) they mediate inhibition of transmitter release. In the
presence of α-blocking agents this inhibition of transmitter release in reduced (Starke, 1971). Starke demonstrated that exogenous NA inhibits the release of neuronal NA from cardiac tissue during nerve stimulation, which was antagonised by α-adrenoceptor antagonist (Starke, 1972). NA, ATP, and NPY are transmitters co-released from post-ganglionic sympathetic autonomic neurones. NA, NPY and adenosine (derived from the break down of ATP) all have pre-synaptic inhibitory action on sympathetic transmitter release. For example, NA and clonidine (α₁-adrenoceptor agonists) both decrease NA release, reduce EJP and fast contraction of rat vas deferens (Stjatrne, 1989). Yohimbine (α₂-adrenoceptors antagonist) increases NA release and fast contraction of rabbit ilocolic artery (Burnstock, and Starke, 1990). In guinea pig vas deferens, yohimbine increased the amplitude of EJPs evoked during train of stimuli. However, yohimbine had no effect on EJPs amplitude in reserpinized tissues (Brock et al., 1990). These results might indicate that yohimbine increases transmitter release by interrupting α-autoinhibition. Studies of occurrence of discrete events in guinea pig vas deferens in the absence of pre-junctional α-adrenoceptors antagonists failed to demonstrate that endogenous transmitter regulates its own release locally (Blakely et al., 1982). This could mean that NA acts at sites remote from its own sites of release (lateral inhibition) (Brock, et al., 1990).

At one stage there was some doubt about the physiological significance of modulation of neurotransmitter by pre-synaptic α-adrenoceptors (Anguse and Korner, 1980). However, it has been suggested that autoreceptors are activated by transmitter released from adjacent varicosities rather than the same varicosity. Autoreceptors are important for regulating the intermittency from individual release sites (Stjarne, 1986). Transmission from autonomic neurones also can be modulated by substance
which are synthesised in near by tissues or circulates as hormones, such as histamine (McGrath and Shephared, 1976).

1-8 *ATP as a neurotransmitter at the sympathetic neuroeffector junction*

There is substantial evidence supporting the role of adenosine 5-triphosphate (ATP) as a neurotransmitter at the sympathetic neuroeffector junction. NA and ATP are co-localized in vesicles isolated from sympathetic nerve terminals (Klein & Leigercrantz, 1981), and following electrical stimulation both are secreted in a Ca²⁺ dependent manner (Kirkpatrick and Burnstock, 1987; Lew & White, 1987). ATP causes contractions mediated via P₂ₓ-purinoceptors subtype. The co-released NA from sympathetic nerves also causes contractions. Released ATP evokes a depolarisation and generation of action potentials, while NA cause little or no depolarisation (Hoyle, 1992). EJP can be recorded in tissues in which the NA has been depleted by pre-treatment with reserpine (Burnstock and Holman, 1962; Brock et al., 1990). As result, according to this finding, it is most unlikely that the transmitter generating EJP is noradrenaline. Brock and Cunnane in 1992, showed that α-adrenoceptor antagonists and β-adrenoceptor antagonists did not alter EJPs amplitude. On the other hand, the P₂ₓ-purinoceptor antagonist ANAPP3 (Sneddon *et al.*, 1982), and suramine (Nally and Muir, 1992; Sneddon *et al.*, 1992) markedly reduced the amplitude of EJPs. Desensitisation of P₂ₓ-purinoceptors by application of the stable ATP analogue α-β-methylene ATP completely abolished EJPs (Sneddon and Burnstock, 1984a,b). A brief application of ATP to either the rodent vas deferens or the rabbit ear artery, produces a depolarisation which resembles the stimulation evoked EJP (Sneddon and Westfall,
1984; Suzuki, 1985; Cunnane and Manchanda, 1988). In these tissues focal application of NA produces non detectable alteration in membrane potential.

ATP released from autonomic nerves may act as a neuromodulator at pre-junctional or post-junctional sites. ATP may act on receptors located on nerve terminals and enhance or inhibit further release of transmitter from these nerves. In some situation ATP may be degraded by ectoenzymes, into adenosine or AMP, which may be responsible for the apparent action of ATP. At post-junctional sites, ATP may affect the responses produced by other transmitters. This is through a change in the affinity of receptors for the transmitters (Hoyle, 1992). However, in the mouse vas deferens, the pre-junctional inhibition of adrenergic transmission by ATP is not affected by blocking P$_1$-purinoceptors (Kügelgen et al., 1989). In the vas deferens, sub-threshold concentrations of ATP potentiate responses to noradrenaline and sympathetic nerve stimulation (Kazic and Milosavljevic, 1980; Huidboro-Toro and Parada, 1988).

1-9 The probability distribution of nerve stimulated responses of the mouse vas deferens

The process of transmitter release at all type of neuroeffector junctions can be described by several types of statistical distribution, but the ones which have been applied to transmitter release are, binomial and Poisson distributions. According to Dell Castillo and Katz (1956), transmitter release is a random manner. And the fate of each quantum of transmitter in response to an action potential has only two outcomes, the transmitter release either does or does not occur. This situation resembles a bino-
The probability of quantum being released by an action potential is independent of the probability of other quanta being released by that action potential (Kendal, 1991). Binomial distribution can only be valid where there is only one quantum available for release at the arrival of each nerve impulse, at a single release site. Release at the neuromuscular junction may fit a binomial distribution, due to the fact that the release of transmitter from sites local to the recording electrode can be relatively easily identified and does not involve complex analysis.

A Poisson distribution, where \((n)\) is the number of event occurring over a length of time, and it is independent of other similar events. If \(n\) is large, but the probability \((p)\) of an event occurring is constant and low, the process is best described by a Poisson distribution. Spontaneous quantal release is a stochastic process of Poisson type (Gage and Hubbard, 1965). This process could happen if the nerve terminal membrane is bombarded with synaptic vesicles, each with small probability of release (Dell Castillo and Katz, 1956). On the other hand, when the vesicle population is greatly depleted, the process remains Poisson (Gage and Hubbard, 1965). A Poisson process is a distribution where the mean and the variance are the same. In this process a large number of quanta are available for release from one release site but the probability of a single quantum being released is less than 0.5. The fact that sympathetic activity is difficult to describe, delayed the possibility of analysing it until recently. A fit to Poisson distribution was made by Blakeley et al., (1982), but only if some of the failures were first excluded from the analysis.
1-10 The rodent vas deferens and its innervation

The vas deferens is a muscular walled tube which connects the epididymis and the urethra. The wall is formed from two distinct muscle layers, the outer longitudinal and the inner circular. The lumen is lined with ciliated columnar epithelial cells. Using electron microscopy, Merrillees (1968) was able to make a detailed study of the arrangement of the muscle cells within the longitudinal layer of the guinea pig vas deferens. The longitudinal layer is organised into small bundles, surrounded by connective tissue sheaths, which in turn frequently anastomose with neighbouring bundles. Muscle cells are up to 450 μm in length. Along the entire length of the muscle cells a basement membrane separates the muscle cells from their neighbours. However at certain points along their length the cells are connected by the protrusion of the membrane of one cell into that of another, adjacent cell. There is an area of close contact between the cells both within these protrusions, and at other sites where the membranes come into close apposition (Burnstock, 1970; Westfall et al., 1975). This gap junction may provide the morphological basis of a pathway between cells.

The vas deferens is innervated by postganglionic sympathetic nerves (Sjostrand, 1965), which originate in the hypogastric ganglia situated within the pelvic plexus, close to the prostatic end of the vas deferens (Sjostrand, 1965; Ferry, 1967). The pre-ganglionic sympathetic fibres innervating these ganglia derive from the hypogastric nerve trunk, which branches before entering the pelvic plexus. The vas deferens nerves are mainly nonmyelinated post-ganglionic sympathetic fibres (0.2-1.7 μm in diameter), and are embedded in Schwann cells together with a small number of fine myelinated fibres (1-2 μm in diameter, originating in the pelvic plexus). The fibres divide into numerous branches as they enter, with the blood vessels, at the
prostatic end (Merrillees et al., 1963). The vas deferens nerve runs within the connective tissue sheath of the vas deferens, branches as it passes into the muscle coat, where these branches split into still smaller bundles of 2 to 8 axons. Fibres within the muscle coat and in the surface bundles become varicose (Bennet, 1972).

The varicosities are thought to be the site of transmitter release, since they are packed with vesicles, and are believed to be the site of transmitter storage (see Smith and Winkler, 1972). The varicosities, which are 0.5-1μm in diameter and approximately 1μm in length, are packed with vesicles and mitochondria, and occur at approximately 5μm intervals. The inter varicose regions are about 0.1-0.2 μm in diameter (see Burnstock, 1970). The Schwann cell covering becomes incomplete in the varicose region, leaving some nerve fibres exposed at points opposite to the smooth muscle cells. The exposed fibres end mostly within a shallow depression in the surface of a smooth muscle cell. The varicosities of these exposed fibres makes close contact with the muscle cells (Merrillees, 1968). In the mouse vas deferens close contact varicosities occur very frequently, to the extent that the majority of the muscle cells form close contacts with nerve cells.

1-11 Receptors in the mouse vas deferens

1- Purinoceptors:

Purinoceptors have been classified as P1 and P2 type by their relative agonist potency order. P1 purinoceptors are most sensitive to adenosine, whereas, P2 receptors are most sensitive to ATP (adenosine 5-triphosphate)
(see Hoyle and Burnstock, 1991). P\textsubscript{1} receptors have been further subdivided into A\textsubscript{1}, A\textsubscript{2} (Burnstock & Buckley, 1985) and A\textsubscript{3} (Ribeiro & Sebastiano, 1986). P\textsubscript{2} receptors have been also further subdivided into contracting P\textsubscript{2x} and relaxing P\textsubscript{2y} (Burnstock and Kennedy, 1985). Both receptor sub types have been identified in the mouse vas deferens (see Boland et al., 1992).

2- Adrenoceptors

Adrenoceptors have been classified into α and β type. This receptors classification was according to their agonist potency order. α-receptors have the order of noradrinaline >adrenaline> isoprenaline. While β-receptors have the reverse order of potency (Mariane Fillenz, 1990).

The α-receptors have been pharmacologically subdivided into α\textsubscript{1} and α\textsubscript{2}. Both types are present in the rodent vas deferens (see Stjarne & Astrand, 1985). The α\textsubscript{1} receptor subtype can be found on the smooth muscle cells membrane, while the α\textsubscript{2}-receptor subtype can be found on the nerve terminals (Starke, 1981). The α\textsubscript{1}-receptor mediates its effect through an intracellular second messenger system. Activation of α\textsubscript{1} receptors by an agonist stimulates the enzyme phospholipase C, which cleaves PIP (phosphatidylinostol biphosphate) into inositol triphosphate and diacylglycerol, influencing intracellular Ca\textsuperscript{2+} binding and leading to the opening of Ca\textsuperscript{2+} channels, by a mechanism which does not involve adenylate cyclase activation (for review see The Noradrenergic Neurone, Mariane Fillenz, 1990).

The contraction of smooth muscle cells in response to sympathetic stimulation in many organs is mediated by adrenoceptors with the pharmacol-
ogical characteristics of the $\alpha_1$ subtype. Among these are the smooth muscle of
the vas deferens, seminal vesicles, and epididymis, the vascular smooth mus­
cle of skin, splanchnic area and skeletal muscle (Hoffman, 1987). Relaxation
of these various smooth muscle cells is due to a decrease in sympathetic tone
(iris, vas deferens, seminal vesicles, epididymis). The mechanism of smooth
muscle control by NA is not fully understood. The rise in intracellular $\text{Ca}^{2+}$, re­
sponsible for the $\alpha_1$ receptor mediated contraction, is due partly to release of
$\text{Ca}^{2+}$ from intracellular stores, and can occur in the absence of extracellular
$\text{Ca}^{2+}$.

The first peripheral $\alpha_2$-receptors to be discovered were the presynaptic
autoreceptors on the noradrenergic nerve terminal. Activation is thought to lead to inhibition of release of NA in peripheral nerve terminals, but the exact mechanisms are still not known. It has been suggested that the physiological
effect of $\alpha_2$-adrenoceptors are via a decrease in intracellular cAMP. However,
this can not fully account for the physiological effect of $\alpha_2$-receptor activation.

1-12 Smooth muscle physiology

Smooth muscle cells which are spindle shaped cells with centrally located nu­
clei, appear circular in cross section. The presence of gap junctions between adjacent
cells allows the electrical continuity, and consequently permits a group of cells to function as electrical syncytia allowing a whole mass of hundreds to millions of mus­
cle fibres to contract as a single unit (Gyton, 1989). Tubular structures such as the di­
gestive tract, blood vessels, and vas deferens depend on layers of smooth muscle cells
in their walls for the control of their function. The change in length and/or diameter of smooth muscle are the basis for smooth muscle activities. Smooth muscle can be stimulated by nerve signals, hormonal stimulation, and local factors. Smooth muscle activities can be achieved by the antagonistic activities of the sympathetic and parasympathetic nervous system. However, in the vas deferens where joint innervation is absent, the local factors play an important role in opposing the activity of the sympathetic system bringing smooth muscle activity into an optimum state of control. Hormonal factors such as angiotensin, vasopresin, and histamine cause contraction of smooth muscle by acting on specific receptors on smooth muscle cell membranes. In small blood vessels where there is little or no nervous supply, yet the smooth muscle is highly contractile and responds to changes in local conditions in the surrounding environment. This is due to the presence of a powerful local feedback control system.

Reduction in oxygen tension, excess carbon dioxide, and high hydrogen ion concentrations are all local factors which lead to local vasodilatation. Other local factors include lactic acid, adenosine, potassium ions, histamine, calcium ions concentration, and temperature all are local modulatory factors affecting smooth muscle contraction.

Most of the current understanding of modulatory factors controlling smooth muscle contraction arise mainly from studies on blood vessels, as a result more is known about vascular rather than non-vascular smooth muscle. Nitric oxide (NO) and histamine have gained interest in the recent years. The interest in histamine arouse mainly because of the development of new agonists and antagonists, which opened the door to more research in this field. The interest in NO started after the discovery of NO-mediated neurotransmission in 1980. The local modulatory action of NO and
histamine are the basis for the research work included in this thesis. In the next sections NO and histamine will be dealt with in some detail.

1-13 Endothelial derived relaxing factor (EDRF)

EDRF was first established as a bioregulatory compound in the endothelium, controlling tone and hemodynamics, but its role has since advanced across almost all physiological systems in mammalian organs. Furchgott and Zawadzki discovered EDRF in 1980. Yet it was not until some years later that its equivalence to Nitric Oxide (NO) was proposed (Furchgot and Vanhoutte, 1988). Furchgot and Zawadzki demonstrated that the relaxation of a precontracted rabbit aortic ring preparation by acetylcholine (Ach) required the presence of intact endothelium. Endothelium dependent relaxation, which was subsequently demonstrated in many vascular preparations (such as veins, arteries and capillaries), occurs in response to a variety of substances e.g. Ach, adenine nucleotide, thrombin, Substance P. Hypoxia also causes endothelium dependent relaxation of vascular tissues in vitro. Other agents such as, nitrovasodilators, atrial natriuretic factor, β-adrenergic agonists, and prostacyclin, induce vascular relaxation by endothelium independent mechanisms (see Furchgot, 1984; Griffith et al., 1984; Buss et al., 1985; Moncada et al., 1986b).

It was established that EDRF is a very short lived substance, with a half-life of only seconds in oxygenated physiological salt solution (Griffith et al., 1984; Cocks et al., 1985). The effects of EDRF were shown to be inhibited by haemoglobin (Hb) and methylene blue (Martin et al., 1985), and to be mediated by stimulation of soluble
guanylate cyclase with the consequent elevation of intracellular cyclic GMP levels (Raport and Murad, 1983).

1-14 Identification of EDRF as NO

The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF release from vascular endothelial cells was detected by the chemical means used to identify NO. NO can be measured directly as the chemiluminescent product by its reaction with ozone. Dawnes et al., (1976), using this method, showed that the concentration of bradykinin that induced the release of EDRF from porcine aortic endothelial cultured cells, also caused a concentration dependent release of NO. In addition, the amount of NO released by these cells was sufficient to cause relaxation of vascular strips (Palmer et al., 1987). Moreover, the levels of NO released by these cells also accounted for the inhibition of platelet aggregation and adhesion induced by EDRF (Radomski, et al., 1987b,c). On the basis of studies on rabbit aorta and bovine pulmonary arteries (Ignarro et al., 1988), it was independently proposed that EDRF is identical to NO. This was based on similarities of properties (Ignarro et al., 1988; Furchgott, 1988):

1- Both are labile.

2- Both are potent relaxants of vascular smooth muscles.

3- Both activate sGC to increase level of cGMP.
4- Both are bound by haemoglobin, which selectively inhibits the relaxing activity of both.

5- Superoxide anion radicals caused inhibition of vasorelaxation of both.

6- Superoxide dismutase potentiated and stabilised the relaxing activity of both.

Despite these data, there is still some controversy as to whether EDRF and NO are equivalent. Some nonvascular muscle preparations were reported to be relaxed by NO but not EDRF (Buga et al., 1989; Dusting et al., 1988; Shikano & Berkowitz, 1987). The vasorelaxation action of EDRF can be lost on passage through anion exchange resin while that of NO is not (Cocks et al., 1985; Long et al., 1987). Therefore, was concluded by these works that EDRF is a more stable form of NO (Mayer, et al., 1990).

1-15 The arginine-NO pathway

In 1988, the amino acid L-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells. Endothelial cells cultured in the absence of L-arginine for 24 hours prior to the experiment, showed a decrease in the release of EDRF induced by bradykinine, which could be restored by L-arginine but not D-arginine (Palmer et al., 1988a). This enhancement only occurred in the presence of L-arginine infusion, suggesting that the formation of NO was dependent on free L-arginine. The conversion of L-arginine to NO is specific because a number of ana-
logues of L-arginine failed as a substrates for NO production (Palmer et al., 1988).

Several studies demonstrated that L-arginine acts as a substrate for NO synthase (nitric oxide synthase), the enzyme involved in the conversion of L-arginine to NO and citrulline (Palmer et al., 1987; Palmer et al., 1988; Moncada et al., 1989).

1-16 Discovery of NO-mediated neurotransmission and neuromodulation

Most of the research leading to the recognition of NO involvement in neuroeffector transmission was carried out with rat anococcygeus and bovine retractor penis muscle (see Gillespie, 1987; Gillespie et al., 1990; Martin and Gillespie, 1991). Field stimulation produced a noradrenaline mediated contraction, but when the contractile response was blocked and the tone was raised, there was a neurogenic non adrenergic, non cholinergic (NANC) relaxation, that was not attributable to any of the other mediators recognised before the discovery of NO-mediated transmission. It had already been shown that there were many similarities between EDRF and the inhibitory transmitter of the anococcygeus muscle and retractor penis muscle (Gillespie, 1987; Furchgott, 1988). Then several different independent groups used inhibitors of NO synthase to provide definitive evidence for NO-mediated transmission in the anococcygeus muscle (Gillespie et al., 1989; Gibson and Mirzazadeh, 1989; Li and Rand, 1989b).

NO is a highly atypical neuronal messenger. NO, unlike any other traditional neurotransmitter cannot be stored and released from lipid lined packets (vesicles), this is similar to PGs (prostaglandin). NO is released by NO synthase, which is the rate limiting step in the regulation of NO. NO synthase is one of most regulated enzymes in
biological systems, its activity is regulated at multiple levels, at post-transcriptional alteration of NO synthase by protein phosphorylation, myristylation, and other modifications, which alter the enzyme activity and its subcellular distribution. NO, once synthesised, readily diffuses through cell membrane where it binds and influences many targets (see Rand and Li, 1989). NO exerts a possible neuromodulatory role in other systems. In the guinea pig ileum and pulmonary artery, inhibition of NO synthase enhances nerve evoked contraction (Gustafsson et al., 1990)

1-17 Criteria for establishing NO as neuromodulator:

Identification of neuromodulator requires the fulfilment of number of criteria defined by Dal in 1933, to be accepted as a biological mediator:

1- A system for synthesis of the modulator should be present at the appropriate site: NO synthase is the enzyme involved in the production of NO from L-arginine. NO synthase is nicotinic adenine dinucleotide phosphatase (NADPH) dependent, and the NO synthase from brain or endothelial sources is calcium and calmodulin dependent (Buss and Mulsh, 1990; Forestermann et al., 1991; Mayer et al., 1991). Three types of NO synthase have been identified, type I, type II, and type III.

a- Type I, or the constitutive NO synthase: this enzyme is present mainly in the brain, it is calcium and calmodulin dependent (Bredt and Snyder, 1990), and requires the addition of NADPH and FAD.
b- Type II, or inducible NOsynthase: this type is Ca^{2+} independent. Induced by various stimuli, for example from activated macrophages. Inducible NOsynthase contains calmodulin tightly bound as a sub unit, suggesting that all NOsynthase isoforms require bound calmodulin for their activity (Chao et al., 1992). The expression of this isozyme is induced by cytokines in macrophages and many other mammalian cells, including brain microglia, suggesting an important role of inducible NOsynthase in neurotoxicity (Boje and Arora, 1992; Chao et al., 1992).

c- Type III, or endothelial NOsynthase: this type is found in endothelial cells. It is Ca^{2+}/calmodulin dependent, and requires NADPH and BH4 for optimal activity (Pollock et al., 1991). Recent work has shown that the enzyme is predominantly localised in KCl-insoluble membrane fractions (Forestermann et al., 1991).

2- Release of modulator substance in appropriate amounts during neurotransmission: there is no fixed system of release of NO. However, NO is formed by NOsynthase on demand by the tissue. NO is a highly diffusible and labile substance, and it is not possible to store, but NO producing substances (such as nitrosothiol), could be stored in transmitter storage vesicles (Ignarro, 1990). However, the rapid action of NOsynthase inhibitors in blocking NO-mediated effects, suggest that there is little or no preformed store, and consequently the transmitter must be formed and diffuse to the effector sites on demand (Li & Rand, 1989b; Starke et al., 1991).
3- The substance should produce an effect specified in vivo and in vitro.

4- Mechanisms for terminating the action of the modulatory substance should exist: NO is a highly reactive free radical with a very short half-life (4-50 ms) (Foresterman et al., 1984). This offer a very effective mechanism for terminating its action.

5- Receptors for the modulatory substance should be demonstrable on or in relevant cells: Results obtained so far prove that NO can be a neuromodulator in some systems and a neurotransmitter in others. Result obtained in this thesis showed that NO imposes a neuromodulatory action on transmission in the vas deferens. This suggest that receptors for NO may be present in this tissues and other tissues. But so far it has not been possible to demonstrate any receptors for NO.

6- A predicted alteration to the response should be produced by drug which interfere with the action or synthesis of the modulator substance. To date several lines of evidence have indicated that L-arginine is the physiological precursor in the formation of NO. NOSynthase inhibitors were developed as competitive inhibitors of NOSynthase, blocking the synthesis of NO. Consequently, they should give effects opposite to that of NO. NOSynthase inhibitors such as M⁶-monomethyl-L-arginine (L-NMMA) (Palmer and Moncada, 1989) and N⁶-nitro-L-arginine (L-NNA) (Bredt and Snyder, 1989) are two examples of NOSynthase inhibitors, both resulted in increase in blood pressure most likely due to inhibition of basal production of NO released from the endothelium (Palmer and Moncada, 1989). L-NMMA re-
duced NANC nerve-induced relaxation in rat and guinea pig anococcygeus muscle. The effect of L-NMMA was reduced by the addition of L-arginine (Gillespie et al., 1989; Li and Rand, 1989).

7- Appropriate increases or decreases in the responses should be produced by experimental techniques or clinical conditions which result in deficiencies of the substance or of the synthesising or metabolising enzyme. L-arginine reduced nerve evoked contraction in the mouse vas deferens. While L-NAME produced the opposite; increasing nerve evoked contraction (R. Patterson, Leicester University, 1996). Clinical experiments showed that \( \text{NG}^\text{G} \)-monomethyl-L-arginine (L-NMMA) caused a transient increase in blood pressure and reduction in heart rate. The study concluded that acute systemic NO synthase inhibition increased blood pressure, reduced heart rate, and reduced cardiac index (Gastellano et al., 1995).

1-18 Mimicry of the NO-mediated mechanism

One of the important pieces of evidence for the existence of a transmitter is the mimicry of the response to nerve stimulation by exogenous administration of the transmitter. It is important to establish whether the properties of NO-mediated transmission, does not correspond to free NO, but to NO donors or producers. NO in aqueous solution, produced by bubbling NO gas through deoxygenated water, mimics the responses to NO transmission, but its potency is rather weak compared to many other NO donors. Many other NO donors are available, for example, inorganic and organic nitrite (Furchgot, 1988; Stambler et al., 1992), Glycerol trinitrate (GTN) (Needleman

1-19 Modifying agents to NO mediated neurotransmission and neuromodulation

**Oxyhemoglobin**: inhibits EDRF in inducing vasodilatation and relaxation (see Gellispie et al., 1990; Luscher and Vanhoutte, 1990, Martin and Gellispie, 1991)

**Hydroxocobalamin**: hydroxocobalamin resulted in inhibition of the relaxation induced by NO and SNP, but had little effect on responses to GTN and SNAP in rat anococcygeus muscles (Li and Rand 1993a).

**Alcohol**: Ethanol resulted in inhibition of relaxation of the rat and rabbit anococcygeus and retractor penis muscles (Gellespie et al., 1982).

**Superoxide and superoxide generators**: superoxide rapidly inactivates EDRFm and elimination of superoxide with superoxide dismutase (SOD) prolonged the half-life of the NO biological system (Gryglewskiet et al., 1986)

**Free radical scavengers**: hydroquinone has free radical scavenging activity. It inhibits relaxation in response to NO in rat anococcygeus muscle, but not in bovine retractor penis muscles (Gellispie and Sheng, 1990).
1-20 Mechanisms of NO-mediated transmission

NO mediated transmission includes activation of intraneuronal NOsynthase by a rise in Ca^{2+} concentration. The activated NOsynthase converts L-arginine to NO, then NO passively diffuses into the effector cells to impose its action, which involves binding the haem group of the soluble guanylyl cyclase, and activation of the enzyme, resulting in the production of cGMP, which mediates relaxation (Sanders and Ward, 1992).

1-21 Modulation of NO-mediated mechanism

Pre-junctional modulation, the automodulation via negative feedback mechanisms, has been demonstrated for many types of transmission, but does NO-mediated mechanism have this property? NO autoinhibitory mechanisms would probably act through a mechanism based on the finding that the activity of NOsynthase was enhanced by oxyhaemoglobin and decreased by NO and NO donors (Rogers and Ignarro, 1992; Assruy et al., 1993). It was thought that the inhibitory effect of NO was due to its combination with haem group associated with the enzyme.

Muscarinic cholinocceptor agonists inhibit NANC mediated relaxation of the rat anococcygeus muscle (Li and Rand, 1989a). The \(\alpha_2\)-adrenoceptor agonist UK-14304 inhibited NANC relaxation of the rat fundus (MacDonald et al., 1990). On the other hand, clonidine had no effect (Lefebure and Begart, 1986). In some tissues where both noradrenergic and NO-mediated transmission was present, NA contraction
was enhanced by NOsynthase inhibitors, e.g. in the anococcygeus muscle (Li and Rand, 1989b; Liu et al., 1991). This could mean that there is a negative feedback mechanism via \( \alpha_2 \)-adrenoceptors regulating NANC mediated transmission, most probably similar to that regulating other transmitters such as NA and ATP, resulting in more tuning of neurotransmission process taking place in those tissues.

1-22 **NOsynthase inhibitors**

The most commonly used NOsynthase inhibitors are the stereospecific analogues of L-arginine. The most widely used in studies on neuroeffector transmission are N-monomethyl-L-arginine (LNMMA), N-nitro-L-arginine (LNNA), and its methyl ester (L-NAME) (Moncada et al., 1991). With high concentrations of these nitroarginine derivatives, interaction with NOsynthase is irreversible, but their inhibitory action is blocked or attenuated by prior exposure to high concentration of L-arginine (Martin et al., 1993). Other inhibitors include NOsynthase cofactors, such as calmodulin inhibitors, haem inhibitors and hypoxia.

1-23 **NO scavengers**

NO scavengers, the imidazolineoxyl N-oxide derivatives, have been shown to react with NO, resulting in inhibition of NO biological function. The derivatives 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (PTIO), carboxy-PTIO, and carboymethoxy-PTIO are the most studied (Akaike et al., 1993; Yoshida et al., 1994). All PTIOs used inhibited NO-related vasorelaxation induced by Ach. Similarly va-
sorelaxation induced with ATP was also markedly inhibited by all PTIOs (Meade et al., 1994). This inhibition of smooth muscle relaxation was not abolished by the addition of L-arginine, whereas L-arginine significantly reverses the inhibition of vasorelaxation by L-NNA. This indicates that the inhibition of vascular relaxation by PTIOs was not due to direct inhibition of NO synthase (Moncada et al., 1989). L-arginine analogues such as L-NNA and L-NMMA, are competitive inhibitors of NO synthase, thus the presence of L-arginine can reverse these inhibitory effects (Kilbourn and Griffith, 1992). An attempt was made in this thesis to test the effect of PTIO on the EJCs of the mouse vas deferens smooth muscle cells.

1-24 The role of NO in different tissues

1-24.1 Vascular regulation

Several endothelium-dependent vasodilators such as acetylcholine (Ach), bradykinine (BK), substance P, histamine, and $\alpha_2$-adrenoceptor agonists, can lead to the release of NO from vascular endothelium. These endothelium-dependent vasodilators cause a rise in intracellular calcium levels, either from internal stores, or from extracellular flux, which will lead to activation of NO synthase. L-arginine-NO pathways have been shown to exist in many parts of the vascular system, including pulmonary, coronary, systemic, mesenteric, and cerebral vasculature. NO synthase activity is the factor responsible for the maintenance of basal dilatory vascular tone (see Kerwine and Heller, 1994). NO regulation is mainly important in large arteries than in arterioles (Toda et al., 1990). However, even in arterioles NO has an important role in regulating vasodilatation. Blockade of NO synthesis enhances contractile responses to
transmural nerve stimulation in guinea-pig pulmonary artery, indicating neuromodulation by endogenous NO (Cederqvist and Gustafsson, 1994).

1-24.2 NO and insulin regulation

It is well established that L-arginine can evoke the release of insulin in the presence of D-glucose (Blachier et al., 1989). L-arginine evoked insulin secretion can be inhibited by L-NNA and L-NMA both in vivo and in vitro (Schmidt et al., 1992).

1-24.3 NO in the central nervous system

In cerebral preparations, L-glutamate resulted in activation of NO synthase, which in turn led to activating sGC and raising cAMP levels (Garthwaite, 1991). Other brain regions and preparations, such as hippocampal neurones and slices, and primary cortical neurones possess similar NO synthase activity.

It has been proposed that NO is neurotoxic and linked to NMDA-receptor activation, and that NO synthase inhibitors could be neuroprotective in vitro. Systemic administration of L-NAME under conditions which do not drastically increase the mean arterial blood pressure in a middle cerebral arterial occlusion model in rats and mice, resulted in reduction in the infarct volume measured histologically (Yamato et al., 1992). The role of NO in the pathophysiology of ischemic stroke is potentially double edged, inhibition of neuronal NO formation may be neuroprotective, inhibition
of endothelial-derived NO formation can be cytoprotective (Kerwine and Heller, 1994).

NO may play a role in other central nervous system actions. This includes a role in long term potentiation (LTP), L-NNA prevented memory formation (Holscher and Rose, 1992). NO synthase has also been implicated in several forms of analgesia (see Duarte and Ferreira, 1992; Kwabata et al., 1992). In addition, NO synthase has been demonstrated in preganglionic sympathetic and postganglionic parasympathetic neurones. The main effect of NO synthase inhibition seems to be related to attenuation of basal endothelial NO production and parasympathetic transmission (Modin et al., 1994).

1-24.4 NO in the peripheral nervous system

Non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission has been a debatable topic for several years regarding the transmitters that mediate it. Gastrointestinal smooth muscle function is regulated by NANC nerves which when stimulated produce relaxation. Immunohistochemistry has shown that NO synthase is expressed in the enteric neurones (Llewellyn-Smith et al., 1991). Evidence regarding the role of NO in neurotransmission is accumulating, for example in canine lower oesophageal sphincter (Bult et al., 1990), pylorus and stomach (Allescher et al., 1992), and mouse anococcygeus muscle (Rand and Li, 1992). NO could be one of NANC pathways present in these tissue.
Penile corpus cavernosum is another tissue which has been investigated for NO pathways (Sjostrand, 1990). Electrical field stimulation of rabbit and human corporeal strips releases NO and increases cGMP levels in this tissue. Further support for this role was obtained by immunohistochemical staining for NOsynthase (Burnett et al., 1992). In guinea-pig vas deferens, exogenous NO affected adrenergic and non-adrenergic non-cholinergic neurotransmission in a complex fashion, without alteration in noradrenaline release (Cederqvist and Gustafsson, 1994). The mouse vas deferens was shown to contain NO. The addition of L-arginine reduced mechanical responses and evoked responses to a significant level. L-AME (NOsynthase inhibitor) resulted in the opposite (R. V. Patterson, Leicester University, 1996). In this thesis the presence of NO-mediated mechanisms will be investigated in some detail.

1-25 Histochemical localisation of NOsynthase

The fact that NOsynthase requires reduced nicotine adenine dinucleotide phosphate (NADPH) as a cofactor, and the electron transferring terminal sequence of NOsynthase gives rise to its NADPH diaphorase activity (Dawson et al., 1991). So the enzyme can reduce a dye such as nitro blue tetrazolium (NBT) to an insoluble, visible reaction product, formazan (Kuhn and Jerchel, 1941). But the question is does all NADPH diaphorase activity represent NOS? In fresh tissue a large fraction of NADPH dependent diaphorase is not NOsynthase (Matsumoto et al., 1993). However, NOsynthase-1 diaphorase activity resists aldehyde fixation (Weinberg et al., 1994). As a result, after fixation, most NADPH staining represents NOsynthase-1. The histochemical procedure of demonstrating NOsynthase activity, when used properly, could
provide a cheap, convenient and reliable marker for detection of multiple isoforms of NO synthase (see Weinberg et al., 1996).

It has been found that NO synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissue. NO synthase immunoreactivity and NADPH diaphorase staining are co-localised in the pedunculopontine nucleus, and large and aspiny neurones (Dawson et al., 1991).

It has been found that NO synthase activity and NADPH diaphorase copurify to homogeneity and that both activities are immunoprecipitated by antibodies recognising neuronal NADPH diaphorase. Furthermore, NO synthase was competitively inhibited by the NADPH diaphorase substrate, nitro blue tetrazolium. Thus, neuronal NADPH diaphorase is NO synthase, and NADPH histochemistry, therefore provides a specific histochemical marker for neurones producing NO (Hope et al., 1991).

Localisation of NO synthase was made possible, through immunohistochemical techniques, using antisera to the purified enzyme (Bredt et al., 1990). Bredt and his co-workers were able to demonstrate that NO synthase in the brain was exclusively associated with a discrete neuronal population. They were able to localise NO synthase in other tissues e.g. autonomic nerve fibres in the retina, in cell bodies and nerve fibres in the myenteric plexus of the intestine, in adrenal medulla and vascular endothelium. These findings of neuronal localisation provided the first conclusive evidence for strong association of NO with neurones.

Some research showed that there is a regional variation between NO synthase containing nerve fibres along the length of the vas deferens. NO immunofluorescent reactivity was mainly in the abdominal and the intermediate portion of the vas def-
rens. These variations were age dependent, being more evident in adult and aged rats compared to immature rats (Ventura et al., 1996).

In this thesis an attempt has been made to demonstrate the presence of NO-synthase, through its NADPH diaphorase activity, by adding NBT to form the insoluble coloured stain formazan. An attempt has been made to demonstrate NOsynthase by electron microscopy.

1-26 Histamine: physiological role

Histamine is closely associated with mast cells in almost all tissues, and as a result it role has been established as a mediator of inflammation, leading to its general description as autocoid or hormone (Rile and West, 1966; Bean, 1982). However, not all histamine is associated with mast cells, histamine has been found in basophils (Graham et al., 1955), platelets (Goth, 1978), enterochromaffin like cells (Hakanson et al., 1974), endothelial cells (Karnishina, 1980), and neurones (Schwartz et al., 1980a., 1986a). These sites of histamine synthesis and storage give us an idea about the multiple roles of histamine other than mediation of inflammatory response.

Histamine is widely distributed within the mammalian central nervous system (CNS) (Adam and Hype, 1966; Taylor and Snyder, 1971). There is strong evidence that histamine might have a role as a neurotransmitter or neuromodulator (Perll and Green, 1986, Schwartz et al., 1986b). Histamine has been found in both neuronal (Schwartz et al., 1980a, 1986b) and non neuronal compartments of mammalian brain (Edrisson et al., 1977).
Some reports have indicated the presence of histaminergic fibres in rabbit reti-
na (Ando-Yamamoto et al., 1987), rat vas deferens (Campos, 1988), and guinea pig
stomach and colon (Hakanson et al., 1983). Several authors have suggested roles for
histamine in the nervous system, such as the control of arousal (Kalivas, 1982), car-
diovascular homoeostasis (White, 1965), temperature regulation (Shaw, 1971) and
neuroendocrine mechanisms (Weir and Gannog, 1978). The presence of histamine in
the mouse vas deferens have been demonstrated in the mouse vas deferens (R. V.
Patterson, Leicester University, 1996). Histamine resulted in reduction of the me-
chanical contractile responses of mouse vas deferens. In this thesis the role of hista-
mine has been investigated in some detail, establishing its modulatory effect on auto-
nomic neurotransmission in the mouse vas deferens.

1-27 Histamine receptors

Histamine receptors have been divided into three major subtypes, H₁, H₂, and
H₃ on the basis of quantitative studies on isolated peripheral tissue and brain slice
preparations. Ash and Schild (1966) were the first to subdivide histamine receptors,
they introduced the term H₁ receptors, to describe the class of histamine receptors that
was sensitive to inhibition by low concentrations of classical antihistamines such as
promethazine and mepyramine. However, some responses to histamine, e.g. stimula-
tion of acid secretion from the gastric mucosa, were resistant to antagonists such as
mepyramine (Ash and Schild, 1966). James Black and his colleagues named those re-
ceptors as H₂-receptors, when they succeeded in developing a selective antagonist,
burimamide. A third group of histamine receptors, H₃, was proposed by Arrang and co
workers (1983), to explain the mechanism of autoregulation of histamine release by autoreceptors, using rat cerebral cortical slices.

1-27.a Histamine H1 receptors:

Smooth muscle contraction is one of the best characteristic of H1 responses. In smooth muscle that freely generates action potentials e.g. intestinal smooth muscle, H1-receptor stimulation leads to an increase in the frequency of action potential discharge in longitudinal smooth muscle of the guinea pig ileum (Bolton et al., 1981). Another action of H1-receptor stimulation in the cardiovascular system is its effect on the leakage of plasma protein from various vascular beds (Majno and Plade, 1961). Histamine has been shown to affect the secretion of catecholamine from adrenal glands (Emmelin and Muren, 1994). Histamine H1-receptor stimulation can produce an increase in the force of cardiac contraction (Levi et al., 1982), and exerts an important role in modulating carbohydrate metabolism (Quach et al., 1978).

1-27.b Histamine H2-receptors:

There are four major functional responses to H2-receptor stimulation: they are gastric acid secretion, smooth muscle relaxation, effect on cardiac muscle and effect on the immune system. H2-receptor stimulation produces relaxation of smooth muscle in a number of tissues including airway (Eyre and Chand, 1982; Foreman et al., 1985b), and vascular smooth muscle (Reinhardt
and Ritter, 1979). Sympathetic excitatory nerves controlling rat vas deferens contractility can be negatively modulated by histamine through the activation of presynaptic receptors which resemble the $H_2$ histamine receptor subtype (Poli et al., 1994).

1-27.c Histamine $H_3$-receptors

The presence of $H_3$-receptors on histamine nerve terminals was first demonstrated by Arrang et al. (1983) working with rat brain tissues. Three major functional responses have been identified as effects of $H_3$-receptors on the effector target.

1- The effect of histamine $H_3$-receptors on histamine release: $H_3$-receptor stimulation may restrict the influx of calcium ions, which is essential for histamine release into the histamine containing nerve terminals. This has been demonstrated by using slices of cerebral cortex loaded with $[^3]H$ histidine (Arrang et al., 1983; 1985b).

2- The effect of histamine $H_3$-receptors on histamine synthesis. Histamine synthesis, induced by depolarising stimuli, can be attenuated by exogenously applied histamine (Arrang et al., 1987b). This effect can be mimicked by (R)-$\alpha$-methylhistamine and antagonised by burimamide, impromidine, and thioperamide, indicating the involvement of $H_3$-receptors (Arrang et al., 1987a,b).
3- The effect of histamine \(H_3\)-receptors on inhibition of the release of other neurotransmitters. Preliminary evidence showed that \(H_3\)-receptor stimulation can inhibit the electrically evoked release of \([^{3}H]5\text{-HT}\) from rat cerebral slices (Schlicker et al., 1987). The inhibitory effect of histamine (10\(\mu\)M) on 5-HT release was antagonised by (32\(\mu\)M) burimamide and (1\(\mu\)M) impromidine, but not by (10\(\mu\)M) pheniramine or (3.2\(\mu\)M) ranitidine (Schlicker et al., 1988).

In addition, histamine regulates substance P release via pre-junctional histamine \(H_3\)-receptors that are located on the peripheral ending of sensory nerves in the rat hind paw, and this is mediated via ATP-sensitive \(K^+\) channels (Ohknbe et al., 1995).

Other studies have indicated that histamine release in mammalian brain can be regulated by muscarinic receptors (Gulat-Marcy et al., 1989a), and \(\alpha_2\)-adrenoceptor stimulation (Hill and Straw, 1988). In guinea pig mesenteric artery nerve, stimulation produces an excitatory junction potential in the vascular smooth muscle cells that can be inhibited by histamine presynaptically (Ishikawa and Sperelakis, 1987). This effect is mimicked by R-\(\alpha\)-methylhistamine, and competitively antagonised by impromidine and burimamide. This indicates that \(H_3\)-receptors may produce vasodilatation by inhibiting the release of sympathetic neurotransmitters (Ishikawa and Sperelikis, 1987).
Electrophysiological studies in the guinea pig ileum indicate that H3-receptors may also regulate neurotransmission at nicotinic synapses of parasympathetic ganglia in the myenteric plexus (Tamura et al., 1988). It was recently reported that the inhibition of noradrenaline release was mediated by the activation of pre-synaptic histamine H3 receptors, which could be related to a decrease in Ca\(^{2+}\) influx through N-type voltage sensitive Ca\(^{2+}\) channels in nerve terminals (Endou et al., 1994). However, in rat brain cortex slices (Schlicker et al., 1989), and in the pig retina (Schlicker et al., 1990), the activation of presynaptic H3 receptors does not inhibit the evoked release of noradrenaline unless the medium contains either phentolamine or a selective \(\alpha_2\)-adrenoceptor antagonist such as raulscine. Furthermore, \(\alpha_2\)-adrenoceptor blockade potentiates the inhibitory effect of histamine H1 receptor agonist in the mouse brain (Schlicker et al., 1992b), and consequently it has been suggested that presynaptic \(\alpha_2\)-adrenoceptors and presynaptic histamine H3 receptors interact, either at the level of the receptors or at a step beyond the receptors (Schlicker et al., 1992). This interaction could be species and/or tissue specific. On the other hand, in the myenteric plexus there is a negative co-operativity between A2-receptors and histamine H3-receptors or \(\alpha_2\)-receptors, which is a consequence of interaction taking place at the postreceptor levels. This is only if the agonists are co-administered, or when one kind of receptors is stimulated before the administration of the agonist of the second receptor. The mechanisms underlying the inhibitory effect of H3-receptor stimulation on neurotransmitter release in central and peripheral tissues remains to be established (Schlicker et al., 1992).
The hypothesis

Despite the large amount of information that has accumulated so far regarding autonomic neurotransmission, more is still remains unknown. In this thesis, the experiments were designed to uncover some of the facts about neuromodulation by local factors in the autonomic nervous system of the rodent vas deferens, in particular Nitric Oxide and histamine. The experiment in this thesis were designed to address the following aspects:

1- Is the mouse vas deferens sensitive to NO?

2- Does the mouse vas deferens have NOsynthase?

3- Does the mouse vas deferens possess a NO pathway?

4- Does the elimination of NO by NOsynthase inhibitors or NO scavengers have any effect on neurotransmission in this tissue?

5- Does NO modulate the release of transmitters in this tissue, and if so, is the modulation pre-junctional or post-junctional or both?

6- Is it possible to demonstrate the presence of NOsynthase histochemically?

7- Does the mouse vas deferens contain histamine receptors?

8- Do those histamine receptors modulate transmitter release in this tissue?
2- METHODOLOGY

2-1 Animals used

Male BKW mice aged between 6 and 8 weeks were used. The animals were anaesthetised with chloroform and decapitated. The abdominal wall was opened and the abdominal cavity was exposed close to the reproductive organs. Both vasa deferentia were dissected and placed in a container containing gassed Krebs saline. Throughout the dissection the abdominal cavity was bathed with 37 °C Krebs saline.

2-2 Mechanical responses, stimulation and recording protocol

The mouse vas deferens was suspended in an organ bath between parallel platinum wires, which formed the stimulating electrodes. The tissue was attached to a transducer from its epidydimal end, while the prostatic end was attached to a curved piece of metal to position the tissue in the middle of the electrical stimulating field (Figure 1). The tissue and Krebs saline were maintained at 37 °C by warm water circulating in a glass envelope surrounding the inner glass tube where the tissue was suspended (Figure 2). The Krebs saline was kept in a container above the level of the organ bath. The organ bath and the container containing the Krebs were both continuously gassed with a mixture of 95% O₂, and 5% CO₂, the pH of the Krebs was 7.4. Krebs saline in the organ bath was changed after each stimulus through a tap at the lower end of the organ bath, and at the same time a fresh Krebs saline replaced it, this was done through another tap at the other end of the lower part of the organ bath.
Figure 1. Diagram showing the electrode assembly used to hold the vas deferens in mechanical response experiments. The isolated vas deferens was suspended between two parallel platinum stimulating electrodes. The vas deferens was suspended with its epydidimal end attached to the isotonic transducer, and the prostatic end attached to a hook. The whole electrode assembly unit was inserted inside the organ bath (figure 2) and stabilised by a holder.
Figure 2. Diagram showing the organ bath where the electrode assembly unit with the vas deferens attached to it was placed during mechanical response experiments.
2-2.1 Evoking contraction

2-2.1.a Exogenous agonist

The agonist was added to the organ bath where the tissue was suspended. The agonist was washed out immediately after the peak contraction. Rapid washing out is important to prevent the development of tissue desensitisation to the drug.

2-2.1.b Field stimulation

Field stimulation was produced by a Square One stimulator. The stimulus was delivered to the tissue by parallel platinum stimulating electrodes in the organ bath. Contraction was recorded by a Linseis penchart recorder, through a transducer connected between tissue and the recorder.

2-2.2 Stimulation parameters

Preliminary experiments were carried out to determine the stimulating parameters that could give measurable and reasonable responses. A train of 10 pulses at 5Hz, 0.6ms pulse width with 10 minute rest interval between trains. Stimulus intensity was between 20-45 Volts. This stimulus protocol was chosen to give real sustained measurable responses.
A number of conditioning stimuli were given at the beginning of each experiment, as trains of 10 pulses at 5Hz, 0.6 ms pulse width at 30 Volts. The response to the fourth stimulus was taken as a control response, all responses were normalised to this (normalising response). Experimental readings were all then expressed as a percentage of this normalised response. Depending on the individual experiment drugs were added. In all experiments parallel responses were recorded after the normalising response, this is referred to as an untreated (control) response. Drugs were added following this step and the proper incubation period was allowed, followed by stimulation with the same stimulation protocol and recording under the effect of the drug (Figure 3).

**Figure 3.** Stimulation protocol for isolated preparation of the mouse vas deferens. Preparations were field stimulated with repeated trains of 10 pulses, 0.6ms pulse width, with 10 minute rest intervals between each train. All contractile responses were normalised to the forth response and the responses were expressed as the percentage change calculated from this normalising response. Addition of the drug was made to the organ bath immediately after the forth normalising response, and the proper time was allowed before recording the contractile responses. The organ bath was washed out with fresh Krebs saline after each response was recorded. Control experiments followed the same protocol, but without the addition of drugs.
The isolated vas deferens was placed in a 3ml organ bath, the tissue was pinned through the connective tissue sheath into the base of the organ bath without stretching the preparation. The base of the organ bath was covered with a Sylgard layer. The bath was continuously perfused with fresh Krebs saline. The Krebs saline was continuously gassed with 95% O₂, and 5% CO₂. The organ bath temperature was maintained at 37°C and the whole surrounding was enclosed in an atmosphere of 95% O₂ and 5% CO₂.

The prostatic end of the vas deferens was sucked into the stimulating electrode. The stimulating electrode as shown in figure 4 consists of 3mm diameter polythene tubing, 2 silver rings 3mm apart and was mounted on the edge of the polythene tubing through which the prostatic end of the vas deferens was sucked.
2-2.1 Stimulation parameters

Preliminary experiments were carried out to choose suitable stimulation parameters that gave reasonable measurable electrical activity without giving a mechanical component, to avoid losing the release site under the recording electrode. The tissue was always stimulated by the following stimulation parameters:

- train of pulses: 20 pulse
- pulse duration: 0.06-0.15 ms
- rest interval: 1 minute
- frequency: 1Hz
2-3.2 Recording by focal extracellular electrode

Glass electrodes were made from a 2.2mm diameter glass tubes pulled using KOPF vertical electrode puller (model 720). The tip of each electrode then carefully broken to give a diameter of about 50μm. The electrode then was filled with Krebs saline. A silver wire electrode and the perfusion tube were inserted into the recording electrode as close to the tip as possible, but without obstructing it. The glass recording electrode was inserted into a holder which was attached to a NEUROLOG AC preamplifier (NL104). The tip of the recording glass electrode was placed very carefully on the surface of the vas deferens making close contact but without depressing it.

Electrical activity under the glass electrode tip was amplified by a NEUROLOG NL 120 AC amplifier. Signals was viewed on a Nicolet 3091 oscilloscope screen and digitised using CED 1401 digital converter. Signals were then passed into an IBM WCP (whole cell program), provided kindly by Dr. John Dempster from Strathclyde University. The analysis and storage was carried out using a WCP program.

2-3.3 Addition of drugs

In this thesis the addition of drugs was made through the recording electrode only. This was done by adding the drug to the reservoir which supplies the recording electrode. About 3 minutes was needed for the drug to reach the tip of the recording electrode (figure 5).
2-3.4 Method of analysis

The analysis of responses was done through the WCP. The first step was to determine and set the zero level to all responses, this can be done automatically through the WCP program. This followed by measuring the amplitude and latency for each response manually. Histograms of latency were constructed, and then the optimum release site was selected, this was done by choosing the latency with the
maximum number of responses. This was followed by selecting an amplitude with the appropriate release site depending on the suitable latency chosen. A histogram of EJC's amplitude was constructed, and the most suitable bin width was selected, the one which give the best fit of almost equally spaced preferred values. The final distribution was reached after several trials of different bin width. Once this was reached then it is evidence that the recording had been made from one release site. The window of preferred values was marked and the number of events in each window was counted (Figure 6).

Figure 6. The graph shows the distribution of responses where the proper bin width had been chosen to give equally spaced preferred values (arrows). The window around each value was identified (dotted lines). Each window represents quanta, the numbers above the arrows represent the quantal content. The number of event within a window is the number of event that quanta.
Once the peaks of preferred value had been identified and the window defining each preferred value marked with the peaks of preferred value in its centre, the number of responses occurring within that window was counted.

2-3.5 Fitting the responses to a Poisson probability distribution

It is possible to determine the effect of drug treatment or any other factor, by fitting the EJCs data to a Poisson distribution. This process assists in the identification of any pre-junctional or post-junctional changes and in turn the possibility of identifying the underlying mechanisms which might had played a role in producing those changes. The number of responses counted was fitted to a Poisson distribution using Excel spread-sheet to accelerate the process. Table 1 shows an example of this spread sheet.
Table 1. Poisson fitter from Excel spread sheet, through which the mean quantal content was counted. Observed column contains raw data (EJCs). Poisson distribution was calculated. The best fit of the calculated data to a Poisson distribution is with minimal errors (E).

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<th>Number of stimuli</th>
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<th>Observed n (o)</th>
<th>E Error</th>
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</table>

<table>
<thead>
<tr>
<th>N. of Responses</th>
<th>Poisson events</th>
<th>Total E</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>84.13</td>
<td>44.01</td>
</tr>
<tr>
<td>Mean(m)</td>
<td>Non P Zero</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>2.86</td>
<td></td>
</tr>
</tbody>
</table>

The above table was used to calculate the best fit of observed raw data to a Poisson distribution this was done as follows:

1- Raw data was entered into observed column

2. Automatically the number of events for each value of quanta was calculated using the following formula $P = \left( \frac{m^x}{x!} \right) e^{-m}$.

Where: $P$ is the probability of an event of $n$ quanta occurring.

$P = \frac{\text{corrected number of events}}{\text{total number of quanta}}$
m is the mean quantal content of released transmitter. This was calculated as follows

\[
\text{Total quanta} / (\text{total number of events} - \text{non Poisson failures})
\]

x is the quantal content of responses

3- At the same time the errors (e) were automatically calculated in the errors column. This was done by subtracting the number of predicted (calculated events) from the numbers of observed events.

4. When the observed probabilities are plotted, they usually cannot be fitted to a Poisson probability distribution. This is due the presence of failures. Blakeley et al. (1982) proposed that the release of excitatory transmitter is determined by two processes. First, the secretion of transmitter which follows a Poisson distribution. Second, they proposed that another process which precedes the secretion of the transmitter which could produce failures in responses and which does not follow a Poisson distribution. Consequently, this could interfere with fitting the data to a Poisson process (Blakeley et al., 1982). As a result, any failures in responses were removed from the analysis. In other words to minimise the total errors, the non-Poisson zeroes were removed.

5- The change in mean quantal content is the final result from the Poisson fitter.
2-3.6 How it was possible to compare EJCs responses from different experiment

In these experiments a normalising approach was adopted to allow us to compare responses between different experiments. This was made possible by recording responses at the beginning of each experiment to a train of 20 pulses at 2Hz, 0.06-0.15 ms pulse width. These responses were taken as a control response (normalising response). The mean of those responses was calculated. And all subsequent responses were normalised to this response, as a result all responses were presented as a percentage of this normalising response.

2-3.7 Testing whether the normalised responses represent the original response

To test whether the normalised responses do actually represent the original responses, the effect of the NO scavenger PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide) on EJCs amplitude experiments will be used as an example. In the original data, thirty minutes following the addition of PTIO, EJCs amplitude had increased from 27µV to 37µV, this is an increase of 40 %. Further exposure to PTIO resulted in a further increase in EJCs amplitude to 40µV, this is an increase of 47 %. The control response which was taken at the beginning of the experiment at 2Hz was 28µV. This is followed by, that the EJCs amplitude responses normalised to the control response (normalising means calculating the percentage response changes of EJCs responses compared to the control response). As a result, EJCs amplitude responses changed from 93 % to 130 %, this is an increase of 40 %, 30 minutes following exposure to PTIO. Further exposure to PTIO caused the EJCs to
increase to 138 %, this is an increase of 47 %, of the original and the normalised data are almost identical (Table 2). Since the normalised data represent the original data, it appears valid to apply it to all electrophysiological data. And by doing so the inter-experimental variation was minimised allowing us to make comparison between different experiments.

Table 2. Comparison between original and normalised data of PTIO effect on EJCs amplitude. Note the prises representation of normalised data compared to the original data.

<table>
<thead>
<tr>
<th>Responses</th>
<th>Mean EJCs amplitude responses of the untreated preparation (μV)</th>
<th>Mean EJCs amplitude responses of preparation treated with PTIO for 30 minutes (μV)</th>
<th>Mean EJCs amplitude responses of preparation treated with PTIO for 60 minutes (μV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original mean EJCs response</td>
<td>27μV</td>
<td>37μV</td>
<td>40μV</td>
</tr>
<tr>
<td>Percentage increase of EJCs amplitude responses compared to the untreated</td>
<td>-</td>
<td>40%</td>
<td>47%</td>
</tr>
<tr>
<td>Normalised EJCs responses (%)</td>
<td>93%</td>
<td>130%</td>
<td>138%</td>
</tr>
<tr>
<td>Percentage increase of EJCs amplitude responses compared to the untreated (%)</td>
<td>-</td>
<td>40%</td>
<td>47%</td>
</tr>
</tbody>
</table>
2-4 Histochemistry

2-4.1 Light microscopy

The animal was anaesthetised with sodium pentobarbitone (sagatal). Then the dissection was carried out. During the dissection the fixative was poured over the abdominal cavity once it had been opened. The vas deferens was dissected then the tissue went through the following steps:

1. Fixation in a mixture of 1% formaldehyde, and 2% glutaraldehyde for 2-3 hours.

2. Tissue then cut transversely into 10µm-30µm section using freezing microtome and thawed onto glass slides.

3. Sections then washed in 0.1M phosphate buffer.

4. Sections then incubated in the staining solution at 37°C for 60 minutes in the following mixture (in the control section the NADPH was omitted from incubation media):

   0.3% Triton X-100 in 0.1M phosphate buffer

   0.4% NBT (Nitro blue tetrazolium)

   2mg/ml NADPH (nicotine adenine dinucleotide phosphate)
5. Sections were rinsed with 0.1M phosphate buffer solution.

6. Sections then counter stained with saffranin for 60 seconds.

7. Sections then washed well with distilled water.

8. Sections then were dehydrated through a graded series of alcohol and cleared with xylene. Finally the sections were mounted in DPX and cover slipped before being examined under the microscope.

2-4.2 Electron microscopy

After several trials using published methods (Kishimoto et al., 1993; Weinberg et al., 1996; Vincent and Johansson, 1983), a slightly modified diaphorase histochemistry procedure was used which gave the best result. The tissue was prepared for electron microscopy as follows:

1. The animal was anaesthetised with sodium pentobarbitone (Sagatal), during dissection the fixative was poured over the abdominal cavity. The fixative consisted of 2% paraformaldehyde, and 2% glutaradehyde made in 0.1M phosphate buffer, pH =7.4.

2. The tissues were fixed in the fridge for 2-3 hours by the same fixative used in step 1.

3. The tissues were then rinsed with 0.1M phosphate buffer.
4. The tissue was then incubated for 90 minutes, in the incubation medium which consists of the following:

- NADHP (nicotine adenine dinucleotide phosphate) 1.2µM
- BPST (2-(2-benzothiazolyl)-3-(4-phthalhydrazidyl)-5-styryl tetrazolium chloride) 1.2µM
- Dimethlformamide 3-4 drops
- Sodium phosphate buffer 0.1M

5. Rinsed with 0.1M phosphate buffer.

6. Post fixed in 2% osmium tetroxide made in veronal acetate buffer. For 1 hour.

7. Dehydrated in graded alcohol series.

8. Embedded in LR white resin for 24-48 hours.

9. Ultrathin sections were cut and collected on Formvar-coated single slot and examined under electron microscopy. Contrast of the section was enhanced by uranyl acetate (1% aqueous solution).
2-5 Test of significance

The evoked responses were expressed as a percentage change compared to the normalising response. The data in the text was given as mean +/- S.E.M. Statistical significance was calculated from Student’s t-test, paired and unpaired, as appropriate.

2-6 Drugs and solution

2-6.1 Drugs used:

\( \alpha \)-methyl histamine (Tocris Cockson)

BPST \( (2-(2\text{-benzothiozolyl})-3-(4\text{-phthalhydrazidyl})-5\text{-styryl tetrazolium chloride}) \)

Dimaprite (Tocris Cockson)

Histamine dihydrochloride (RBI)

L-arginine (Sigma)

L-arginine methyle ester (Sigma)

NADPH (nicotine adenine dinucleotide phosphate)

NBT (nitro blue tetrazolium)

Noradrinaline hydrochloride (Sigma)

Pentobarbitone sodium (Rhone Merieux)

Prazosine hydrochloride (Sigma)
PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide) (Sigma)

Ranitidine hydrochloride (Sigma)

Thioperamide maleate (RBI)

Yohimbine hydrochloride (Sigma)

2-6.2 Krebs saline

The solution was made from the following concentration:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.4</td>
</tr>
<tr>
<td>KCl</td>
<td>4.7</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.4</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.3</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1.13</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.1</td>
</tr>
</tbody>
</table>

The pH of the solution must be 7.4. This is achieved by gassing the solution with a mixture of 95% O₂ and 5% CO₂ for about 30 minutes.
3- RESULTS

3-1 Mechanical responses

Evoked responses of mouse vas deferens smooth muscle can be produced in two main ways, either by nerve stimulation, or by application of exogenous agonist. Nerve evoked contraction in the mouse vas deferens varies with the intensity of stimulus frequency. The response consists of two components. First, a rapid twitch (phase I), followed by a slow sustained contraction (phase II). The relative magnitude of these components is frequency dependent. Phase I is most evident at 10Hz frequency, whereas phase II is produced by a lower frequency stimulus (Swedin & Astrand, 1985; Stjarne & Astrand, 1985). It has been established that the first component is non adrenergic, mainly purinergic, whereas, the second component is mainly adrenergic in nature (Hedguist & Von Euler, 1976; Mc Grath, 1978; Booth et al., 1978). Other transmitters may be involved. Contractile responses produced by low stimulus intensity are prazosine sensitive (prazosine is an adrenergic antagonist). As stimulus intensity increases the effect of prazosine on the contractile response decreases (Singh et al., 1991).

3-1.1 The effect of L-arginine on the nerve evoked contractile response

L-arginine is the natural substrate for NOsynthase, the enzyme which produce NO in biological systems. Citrulline is a coproduct (Knowles et al., 1989; Palmer et al., 1988a,b). NO has an effect on a number of systems, including vascular smooth muscle (Rees et al., 1989), non vascular smooth muscle (Katsuki, 1977), neuronal tis-
sues (Knowles et al., 1993), and macrophages (Hibbs et al., 1987). The effect of L-arginine on the autonomic neurotransmission in the mouse vas deferens has been investigated briefly previously. The results showed that the presence of NO through the addition of L-arginine resulted in reduction in mechanical responses as well as a reduction in EJC's amplitude responses in the mouse vas deferens (Ph.D., R. V. Patterson, Leicester University. 1996). In this thesis an attempt was made to test the effect of L-arginine on the contractile response in the mouse vas deferens.

3.1.1.a Experimental protocol

Responses were recorded both in the absence and in the presence of L-arginine (3x10^{-6}M). 40 minutes were allowed for L-arginine to establish its action (this was determined from preliminary experiments). The preparations were field stimulated with repeated trains of 10 pulses, of 0.6ms width, 5Hz, at 15, 25, 35, and 40 Volts. 10 minutes rest intervals were allowed between each stimulus train. Before the addition of any drug the fourth response was taken as control response, subsequent responses were expressed as a percentage change from this control response. Figure 1 shows the experimental protocol.
Figure 1. Stimulation protocol for isolated preparation of mouse vas deferens in the absence and in the presence of L-arginine (3x10^-6M). Preparations were field stimulated with repeated trains of 10 pulses, 0.6ms pulse width, 5Hz. 10 minutes rest interval were allowed between each train, at 15, 25, 35, and 40 Volt. All contractile responses were normalised to the fourth control response, and responses expressed as a percentage of this normalising response. Addition of L-arginine was made to the organ bath immediately after the fourth control response. 30 minutes incubation period was allowed before recording the contractile responses. The organ bath was washed out with fresh Krebs saline containing L-arginine 3x10^-6M after each response was recorded. Control experiments followed the same protocol, but without the addition of L-arginine.

3-1.1.b The effect of L-arginine (3x10^-6M) at 15, 25, 35, and 40 Volts on nerve evoked contractile response of mouse vas deferens

L-arginine was added to the organ bath immediately after the fourth normalising response. A 40 minute incubation period was allowed for L-arginine to start acting. Stimulus intensity varied as follows 15, 25, 35, and 40 Volts. L-arginine resulted in a noticeable reduction in the contractile activity. The greatest differences was with the 40 Volts stimulus intensity. The mean differences was up to 28 % depression of responses. This difference is highly significant (P < 0.0001, d.f. = 38). This result suggests that L-arginine may have caused an increase in NO production, resulting in modulating the contraction through NO-mediated transmission (Figure 2).
Figure 2. The effect of L-arginine 3x10^4M on the contractile response of mouse vas deferens. The graph shows mean response +/- SEM. Responses were recorded in the absence (n = 5), and in the presence of L-arginine 3x10^4M (n = 5). All responses were normalised to the control response and expressed as a percentage of it. The addition of L-arginine resulted in reduction in the contractile responses compared to the untreated preparation. There was a significant differences between L-arginine treated and untreated responses (P < 0.0001).

3-1.2 The effect of NOsynthase inhibitor L-AME (2.3x10^4M) on the contractile response of stimulated mouse vas deferens

Previous results in section (3-1.1) showed that the autonomic neuroeffector junctions in the mouse vas deferens smooth muscle are sensitive to L-arginine, possibly due to the production of NO. Thus it is reasonable to use NOsynthase inhibitors to establish whether NO is normally synthesised in this tissue. L-AME (L-arginine
methyl ester), the NO synthase inhibitor was used in this experiment. L-AME was added to Krebs after the fourth control response. The vas deferens was field stimulated by 30 Volts, train of 10 pulses at 5Hz, 0.06ms pulse width, with 10 minute rest intervals between each train (figure 3). A set of six experiments was carried out in the presence of L-AME, and a set of six control experiments was carried out in the absence of drug treatment.

![Experimental protocol](image)

**Figure 3.** Experimental protocol. Field stimulation of train of 10 pulses at 30 Volts, 5Hz, of 0.6ms pulse width, with 10 minute rest intervals between each stimulus. The contractile response were normalised to the fourth response and L-AME (2.3x10^-4M) was added immediately after the fourth normalising response. Stimuli were given 10 minutes later to observe the onset of the effect of L-AME. The Krebs containing L-AME was changed after each stimulus. Control experiments followed the same protocol without the addition of the drug (n

The effect of inhibiting NO synthase on nerve evoked contraction

Figure 4 shows that the amplitude of nerve evoked contractions were elevated in the presence of L-AME (2.3x10^-4M), compared to control recorded in the absence
of L-AME. The elevation of the responses was clearly established after 60 minutes of incubation with L-AME, but became more obvious and significant after 80 minutes incubation. The difference at 60 and 75 minutes were as follows, +43% and +60% respectively. The differences at 75 minutes is highly significant (P < 0.0001, d. f. = 10). This suggests that the inhibition of NOsynthase by L-AME caused a significant elevation of nerve evoked contraction.
Figure 4. The effect of NOsynthase inhibitors L-AME (2.3x10^{-4} M) on contractile responses of isolated mouse vas deferens. The graph shows mean responses +/- SEM. Responses were recorded in the absence (n = 6), and in the presence of L-AME (n = 6). The addition of L-AME resulted in an increase in responses to a significant level compared to the untreated preparation (control). The difference was noticeable at 60 minutes of incubation where responses increased by + 43 %. The differences were highly significant at 70 minutes of incubation where responses increased by 60 %, (P < 0.0001).

3-1.3 The effect of L-arginine and NOsynthase inhibitor L-AME on the noradrenaline Dose-Response curve (D-R curve)

As mentioned before, smooth muscle contractile responses can be produced by two methods, either by nerve stimulation, or by the application of agonists acting on post-junctional receptors. The effect of L-arginine on noradrenaline D-R curve was examined, to see whether NO has any effect on the post-junctional responses of iso-
lated vas deferens. The effect of L-AME, the NOsynthase inhibitor was also tested in another set of experiments to see whether inhibition of NOsynthase produced any effect on the contractile response evoked by exogenous addition of noradrenaline. This should establish whether NO is synthesised in this tissue.

3-1-3.a The effect of L-arginine 3x10^4M

In the presence of L-arginine (3x10^4M) the EC_{50} of noradrenaline was shifted to the right from 1.6x10^{-5}M (n = 8) to 1.7x10^{-3}M (n = 12) (the EC_{50} was determined using Ultrafit program from Macintosh). The plateau of noradrenaline was not reached in the presence of L-arginine (3x10^4M). As a result, it is not clear if the maximum response was altered or not in the presence of L-arginine (Figure 5).
Figure 5. The effect of L-arginine (3x10^{-8}M) on responses of isolated mouse vas deferens evoked by exogenous application of noradrenaline (NA). All concentrations were applied in random order, and responses were normalised to the maximum response of each preparation. The D-R curve was shifted to the right under the effect of L-arginine. EC_{50} changed from 1.6x10^{-5}M to 1.7x10^{-3}M. It is not possible to comment on the maximum response in preparations treated with L-arginine, due to the fact that the plateau of the D-R curve was not reached with the maximum concentration used.

3-1.3.b The effect of L-AME (2.3x10^{-4}M) on Noradrenaline D-R curve

In the presence of the NOsynthase inhibitor L-AME (2.3x10^{-4}M), the EC_{50} was shifted to the left from 1.6x10^{-5}M in the absence of L-AME to 8.6x10^{-6}M in the presence of L-AME (EC_{50} was determined using Ulltrafit program). Additionally, there
was a noticeable increase in the maximum response where it reached up to 120 % in the presence of L-AME compared to that in its absence (Figure 6). This result indicates that nitric oxide does normally modulate post synaptic responses in this tissue.

Figure 6. The effect of the NOsynthase inhibitor L-AME on the responses of isolated vas deferens evoked by exogenous application of a range of concentrations of noradrenaline (NA). All concentrations were applied in random order, and responses were normalised to the maximum response of each preparation. There was a shift in the dose response curve to the left, with a change in the maximum response by up to +20 % +/- 10 in the presence of L-AME. In addition, the maximum response increased under the effect of L-AME. Vertical lines represent error bars (SEM).
3-1.3.c The effect of L-arginine (3x10^{-6}M) and L-AME (2.3x10^{-4}M) on noradrenaline D-R curve

The presence of both L-arginine (3x10^{-6}M) and L-AME (2.3x10^{-4}M) (both added at the same time) had no significant effect on the noradrenaline Dose-Response curve compared to the control. The EC_{50} in the absence of L-arginine + LAME was 1.6x10^{-5}M and the same in the presence of L-arginine + L-AME. This might suggest that the effect of L-arginine was due to the presence an active form of NO synthase. Therefore, the effect was absent when L-AME was added, due to its inhibitory effect on L-AME. As a result, the added L-arginine was not converted into NO. In a another word that the effect of L-arginine requires the presence of active form of NO synthase. The other thing which might be concluded is that L-arginine did not have any action itself (Figure 7).
Figure 7. The effect of both L-arginine (the natural substrate in the production of NO), and L-AME (NOSynthase inhibitor), on responses of isolated vas deferens evoked by exogenous application of a range of concentration of noradrenaline. All concentrations were applied in random order, and responses were normalised to the maximum response of each preparation. Data shows represent mean response +/- SEM. The graph shows that the addition of L-AME and L-arginine (solid line)did not have any significant effect on responses from a range of concentrations of noradrenaline compared to the control (dotted line). L-arginine (L-arg), noradrenaline (NA).

3-1.3.d Conclusion

The above results proved that NO produced from L-arginine did have an inhibitory effect on the evoked contraction. Another piece of evidence came from the application of the NOSynthase inhibitor L-AME to the preparation. In the presence of L-AME the contractile response increased to a significant level. This indicates that NOSynthase is present in this tissue. It is possible to conclude that L-arginine acts
through NOsynthase because L-AME reverses its effect. As NO is possibly a mediator of inhibitory transmission in this tissue, the effect of L-arginine requires the presence of the active form of NOsynthase.
3-2 Electrophysiological responses of the mouse vas deferens smooth muscle cells (extracellular recording)

By applying a focal recording electrode on the outer surface of the vas deferens, it is possible to record the electrical activity of number of varicosities located under the tip of the electrode, this is termed extracellular recording. The amplitude of the recorded electrical activity is dependent on the electrode tip diameter. A small tip diameter (4.6 \( \mu \text{m} \)) gave recordings of 59-67\( \mu \text{V} \), whereas larger tip diameters (20-50\( \mu \text{m} \)) gave recordings of 25-29\( \mu \text{V} \) of (Lavidis & Bennett, 1992). Recordings made in this thesis were 25-45\( \mu \text{V} \) in amplitude, with electrode tips approximately 50\( \mu \text{m} \) in diameter.

In the absence of any electrical stimulation a spontaneous junction current (SEJCs) can be recorded, which represents the spontaneous release of transmitter. During stimulation an excitatory junction current (EJCs) can be recorded, which resembles the SEJCs in time course and amplitude. The EJCs are calcium dependent, and tetrodotoxin (TTX) sensitive. They represent the action of ATP on the post synaptic P_2x receptors (Brock & Cunnane, 1988). EJCs when evoked within the tip of the electrode a gives a negative going current. On the other hand, if these electrical activities are evoked outside the recording electrode tip, they give rise to a positive going current (Stjärne, et al., 1990), (Figure 8).
Performing a quantal analysis on the EJCs made it possible to distinguish to some extent the pre- or post-junctional effect of a given pharmacological manipulation. Pre-junctional effect manifests as a change in the number of failures and the relative size of peaks (of amplitude frequency distribution) at unchanged preferred values. Whereas, post-junctional effects affect the quantal effect. This represents as a change in the interval between preferred values of peaks (Kullman & Nicoll, 1992).

The mechanical responses of isolated vas deferens, initially performed in this thesis, indicated the possibility that a NO-mediated modulatory mechanism of transmission might be present in the vas deferens, and confirmed the possibility that
NO is synthesised in the mouse vas deferens. This was proved both by field stimulation of the vas deferens and by applying an agonist acting post junctionally. In the case of nerve evoked contraction by field stimulation, the NO synthase inhibitor L-AME, proved effective in elevating the responses to a significant level. NO produced from L-arginine proved successful in reducing the nerve evoked contraction. Post junctional responses evoked by exogenous addition of noradrenaline, were elevated following the addition of L-AME, and depressed following the addition of L-arginine. Such results support the idea that NO-mediated modulatory mechanisms of transmission do exist in the mouse vas deferens, and NO is synthesised naturally in this tissue.

To study the effect of NO as a modulator of transmitter secretion, EJCs were recorded in the presence of L-arginine testing its effect on neurotransmission in this tissue. The effect of NO scavengers, such as PTIO was studied in some detail, as an additional way for determining the presence of NO-mediated modulatory mechanisms of transmission, and to prove that NO is synthesised in this tissue. PTIO known to act on NO and not NO synthase, scavenging any NO produced. The addition of PTIO should produce an effect opposite to that produced by L-arginine. EJCs were recorded in the presence of PTIO, in the presence and absence of L-arginine, and in the presence of both. Another set of experiments was conducted where the effect of PTIO was examined after incubation with L-arginine.
3-2.1 The effect of L-arginine (3.4x10⁻⁴M) on the EJCs

Evoked contractile responses of mouse vas deferens discussed earlier, demonstrated that NO was produced naturally in this tissue. This is proved when NO production was inhibited by the exogenous application of NO synthase inhibitor L-AME, which resulted in enhancement of the contractile evoked responses. In addition, NO produced by incubation with L-arginine had an inhibitory effect on the contractile evoked responses by up to 29 +/- 3%. To test this result electrophysiologically, EJCs were recorded in the presence and in the absence of L-arginine (3.4x10⁻⁴M). In addition, quantal analysis was carried out to determine the site of action of L-arginine.

3-2.1.a Experimental protocol

As discussed earlier in methodology, EJCs were first recorded in the absence of L-arginine. A train of 20 pulses at 1Hz, 0.06-0.15ms pulse width, and allowing 1 minutes rest intervals in between trains. Initially a number of pulses were given at 2Hz, as a control response (all EJCs collected were normalised to this response). This procedure was done to reduce the experimental variation, and as a result to make it possible to compare the effect obtained from different experiments. EJCs were collected at the beginning in the absence of L-arginine, followed by 40 minutes incubation with L-arginine 3.4x10⁻⁴M. Finally another set of EJCs were collected. Preliminary experiments carried out to determine the onset of the effect of L-arginine, showed that L-arginine needed 40 minutes to establish its effect.
3-2.1.b *The effect of L-arginine (3.4x10^-4M) on EJCs amplitude*

The effect of L-arginine initially started as a gradual reduction in EJCs. EJCs were reduced from 97 +/- 6% (n=3), to 57 +/- 7% (n=3). This is a reduction of 41%. These changes are significant (P < 0.002). Further 30 minutes reduced the mean EJCs amplitude up to 10 +/- 10 %. This is a significant reduction of 87 % (P < 0.001). As the experiment progressed, the EJCs continued to be reduced until they were undetectable in most experiment.

As the EJCs reduce in size, a current of positive polarity appeared. The threshold of the new positive polarity current was smaller than that of the original EJCs. And the latency was shorter than that of the EJCs. Within about 70 minutes of incubation, the EJCs became undetectable in almost all experiments, and the positive polarity current was well established by this time (Figure 9). Figure 10(a) shows electrical recordings of the new current, figure 10(b) shows that the new current continues to increase until approaching its maximum after about 70 minutes.
Figure 9. The effect of L-arginine ($3.4 \times 10^4 \text{M}$) on EJCs of isolated mouse vas deferens. EJCs were first collected from untreated preparations. This was followed by incubating the preparation with L-arginine ($3.4 \times 10^4 \text{M}$) for 40 minutes, then EJCs collected. 30 minutes further incubation was allowed, followed by another collection of EJCs. Stimulation protocol was as follows, train of 20 pulses at 1Hz, 0.06-0.15 ms pulse width, stimulus intensity was about 25% above threshold. Control experiments followed the same protocol without the addition of L-arginine. All EJCs were normalised to a control EJCs taken at the beginning of each experiment at 2Hz and expressed as the percentage of it. The graph shows that L-arginine resulted in a reduction in EJCs amplitude until they almost disappeared in most experiment ($n = 3$). Untreated preparation showed a slight reduction ($n = 3$). The graph shows mean value of EJCs vs time.
Figure 10 (a). Example of the new current (b) revealed by L-arginine (3.4x10^{-4}M) below the threshold for EJC (a) in two different preparations (1, and 2). Twenty minutes incubation with L-arginine was needed for the new current to be revealed. The new current was of opposite sign and evoked at a lower threshold compared with EJCs recorded from the same electrode (without moving the focal recording electrode).
Figure 10 (b). The effect of time on the new positive polarity current induced by incubating the preparation with L-arginine ($3.4 \times 10^{-6}$M). The new currents was recorded at 40, 70, and 100 minutes incubation time. All new currents recorded were normalised to a recording of the new current made at the beginning of the appearance of the new current at 2Hz and normalised to this. The new current increased in amplitude from 98% +/- 8 at 40 minutes to 119% +/- 6 at 70 minutes incubation (n = 3). Further incubation had no significant effect on the amplitude of the new current. The graph shows mean new current amplitude vs. time.

Mechanical response experiment evoked with exogenous application of noradrenaline, performed earlier in this study showed that NO does modulate transmission in the vas deferens. Noradrenaline acts on $\alpha_1$-postsynaptic receptors. NO caused the noradrenaline dose-response curve to shift to the right, therefore this modulation is post-junctional. Quantal analysis of the effect of $3.4 \times 10^{-6}$M L-arginine on EJCs amplitude showed that the effect of L-arginine was pre-junctional. The effect was a reduction in relative size of the peak of preferred values (peaks represent the frequency occurrence of the EJCs amplitude), this is a pre-junctional effect. Furthermore, there was a reduction in the mean EJCs amplitude by up to 87 +/- 10% (n = 4). In addition, there was no change in the size of the interval between peaks of preferred values. This will be further discussed in section 3.2.1e.
3-2.1.c Effect of time on the new current revealed by L-arginine

The new current revealed by L-arginine continued to increase in size for about one hour. Approximately 40 minutes incubation time was needed for the new current to start developing and become detectable. But only about 20 minutes incubation with L-arginine was needed to start the modulation of EJCs amplitude. By 70 minutes incubation time, almost all EJCs disappeared (Figure 9 & 10 (a, b), section 3.2.1.b).

3-2.1.d Effect of L-arginine on the mean quantal content

Figure 11 shows the mean quantal content recorded in the absence and in the presence of L-arginine (3.4x10^{-4}M). The mean quantal content in the absence of L-arginine was 3.1 +/-0.9 (n = 4), and 1 +/-0.6 (n = 4) in its presence. There was a 68% reduction in the mean quantal content. This is a significant change (P < 0.001), this suggesting that L-arginine may have depressed the quantal content of transmitter release.
Figure 11. The effect of L-arginine on the mean quantal content. Responses were first recorded in the absence of L-arginine (3.4x10^4M) and then it was added to the recording electrode tip. The data represent the mean quantal content calculated from a single release site in three different preparations. The figure shows that the quantal content was reduced in preparations treated with L-arginine. There was a significant reduction of 68% in the mean quantal content (P < 0.01), which could partially explain the reason for the reduction in EJCs amplitude. This is a pre-junctional effect.

3-2.1.e The effect of L-arginine (3.4x10^4M) on EJCs amplitude distribution

Figure 12 shows the effect of L-arginine (3.4x10^4M) on EJCs amplitude distribution. The effect was derived from EJCs amplitude distribution in the absence and the presence of L-arginine. The graph shows that the distance between relative peaks of preferred values of amplitude distribution in both situation did not change. There was an increase in number of failures under the effect of L-arginine. The mean reduction in EJCs amplitude was -78 +/-7 % (n = 4) in the presence of L-arginine. These changes imply that the effect of L-arginine is not post-junctional.
Figure 12. The effect of L-arginine on EJC's amplitude distribution. Preparation was field stimulated by a train of 20 pulses at 1Hz, 0.06-0.15 ms stimulus width, with 10 minutes rest interval between trains. The same stimulation protocol was followed in the absence (a), and in the presence (b), of L-arginine. The arrows represent the peak of preferred values, and the numbers represents the quantal content of the released transmitters. Relative peak heights of preferred values changed, but the separation between peaks of preferred values of amplitude distribution did not change. There was an increase in the number of failures. These changes indicate that the effect of L-arginine is not a post-junctional effect, favours a pre-junctional effect.
3-2.2 The effect of NO scavenger PTIO (phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide) on EJCs

It has been shown by using electron spin resonance, that imidazolineoxyl N-oxides (such as PTI, PTIO & carboxyPTIO) react avidly with NO. Furthermore, vasorelaxation was inhibited by the imidazolineoxyl N-oxide (Akaike et al., 1993). The inhibitory action of imidazolineoxyl N-oxides was not due to direct inhibition of NO synthase, but due to direct reaction with NO (Maeda et al., 1995). The imidazolineoxyl N-oxides selectively react with NO and convert it to NO₂, as a result they inhibit the biological effect of NO. The effect of PTIO (3x10⁻⁸M) on EJCs was tested in the presence and in the absence of L-arginine (3.4x10⁻⁴M).

3-2.2.a Experimental protocol

EJCs were recorded first in the absence of PTIO. Following incubation with PTIO (3x10⁻⁸M) for 40 minutes, another set of EJCs was recorded (preliminary experiments showed that the significant action of PTIO develop within 40 minutes incubation). In order to reduce the inter experimental variation, all EJCs were normalised to a response taken at 2Hz at the beginning of each experiment, allowing for comparison between different experiment to be made. All EJCs were expressed as a percentage following normalisation.
3-2.2. The effect of PTIO on EJC's amplitude

The effect of PTIO reached a significant level after 40 minutes incubation. The mean EJC's increased by 35 +/- 10%, this is a significant changes (P < 0.04). By 70 minutes incubation time the EJC's had increased by 55 +/- 10%, this is highly significant (P < 0.009). In the absence of PTIO the mean amplitude of the responses fell slightly (Figure 13). It is clear that the NO scavenger PTIO did have an effect on EJC's, there was a significant increase in EJC's as a result of incubation with PTIO.

![Figure 13. The effect of PTIO (3x10^-4M) on the mean EJC's amplitude calculated from preparations in the absence and in the presence of PTIO. The preparations were field stimulated with trains of 20 pulses at 1Hz, 0.06-0.15ms pulse width, with 1 minutes rest intervals. Responses were first recorded at 2Hz as a normalising response, followed by recording in the absence and in the presence of PTIO. The mean quantal effect was calculated from the same release site in 4 different preparation. The graph shows mean EJC's. The EJC's amplitude increased to a significant level (P < 0.009) under the influence of PTIO, compared to the untreated preparation, where responses were slightly reduced.](image-url)
3-2.3 The effect of PTIO (3x10^4M) and L-arginine (3.4x10^-4M)

It is well established that L-arginine is the natural substrate for NO synthase in the production of NO, as demonstrated in the above results, where PTIO, the NO scavenger enhanced EJCs amplitude. L-arginine, on the other hand, caused a gradual reduction in EJCs amplitude until they disappeared completely in some preparations. The question is does PTIO exert any significant effect on the preparations where NO effect is established? The effect of L-arginine on preparations previously treated with PTIO was tested, in order to establish whether L-arginine can reverse any effects produced by PTIO.

3-2.3.a The effect of PTIO (3x10^4M) on EJCs reduced amplitude in preparation previously treated with L-arginine (3.4x10^-4M)

EJCs were first recorded as untreated preparations, followed by the addition of L-arginine to the electrode tip. 40 minutes incubation periods were allowed for L-arginine to establish its effect, followed by a recording of EJCs. Finally, PTIO and L-arginine were both added to the electrode tip. A further 40 minutes incubation period was allowed, and EJCs collected. All EJCs were normalised to a control response taken at the beginning of each experiment at 2Hz as a normalising response.

L-arginine reduced EJCs to a significant level from 103 +/- 6 % in the absence of L-arginine to 26 +/- 15 % in the presence of L-arginine, (P < 0.004) (n = 4). In some preparations the EJCs completely disappeared. PTIO reversed the inhibitory ac-
tion of L-arginine, and EJCs were greatly increased from 26 +/- 15 % in the presence of L-arginine to 106 +/- 9 % in the presence of L-arginine and PTIO, (P < 0.004) (n = 4). From the above result it is clear that PTIO reversed the inhibitory effect of L-arginine, most probably through the elimination of NO (Figure 14).

![Diagram](image)

**Figure 14.** The effect of PTIO (3x10^-8M) on EJCs amplitude reduced by L-arginine (3.4x10^-4M). The preparations were field stimulated with a train of 20 pulses at 1Hz, 0.06ms pulse duration and 1 minutes rest intervals. Responses were first recorded in the absence of L-arginine and then in the presence of L-arginine which was added to the recording electrode tip. Finally, responses were recorded in the presence of both L-arginine and PTIO. All responses were normalised to a control responses taken at the beginning of each experiment at 2Hz. Responses were expressed as a percentage of this control response. The data were collected from a single release site in four different preparation. The graph shows mean changes in EJCs. L-arginine significantly reduced EJCs amplitude by 96 % P< 0.004. This was reversed by the addition of PTIO, where EJCs increased in size by up 99 %, P< 0.004.
3-2.3.b The effect of L-arginine (3.4x10^{-4}M) on preparation treated with PTIO (3x10^{-4}M)

These experiments followed the same protocol as in section 3-2-3.a, except that PTIO was first added to the preparation, followed by the addition of L-arginine and PTIO to the electrode tip. PTIO increased EJCs amplitude from 99 +/- 6 % in the absence of PTIO to 133 +/- 7 %, in its presence. This is an increase of 25%, (P < 0.02). The addition of L-arginine did not have any effect in reducing the EJCs amplitude, EJCs continued to increase in amplitude until they reached 154 +/- 7 %, (P < 0.004), which is an increase of 55% (Figure 15). This is a highly significant changes. The reason for this could be that PTIO continued its scavenging effect on naturally and newly produced NO.

![Figure 15](image-url)  
*Figure 15. The effect of L-arginine (3.4x10^{-4}M) on EJCs responses in preparations previously treated with PTIO (3x10^{-4}M). PTIO increased EJCs amplitude to a significant level (P < 0.02). The addition of L-arginine failed to have any significant effect in reducing the enhanced EJCs amplitude, compared to the untreated preparation. The EJCs continued to increase significantly (P < 0.004) despite the addition of L-arginine and PTIO. One reason for this may be continuation of the scavenging effect of PTIO.*
The above results suggest that NO is naturally produced in this tissue. The effect of NO is NO-mediated inhibitory effect exerted on neurotransmission in this tissue. Removing NO from this tissue produced a significant increase in EJCs amplitude. This is a result of removing the inhibitory effect (by reducing the tissue content of NO). Figure 16 summarises the above results.

Figure 16. Summary of results from four different experiments. In set one of the graph the preparations were all untreated with any drug. In set two and three the preparations were treated as indicated in the legend of the graph. PTIO increased EJCs amplitude probably by eliminating NO from the tissue. This was true both in tissue treated only with PTIO, and with tissue previously treated with L-arginine. On the other hand, L-arginine could not reverse the effect produced by PTIO. The graph shows mean EJCs response.
3-2.4 The effect of manipulating presynaptic $\alpha_2$-adrenoceptors on neurotransmission in mouse vas deferens

Autonomic nerve terminals contain a wide range of pre-junctional presynaptic receptors. These receptors when activated by the appropriate agonists can either increase or decrease transmitter release (Starke, 1977, 1987; Gillespie, 1980). $\alpha_2$-adrenoceptors plays an important role in regulating transmitter release from nerve terminal. Various experiments showed that $\alpha_2$-adrenoceptors antagonists increase noradrenaline overflow during sympathetic nerve stimulation (Starke, 1977, 1987; Gillespie, 1980). When these presynaptic receptors react with noradrenaline, mediates an inhibition of transmitter release occurs. Exogenous noradrenaline inhibits release of neuronal noradrenaline from cardiac tissue during nerve stimulation, and its action can be antagonised by $\alpha_2$-adrenoceptors antagonists (Starke, 1972). In sympathetically innervated tissue $\alpha_2$-adrenoceptor agonists decrease electrically evoked release of noradrenaline (Starke, 1977; Gillespie, 1980). This mechanism of regulation of noradrenaline release by pre-synaptic $\alpha_2$-adrenoceptors is referred to as autoregulation. Transmitter release in rodent vas deferens and various blood vessels support the hypothesis that transmitter release is autoregulated through pre-junctional $\alpha_2$-adrenoceptors (Starke, 1987). EJC's in these tissue are a measure of the release of ATP and not noradrenaline.

It is has been proved that yohimbine ($\alpha_2$-receptor antagonist) increases EJC's amplitude evoked by a train of stimuli. Yohimbine had no effect on EJC's amplitude in reserpinized tissue, as a result, yohimbine increased transmitter release by interrupting $\alpha$-autoinhibitory mechanisms presynaptically and not via a non-selective action (e.g.
K⁺ channel block in nerve terminal). This result strongly supports the view that ATP release is modulated by the co-released noradrenaline.

Yohimbine and idazoxan (α₂-receptor antagonists) affect neurotransmitter release, and this is frequency dependent. Yohimbine and idazoxan did not affect EJC amplitude at 0.1 Hz, but enhanced EJC amplitude and NA release at 2 Hz to the same extent (Msghina et al., 1992). In addition, it is thought that ATP and noradrenaline are released in parallel from sympathetic nerve terminals in rat tail artery (Msghina et al., 1992). Does this autoregulatory mechanism via presynaptic α₂-adrenoceptors exist in the mouse vas deferens. In this study the effect of the α₂-adrenoceptor antagonists yohimbine and idazoxan have been investigated. Their action on the new current has been studied.

3-2.4. a The effect of yohimbine on EJC amplitude

In order to investigate the mechanism of α₂-adrenoceptor mediated inhibition of transmitter release, the effect of a range of concentrations of the selective α₂-adrenoceptor antagonist yohimbine was first investigated.

3-2.4. a.1 Experimental protocol

The effect of a range of concentration of yohimbine was tested (10⁻⁹M-10⁻⁴M) on EJC, and a dose-response curve was constructed. This was achieved by adopting the same approach that has already been described in this thesis to reduce inter experi-
mental variation, by normalising responses to a control response taken at the beginning of each experiment. Thus, all EJCs were expressed as percentages of this response. The normalising responses were recorded at 2Hz, followed by recording of EJCs at 1Hz as the untreated response, then yohimbine ($10^{-9}$M - $10^{-4}$M) was added to the electrode tip, followed by electrical stimulation and recording of EJCs under the effect of yohimbine.

3-2.4.a.2 The effect of a range of concentration of yohimbine

Generally yohimbine increased the size of EJCs amplitude. This increase in EJCs amplitude was dose dependent, it varied from 9 +/- 7% with yohimbine $10^{-9}$M ($n=8$), to 84 +/- 8% with yohimbine $10^{-4}$M ($n=8$). EC$_{50}$ was $10^{-7}$M ($n=7$), (Figure 17).
Figure 17. Dose-response curve of yohimbine. Responses were collected in the absence and in the presence of a range of concentrations of yohimbine (10^{-9}M-10^{-4}M). The graph shows mean EJCs responses +/- SEM. Yohimbine resulted in an increase in EJCs responses which varied with different concentrations. EJCs amplitude increased by 9% (n = 4) with yohimbine 10^{-9} M, and up to 84.17% (n = 4) in preparation treated with yohimbine 10^{-4} M.

3-2.4.a.3 The effect of Yohimbine (10^{-7}M) on EJCs amplitude distribution

Figure 18 shows the effect of yohimbine on EJCs amplitude distribution in the absence and in the presence of yohimbine 10^{-7}M. There was no change in the separation between the relative peaks of preferred values of amplitude distribution under the effect of yohimbine. But in the presence of yohimbine the distribution appeared to shift slightly to the right, as a result of increase in EJCs amplitude. There was an increase in EJCs mean amplitude of 59 +/-11 % (n = 8) in the presence of yohimbine, compare to in its absence. This is a significant changes (P < 0.0001). In addition, there was a reduction in number of failures. Those changes therefore, favour a pre-
junctional rather than a post-junctional effect, since there was no change in the quantal effect of secreted transmitter.

Figure 18. The effect of yohimbine $10^{-7}$M on EJCs amplitude frequency distribution. EJCs was recorded from preparation in the absence (a), and in the presence (b) of yohimbine. The arrows indicate the peak of preferred values, and the numbers indicate the quantal content. Note that there was no change in the separation between preferred values, which might suggest a pre-junctional change. This is compatible with the current knowledge regarding the site of $\alpha_2$-adrenoeceptors.
3-2.4.a.4 The effect of yohimbine (10^{-7}M) on the mean quantal content

Figure 19 shows the effect of yohimbine (10^{-7}M) on the mean quantal content of secreted transmitter in three preparations following treatment with yohimbine. Mean quantal content changed from 2.4 +/- 0.05 (n=3) in the absence of yohimbine, to 3.1 +/- 0.115 (n=3) in its presence. These changes are significant (P < 0.006). The above results suggests that the increase in the EJCs amplitude resulted from an increase in the quantal content of secreted ATP, reflecting a pre-junctional effect of yohimbine.

![Figure 19. The effect of yohimbine on the mean quantal content calculated from responses in the presence and in the absence of yohimbine. Responses recorded from three different preparations. Each line represent quantal content from a single preparation. There was an increase of 29.16 % in the quantal content under the effect of yohimbine (P < 0.006). This result suggests that the change in EJCs amplitude during treatment with yohimbine resulted from an increase in the secreted quanta.](image)
3-2.4.b The effect of Idazoxan $10^4$M ($\alpha_2$-adrenoceptor antagonist) on EJCs amplitude

Yohimbine and Idazoxan are both $\alpha_2$-adrenoceptor antagonists, they have no effect on EJCs at 0.1 Hz, but enhance EJCs amplitude at 2 Hz to the same extent in rat tail artery (Mshina et al., 1992). The effect of idazoxan in the mouse vas deferens has not been tested before. Consequently, the effect of idazoxan $10^4$M at 1 and 2 Hz was tested in this thesis.

Figure 20 shows that idazoxan increased the size of EJCs amplitude at both 1 and 2 Hz. However, its effect at 2 Hz was more prominent compared to its effect at 1 Hz. At 1 Hz the mean amplitude increased by $+31 \pm 6\% (n = 3), (P < 0.001)$, and at 2 Hz the EJCs mean amplitude increased by $+70 \pm 10\% (n = 3), (P < 0.001)$. Both results are highly significant. It is not possible at this stage to state whether the effect of idazoxan is pre- or post-junctional.
Figure 20. The effect of idazoxan $10^{-6}$M on EJCs amplitude at 1 and 2Hz. Responses were recorded in the presence of idazoxan which was added to the electrode tip, recordings were made at 1Hz and at 2Hz. The data shown in the graph represent mean response changes. There was an increase in the EJCs amplitude under the effect of IDZX, both at 1 and 2 Hz. Both changes were significant (P < 0.001). The effect on EJCs amplitude was maximum at 2Hz compared to the mean amplitude difference at 1Hz.

3-2.5.a The effect of Yohimbine ($10^{-7}$M) on the new current revealed by L-arginine ($3.4 \times 10^{-4}$M)

The effect of the $\alpha_2$-adrenoceptor antagonist yohimbine ($10^{-7}$M) was tested on the new current revealed by L-arginine ($3.4 \times 10^{-4}$M). The above experimental results showed that yohimbine and idazoxan increased EJCs amplitude. This was mostly via presynaptic $\alpha_2$-adrenoceptors, resulting from interrupting the autoinhibitory mechanism regulating neurotransmitter release. As discussed earlier, EJCs is a reflection of released ATP. Since ATP and noradrenaline are co-released, any changes in ATP release represented by changes in EJCs amplitude reflect a change in released noradrenaline and ATP. If this feedback inhibitory mechanism does exist, does it impose any effect on the new current? In this experiment the effect of yohimbine and ida-
zoxan on the new current was examined. Preparations were first incubated with L-arginine (3.4x10⁻⁴M) to reveal the positive polarity new current. This was followed by the addition of yohimbine to test its effect on the positive polarity new current.

Figure 21 shows the effect of yohimbine 10⁻⁷M on the new current. There was an increase in the mean amplitude of the new current by + 28 +/- 4 % (n = 6) (P < 0.002), two minutes after exposure to yohimbine, and + 46 +/- 8 % (n = 6) (P < 0.001), 30 minutes after exposure to yohimbine. Both changes are highly significant. In the absence of yohimbine the new current increased with time when exposure to L-arginine was extended. But this increase was only of a limited extent, the new current amplitude increased by + 21 +/- 6 % (n = 3), (P < 0.07), and + 23 +/- 5 % (n = 3), (P < 0.024), following the same time base as above.

Figure 21. The effect of Yohimbine 10⁻⁷M on the new current revealed by L-arginine 3.4x10⁻⁴M. The data shown in the graph represent mean response changes. In set one, the preparation was treated with L-arginine alone. In set two the control preparation continued to be treated only with L-arginine, whereas yohimbine was added to the other preparation. Recording was started 2 minutes after the addition of yohimbine. The same treatment followed in set three. Recording was also made about 30 minutes following the treatment with yohimbine. Yohimbine resulted in an increase in the new current amplitude, both 2 and 30 minutes after the addition of yohimbine. Both results were highly significant. Compare with the control there was a slight increase in the new current amplitude but not to the same extent as that under the effect of yohimbine.
3-2.5.b The effect of Idazoxan (10^4M) on the new current revealed by L-arginine
(3.4x10^4M)

Figure 22 shows the effect of idazoxan on the new current revealed by L-arginine. There was a significant increase in the amplitude of the new current of +33 +/- 4 %, (P < 0.003), and + 54 +/- 4.4 % (P < 0.001) at 2 and 30 minutes of exposure to idazoxan, respectively. Both changes are highly significant. The new current amplitude increased under the effect of idazoxan compared to the amplitude in its absence. It is probable that the effect of idazoxane was through α2-adrenoceptors.

Figure 22. The effect of idazoxane 10^4M on the new current revealed by L-arginine 3.4x10^4M. Data shown in the graph represent mean new current responses. In set one both preparations were treated with L-arginine alone. In set two idazoxane was added to one preparation. The same procedure was followed in set three. Recording was made at 2 and 30 minutes in set two and three respectively, following the addition of drugs. The figure shows that idazoxane (10^4M) did increased the size of the new current amplitude to a significant level (P < 0.001), compared to the preparation treated with L-arginine alone.
3-2.6.a The effect of noradrenaline on EJCs

The addition of yohimbine resulted in a reduction in the size of EJCs amplitude, through a pre-synaptic mechanism, most probably through pre-synaptic $\alpha_2$-adrenoceptors. The addition of an $\alpha_2$-agonist should produce the opposite effect. Noradrenaline were used and its effect on EJCs amplitude was investigated. Noradrenaline was added to the recording electrode tip. EJCs was first collected in the absence of noradrenaline, followed by the addition of $10^4$M noradrenaline. A second set of EJCs was then collected. Both recording events followed the same stimulation protocol. All EJCs were normalised to a control response taken at the beginning of each experiment at 2Hz, and all responses were expressed as a percentage of the normalising response.

3-2.6.a.1 The effect of noradrenaline on EJCs amplitude

Figure 23 shows the effect of noradrenaline on EJCs amplitude. EJCs were decreased by $57 \pm 7\%$ ($P < 0.0001$), compared to the untreated preparation where EJCs amplitude dropped only by $10 \pm 8\%$, ($P < 0.26$). The reduction in EJCs amplitude under the effect of noradrenaline is highly significant, whereas that of the untreated preparation was not. This result is compatible with the results obtained using yohimbine as an antagonist, where EJCs were increased in size (opposite to the effect of noradrenaline).
Figure 23. The effect noradrenaline (NA) $10^{-6}$M on EJCs amplitude. EJCs were recorded first in the absence of NA followed by recording in the presence of NA. The data shown in the graph represent mean EJCs responses. The figure shows that the addition of noradrenaline resulted in a reduction of EJCs amplitude by up to $56 \pm 7\%$, ($n=6$), ($P < 0.0001$), compared to the untreated preparation where the EJCs amplitude fell slightly, (not significant).

3-2.6.a.2 The effect of noradrenaline on the mean quantal content

Figure 24 shows the effect of noradrenaline ($10^{-6}$M) on the mean quantal content of preparations in its absence and its presence. The mean quantal content reduced from $3 \pm 0.5$ ($n = 3$) in untreated preparations, to $2.4 \pm 0.2$ ($n = 3$) in preparation treated with noradrenaline. This is a reduction of 24\%, but it is not significant, ($P = 0.21$, d.f. = 4). This indicates that the reduction in EJCs amplitude could be as a result from a reduction in quantal content of transmitter released.
Figure 24. The graph shows the effect of noradrenaline on the mean quantal content of secreted transmitter. The graph represents the quantal content from three different preparations. Responses were recorded in the absence and in the presence of noradrenaline (NA) which was added to the electrode tip. Each line represents the quantal content from a single preparation. Quantal content was calculated from Poisson fitter using Excel program. The graph shows that the mean quantal content reduced by 76% under the effect of noradrenaline, but this reduction is not significant (P < 0.21, d.f. = 4).

3.2.6.3 The effect of noradrenaline (10−4M) on EJC's amplitude distribution

Figure 25 shows the quantal analysis of the effect of noradrenaline. There was no change in the separation between peaks of preferred values. The relative size of each peak was reduced. Generally noradrenaline resulted in a reduction of EJC's mean amplitude, and as seen in the previous section (3.2.6.a.2) noradrenaline also produced a reduction in the mean quantal content by up to 58%. These changes mainly follow a pre-junctional effect. This can be explained as by noradrenaline acting presynaptically on α2-adrenoceptors. This would lead to the reduction in the release of both neuro-
transmitter present in this tissue, noradrenaline and ATP. EJCs amplitude change reflects a change in ATP released, and since both are co-released, then EJCs is in a way represent released ATP and noradrenaline.

Figure 25. The effect of noradrenaline $10^{-6}$M on the quantal effect of secreted transmitters. Responses were recorded in the absence (a), and in the presence (b) of noradrenaline. Both situations followed the same stimulation protocol. Noradrenaline was added to the electrode tip. The arrows show preferred values of released transmitter, and the numbers show the quantal content. Noradrenaline resulted in a reduction in EJCs amplitude. In addition, there was a shift of the amplitude frequency distribution to the left. There was no change in the separation between preferred values. Those changes suggest a pre-junctional effect.
3-2.6.b The effect of noradrenaline (10^4M) on the new current amplitude

The effect of noradrenaline (10^4M) on the new current revealed by L-arginine (3.4x10^4M) was tested to investigate the possibility that the new current might be subjected to the inhibitory regulatory mechanisms regulating transmitter release. In this experiment the preparation was first incubated with L-arginine until the new current revealed, followed by the addition of noradrenaline to the recording electrode tip. As figure 26 shows, there was a reduction in the new current amplitude of -83.28 +/- 13 %, (P < 0.0001, n=10) 2 minutes after exposure to noradrenaline. Furthermore, all the new current responses disappeared after 30 minutes of exposure to noradrenaline. This can be explained as either that noradrenaline acted post-synaptically on α₁-adrenoceptors, causing the tissue to contract, resulting in the loss of the release site under the recording electrode. Or the other possibility is that noradrenaline acted presynaptically on α₂-adrenoceptors, hence causing a negative feedback autoinhibitory mechanism towards the new current. To test the above two assumptions, prazosin (an α₁-adrenoceptor) antagonist was added. Prazosin should result in desensitisation of post-synaptic α₁-adrenoceptors. As a result, added noradrenaline should not produced any tissue contraction. Such a test should rule out the possibility that the release site was lost due to tissue contraction.
Figure 26. The effect of noradrenaline on the new current. The graph shows the mean reduction in amplitude of the new current before and after the addition of noradrenaline (NA). Preparations were stimulated with the same stimulation protocol in both situations. In set one the preparations were only treated with L-arginine. In sets two and three NA was added to half of the preparation. Recording was made 2 and 30 minutes following the addition of NA. Responses reduced after the addition of noradrenaline, until they disappeared.

3-2.7. **The effect of Prazosin and noradrenaline on the new current revealed by L-arginine**

The effects of prazosin ($10^{-6}$M) (the $\alpha_1$-adrenoceptor antagonist) and noradrenaline ($10^6$M) on the new current were tested. Prazosin was added to inhibit the action of presynaptic $\alpha_1$-adrenoceptors. This is to test whether the responses were lost due to tissue contraction by the action of noradrenaline.

Figure 27 shows the effect of prazosin and noradrenaline on the new current. As the figure indicates, the mean amplitude was reduced from $99 +/- 6\%$ ($n = 6$) in the presence of L-arginine to $32 +/- 12\%$ ($n = 6$) in the presence of L-arginine, noradrena-
line, and prazosin after few minutes incubation. This reduction of 67% is highly significant, \( P < 0.0001 \). Further exposure abolished the new current completely. This result was compatible with results from previous sets of experiments where only noradrenaline was added. This result conforms that the reduction in the new current amplitude is attributable to noradrenaline acting on pre-synaptic \( \alpha_2 \)-adrenoceptors and not due to noradrenalin mediated tissue contraction through \( \alpha_1 \)-postsynaptic adrenoceptors.

![Graph showing the effect of prazosin (Praz.) and noradrenaline (NA) on the new current revealed by L-arginine.](image)

**Figure 27.** The effect of prazosin (Praz.) and noradrenaline (NA) on the new current revealed by L-arginine. The graph shows mean response changes. Recordings were made first in the presence of L-arginine (set one), followed by recording in the presence of L-arginine noradrenaline and prazosin 2 and 30 minutes after the addition of the drugs according to the legend, (set two and three respectively). New currents reduced in size until they disappeared after the addition of NA+Praz. This result is compatible with the result obtained when NA only was added to the electrode tip. This means that the disappearance of the new current is not due to NA induced tissue contraction, because \( \alpha_1 \)-adrenoceptors is desensitised by the presence of Praz. These result might indicate that the disappearance of the new current is due to NA acting on the nerve responsible for the new current.
Figure 28 summarises the results of the above four experiments. Since the new current disappeared after exposure to noradrenaline and increased in the presence of yohimbine and idazoxan (the \(\alpha_2\)-adrenoceptor antagonists). This might indicate that there might be \(\alpha_2\)-adrenocptors on the new current.

Figure 28. Summary of the effect of L-arginine, yohimbine, idazoxan, noradrenaline, and noradrenaline and prazosin. All preparations followed the same experimental protocol. The data shown represent mean response changes. The preparations in set one were always treated with L-arginine. In sets two and three preparations were treated according to the drug mentioned in the legend. The new current amplitude in the presence of L-arginine increased, but to a limited extent. Yohimbine and idazoxan both increased the new current amplitude to a highly significant level. Noradrenaline on the other hand abolished it completely.
3-2.8 Electrophysiological analysis of the effect of histamine

Histamine as a mediator of neurotransmission has been shown to act in a number of biological tissues. There is strong evidence that histamine may have a role as a neurotransmitter or neuromodulator (Prell & Green, 1986; Schwartz et al., 1986b). Histamine roles as a neurotransmitter or neuromodulator have been investigated in many tissues but not to the same extent in the mouse vas deferens. In this thesis the role of histamine will be investigated in some detail. The aim of this section is to answer two questions:

1- Does histamine exert any effect in this tissue?

2- If so, which histamine receptors exist in the mouse vas deferens and what is their action?

3-2.8.a The effect of histamine \((10^9-10^3M)\) on EJCs

The effect of a range of concentrations of histamine was tested on EJCs amplitude. In addition, a dose-response curve of histamine was constructed. EJCs were first collected before the addition of any drug. This was followed by the addition of histamine by internal perfusion of the electrode tip, followed by collection of EJCs under the effect of histamine. The tissue was field stimulated by train of 20 pulses at 1Hz, 0.06-0.15 ms pulse width, with 1 minute rest intervals were allowed in between trains.
There was a reduction in EJCs amplitude following the addition of histamine. The reduction varied from 14 +/-5 % (n = 4), (P < 0.09) with histamine 10^{-9}M. And up to a reduction of 82 +/- 20 % (n = 4), (P < 0.0001) with histamine 10^{-5}M. This is a highly significant effect. Figure 29 summarises the effect of histamine.
3-2.8.b The effect of histamine ($10^6$M) on the mean quantal content

Figure 30 shows the effect of histamine ($10^6$M) on the mean quantal content of responses from preparations in its absence and presence. There was a reduction in the quantal content of secreted transmitter. The mean quantal content changed from $5 \pm 0.12$ (n=3) in the absence of histamine to $2.7 \pm 0.2$ (n=3) in its presence. This is 53% reduction in the mean quantal content. This reduction is highly significant (P<0.0001). These results suggest that the reduction in EJCs amplitude is partly due to a reduction in the quantal content of released transmitter.

![Figure 30. The effect of histamine ($10^6$M) on the mean quantal content. Responses were recorded from preparations in the presence and in the absence of histamine, from three different preparations. Each line represent quantal content change from a single preparation. There was a 53% reduction in the quantal content. This reduction is highly significant (P < 0.0001). The figure shows that the reduction in EJCs amplitude responses were in part due to a reduction in the transmitter released.](image-url)
3-2.8.c The effect of histamine ($10^4$M) on EJC's amplitude distribution

Figure 31 shows the quantal effect of histamine on EJC's amplitude distribution. There was a reduction of 60 +/-6 % in the mean amplitude distribution in the presence of histamine, which resulted in a general shift of distribution of amplitude to the left. Additionally, there was a change in the separation between preferred values, also there was a change in the peaks of preferred value which reflects a pre-junctional effect. These changes together reflect both a pre- and a post-junctional effect.
Figure 31. The effect of histamine (10^6 M) on the mean quantal effect of responses. EJCs were recorded by focal extracellular electrodes from the surface of the vas deferens in the absence and in the presence of histamine (10^6 M). (a) distribution of response in the absence of histamine, it was rather not possible to identify the quantal content due to most probably the presence of more than one release site. (b) distribution of responses in the presence of histamine. The arrows show the peaks of preferred values, and the numbers represent the quantal effect of the released transmitter. The mean quantal effect reduced by 60 +/- 6% in the presence of histamine. The reduction in the EJCs amplitude resulted in shifting the frequency distribution to the left. In addition, there was a change in the relative separation between peaks of preferred values, which reflects a post-junctional effect. This indicates that histamine manifested a pre- and a post-junctional effect on transmitter release in this tissue.
Conclusion

The quantal analysis showed that there was a combination of both pre- and post-junctional modulation by histamine. However, the overall effect of histamine was a reduction of EJC's amplitude, reflecting either a change in transmitter release or a change in the post-synaptic effect of the transmitter. Since the effect of histamine was not immediate, it suggests a modulatory effect related to transmitter release and synthesis.

The above results clearly indicate that mouse vas deferens does contain histamine receptors. In the next section of this thesis a number of H₂ and H₃ receptor agonists and antagonists will be tested in an attempt to established some of mechanisms of action of histamine (the results obtained by manipulating H₁ histamine receptors were not conclusive).

3-2.9.a The effect of H₃ histamine receptor manipulation

Do H₃ histamine receptors exist in this tissue? In order to answer this question, the effect of thioperamide was tested. Thioperamide is a highly selective H₃ histamine receptor antagonist (Hill, 1992). Figure 32 shows the effect of thioperamide on EJC's amplitude. EJC's amplitude was increased in size by 13 +/- 4 % (n = 3), (P < 0.03) 20 minutes after the addition of thioperamide, and up to 35 +/- 7 % following 40 minutes of exposure to thioperamide. This is a significant change (P < 0.008). This is an indi-
cation that histamine H3-receptors do exist in this tissue, and they might account for some of the effects induced by histamine.

Figure 32. The effect of thioperamide $10^{-7}$M on EJC's amplitude. Thiopiramide is a highly specific histamine H3-receptor antagonist. The graph shows the changes in EJC's amplitude in the presence and in the absence of thioperamide which was added to the electrode tip. In set one both preparations were untreated. In sets two and three some preparations were treated with thioperamide and the others were not (and taken as control). In set two and three recordings were made 20 and 40 minutes following the treatment with thioperamide respectively. The graph shows that thioperamide resulted in an increase in EJC's amplitude. The increase varied from 13% 20 minutes after exposure to thioperamide. And up to 35% following 40 minute of exposure to thiopiramide, this being highly significant effect ($P < 0.008$).

3-2.9.b The effect of thioperamide on mean quantal content

Figure 33 shows the effect of thioperamide ($10^{-7}$M) on the mean quantal content. There was an increase in the quantal content of secreted transmitter after the addition of thioperamide. The quantal content increased from 2.23 +/- 0.05 ($n = 2$) in the
absence of thioperamide to 3.05 +/- 0.05 (n = 2) in its presence. This is an increase of 37%. This is a significant increase (P < 0.008).

![Graph showing the effect of thioperamide on mean quantal content of EJCs](image)

Figure 33. The effect of thioperamide $10^{-7}$M on the mean quantal content of EJCs recorded from preparations in its absence and presence. The mean quantal content increased by 37% in the presence of thioperamide. This increase is significant (P< 0.008). The mean quantal information was extracted from Excel. The lines represents the change in the mean quantal content of responses from preparations in the absence and in the presence of thioperamide.

3-2.9.c The effect of thioperamide on EJCs amplitude distribution

Figure 34 shows the effect of thioperamide on EJCs amplitude distribution. There was an increase in EJCs amplitude by up to 35 +/- 7%, (P < 0.0008). Furthermore, there was a shift of responses to the right as a result of increase in EJCs amplitude. There was no change in the relative separation between peaks of preferred values of EJCs amplitude distribution. There was a reduction in number of failures. These changes indicate that the effect of thioperamide is not post-junctional. The effect of
thioperamide was not an immediate effect. The sum of all these changes predicts that thioperamide exert a pre-junctional modulatory effect.

Figure 34. The effect of thioperamide $10^{-7}$M on EJCs amplitude distribution. (a) responses distribution in the absence of thioperamide. (b) response distribution in the presence of thioperamide. Arrows showed preferred values, and the numbers represent the quantal content. There was no change in the separation between peaks of preferred values. There was an increase in the EJCs amplitude, which resulted in shifting the frequency distribution to the right. This suggests that the effect of thioperamide is not post-junctional.
3-2.10 The effect of the $H_3$-histamine agonist, $R-(\alpha)$-methyl histamine

3-2.10.a The effect of $R-(\alpha)$-methyl histamine ($10^4 M$) on EJCs amplitude

The effect of $R-(\alpha)$-methyl histamine, an $H_3$-receptor agonist on EJCs amplitude was tested. Results from the previous set of experiment through manipulation of histamine $H_3$-receptors by the highly selective $H_3$ antagonist thioperamide, indicated the presence of histamine $H_3$-receptors most probably in a pre-junctional site. Thioperamide resulted in an increase in EJCs amplitude by up to 35 +/-7 %. To prove by a different method that this increase was due to the thioperamide acting on $H_3$ histamine receptors, the effect of $R-(\alpha)$-methyl histamine was tested on EJCs. ($R-(\alpha)$-methyl histamine is a highly selective $H_3$ receptor agonist). Figure 35 shows the effect of $R-(\alpha)$-methyl histamine, which resulted in reduction in the mean EJCs amplitude by 50 +/- 6.6 % (n = 3). This is highly significant (P < 0.0001). Further exposure to the drug did not have any significant reduction in the EJCs amplitude. This is further evidence that $H_3$-receptors do exist in this tissue, they probably modulate neurotransmitter release. The effect of $H_3$-receptor agonists and antagonists is not immediate. It needs some time to produce a significant effect on EJCs amplitude responses and this could suggest a pre-synaptic modulation towards transmitter synthesis.
Figure 35. The effect of α-methyl histamine $10^4$M (H$_3$-receptor agonist), on the mean EJCs amplitude. Data shown represent mean EJCs responses change. α-methyl histamine was added to the electrode tip. In set one both preparations were not treated with α-methyl histamine. In set two (20 min), and three (40 min) half of the preparations were treated with α-methyl histamine, and the other half not and was taken as a control. α-methyl histamine resulted in a reduction in EJCs amplitude by up to 50%. This is highly significant effect ($P < 0.0001$). EJCs in untreated preparation fell slightly, which was not significant. This is an indication that histamine H3-receptors exist in this tissue, and it may account for some of the effects of histamine in reducing EJCs.

### 3.2.10.b The effect of R-(α)-methyl histamine ($10^4$M) on the mean quantal content

Figure 36 shows the change in mean quantal content of preparations treated with R-(α)-methyl histamine ($10^4$M). The mean quantal content reduced from 3.4 +/- 0.05 (n = 2) in the absence of R-(α)-methyl histamine, to 2.4 +/- 0.05 (n = 2) in its presence. This is a reduction by 71%, which was significant ($P<0.008$). This indicates that the reduction in EJCs amplitude was in part due to a reduction in secreted quanta. This means that the effect of R-(α)-methyl histamine is pre-junctional.
Figure 36. The effect of αmethyl histamine $10^{-8}$M on the mean quantal content. Responses were recorded in the absence and in the presence of αmethyl histamine. The graph shows results from two preparations. αmethyl histamine resulted in 71 % reduction in the quantal content of transmitter secreted. This was significant ( $P<0.008$). This indicates that the reduction in EJCs amplitude resulted from reduction in secreted quanta. This effect is a pre-junctional effect. The data extracted from Poisson fitter using ECXEL.

3.2.10.c The effect of R-(α)-methyl histamine ($10^{-4}$M) on EJCs amplitude distribution

Figure 37 shows the effect of R-(α)-methyl histamine ($10^{-4}$M) on EJCs amplitude distribution. Generally there is a reduction in EJCs amplitude. The relative separation between peaks of preferred values of EJCs amplitude distribution did not change. There was an increase in the number of failures. This does not favour a post-junctional effect.
Figure 37. The quantal effect of R-(\(\alpha\))-methyl histamine 10^{-4}M on EJCs amplitude. EJCs recorded in the absence (a), and in the presence (b) of (\(\alpha\))-methyl histamine, which was added to the electrode tip. Both situations followed the same stimulation protocol. The arrows show the peaks of preferred values, and the numbers represent the quantal content. There was a reduction in EJCs amplitude following the addition of R-(\(\alpha\))-methyl histamine. There was no change in the separation between peaks of preferred values. Those changes indicate that the effect of R-(\(\alpha\))-methyl histamine is not post-junctional.
3-2.10.d Does thioperamide (10^-7 M) have the ability to reverse the inhibitory effect exerted by α-methyl histamine (10^-4 M)

In this set of experiments R-(α)-methyl histamine (10^-8 M) was added first followed by the addition of thioperamide (10^-7 M). This is to test if thioperamide can reverse the inhibitory effect of R-(α)-methyl histamine. Figure 38 shows that thioperamide (10^-7 M) reversed the inhibitory effect exerted by R-(α)-methyl histamine (10^-8 M). EJC's were reduced by 40%, (P < 0.006) (n = 5) in the presence of R-(α)-methyl histamine. The addition of thioperamide resulted in an increase of EJC's amplitude by 41%, which was significant (P < 0.02) (n = 5). The above results suggest that H3 histamine receptors do exist in this tissue. This is because both the agonist and the antagonist produced an effect, and the fact that the antagonist did reverse the effect produced by the agonist.
Figure 38. The effect of thioperamide (10^{-7} M) on the inhibitory effect induced by R-(α)-methyl histamine (10^{-4} M). Data shown represent mean EJC's response changes. In set one both preparation were not treated. In set two half of the preparation were treated with R-(α)-methyl histamine, and the other half were not and taken as a control. In set three thioperamide was added to preparation which was treated with R-(α)-methyl histamine, and the other half kept untreated. Thioperamide was able to reverse the inhibitory effect induced by R-(α)-methyl histamine, suggesting most probably that both work on the same receptors, the H_3 histamine receptors. both effects being significant. All responses were expressed as a percentage of a normalising response taken at the beginning of each experiment.

**Conclusion**

Figure 39 shows the summarised results regarding histamine H_3-receptor. H_3-receptor stimulation by the addition of an H_3 agonist resulted in inhibition of responses. Whereas, the inhibition of H_3 receptors by an antagonist such as thioperamide, resulted in the opposite effect, an enhancement of the responses. Thioperamide reversed the inhibitory action produced by the agonist αmethyl histamine. Since both drugs are highly specific in their action, consequently the above result is proof that H_3 receptors do exist in this tissue and their action is most probably due to regulation of transmitter release and synthesis.
Figure 39. The effect of thioperamide (10^(-7)M), 3-methyl histamine (10^(-8)M), and thioperamide + 3-methyl histamine on EJCs mean amplitude. Preparations were recorded in the absence and in the presence of drug treatment. All responses expressed as a percentage of a control response taken at the beginning of each experiment before the drug treatment. Data shown represent mean EJCs response changes. In set one all preparations were untreated. In sets two and three preparations were treated according to the drug mentioned in the legend. Thioperamide resulted in an increase in EJCs amplitude, 3-methyl histamine resulted in reduction in EJCs amplitude. Thioperamide reversed the inhibitory effect induced by 3-methyl histamine.

3-2.11 The effect of manipulating H₂ histamine receptors by an agonist.

3-2.11.a The effect of the H₂ receptor agonist dimaprine (10^(-6)M) on EJCs amplitude

The results obtained through manipulating histamine H₃-receptors are not enough to explain the effect of histamine on EJCs. Therefore, the effect of manipulating H₂ receptors (if they do exist in this tissue) was tested. Dimaprine (10^(-6)M), an H₂ receptor agonist was used. The protocol followed was similar to that of H₃ receptors experiment. Figure 40 shows the effect of dimaprine on EJCs amplitude. There was a
reduction in EJCs amplitude of -48 +/- 6 % (n = 5) following exposure to dimaprite. This change is significant (P < 0.01). This suggests that H₂ histamine receptors do exist in this tissue and it could account for some of the effects exerted by histamine in reducing EJCs responses amplitude.

![Graph showing the effect of dimaprite on EJCs amplitude](image)

Figure 40. The effect of dimaprite on EJCs amplitude. In set one both preparations were not treated with any drugs. In set two (15 min) and three (30 min) half of the preparations were treated with dimaprite and the other half were not and taken as a control. Data shown represent mean EJCs response changes. The graph shows that dimaprite reduced the EJCs amplitude by 48 %. This is a significant effect (P < 0.01). In the untreated preparations, EJCs amplitude fell slightly, which was not significant.

3-2.11.b The effect of dimaprite on the mean quantal content

Figure 41 shows the effect of dimaprite on the mean quantal content of EJCs recorded from preparations in the absence and in the presence of dimaprite. There was a reduction of mean quantal content from 3.3 +/- 0.05 in the absence of dimaprite to
2.5 +/- 0.06 in its presence (n=2). This is a reduction of 25 % in the mean quantal content. This effect is not significant (P < 0.07).

Figure 41. The effect of dimaprite on the mean quantal content calculated from two preparations in the presence and in the absence of dimaprite. Both situations followed the same stimulation protocol. Dimaprite was added to the recording electrode tip. The graph shows that the reduction in EJC's amplitude in the previous experiment is in part due to a reduction in quantal content of transmitter released. The mean quantal content reduced by 75 %, but this reduction is not significant (P < 0.07).

3-2.11.c The effect of dimaprite on EJC's amplitude distribution

Figure 42 shows the effect of dimaprite on the EJC's amplitude distribution recorded from preparation in its absence and presence. Dimaprite generally resulted in
the reduction of EJCs amplitude by 48%. In addition, the preferred values changed in the presence and in the absence of dimaprite, suggesting a post-junctional effect, considering the fact that there was a change in quantal content, and a change in the separation between EJCs amplitude preferred values. These two changes taken together indicate that there is pre- and post-junctional $H_2$ receptors.
Figure 42. The effect of dimapride on EJCs recorded from preparations in its absence (a), and presence (b). Both situations followed the same stimulation protocol. Dimapride was added to the recording electrode tip. The arrows show the peaks of preferred values, and numbers represent the quantal content. As shown, the EJCs amplitude reduced in the presence of dimapride by 48%. This reduction is significant (P<0.01, d.f. = 2). In addition, there was a change in the separation between the relative peaks of preferred values. Those two effects added together mean that dimapride exerted both pre- and post-junctional effect.
3-2.12 The effect of manipulating $H_2$ histamine receptors by an antagonist

3-2.12.a The effect of ranitidine ($10^{-5} M$) on EJCs amplitude

The effect of histamine on EJCs amplitude performed earlier could not be fully explained by thioperamide acting on $H_3$ histamine receptors. This experiment was designed to determine whether histamine $H_2$ receptors exist in this tissue, and if they do, does their action explain some of the inhibitory effect produced by histamine. Ranitidine ($10^{-5} M$) was used, it is a highly selective antagonist of $H_2$ receptors. The protocol used is as follows: EJCs were recorded in the absence of ranitidine. Ranitidine was added to the electrode tip. Recording were made 15 and 30 minutes following the addition of ranitidine. Control preparations followed the same protocol but without the addition of ranitidine. Figure 43 shows that ranitidine increased EJCs amplitude by 47% ($n = 3$). This is a significant increase ($P < 0.007$), compared to the untreated preparations where responses fell slightly.
Figure 43. The effect of the H₂ histamine receptor antagonist ranitidine (10⁻⁷M) on the mean EJCs amplitude. In set one all preparations were untreated. In sets two and three ranitidine was added to the electrode tip in half the preparation and the other half was used as control. Recordings were made 15 and 30 minutes following the addition of ranitidine. Data shown represent mean EJCs response changes. The graph shows that ranitidine resulted in an increase in the EJCs amplitude. This increase is significant (P < 0.007), (n = 3). The control response fell slightly.

3-2.12.b The effect of ranitidine (10⁻³M) on the mean quantal content

Figure 44 shows the effect of ranitidine on the mean quantal content of secreted transmitter. From the above results ranitidine caused an increase in EJCs amplitude to a significant level. Ranitidine also resulted in an increase of the quantal content from 2.25 +/-0.05 (n = 2) in the absence of ranitidine, to 3.35 +/-0.05 (n = 2) in its presence, this is an increase of 49%. This increase is not significant (P < 0.05).
Figure 44. The effect of ranitidine on the mean quantal content. Responses recorded in the presence and in the absence of ranitidine, which was added to the recording electrode tip. Both situations followed the same stimulation protocol. The graph shows the there is an increase in the quantal content of secreted transmitters as a result of ranitidine treatment by up to 49%. This increase was not significant (P < 0.05).

3-2.12. c The effect of ranitidine on EJC's amplitude distribution

Figure 43 shows the effect of ranitidine on the distribution of EJC's amplitude. Ranitidine generally resulted in an increase in the EJC's amplitude. Figure 44 shows that ranitidine did increase the quantal content of secreted transmitter. Figure 45, the effect of ranitidine on EJC's amplitude distribution, shows that ranitidine resulted in a change in the separation between peaks of preferred value. This is an indication on change in the quantal effect post synaptically. The sum of these results indicates that ranitidine has a post- and a pre-synaptic effect.
Figure 45. The effect of ranitidine on EJCs amplitude distribution. Response recorded from preparations in the absence (a), and in the presence (b) of ranitidine. Ranitidine was added to the recording electrode tip. The arrows show the peaks of preferred values, and the numbers represent quantal content. There was a shift of responses to the right as a result of increase in EJCs amplitude. In addition, there was a change in the separation between peaks of preferred values, which reflects a post-junctional effect.
3.2.13 Does ranitidine reverse the inhibitory effect of dimaprite on EJC's amplitude

The previous experiments showed that histamine resulted in a depression of responses. This could be explained by either histamine acting on presynaptic receptors, thus resulting in an alteration of transmitter released, or on the other hand, that histamine acted on post synaptic H₂-histamine receptors resulting in an alteration of responses. The latter possibility was partially tested by examining the effect of dimaprite and ranitidine on responses. Dimaprite (10⁻⁴M) (a H₂-histamine receptors agonist) resulted in a reduction of EJC's amplitude. Whereas ranitidine (a H₂-histamine receptors antagonist) resulted in enhanced responses. This experiment was designed to test whether ranitidine could reverse the inhibitory effect exerted by dimaprite.

Figure 46 shows the effect of ranitidine on the depressive effect of dimaprite. Dimaprite reduced the EJC's amplitude by 78 +/- 10 % (n = 4). This reduction is significant (P < 0.001). The addition of ranitidine resulted in a reversal of this inhibitory effect of dimaprite. EJC's increased by 59 +/- 11 % (n = 4). The effect of ranitidine is significant (P < 0.003). This means that dimaprite, acting on H₂-histamine receptors resulted in a reduction in EJC's amplitude, and ranitidine probably acted on the same receptors resulting in reversing the action imposed by dimaprite.
Figure 46. The effect of ranitidine on the depressive effect of dimaprite (10^4M). In set one all preparations were untreated. In set two half of the preparations were treated with dimaprite and the other half were not. In set three ranitidine was added to the preparation which contained dimaprite. Both situations followed the same stimulation protocol. Data shown represent mean EJC’s response changes. Dimaprite resulted in reduction in EJC’s amplitude by 78% +/- 10, this is a significant effect (P < 0.001). Ranitidine was able to reverse the inhibitory effect exerted by dimaprite through acting on histamine H2-receptors. Ranitidine resulted in an increase in EJC’s amplitude by up to 59% +/- 11, which was significant (P < 0.003).

**Conclusion**

Figure 47 summarises the effect of manipulating H2-histamine receptors. Ranitidine an H2-histamine receptor antagonist, resulted in enhancing the EJC’s amplitude. Whereas dimaprite caused the opposite to happen, it reduced EJC’s amplitude. Both drugs altered the mean quantal content, this reflects a pre-junctional effect. On the other hand, EJC’s amplitude distribution showed that there was a mixture of both pre and post-junctional modulation. Ranitidine was able to reverse the inhibitory action of dimaprite, suggesting that both acted on the same receptors.
Figure 47. Summary of the effect of manipulating H₂-histamine receptors on EJCs amplitude. All preparations followed the same stimulation protocol. The drugs in all experiments were added to the recording electrode tip. In set one all preparations were not treated. In sets two and three, preparations were treated according to the drug mentioned in the legend. Data shown represent mean EJCs response changes. Ranitidine increased the EJCs amplitude, whereas, dimaprit did the opposite, it reduced EJCs amplitude. Ranitidine was able to increase EJCs that had been depressed by dimaprit. All effects were statistically significant.
3-3 Histochemical demonstration of NOsynthase

The fact that NOsynthase possesses NADPH-diaphorase activity, made it possible to demonstrate the presence of NOsynthase by using a NADPH-diaphorase histochemical technique. This technique is based on the presence of an enzyme that can catalyse the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble, visible formazan pigment (Schere Singler et al., 1983). The important question is: does all NADPH-diaphorase activity represent NOsynthase, in fresh tissue, a large fraction of NADPH-dependent diaphorase activity is not NOsynthase (Matsumoto et al., 1993). However, the diaphorase activity of NOsynthase-I is exceptionally resistant to aldehyde fixation (Weinberge et al., 1994). After fixation, most NADPH-diaphorase positive areas is co-localised with NOS-I immunoreactivity. In neostriatum NOsynthase-I immunoreactivity is 100% co-localised with NADPH-diaphorase staining, in cerebellum 99% of NADPH-diaphorase positive neurones are co-localised with NOsynthase-I immunoreactivity (Kharazia et al., 1994). This procedure when used properly provide a simple, convenient and reliable marker for detecting multiple isoforms of NOsynthase (Richard et al., 1996). Three major techniques are now available to use for the localisation of NOsynthase:

1- NADPH-diaphorase histchemistry

2- NOsynthase immunocytochemistry

3- In situ hybridisation

In this thesis an attempt was made to demonstrate the presence of NOsynthase by its NADPH-diaphorase activity. NADPH-diaphorase histochemistry technique was used, it is a rapid method with excellent sensitivity, but the staining might not be permanent.
**Results**

3-3.1 **Light microscopy**

Histochemical staining with nitro blue tetrazolium (NBT) revealed a distinctive dark blue stain scattered throughout the smooth muscle layer. It was not possible to tell whether this stain was located inside or in between the smooth muscle cells. It was very easy clear to distinguish the positively stained areas in the stained tissue from those which are not in the controls. The stain within the muscular layer was more intense towards the lumen of the vas deferens. Under higher magnification the stain appears to be blue oval shaped deposits scattered intra- and extracellularly (Figure 48).

![Figure 48. NADPH-diaphorase histochemistry. Light microscopy photograph of mouse vas deferens smooth muscle cells (40x magnification), positive area appear as irregular dark deposits scattered all over the smooth muscle layer (arrow).](image-url)
The distribution of NADPH-diaphorase histochemical positive areas was almost identical in different parts of the vas deferens. Suggesting that the function of NO synthase is evenly distributed along the vas deferens. This is unlike the distribution of some neurotransmitters such as acetylcholine the majority of which is at the testicular end of the vas deferens (Dixone et al., 1976). In the control section there were no NADPH-diaphorase positive deposits and the tissue was simply stained with the red pink safranine dye, the counterstain (Figure 49).

Figure 49. Light microscopy photograph of vas deferens smooth muscle cells (control) in which the NADPH was omitted from the incubation medium (25x magnification). The irregular oval deposits are absent (compare with figure 48).
3-2.2 Electron microscopy

Tissue processing for electron microscopy was rather difficult. One of the early problems faced was that the epoxy propane used (epoxy propane is a solvent used in tissue processing for electron microscopy), caused the stain to leach out at the stage of infiltration with resin. LR white (LRW) was used instead. It is a type of resin (methacrylate) which does not require the use of epoxy propane. On the other hand, another problem emerges, which was when thin sections were examined in the electron microscope, they were often very unstable in the electron beam. To get over this problem sections have to be examined and photographed as quickly as possible.

Deposits of formazan were seen associated with endoplasmic membrane but only at a few sites. The stain is more prominent on parts of the outer mitochondrial membrane. The same stain was also associated with other cellular organelles which were difficult to identify (Figure 50). The stain was also associated with parts, but not the whole of the nuclear membrane.

BPST (2-(2-benzathiazolyl)-3-(4-phthalhydrazidyl)-5-styryl tetrazolium chloride) was used in this technique. It is a monotetrazolium salt, which was specifically designed for electron microscopy. Formazan the reaction product is lipophobic and tends not to dislocate or dissolve. As a result, a very precise localisation of the enzyme was seen in our electron microscopy specimen, It is reasonable to conclude that NADPH-dihaporase (smooth muscle type)is primarily bounded to membrane. In particular to those of the mitochondria, and to a lesser extent to the nucleus.

The present experiment confirmed the presence of NOsynthase enzyme in the mouse vas deferens. Under light microscopy the NADPH-positive areas are present
throughout the muscular layer, suggesting that NO synthase activity does indeed exist in the mouse vas deferens.

Figure 50. Visualisation of formazan deposits using electron microscopy preparation following NADPH-diaphorase reaction. (a) NADPH-diaphorase positive smooth muscle cells, shows either all or part of the outer layer of the mitochondrial membranes are positively stained (arrow). Nuclear membrane displays sites of formazan precipitation (arrow). (b) (next page) control section in which BPST was excluded from the incubation medium, shows no deposits of the stain.
My hypertension are the two local treatments that have been
response. In addition, results showed that the effect of hypertension is mediated through
H1 and H2 receptors. These receptors are part of a group complex modulated
system participate in by several different systems relevant to this tissue.
4- DISCUSSION

NO and histamine are the two local neuromodulatory agents that have been dealt with in this thesis. The role of nitric oxide (NO) in modulating the release of neurotransmitters has been demonstrated in some tissues, such as anococcygeus muscle (Li & Rand, 1989; Leiu et al., 1991). NO modulatory effect in the mouse vas deferens has not been fully established, but some early experiments from our laboratory showed that NO is a modulator of neurotransmission in the mouse vas deferens. NO depressed pre- and post-junctional activities. In addition, experiments using histamine showed that it modulates neurotransmission processes (Ph.D. thesis, R. Paterson, Leicester University, 1996). In the current study, the role of NO and histamine in modulating sympathetic neurotransmission in the longitudinal smooth muscle cell of the vas deferens has been investigated in some detail. Results of experiments included in this thesis showed that NO is a modulator of neurotransmission in the mouse vas deferens. NO depressed pre- and post-junctional responses. In addition, results showed that the effect of histamine is mediated through H₂ and H₃ receptors. Those receptors are just a part of a more complex modulatory control system participated in by several different innervations found in this tissue.
The establishment of a role for NO in modulating tissue function

Furchgott and Zawadzki (1980) were the first to discover endothelium derived relaxing factor (EDRF). They demonstrated that the relaxation of precontracted rabbit aortic ring preparations by acetylcholine required the presence of intact endothelium (Furchgot and Zawadzki, 1980). Endothelium dependent relaxation was subsequently demonstrated in many vascular preparations, such as veins, arteries and capillaries (Furchgot, 1984; Griffith et al., 1984). EDRF was first established as a boiregulatory compound in the endothelium in controlling tone and hemodynamics. Since then this role has been established across almost all physiological systems in the mammalian organs. It is proposed that EDRF is equivalent to NO (Furchgot & Vanhoutte, 1988).

The main bulk of research work to establish the role of NO in biological system was performed on vascular smooth muscle cells. Little has been done to established the role of NO in non-vascular tissues, and in particular in the mouse vas deferens smooth muscle cells. However, NO synthase has been demonstrated in some neuronal tissues. For example, in the guinea pig vas deferens exogenous NO exerted an inhibitory effect on adrenergic and non-adrenergic non-cholinergic neurotransmission, supporting a role for NO as a neuromodulator (Cederqvist & Gustafson, 1994).

NO is produced from L-arginine, the natural substrate for NO synthase, and citrulline is a co-product. NO mediates its action through activation of soluble guanylate cyclase (s-GC) resulting in production of c-GMP, which mediates relaxation of muscle cells (Sanders & Word, 1992). Pre-junctional modulation of transmission has been demonstrated in many tissues, but whether NO is playing any role in this mechanism is to be established.
4-1.2 The existence of NO mediated neuromodulatory mechanism in the mouse vas deferens

Over the last few years evidence has been accumulating to suggest that NO may be the transmitter in some non-adrenergic non-cholinergic nerves (NANC), and may be a modulator in others. In rat and mouse anococcygeus muscle, L-NMMA inhibited NANC-mediated relaxation (Li and Rand, 1989; Gibson et al., 1989). Others showed that NO synthase inhibitors such as L-NMMA enhance the contractile response to noradrenergic stimulation (Li and Rand, 1989; Gibson et al., 1989) suggesting a neuromodulatory role for NO. NO is responsible for NANC-mediated relaxation of gastric fundus (Li and Rand, 1990). NO also mediates NANC inhibition of lower oesophageal sphincter (Tottrup et al., 1991) suggesting its role as a neurotransmitter. The L-arginine:NO pathway has been shown to have an important role in relaxation of human (Holmquist et al., 1991) and rabbit (Ignarro et al., 1990) corpus cavernosum. In those tissues, electrically evoked relaxation is inhibited by L-NNA and mimicked by the NO donor sodium nitroprusside (SNP).

A possible neuromodulatory role for NO has been proposed in other systems. In the guinea-pig ileum and pulmonary artery, inhibition of NO synthesis enhanced nerve-evoked contraction (Gustafsson et al., 1990). Furthermore, NO suppressed the relaxant response of cerebral artery strips to transmural nerve stimulation (Toda and Okamuras, 1990a). Moncada suggested as an explanation for NO-mediated neuromodulation or neurotransmission, that there is a widespread system of nerves which contribute to the regulation of function of gastrointestinal, respiratory, cardiovascular, and genito-urinary systems. He suggested that these nerves are as
important as the classical adrenergic, cholenergic, and peptidergic systems, and their
dysfunction may lead to a variety of disorders (Moncada, 1991).

The results of experiments described in this thesis showed that NO modulates
neurotransmission in the mouse vas deferens, both pre- and post-synaptically.
However, what is described in this thesis is just a small part of a more complex
control system maintained by NO and other neuromodulators on sympathetic
neurotransmission in this tissue.

4-1.3 NO mediated neuromodulatory mechanism

The presence of NO synthase and NO-mediated neuromodulatory mechanism
was proved in this thesis by two methods, by pharmacological manipulation (dealt
with in sections 4-3.2 & 4-3.3). And by histochemical identification (dealt with in the
next section).

4-1.3.1 Histochemical evidence for the existence of NO synthase

NO synthase requires a reduced nicotine adenine dinucleotide phosphate
(NADPH) as a co-factor, and the electron transferring terminal sequence of
NO synthase gives rise to its NADPH-diaphorase activity. As a result, NO synthase can
reduce a dye such as nitro blue tetrazolium (NBT) to an insoluble, visible formazan
(Kuhn and Jerchel, 1941). Accordingly, it was quite possible to demonstrate the
presence of NO synthase through its NADPH-diaphorase activity, by using NADPH-
diaphorase histochemical techniques. Fixation is an important step in demonstrating the existence of NOsynthase, this is because a large fraction of NADPH-dependent diaphorases are not resistant to aldehyde treatment. NOsynthase is exceptionally resistant to fixation. As a result, following fixation a large fraction of NADPH-diaphorase positive areas represents NOsynthase (Schere-Singler et al., 1983). Furthermore, it is possible to identify NOsynthase using electron microscopy by employing BPST (2-(2-benzothiozolyl)-3-(4-phthalhydrazidyl)-5-styryl tetrazolium chloride) which forms an electron-dense reaction product under the influence of this enzyme (Wolf et al., 1992).

The enzyme NADPH-diaphorase is a synonymous with NOsynthase in rat brain. It has been found that NOsynthase and neuronal NADPH-diaphorase are identical in rat brain and peripheral nerve tissue. NOsynthase immunoreactivity and NADPH-diaphorase staining are co-localised in the pedunculopontine nucleus, and large and aspiny neurones (Dawson et al., 1991, Hope et al., 1991). It has been found that NOsynthase and neuronal NADPH-diaphorase copurify to homogeneity and that both activities could be immunoprecipitated with antibody recognising neuronal NADPH-diaphorase. Furthermore, NOsynthase was competitively inhibited by the NADPH-diaphorase substrate, NBT. Thus neuronal NADPH-diaphorase is NOsynthase, and NADPH histochemistry, therefore, provides a specific histochemical marker for neurones producing NO (Hope et al., 1991).

Under light microscopy it was possible to demonstrate a positive NADPH-diaphorase area, which appeared as a distinctive dark blue stain scattered throughout the smooth muscle layer. The intensity of the stain was more towards the lumen of the vas deferens. There were no obvious differences in the distribution of the NADPH-
diaphorase positive areas between the prostatic and the urethral ends of the vas deferens. Unlike other tissues such as rat vas deferens where NO-immunoreactivity was mainly in the lamina propria of the abdominal portion of the vas deferens (Ventura & Burnstock, 1996). Results from the current study are clear evidence for the existence of NO synthase in this tissue, which is an additional support for the possibility of the existence of NO-mediated neuromodulatory mechanisms in this tissue.

Further evidence came from the demonstration of NADPH-diaphorase positive areas under the electron microscope. BPST was used, as it is specially designed for electron microscopy. The reaction product formazan is lipophobic and tends not to be displaced or dissolve. As a result, this gives a highly precise location of the enzyme. Deposits of formazan were associated with (endoplasmic membrane) of the outer mitochondrial membrane, of some but not all mitochondria. The stain was also associated with other cellular organelles which were difficult to identify. Some parts of the nuclear membrane were also stained. It is possible to conclude that NO synthase is present in this tissues, and is associated with endoplasmic membranes (that of mitochondria and nucleus). It would be rather interesting to perform a NO synthase immunochrometry experiment and demonstration of the presence of NO synthase in this tissue through its binding with antibody recognising NO synthase. This could provide further evidence for the existence of NO synthase.
4-1.3.2 The effect of L-arginine (the natural substrate for NOsynthase) on nerve evoked contractions

L-arginine, the natural substrate for NOsynthase and the precursor of NO (Palmer et al., 1988), significantly reduces nerve evoked contractions when applied to isolated preparations of mouse vas deferens. Mechanical responses were depressed by 28% (P<0.0001). The application of exogenous agonist (noradrenaline or ATP) causes the smooth muscle of mouse vas deferens to contract. Analysis of these responses gives direct information about post-junctional mechanisms of agonist/receptors interaction. L-arginine resulted in shifting noradrenaline Dose-Response curve to the right, the EC$_{50}$ changed from 1.6x10$^{-5}$M to 1.7x10$^{-3}$M. This was not associated with any change in the maximum response. The above results suggest that there is a post-junctional, NO sensitive receptor mechanism in this tissue.

4-1.3.3 The effect of L-AME on nerve evoked contractions

Theoretically L-arginine methyl ester (L-AME), the NOsynthase inhibitor should result in reduction in the production of NO by competing with L-arginine (the natural substrates for NOsynthase in the production of NO). L-AME was used to inhibit NOsynthase, assuming it is present in this tissue, and as a result, the production of NO should be reduced. The use of L-AME resulted in a significant increase in nerve evoked contractions by up to 29 +/- 7%. The effect of L-AME on nerve evoked contractions reached a detectable level after about 60-70 minutes incubation period. In addition, L-AME resulted in shifting the noradrenaline dose-response curve to the left, the EC$_{50}$ changed from 1.6x10$^{-5}$M to 8.6x10$^{-6}$M, the maximum response increased
by 20 +/-10 %. These results indicate that NO is produced naturally in this tissue, and there is a system of NO-mediated neuromodulation in this tissue and it most probably acts post-synaptically.

4-1.4 Quantal analysis permits the assessment of pre- and post-junctional effect of a given treatment

EJCs recorded by focal extracellular electrodes are produced by the action of secreted ATP on post junctional P2x receptors on smooth muscle membranes (Åstrand et al., 1988). Both ATP and noradrenaline are co-released upon nerve stimulation, and since released transmitters cause cross inhibition of subsequent release, then EJCs are a direct indication of secreted ATP and an indirect indication of secreted noradrenaline. Quantal analysis of these currents gives information about pre- and post-junctional modulation of transmission. In addition, it gives information about quantal content and the number of quanta released. Under normal conditions, the EJCs amplitude distribution falls into preferred values which are thought to be related to the number of quanta released. Following drug treatment two types of changes can occur:

1- A change in quantal effect: This is a measure of post-junctional responses to released transmitter. This can manifest as a change in the magnitude and limits of preferred values compared to the control. In the short term this change reflects a change in the amplitude of post-junctional responsiveness. But these changes may occur slowly and in this case could reflect a pre-junctional modification, for example, a change in quantum size. Any
change in quantum size is a measure of a change in the number of transmitter molecules in each quantum due to a change in the synthesis of transmitter.

2- A change in quantal content, which is a change in the number of packets of transmitter released from each release site following stimulation. This manifests as a change in the number of peaks occurring, with no change in the separation between them.

4-1.5 Electrophysiological analysis of the effect of L-arginine

The addition of L-arginine to the extracellular electrode tip resulted in two types of changes. First, about 20 minutes following the application of L-arginine, EJCs started gradually to reduce in amplitude and in most experiments they disappeared by the end of the experiment. Second, about 40 minutes following exposure to L-arginine a new positive polarity current appeared, with shorter latency and lower threshold. The new positive polarity current continue to develop and increase in amplitude for about a further 30 minutes, by which time they had became fully developed. In almost all experiments all EJCs disappeared.

L-arginine resulted in a reduction in EJCs amplitude response by 87 %. Quantal analysis showed that there was a reduction in mean quantal content by 68%. EJCs distribution revealed a reduction in the size of the peaks of preferred values (peaks represent the frequency of occurrence of the EJCs amplitude). In addition, there was no change in the size of the interval between peaks of preferred values. The
above results, and the results obtained from the mechanical response experiments (Section 4-2.2) show that the effect of L-arginine is both pre- and post-junctional. Control experiments carried out in this thesis showed that the quantal effect and content of released transmitters remain unchanged throughout the experiments.

4-1.6 Characteristic of the new positive polarity currents

When L-arginine (3.4x10^{-4}M) was applied locally through the extracellular recording electrode for about 40 minutes a new current was revealed. The new current is of positive polarity, with threshold lower than that of EJCs recorded from the same site, indicating that a different nerve population is responsible for the existence of the new current. The latency of the new current was shorter than that of EJCs, indicating a faster conduction along the nerve population responsible for the new current.

4-1.7 Evidence that the new current is not EJCs activity outside the recording electrode tip

It is might be argued that this positive polarity new current is just a negative polarity (EJCs) outside the recording electrode tip, but this is not tenable for the following reasons:

1- During control experiments carried out as part of this thesis where no drug was added, the EJCs continued to be evoked throughout the experiment. In those experiments negative polarity currents were never seen. In some experiments a
complex current (positive and negative polarity) was sometimes recorded when the stimulus intensity was increased. This can be explained by the fact that a higher stimulus intensity leads to activation of a number of nerve population, each with different thresholds and latency. As a result EJCs are evoked both inside and outside the electrode tip and give rise to the complex current. The stimulus intensity used in experiments in this thesis was only 20-25% above threshold, to allow a minimum number, as far as possible, of the nerve population to be stimulated.

2- The threshold of the new positive polarity current was below the threshold of EJCs, indicating that it is produced by a distinct nerve population. The possibility that the new current is an EJC outside the electrode tip is very unlikely. In experiment where the new current was revealed by L-arginine, there was no sign of any positive polarity current at the beginning of the experiment. In addition, the new current has a shorter latency than that of EJCs, suggesting that it is being produced by a nerve population with a faster rate of conduction.

3- The presence of an inhibitory mechanism controlling the new current: receptors on nerve terminals can be divided into autoreceptors and heteroceptors. Autoreceptors respond to the sympathetic neurones own transmitter, this include purines, peptides and noradrenaline. Heteroceptors respond to transmitters released by adjacent neurones and those circulating in the blood stream (Fillenz, 1992). It is generally accepted that one of the inhibitory pre-synaptic receptors is the $\alpha_2$-adrenoceptor, and there are two possible ways for its inhibitory action. Either by depression of propagation of action potential in terminal varicose fibres, or through an action on the release mechanism (Fillenz, 1992).
The release of transmitter is regulated by an inhibitory presynaptic mechanism through binding of noradrenaline to presynaptic α₂-adrenoceptors, which triggers a series of mechanisms leading to the inhibition of transmitter release. This could be either through a second messenger, or through interfering with Ca²⁺ entry to the presynaptic nerve membrane and consequently interfering with vacocytosis of transmitter vesicles. L-arginine revealed a new current which is of a positive polarity with a shorter latency and lower threshold. Furthermore, L-arginine inhibited EJCs and in most cases they were completely abolished. Consequently, L-arginine is exerting an inhibitory mechanism on neurotransmission in this tissue. The effect of α₂-adrenoceptor agonists and antagonists on the new current was tested. As mentioned before once the new current revealed it continues to increase in amplitude until it reaches a complete establishment. where further incubation induced no further change in the new current amplitude. The addition of yohimbine (10⁻⁷M) through internally perfused recording electrode resulted in an increase of the new current amplitude by up to 46 +/- 8%. Almost the same result was obtained by using idazoxan (10⁻⁶M), where the new current amplitude increased by up to 54 +/- 4%. This might suggest that the same autoinhibitory mechanism of transmitter release, which is controlling ATP and noradrenaline release, is also controlling the new nerve population responsible for the existence of the new current.

As mentioned earlier the new current has a different latency and threshold from that of EJCs. This suggests that it is produced by a different nerve population from that producing EJCs. Since EJCs are a reflection of secreted ATP, any changes in ATP release is indicated in a change in EJCs amplitude. It is important to bear in mind that ATP and noradrenaline are both co-released. NO
produced from L-arginine resulted in a reduction of EJCs amplitude until they disappeared, and at the same time the new current emerged. It is possible to explain this as follows: the new current is produced by a distinct nerve population other than that responsible of EJCs, under normal conditions the nerve producing the EJCs is in a dominant state of control, and the other nerve are dormant. As the concentration of NO is increased by the addition of L-arginine, this will activate the nerve which were dormant to exert its inhibitory action on the other nerves, resulting in a reduction of EJCs amplitude and the appearance of the new current.

The other question which arises is does the nerve responsible for the new current have the same type of $\alpha_2$-adrenoceptors as the others? The answer to this question is possibly yes. Yohimbine and idazoxan are both $\alpha_2$-adrenoceptor antagonists, and both resulted in an increase in the amplitude of the new current. If this is true then the addition of $\alpha_2$-agonists should produce the opposite effect. For this purpose noradrenaline ($10^{-6}$M) was added locally through an internally perfused recording electrode, and after few minutes the new current was completely abolished. There are two explanations for this result; it could be that noradrenaline was acing on post-junctional $\alpha_1$-adrenoceptors and leds o tissue contraction, and as a result loss of the release site occurred due to movement of the tissue. The other explanation is that noradrenaline was acting on pre-junctional $\alpha_2$-adrenoceptors on the new nerve, producing a mechanism similar to that controlling the other transmitters release, in other words a feedback inhibitory mechanism. It is possible to rule out the first explanation by blocking post-junctional $\alpha_1$-adrenoceptors through the addition of prazosine. Noradrenaline and prazosine were added through internal perfusion of the recording electrode tip. This resulted in the complete disappearance of the new current, just as
happened when noradrenaline alone was added. These results are compatible with the previous results. This indicates that the disappearance of the new current is very likely to be through noradrenaline acting on pre-junctional α₂-adrenoceptors on the new nerve. And it is also possible that these α₂-adrenoceptors are part of the inhibitory mechanism regulating the release of transmitter from the new nerve in this tissue. These results rule out the possibility that the disappearance of the new current was due to tissue contraction through α₁-adrenoceptors the moment noradrenaline comes into contact with the tissue.

It is well known fact that purinergic and adrenergic nerve populations contain a wide range of receptors. It would be important to find out, in the future, whether the new nerve population contains other types of receptors, and whether they are similar to those on other known nerve populations.

4-1.8 Local regulation of transmitter release

Autonomic nerve terminals contain a wide range of presynaptic receptors. According to the hypothesis of local inhibition of transmitter release, it is assumed that presynaptic α₂-adrenoceptors are one of those receptors, and they mediate inhibition of transmitter release. This is thought to happen through binding of noradrenaline to pre-junctional α₂-adrenoceptors and as a result reducing the probability of release from its own and surrounding release sites. However, studies of the occurrence of discrete events, in the absence of pre-junctional α-adrenoceptor antagonists, showed that discrete events occurring at one latency have no influence on the discrete events at longer latencies. (Blakeley et al., 1982). This study failed to
demonstrate that endogenous transmitters regulate their own release locally. Almost the same result was obtained by Blakely et al. (1989), which showed that in the absence of drug treatment the occurrence of EJCs in the guinea-pig and mouse vas deferens have no effect on the probability of observing subsequent EJCs. Brock and Cunnane (1990) used extracellular recording techniques to demonstrated that the occurrence of EJCs in the guinea-pig vas deferens does not reduce the probability of observing subsequent EJCs. Apparently transmitter released by nerve stimulation does not inhibit subsequent release from the same or closely related groups of varicosities, but rather acts at sites remote from its own site of release (lateral inhibition) (Brock and Cunnane, 1990). Another possibility is that the inhibitory effects via $\alpha_2$-adrenoceptors are located at sites upstream of the release sit (Stjärne, 1981).

It has been proved that exogenous noradrenaline inhibits the release of neuronal noradrenaline from cardiac tissues and its action can be antagonised by $\alpha_2$-adrenoceptor antagonists (Starke, 1972). This is true for almost all sympathetically innervated tissues. $\alpha$-adrenoceptor agonists such as clonidine, decrease electrically evoked release of noradrenaline (Starke, 1977).

In electrophysiological experiments the effect of $\alpha_2$-adrenoceptor agonists and antagonists on ATP-mediated EJPs and EJCs were studied. Clonidine ($\alpha_2$-adrenoceptor agonist) suppresses, whereas, yohimbine ($\alpha_2$-adrenoceptor antagonist) enhances the probability of release of transmitter (Blakeley et al., 1986), and increase the occurrence of evoked EJCs (Stjärne and Stjärne, 1989). ATP released from autonomic nerves may act as a neuromodulator at pre-junctional or post-junctional sites. At pre-junctional sites ATP may act on receptors located on nerve terminals and enhance or inhibit further release of transmitter substance from these nerves (Hoyle,
However, in the mouse vas deferens, the pre-junctional inhibition of adrenergic transmission by ATP is not affected by blocking P1-purinoceptors (Kugelgen et al., 1989).

Studies on mouse vas deferens have demonstrated that $\alpha_2$-adrenoceptor antagonists cause an increase in the amplitude of EJCs recorded during a short train of stimuli (Blakeley et al., 1984; Illes & Starke, 1983). In this thesis yohimbine increased the EJCs amplitude, and the effect was dose-dependent. The increase in EJCs amplitude varied from 5% with yohimbine $10^{-9}$M up to 84% with yohimbine $10^{-4}$M, the $EC_{50}$ was $0.7 \times 10^{-7}$M.

Quantal analysis of the effect of yohimbine on EJCs suggests a pre-junctional site of action. There was a reduction in the number of failures, and no change in the separation between peaks of preferred values. In addition, the effect of yohimbine manifested as a change in the mean quantal content where it increased by 29%. The above results are also evidence of pre-junctional $\alpha_2$-adrenoceptors mediating inhibition of transmitter release.

The effect of idazoxan $10^{-4}$M ($\alpha_2$-adrenoceptor antagonist) was tested with nerve stimulation at 1 and 2Hz. The results, showed that at both frequencies the EJCs amplitude increased, but the increase in EJCs amplitude was more prominent at 2Hz. The mean increase in EJCs amplitude at 1Hz was +31%, whereas at 2Hz it was +70%. This is additional evidence of autoinhibition through pre-junctional $\alpha_2$-adrenoceptors. And since this effect is induced by local application of $\alpha_2$-adrenoceptor antagonists through internal perfusion of the recording electrode, it appeared that the pre-junctional $\alpha_2$-adrenoceptors are located under the electrode tip.
The α-adrenoceptor agonist noradrenaline when applied locally by internal perfusion of the recording electrode, resulted in a reduction of EJCs amplitude recorded during a train of stimuli. EJCs were reduced by 57 +/- 7 %, compared with the untreated preparation. This reduction occurred as a change in the mean quantal content by -76 %, suggesting a pre-junctional modulatory effect. Quantal analysis also suggests a pre-junctional effect as mentioned above.

4.1.9 The interaction between NO-mediated neuromodulatory mechanism, the new nerve population and neurotransmission present in this tissue

The new current which was revealed by L-arginine was only present in preparations treated with L-arginine. During the initial phase of the experiment when the tissue was incubated with L-arginine, the EJCs started to reduce in amplitude and as they are reduced the new current appeared. Its threshold was below that of the EJCs indicating a different nerve population is responsible for the new current. Further incubation leads to further decrease in EJC amplitude until they completely disappeared, when the new current becomes more evident. This observation might suggest that there is a cross-inhibition between the new nerve and the purinergic nerves. Under normal physiological conditions there is a state of balance between the activity of those two nerves. The addition of L-arginine, leads to the production of NO which disturb this balance. NO act as an inhibitory modulator towards the purinergic nerve, and consequently EJCs are reduced in amplitude. This in turn will result in a reduction in the inhibitory effect exerted by the purinergic nerve through secreted ATP towards the new nerve, to a stage where the new current is revealed. The production of more NO leads to an increased inhibitory modulatory effect by NO on
the purenergic nerve, which manifests as a further reduction in EJCs amplitude until they completely disappear and the new current is well established. An important experiment which might be needed to be conducted in the future is the demonstration of hyperpolarisation through intracellular recording techniques after the addition of L-arginine.

From our observations the tissues needed to be incubated for about 20 minutes before any effect on EJCs can be observed. There are several reasons which may account for the slow onset of the effect of L-arginine but some of them can be ruled out. The molecules should reach the tissue and into the cell with no difficulty because the molecular weight of L-arginine is low (M.W. = 174.2). Compared to, for example noradrenaline (M.W. = 319.3) which gives an effect in about few seconds following it contact with the tissues. NO is a labile short-lived molecule with half life 4-50 sec. and cannot be stored, and therefore there is no need for building NO stores. The most likely explanation is that the enzyme system (NOsynthase) is only present in small quantities in the mouse vas deferens, and accordingly, it needs some time to produce enough NO to give rise to a detectable effect over neurotransmission in the tissue.

L-arginine may have an effect on other transmitters secreted in this tissue, such as noradrenaline. Noradrenaline is known for not producing any electrical changes on smooth muscles membranes, consequently, their activities cannot be recorded by either extracellular or intracellular recording methods. It is possible that the secretion of noradrenaline could be depressed by L-arginine. EJCs are produced by ATP acting on \( P_{2x} \) post-synaptic purinergic receptors. L-arginine resulted in a reduction in EJCs amplitude which is a reflection of secreted ATP. ATP and noradrenaline are probably co-released, and there is a cross-inhibition between nerves secreting those two
neurotransmitters. ATP and its degradation product adenosine inhibit the release of noradrenaline in the mouse vas deferens through acting on pre-junctional P₁ and P₂ receptors (Vonkügelgen et al., 1989; Vonkügelgen & Starke, 1993). It is possible that noradrenaline is involved in the effect of L-arginine. Since L-arginine reduce the EJC amplitude, reflecting an effect on ATP secretion, and this could mean a change in secreted noradrenaline. It is possible to evaluate the effect of L-arginine on noradrenaline by measuring the release of noradrenaline into the extracellular fluids (this has not been done in this thesis).

4-1.10 Other evidence suggesting the existence of NO mediated neuromodulatory mechanism

4-1.10.a Removing NO from the tissues enhances neurotransmission in the mouse vas deferens

PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide) is a powerful NO scavenger. Studies showed that it reacts with NO resulting in elimination of any free NO, and accordingly minimises any NO-related mechanisms. Vasorelaxation was inhibited by the imidazolineoxy N-oxide (Akaike et al., 1993). Vasorelaxation induced with ATP or acetylcholine was also markedly inhibited by PTIO (Meade et al., 1995). This inhibition of vasorelaxation was not reversed by the addition of L-arginine, whereas L-arginine significantly reverses the inhibition of NO synthase inhibitors such as L-NNA. This is an indication that the inhibition of vasorelaxation by PTIO was not due to direct inhibition of NO synthase.
During experiments carried out as part of this thesis in which EJCs were recorded, PTIO (10⁻⁸M) was applied to isolated preparations of mouse vas deferens. Following exposure to PTIO, EJCs amplitude were increased by up to 35 +/-10 %, and further exposure to PTIO increased the EJCs amplitude by up to 55 +/-10 %. This suggests that NO is present in this tissue, and it is modulating neurotransmission most probably by an inhibitory mechanism. PTIO needed some time to induce a measurable and significant effect over transmission in this tissue. One explanation is that a reduction in NO effect must a reach certain level to give less inhibitory action over any neurotransmission process present in this tissue. A higher concentration of PTIO might give a faster onset of action. One important thing that should be remembered is that by using extracellular recording techniques, drug dilution might occur at the electrode tip and it might be the reason for the slow onset in the effect of PTIO.

4-1.10.b The ability of PTIO (NO scavenger) on reversing the prominent inhibitory mechanism produced by L-arginine

As mentioned above that PTIO increased EJC amplitude to a significant level under normal conditions. On the other hand, will PTIO impose any effects on NO mediated mechanisms that are already well established, this is done by adding L-arginine prior to the addition of PTIO. L-arginine reduced the EJCs amplitude by 76 %, the addition of PTIO resulted in an increase in EJCs amplitude from 26 +/-15 % to 106 +/-9 %, this is an increase by 44 %. As a result, the addition of PTIO completely abolished the inhibitory effect of L-arginine.
This is evidence that NO-mediated neuromodulatory mechanisms exist in this tissue, and L-arginine produces its action through NO. The effect of NO is inhibitory on neurotransmission. Removing any naturally produced NO leads to enhancement of the excitatory neurotransmission mechanism present there. NO mediated inhibitory action is not naturally dominant in this tissue, but bringing this mechanism into dominance is achieved by the addition of L-arginine. This leads to inhibition of the excitatory mechanism and prominence of the inhibitory mechanism, through the production of NO, and even at this stage, removing any NO by PTIO results in reversing the inhibitory mechanism.

The addition of L-arginine to preparations previously treated with PTIO did not have any significant inhibitory effect. EJCs increased by 34 +/-7 % under the effect of PTIO, the addition of L-arginine did not reverse this, EJCs continued to increase in amplitude by up to 55 %. Despite the presence of L-arginine EJCs continued to increase under the influence of PTIO, this suggests that any produced NO was immediately and continuously scavenged by PTIO, which leads to removal of the inhibitory effect of NO. The mechanism of action of PTIO is different from that of NOsynthase inhibitors. NOsynthase inhibitors act on NOsynthase by competitively inhibiting the enzyme, and as a result the addition of L-arginine is capable of reversing the effect of NOsynthase inhibitors. PTIO, on the other hand, acts by removing any NO, and as a result the addition of L-arginine does not reverse the effect of PTIO.
4-2 Histamine as a neuromodulator

The idea that peripheral histaminergic fibres may exist is relatively old. Histamine as a mediator of neurotransmission has been shown to act in a number of biological tissues. In the guinea pig ileum, histamine produces a well characterised contractile response via \( H_1 \)-receptor stimulation (Hill, 1990; Hill and Young, 1981). In addition, evidence regarding the possibility that histamine may have a role as a neurotransmitter or neuromodulator is accumulating (Prell & Green, 1986; Schwartz et al., 1986b). Histamine is widely distributed within the mammalian central nervous system (Adam and Hype, 1966; Taylor and Snyder, 1971), in both neuronal and non neuronal components of mammalian brain (Schwartz et al., 1980a, 1986b; Edrission et al., 1977). Some reports have indicated the presence of histaminergic fibres in mouse vas deferens (Campos, 1988). Three types of histamine receptors have been identified so far: \( H_1 \), \( H_2 \), and \( H_3 \).

Results described in this thesis showed that exogenously applied histamine resulted in a depression of the nerve evoked EJCs. Histamine did not act as an agonist to evoke contraction. The depressive effect of histamine occurred in a concentration dependent manner, which varied from a reduction by 14 % with histamine \( 10^{-9} \)M, and a reduction of up to 82 % with histamine \( 10^{-4} \)M, the \( EC_{50} = 0.6x10^{-4} \)M. There was a reduction in the mean quantal content from 5 +/- 0.17 (n = 3) in the absence of histamine \( 10^{-6} \)M to 2.66 +/-0.17 (n = 3) in its presence, this is a reduction of 53 %. This indicates that the modulatory effect of histamine is via pre-synaptic sites; to be more precise the effect is on transmitter release.

Quantal analysis performed suggested both pre- and post-junctional modulatory effects. There was a change in the separation between peaks of preferred values,
which reflects a post-junctional effect. In addition, there was a change in the size of the relative peaks of preferred values which reflects a pre-junctional effect. Adding all these findings together, it is reasonable to conclude that histamine resulted in reduction of EJC's amplitude through its modulatory effect on both pre- and post-junctional sites. Its effect on pre-junctional sites manifested itself on the transmitter release mechanism. Furthermore, histamine had a modulatory effect on post-junctional responses of the released transmitter, this is either effecting directly the receptors which are responsible for smooth muscle contraction, or possibly by acting on ion channels which aid in smooth muscle contraction.

4-2.1 The presence of histamine H3-receptors in the mouse vas deferens

Arrange et al., in 1983 were the first to demonstrate the presence of H3-histamine receptors on nerve terminals, since then three major functional characteristics of these receptors have been identified:

1- Histamine H3-receptors have an effect on the inhibition of histamine release, possibly by restricting the influx of Ca²⁺ ions, a step essential for histamine release from histamine containing nerve terminals (Arrange et al., 1983; 1985b).

2- Histamine H3-receptors affect the synthesis of histamine which is increased by R-(α)-Methyl histamine and antagonised by burimamide, impromidine, and thioperamide (Arrange et al., 1987a,b).
3- Histamine H₃-receptor stimulation can lead to inhibition of the release of other transmitters. Recently the pre synaptic modulation by histamine has been highlighted by the discovery of the pharmacological characteristics of histamine H₃-receptors. A reduction in noradrenaline release, as well as a reduction of the associated functional responses coupled to histamine H₃-receptors in isolated tissues or in vivo preparations have been demonstrated (Hay et al., 1992; Ishukawa & Sarelakis, 1987). The mechanism underlying the inhibitory effect of histamine H₃-receptors stimulation on transmitter release in the central and peripheral tissues remains to be established.

4-2.1.a The effect of manipulating histamine H₃-receptors by an antagonist

Results obtained in this thesis showed that thioperamide, a highly selective H₃-histamine receptor antagonist (Hill, 1992) increased the EJCs amplitude. The increase in EJCs amplitude was up to 35 +/- 7%, 40 minutes following the addition of thioperamide. This indicates that there are H₃-histamine receptors in this tissue, and that they probably impose a modulatory effect on sympathetic transmission in this tissue. Thioperamide 10⁻⁷M increased the mean quantal content from 2.2 +/- 0.05 (n = 2) in the absence of thioperamide to 3.05 +/- 0.05 (n = 2) in its presence. This is an increase by up to 37% in the mean quantal content. The change in the mean quantal content indicates a pre-junctional site of action. Quantal analysis suggests a pre-junctional modulatory effect, as well as, there was an increase in the EJCs amplitude by up to 35 +/- 7%, and there was no change in the separation between relative peaks of preferred values.
The above results indicate the existence of H₃-histamine receptors in this tissues. They are most probably located pre-synaptically, and they impose a pre junctional modulatory effect on neurotransmission. It is possible that thioperamide acted on those pre junctional H₃-receptors which are present on nerve terminals in that site, resulting in modulation of transmitter release. The effect of thioperamide through H₃-histamine receptors could work either by affecting ion channels, or through a second messenger system. The above results are evidence indicating the presence of pre-junctional histamine H₃-receptors in the mouse vas deferens.

4-2.1.b The effect of manipulating histamine H₃-receptors by an agonist

R-(α)-Methyl histamine is a highly selective histamine H₃-receptor agonist. Its effect can be summarise as follows: R(α)-Methyl histamine (10⁻⁸M) resulted in a reduction of EJCs amplitude by up to 50 +/- 7 %. This was associated with a reduction in the mean quantal content from 3.35 +/- 0.05 to 2.35 +/- 0.05, this is a reduction of 30 %. The change in EJCs amplitude reflects a change in either transmitter release or a change in post-synaptic responses to released transmitter. Quantal analysis suggested a pre-junctional effect, this is because there was no change in separation between relative peaks of preferred values. And there was a general reduction in EJCs amplitude and in quantal content of transmitter released. Considering all the above results, it is possible to conclude that the histamine H₃-receptor agonist R-(α)-Methyl histamine reduced EJCs responses through a pre-junctional mechanism involving H₃-receptors. H₃-receptors are most likely have a modulatory effect on other transmitter release, through their pre-junctional sites.
Results obtained in this thesis showed that thioperamide $10^{-7}$M reversed the inhibitory effect of R-(α)-Methyl histamine $10^{-8}$M. The effect of both R-(α)-Methyl histamine and thioperamide were not immediate. This indicates that their action is through a complex system which affects the release of transmitters, most probably ATP, because it is the transmitter responsible for EJCs. ATP and noradrenaline are co-released, consequently histamine $H_3$-receptors probably effect the release of ATP and noradrenaline. The best way to demonstrate the effect of noradrenaline is by measuring its concentration in the extracellular fluid, a method that has not been used in this thesis. In rat spinal cord slices histamine inhibited, in a concentration-dependent manners the evoked release of noradrenaline elicited by electrical field stimulation, which was mimicked by R-(α)-Methyl histamine (Stella, 1995). Recently Enduo et al., (1994) reported that inhibition of noradrenaline release mediated by activation of presynaptic histamine $H_3$-receptors could be related to a decrease in Ca$^{2+}$ influx though N-type voltage sensitive Ca$^{2+}$ channels in nerve terminals. In rat skin inhibition of Substance P release from sensory nerve terminals via pre-junctional histamine $H_3$-receptors may be achieved by activation of ATP-sensitive K$^+$ channels coupled to the histamine $H_3$-receptors (Tsuyako & Manabu, 1995).

4-2.2 Histamine $H_2$-receptors and their action in the mouse vas deferens

Gastric acid secretion, smooth muscle relaxation, effects on cardiac muscle, and an effect on the immune system are four major functional responses to histamine $H_2$-receptor stimulation. Histamine $H_2$-receptor stimulation produce relaxation of airways smooth muscles cells (Ayre & Chand, 1982), and vascular smooth muscle (Reinhard & Ritter, 1982). Sympathetic excitatory nerves controlling rat vas deferens
contractility can be negatively modulated by histamine though the activation of presynaptic receptors which resemble histamine H2-receptors sub type (Poli et al., 1994). Pre- and post-junctional H2-receptors have been demonstrated, the prejunctional H2-receptors are related to transmitter release. Post-junctional H2-receptors are involved in adenylate cyclase activation in many cells (Johnson, 1989).

Results obtained in this thesis indicated the presence of histamine H2-receptors in this tissue. H2-histamine receptors could account for some of the pharmacological effects of histamine which were observed early on in this thesis and which could not be fully explained by the histamine H3-receptors experiments. The H2-receptor agonist dimaprite resulted in a reduction in the EJCs amplitude by up to 47 +/- 6 %, this was associated with a reduction in the mean quantal content from 3.25 +/- 0.05 in the absence of dimaprite, to 2.45 +/- 0.06 in its presence, this is a reduction by -75 % in the mean quantal content. Indicating a pre-synaptic modulatory inhibitory effect, through pre-junctional histamine H2-receptors.

Quantal analysis revealed pre- and post-junctional changes. This indicates that it is most likely that H2-receptors have both pre- and post-junctional location, and accordingly they have a combination of pre- and post-synaptic modulatory effects. Pre-junctional H2-receptors are probably linked to other neurotransmission processes. Dimaprite resulted in a reduction in quantal content, this indicates that H2-receptors are linked to the transmitter release process in this tissue. H3 and H2 histamine receptors are both linked in one way or another to pre-junctional processes of transmitter release. It is very likely that both receptors provide a more precise modulatory effect on transmitter release. This assumption should not be ruled out.
Ranitidine ($10^{-5}$M) is a highly specific histamine $H_2$-receptor antagonist. It is resulted in a $45 \pm 9\%$ increase in the EJCs amplitude compared to the untreated preparation. In addition, ranitidine resulted in a $49\%$ increase in the mean quantal content, indicating that the increase in the EJCs amplitude is due to a change in the transmitter secreted. Quantal analysis, on the other hand, showed that ranitidine resulted in both pre- and post-junctional modulatory effects, which manifested as an increase in EJCs amplitude, and as a result, shifting the EJCs amplitude frequency distribution to the right. This was associated with a change in the separation between peaks of preferred values. The addition of ranitidine to preparations previously treated with dimapride resulted in reversing the inhibitory effect on EJCs amplitude brought about by dimapride. The above results suggested the presence of $H_2$-receptors pre- and post-junctionally.

4-2.3 The possibility that nerve populations interact, producing a fine modulatory effect in this tissue

The analysis of results under the effect of histamine, histamine $H_2$ agonist and antagonist, histamine $H_3$ agonist and antagonist, noradrenaline, yohimbine, idazoxan, and L-arginine, showed that there is a multiple control system exerting a precise control over neurotransmission processes in this tissue. This is most probably just a part of a more complex system which exists in this tissue to control transmitter release processes, bringing the inhibitory and the excitatory mechanisms into a balanced state. Analysis of EJCs and new current revealed that these currents were produced by a different nerve population. The EJCs were produced by excitatory nerves while the
new current was produced by a nerve population most probably inducing an inhibitory action over transmission.
5- Conclusion

Sympathetic neurotransmission in this tissue is controlled by the action of a number of neurotransmitters and neuromodulators, in which NO and histamine play an important role. The following points are the final conclusions of the current study:

1- NO is naturally produced in this tissue. NO has an inhibitory mechanism of action on other neurotransmission processes in this tissues.

2- L-arginine revealed a nerve population with a lower threshold and faster conduction velocity compared to the excitatory nerves.

3- The new nerve population which has been revealed by L-arginine contains $\alpha_2$-adrenoceptors. Activation of $\alpha_2$-adrenoceptors leads to an inhibitory mechanism of action on the nerves activity. The mechanism is probably similar to that controlling other neurotransmitters in this tissue.

4- NO has both pre- and post-junctional site of action.

5- The presence NO synthase has been proven histochemically through its NADPH-diaphorase activity by reacting with a formazan product, producing a coloured insoluble formazan deposits visible by light and electron microscopy.

6- Autoinhibition of transmitter release through pre- synaptic $\alpha_2$-adrenoceptors was confirmed in this tissue, by the use of yohimbine and idazoxan.
7- Histamine plays an important role in neurotransmission in this tissue. Histamine is an amine which is produced naturally in this tissue.

8- Two types of histamine receptors exist in the mouse vas deferens: H₂ and H₃. H₂ receptors are located both pre- and post-junctional. Pre-junctional H₂ receptors play an important role in transmitter release, such as noradrenaline and ATP. Activation of H₂ receptors produce inhibition of excitatory mechanisms in this tissue. Quantal analysis revealed that this effect is a combination of a pre-junctional effect on transmitter release, and a post-junctional effect on transmitter effect.

9- H₃ receptors are present only at pre-junctional sites. Their main role is a regulatory action on transmitter release.
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