A study of some agents which modulate neurotransmission in the mouse vas deferens

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by

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Abstract

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The effects of nitric oxide (NO) and histamine as modulators of neuromuscular transmission in the mouse vas deferens have been studied. Controls exerted by these agents have not previously been identified in this tissue.

A pharmacological approach was taken to investigate the various activities of NO. The presence and identity of histamine receptors in this tissue was also determined by this method. The use of specific antagonists also showed that histamine is secreted during nerve stimulation.

Contractions evoked by low frequency (5Hz) electrical nerve stimulation (5-50V) were recorded to give information about pre and postjunctional effects. Contractions evoked by the agonists noradrenaline or αβ-methylene ATP (αβ-met. ATP) gave information about postjunctional responses.

Intracellular and focal recording techniques recorded evoked electrical activity (EJPs and EJCs); these reflect the neuronal release of ATP. Quantal analysis of EJC amplitudes permitted pre and postjunctional effects to be determined.

Sodium nitroprusside (4x10^-6M) depressed nerve evoked contractions suggesting a NO sensitivity. These contractions were also depressed by L-arginine (L-Arg), the natural precursor of NO. The nitric oxide synthase (NOS) inhibitor L-arginine methyl ester (L-AME) (2.3x10^-4M), caused all mechanical responses to increase in amplitude suggesting a physiological depressive role for NO synthesis. An elevation of EJC amplitudes by L-AME (2.3x10^-4M) was rapidly reversed by L-Arg (3.4x10^-4M) suggesting that endogenous NO normally depresses ATP secretion. In the longer term L-Arg (3.4x10^-4M) revealed a new RB2 sensitive low-threshold current of positive sign.

L-Arg (3.4x10^-4M) may have degranulated mast cells. This histamine may have been taken up and released as a neuro-transmitter. Thioperamide (6x10^-6M) and ranitidine (1x10^-4M) showed that endogenous histamine acts at H3 and H2 receptors to depress contractions but not those evoked by exogenous αβ-met.ATP. Exogenous histamine (1x10^-6M) also depressed EJC amplitudes by a physiological mechanism.

The results showed that nitric oxide is a modulator of transmission in this tissue which depresses pre and postjunctional activities. Endogenous histamine also modulates transmission processes with effects similar to those of NO.
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Introduction

The aim of the work comprising this thesis was to investigate the fundamental modulation of sympathetic neurotransmission using the mouse vas deferens as a model system. In particular the roles of nitric oxide and histamine on the secretion of ATP and the modulation of its postjunctional effects were studied.

In the following sections the structure and function of the sympathetic nervous system and smooth muscles innervated by it will be reviewed. Special reference will be made to the rodent vas deferens its innervation, structure and function in the context of an organ innervated by the autonomic system. An historical overview of the development of the methods used during this current study will be described. The development of the concepts of chemical neurotransmission will be included and the current concepts concerning the modulatory roles of nitric oxide (NO) and histamine in other tissues will also be described.

What is chemical neurotransmission?

The process of chemical neurotransmission is defined as the passage of information from a neurone to an effector cell. In most cases this transfer of information occurs across a narrow gap, the synaptic space, by a chemical substance released from the neurone. Where the nerve cell is fused to a second cell, the message is transmitted by ions flowing directly between them. This is described as electrical transmission. This kind of junction is sometimes found between two neurones but not where muscle cells represent the targets of the transmission process. Chemical transmission necessarily involves a delay. This is extremely short and does not prejudice the process. The major advantage of this kind of transmission is that sites for the modulation of the mechanism are available. These may be found on or in the nerve. This is described as prejuncional modulation. Where these sites are found on the target cells the modulation is described as postjuncional. The skeletal neuromuscular junction has evolved to provide a fail-safe mechanism in which chemical transmitter (acetylcholine) always evokes a contraction of the target tissue (skeletal muscle). This system has also evolved to minimise the roles of modulatory influences within the synaptic space.
In contrast transmission between autonomic nerves and their target cells is not a fail-safe system and is open to many different forms of modulatory influences. These may vary with the type of tissue studied or may possess similar elements. Some of these mechanisms will be discussed later in the sections of NO and histamine.

The quantal hypothesis

Our present understanding of the fundamental mechanisms underlying the release of neurotransmitter originates from studies of the amphibian somatic neuromuscular junction. When a muscle fibre at the end-plate region was penetrated by Intracellular recording electrode, small random depolarisations were recorded (Fatt and Katz 1952). These potentials were potentiated by the anticholinesterase prostigmine, reduced by curare and absent in muscle that had been previously denervated. This suggested that these potentials, termed miniature end-plate potentials (MEPPs), resulted from the release of acetylcholine from the motor nerve terminals. It was also demonstrated that the MEPP resulted from the release of a multimolecular packet of acetylcholine rather than a single molecule. This model of transmission mechanisms has been adopted to explain processes at other nerve-muscle junctions.

Following the stimulation of the motor nerve innervating the muscle a large EPP was recorded in the impaled cell. This had a similar time course to the MEPP. The relationship between the MEPP and the EPP was determined by reducing the Ca$^{2+}$ content of the bathing solution, which resulted in a marked reduction in the EPP amplitude with no corresponding effect on the amplitude of MEPPs. Individual EPPs fluctuated in amplitude in a random stepwise manner. The size of this step as the EPP approached the amplitude of the MEPP had resembled that of the MEPP. Additionally, a proportion of the stimuli failed to elicit EPPs. These observations led to the idea that the EPP is made up of several hundred all or none units (quanta) which are identical in size to the MEPP. This was the quantal hypothesis (Fatt & Katz, 1952).

Evidence for quantal release of transmitter came from mammalian skeletal neuromuscular junction of feline tenuissimus muscle (Boyd & Martin 1956). This showed that the EPP was made up of discrete units (i.e. quanta) of the same size as the MEPP. The normal
The random nature of MEPP discharge at the amphibian neuromuscular junction has been questioned, since the discharge of a single MEPP transiently increases the probability of observing subsequent MEPPs (Bennett & Pettigrew, 1972; Cohen et al. 1974a, & b). The occurrence of an event also appears to transiently increase the probability of observing subsequent events at other neuromuscular junctions (Usherwood, 1972; Cohen et al. 1974c; Rees, 1974; Bornstein, 1978). The sensitivity of the MEPP and EPP to changes in the external Ca$^{2+}$ and Mg$^{2+}$ concentration, osmotic pressure and drugs also differ (see Hubbard 1973). This suggest that evoked release of quanta may not be simply related to the release process underlying the MEPP discharge.

Studies at other neuromuscular junctions and at chemical synapses have confirmed the quantal nature of the transmitter release process (see Korn & Faber, 1987). The assumption that all the potential release sites have the same probability of discharging a quantum may not be valid. A considerable non-uniformity has been demonstrated in the probability of release at the amphibian neuromuscular junction under conditions of reduced external Ca$^{2+}$ concentration (Bennett & Lavidis 1979). The focal extracellular electrodes employed in this study recorded the activity from a 30 μm length of the nerve terminal, which may be up to 1000 μm in length. The area of the nerve terminal "seen" by the extracellular electrode would be expected to contain only a small proportion of the total number of release sites. However, on occasions they found that the estimated mean quantal content of release at some of these sites was comparable to that of the whole junction. Therefore, it was concluded that under these conditions the majority of the released quanta came from only a small population of the available release sites which had a relatively high probability of
release. Raising the external Ca\(^{2+}\) concentration and delivering short trains of electrical stimuli increased the probability of release at each of the release sites in a manner which was independent of their initial probability. Therefore, under normal conditions non-uniform release would be expected and suggested that the number of voltage gated Ca\(^{2+}\) channels associated with a particular release site may determine its probability of secretion (Bennett and Lavidis, 1979).

**Morphological correlates of the quantum**

In the mid 1950's several workers reported the presence of membrane bound vesicles in close association with the prejunctional or presynaptic membrane in number of different nerve terminals (Sjöstrand, 1953; Palade, 1954; Palay, 1954; De Robertis & Bennet, 1955; Robertson, 1956). It was suggested that these vesicles might be involved in transmitter storage and transport (De Robertis & Bennet, 1955).

"it is possible to imagine a mechanism by which each particle (i.e. vesicle) loses its charge of ACh ions in an all-or-none manner when it collides with, or penetrates the membrane of the nerve terminal" (Del Castillo & Katz, 1955 also Robertson, 1956). This idea gave rise to the idea that the transmitter content of a "synaptic vesicle" represents the quantum. This is the basis of the vesicle hypothesis.

Synaptic vesicles prepared from several different tissues by homogenisation and centrifugation contain neurotransmitters (De Robertis, 1967). Prolonged electrical nerve stimulation can deplete the vesicle numbers in the nerve terminals of several different tissues (see Zimmermann, 1979). The morphological changes associated with transmitter release was investigated by rapidly freezing the tissue shortly after stimulation (Heuser, Reese and colleagues, 1979,1981). The number of vesicle openings at the active zones is correlated with the quantal content of the EPP following treatment with 4-aminopyridine (4-AP) to increase the amount of transmitter released. A good agreement between the number of vesicles fused with the surface of the nerve terminal and the estimated quantal content of the evoked potential in the presence of a range of 4-AP concentrations suggests that the vesicle is the morphological correlate of the quantum of transmitter.
The presence of vesicles is now used to define the regions of the nerve terminal specialised for transmitter release (Kuffler et al. 1984). This assumption has been challenged (Tauc, 1982; Dunant & Israel, 1985). Studies of transmitter release from the cholinergic nerves innervating the Torpedo electric organ following treatment with [\(^3\)H]choline showed that recently synthesised acetylcholine was preferentially released (Dunant & Israel, 1985). Furthermore, prolonged stimulation depressed electrical responses but caused no change in the number of vesicles or in the specific radioactivity of vesicular acetylcholine. Newly synthesised acetylcholine may not be taken up into vesicles during the period of stimulation. This would be expected if vesicles were recycled. The cytoplasmic pool of acetylcholine was depleted to about 50% of its initial value, and the specific radioactivity of the remaining transmitter was high, indicating a high rate of synthesis. These findings suggest that the released transmitter comes from the cytoplasm and not from the vesicle. In this case the vesicle is not the morphological correlate of the quantum but instead a membrane bound macromolecular structure which binds several hundred molecules of acetylcholine. Release follows a conformational change in the macromolecule resulting in its exocytosis (Tauc, 1982). However, the vesicle population of the nerve terminals of the Torpedo electric organ changes upon electrical stimulation with the appearance of a second type of vesicle. This is smaller in diameter and may be formed from vesicles which have already undergone one or more cycles of endo-/exocytosis (Zimmermann & Whittaker, 1977). Following stimulation of the nerves, in the presence of tritiated ([\(^3\)H]) acetate, these smaller vesicles showed a marked accumulation of labelled acetylcholine, while that of the larger vesicles was reduced. This has recently been supported by the discovery of a third population of vesicles (Stadler and Kiene, 1987).

The drug AH5183 (2-(4)phenylpiperidino) cyclohexanol), prevents the uptake of acetylcholine by the vesicles (Marshall, 1970). This provides further evidence for a heterogeneous population of synaptic vesicles. Following treatment with AH5183, stimulation at 10 Hz for a period of 6 minutes causes a uniform reduction in the amplitude of MEPPs recorded at the frog neuromuscular junction. AH5183 did not affect MEPP amplitude if the preparation was left unstimulated but in the absence of the drug, stimulation caused a small increase in the amplitude of MEPPs. The decrease in MEPP
amplitude represents a reduction in the size of the transmitter quantum and occurs even though there is thought to be a large reserve of vesicles (Van der Kloot, 1986). AH5183 does not inhibit acetylcholine synthesis (Michaelson et al. 1987) but produces a marked decrease in EPP amplitude and mobilisation of transmitter during short trains of stimuli at the frog neuromuscular junction (Suszkiw & Manalis, 1987). This suggests that there is a sub-population of "active" vesicles which are involved in exocytosis, the remainder provide a reserve transmitter pool which can be used to maintain the cytoplasmic levels of transmitter during periods of prolonged activity. In Torpedo electric organ synaptosomes, AH5183 selectively inhibits the release of tritium in tissues treated with $^3$H-acetate. This indicates that newly synthesised acetylcholine requires uptake into the vesicles prior to its release (Michaelson et al. 1987). AH5183 also inhibits the spontaneous non-quantal release of transmitter at the mammalian neuromuscular junction which accounts for over 95% of the transmitter overflow in the absence of stimulation (Edwards et al. 1985). Non-vesicular release of transmitter may result from the inclusion of the vesicular acetylcholine carrier into the nerve terminal membrane following exocytosis (Edwards et al. 1985).

The fundamental idea that the transmitter contents of a single vesicle represents the quantum has also been questioned (see Trembley et al. 1983). When recordings were made from end-plate region of high resistance muscle cells a second population of sub miniature potentials were identified which are between 1/7th and 1/15th the size of the MEPP (Kriebel & Gross, 1974). These potentials have been termed sub miniature end-plate potentials (s-MEPPs). The MEPP amplitude distribution displays multiple peaks which are regularly spaced suggesting that they were made up of sub-units the same size as the s-MEPP (Kriebel & Gross, 1974; Kriebel et al. 1976; Wernig & Stirner, 1977). These observations have led to the suggestion that the s-MEPP represents the release of a single vesicle and that the MEPP (i.e. quantum) results from the synchronous release of approximately 10 vesicles (the multivesicular hypothesis). This hypothesis was vigorously challenged and the conclusion drawn that the intervals between the amplitude classes in the MEPP amplitude distribution were not uniform (Magleby & Miller, 1981). In a recent study of the amplitude distributions of miniature end-plate currents (Erxleben & Kriebel, 1988), the intervals between the amplitude
classes both within and between different preparations were found to be highly uniform.

An alternative view is that transmitter released is not vesicular (Erxleben & Kriebel, 1988). Experiments on the transmitter release mechanism at the inhibitory synapse innervating the teleost Mauthner cell, showed that the evidence was consistent with the idea that only a single vesicle was released following the activation of the release mechanism in a bouton (Korn, 1984; also see Korn & Faber, 1987). There may also be physical constraints on the release mechanism whereby only a single vesicle can undergo exocytosis at a particular release site at any one time. Under normal conditions the value of \( n \) derived using binomial statistics at several neuromuscular junctions and synapses was similar to the number of putative release sites described morphologically (see Korn & Faber, 1987), it has been suggested that the "one vesicle hypothesis" may be a universal characteristic of release sites in both the peripheral and central nervous systems (Korn, 1984).

Choosing a probability distribution which would best fit the nerve stimulated responses of the mouse vas deferens

The release of transmitter at all types of neuroeffector junctions can be described by a probability distribution. These can be of several types but those which have been applied to transmitter release include binomial and Poisson distributions.

A binomial distribution best describes a process in which there are only two possible outcomes. The event either does or does not occur and \( p \) is large. This can only be used to describe the release of transmitter where one quantum is available for release at the arrival of each nerve impulse at a single release site. In this instance the probability of release is equal to or greater than 0.5.

In a random or stochastic process the probability of an individual event (n) occurring over an appreciable length of time is constant and is not affected by any other factor such as the simultaneous release of other vesicles. Where \( n \) is large but the probability (P) of an event occurring is constant and low, the process is best described by a Poisson distribution. In such a process a variable 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 examples which may fit binomial distributions have described skeletal neuromuscular junctions where the release of transmitter from sites local to the recording electrode can be relatively easily identified and do not involve complex analysis.

Sympathetic nerve activities are more difficult to describe and for this reason were not statistically analysed until more recently. Releases from individual sites are intermittent (Cunnane & Stjärne, 1984) which makes the distinction between Poisson and binomial distributions more difficult to assess. A fit to a Poisson distribution could be made (Blakeley et al 1982) but only if some of the failures were first excluded from the analysis. The conclusion was made that in the mouse vas deferens the release of transmitter could be described by a Poisson model but that some other independent factor increased the probability of failure (Blakeley et al 1982).

**Nervous systems and their target tissues: structure-function relationships and the modulation of the neuronal response with special reference to the rodent Sympathetic nervous system**

Neuro-muscular systems of mammals are structurally diverse. They include skeletal muscles innervated by somatic nerves and a variety of smooth muscles supplied by nerves of the autonomic system. Modulatory mechanisms exerted on neuromuscular transmission are complex. These may be controlled centrally or peripherally or by both depending on the tissue in question.

The nerves supplying skeletal muscles have evolved to minimise the effects of local conditions on transmission. Autonomic nerves, however, have evolved to allow a combination of central and peripheral factors to determine the response of the effectors. These represent systems well suited to studies of peripheral modulation of neurotransmission.

**The parasympathetic system**

Preganglionic nerves of this system arise in the brainstem and sacral spinal cord. Parasympathetic outflow from the CNS is more restricted than that of the sympathetic with cranial nerves 3, 7 and 9 supplying the smooth muscle of the eye, the lachrymal and submaxillary and parotid glands. The lower colon, rectum, bladder and genitalia are supplied by sacral parasympathetic outflows. By far the largest outflow
of preganglionic parasympathetic nerves is the Vagus nerve (cranial nerve 10) which supplies the viscera of thorax and abdomen.

All ganglia which contain synapses between parasympathetic preganglionic nerves and the cell bodies of post ganglionic fibres are situated close to the effector organ: post ganglionic fibres are therefore, short.

In the first demonstration of cholinergic transmission the release of a muscarine-like substance was shown to occur from cranial and sacral 'involuntary' (now known to be parasympathetic) nerves (Dixon, 1906). This substance (acetylcholine) is now known to be the transmitter at autonomic ganglia (Feldberg & Vartiainen 1934), the skeletal neuromuscular junction (Eccles, Katz, & Kuffler, 1942, Eccles & McFarlane 1949) and at parasympathetic nerve terminals (see Zaimis, E. 1976).

Parasympathetic nerves in the vas deferens.

The rat vas deferens has a cholinergic innervation (Furness & Iwayama 1972, Dixon & Gosling, 1972, Fedan, 1982). These nerves, which release acetylcholine from their terminals, comprise 30% of all axons supplying the vas deferens and are localised in the circular smooth muscle of this organ (Furness & Iwayama 1972) predominantly towards its epididymal end (Dixon & Gosling, 1972). Most cholinergic axons are associated with adrenergic neurones rather than with smooth muscle cells. This may suggest a role for cholinergic nerves as modulators of sympathetic activity (Furness & Iwayama 1972). Following treatment of the vas deferens with 6-hydroxidopamine which destroys adrenergic nerve terminals a small residual nerve evoked contraction remains. This is sensitive to atropine suggesting an excitatory activity of cholinergic nerves in this organ (Fedan et al 1981). The activity of these nerves would offer interesting scope for further experiments but was not included in this current study.

The sympathetic system origins and structure

The sympathetic nervous system originates within the CNS and is centrally controlled by the hypothalamus, brainstem and spinal chord. This system is conventionally defined as wholly motor in composition and function but receives a sensory input which enters the spinal chord through dorsal root ganglia with somatic sensory nerves. These ganglia contain sympathetic sensory cell bodies (Holton & Holton, 1954).
Sympathetic sensory neurones are purinergic with ATP and adenosine as their transmitters. An example of this is seen in feline spinal chord laminae of the substantia gelatinosa (Fyffe & Perl, 1984).

Sympathetic outflow from the cord is from T1 to L2 or L3 segments of the cord with short preganglionic motor fibres. The cell bodies of preganglionic fibres are found in the intermiolateral columns. Axons leave via the ventral horn with somatic nerves but diverge to form white rami communicantes (myelinated). These join a bilateral chain of paravertebral ganglia running close to and parallel with the spinal chord. These contain the synapses with post ganglionic cell bodies. Other axons pass to prevertebral ganglia which lie ventral to the abdominal aorta. These also contain cell bodies of post ganglionic neurones. Postganglionic fibres emerge as grey (unmyelinated) rami communicantes, join somatic nerves and pass to the periphery with them. In addition to these some preganglionic nerves pass further to the paraviceral and intramural ganglia.

Conduction along sympathetic nerve fibres

Preganglionic nerves of the sympathetic system are small myelinated B fibres which have a conduction rate of about 10 metres/sec. Postganglionic nerves are all unmyelinated C fibres which conduct at about 1 metre/sec. or less. In the vas deferens these are 0.2-1.8\(\mu\)m in diameter embedded in Schwann cells together with a small number of fine myelinated fibres (1-2\(\mu\)m in diameter).

Postganglionic sympathetic nerve fibres

These are always unmyelinated and become highly branched in their terminal regions. In the terminal regions the axons become punctuated by small swellings known as varicosities. These are 0.5-2\(\mu\)m in diameter and about 1\(\mu\)m in length. Varicosities are packed with vesicles and mitochondria, and usually occur at 3-5\(\mu\)m intervals over hundreds of microns of axonal length. Axons between varicosities are usually about 0.1-0.5\(\mu\)m in diameter. Since vesicles are believed to be the sites of transmitter storage, varicosities are assumed to be the sites of transmitter release in the terminal nerve network. Varicosities can therefore be used to identify the terminal region of an axon. One varicosity in each branch is the terminal one, while all the others are ‘boutons-en-passage’ which are potential specialised points of transmitter release (Gabella, 1992). Individual
sympathetic nerves may give rise to as many as 30,000 varicosities spread over the total length of the terminal arborization (Dahlström & Häggebdal, 1966, Gabella, 1992). Postganglionic nerves for most of their length occur in bundles. The individual fibres are surrounded by a Schwann cell wrap but in the varicose regions the Schwann cell covering becomes incomplete which leaves some parts of the nerve fibre naked. These points are apposed to individual smooth muscle cells.

**Sympathetic neurotransmitters.**

Dale (1933) in order to promote "clear thinking", proposed that nerves releasing an adrenaline-like substance be termed adrenergic, whereas those which release an acetylcholine-like substance be termed cholinergic. Baeq (1934; see Baeq, 1975) was the first to suggest that noradrenaline may be the sympathetic neurotransmitter. However, this view was not widely accepted until the demonstration that noradrenaline was the predominant catecholamine in mammalian sympathetic nerve terminals (von Euler, 1946). This finding was followed by the demonstration that noradrenaline was the major active component collected in the perfusion fluid following splenic nerve stimulation (Peart, 1949).

Recent studies have demonstrated that sympathetic nerves store and release a number of other substances which act as neurotransmitters. These principally include ATP and neuropeptide Y (NPY). Sympathetic nerves also contain several other neuropeptides such as opioids for which no neurotransmitter function at present has been described (see Morris & Gibbons, 1992). The presence of co-transmitters has been used to explain the resistance of responses evoked by sympathetic nerve stimulation to \( \alpha \)- and \( \beta \)-adrenoceptor antagonists (this is described later in the section on **ATP as a neurotransmitter at the sympathetic neuroeffector junction**).

**Biochemical studies of noradrenaline storage and secretion**

Biochemical studies have revealed that the majority of the noradrenaline stored in nerve terminals of postganglionic sympathetic nerves is present in vesicles. At the sympathetic neuroeffector junction there is good pharmacological evidence to suggest that only noradrenaline stored in vesicles is released by nerve impulses. Reserpine blocks the uptake of noradrenaline into storage vesicles and
thereby depletes the vesicular store. However, in the presence of monoamine oxidase inhibitors, which prevent the cytosolic metabolism of noradrenaline, reserpinized tissues are able to sequester [3H]noradrenaline through uptake to an extent similar to control tissues. The available evidence indicates that the sequestered noradrenaline is free in the cytosol and is not taken up by the vesicles (Iversen, Glowinsky & Axelrod, 1965). In these tissues the electrically evoked release of tritiated noradrenaline is markedly inhibited compared to control tissues (Potter, 1967), suggesting that only noradrenaline present in vesicles can be released by nerve impulses.

Additional support for the vesicular origin of the released noradrenaline comes from the demonstration that two vesicular proteins, dopamine β-hydroxylase and chromogranin A, present in the noradrenaline 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). By analogy with the adrenal medulla where all the soluble constituents of the chromaffin granule are secreted during stimulation (see Smith & Winkler, 1972) this evidence supports the view that noradrenaline is released by an exocytotic mechanism. Electron microscopical studies have revealed vesicles fusing with the plasma membrane in the varicosities of sympathetic nerves providing further support for an exocytotic mechanism (Thureson-Klein, 1983; Thureson-Klein & Klein, 1990; see also Fillenz, 1992).

Another substance present in vesicles is the purine nucleotide adenosine 5'-trisphosphate (ATP). In the adrenal medulla adrenaline, ATP and its metabolites are secreted in a molar ratio similar to the that found in the chromaffin granule, there being about four molecules of adrenaline for each molecule of ATP (see Smith & Winkler, 1972). This finding strongly supports the concept that the entire contents of a storage vesicle is released by an exocytotic mechanism. Originally it was suggested that vesicular ATP may act solely to stabilise the stored noradrenaline, however it is now clear that ATP acts as a neurotransmitter in its own right (see section ATP
The vas deferens and its nerve supply

The vas deferens which is an easily obtainable tissue receives a dense sympathetic innervation (Ferry, 1967). This makes it an ideal subject for studies investigating some of the factors involved in the modulation of sympathetic transmission. The vas deferens is a muscular tube connecting the epididymus with the urethra with the function of transporting spermatozoa. The muscle coats consist of outer longitudinal and inner circular layers. Nerve evoked contractions recorded during this current study involve both types of these muscles. There is some uncertainty concerning the site of action of exogenous agonists. This may be at both types of muscle or their action may be localised to the outer longitudinal layers. Electrophysiological recordings of intra and extracellular electrical activity carried out during this current study were recorded from longitudinal smooth muscle cells just beneath the surface connective tissue sheath.

The muscle cells of the longitudinal layer are arranged in small bundles surrounded by connective tissue sheaths, these bundles frequently anastomosing with neighbouring bundles. Individual muscle cells are 3μm in diameter and up to 450μm long. Each cell makes contact with at least 6 others when viewed in any one plane. Along their length they are also apposed by up to 12 other cells. This structure as seen in the guinea pig vas deferens was described by Merrillees (1968). For most of their length smooth muscle cells are separated from their neighbours by a basement membrane-filled gap (50-80nm). In places some cell membranes make small protrusions into neighbouring cells. These protrusions are <1μm and attach to the parent cell by a narrow neck (about 0.25μm wide). In the mouse and rat gap junctions occur. These are regions of smooth muscle membrane which are closely apposed (2-3nm). Hexagonal arrays of structures join the two cells together and may provide the basis of a low resistance electrical coupling between the cells (Fry, 1977).

The vas deferens is innervated by postganglionic sympathetic nerves. These arise from the hypogastric ganglia situated within the pelvic plexus (Sjöstrand, 1965, Ferry, 1967, Costa & Furnace, 1973). In the guinea pig a mass of small ganglia are present approximately 2 cm from the prostatic end of the tissue (Ferry, 1967). This may be the same in the mouse. This differs from other postganglionic nerves which
originate within the paravertebral ganglia of the sympathetic chain. The nerves supplying the vas deferens originate in the pelvic plexus and divide into multiple branches as they enter the organ at its prostatic end (Merrillees et al 1963). These fine nerve bundles run within the connective tissue sheath and enter the muscle layers. In the varicose terminal regions the nerves lose their Schwann cell covering and make close contacts (20nm) with the smooth muscle cells (Merrillees, 1968). In the adult rat and mouse every smooth muscle cell forms a close contact with at least one nerve cell.

The vas deferens most probably experiences a high degree of central control which may be modified in the periphery but the extent of this is unknown. During this current study some mechanisms involved in the modulation of sympathetic transmission in the vas deferens of the mouse were identified. These include the depressive actions of NO and histamine (for a further discussion of the mode of action of these two agents in other tissues see later sections on NO and histamine). Contractions (mechanical responses) and electrical responses of smooth muscle cell membranes were recorded by various methods which will be described later. These recordings permitted both pre and postjunctional aspects of the transmission process in the vas deferens to be studied.

**Mechanical responses of the rodent vas deferens**

Mechanical responses have long been recorded in experiments designed to investigate mechanisms involved in the production of smooth muscle contractions. This method which was available before the development of more sophisticated techniques is still currently in use and was used extensively during this current study. When evoked by nerve stimulation, contractions of the mouse vas deferens are composed of all components of excitation-contraction coupling from the release of transmitter to the interaction of contractile proteins. A pharmacological approach is the only method by which pre and postjunctional components can be identified. When evoked by agonist application contractions are purely postjunctional in composition but are still a complex mix of intramuscular responses. The method of mechanical response recording is, therefore, not sensitive enough to give information concerning transmitter release mechanisms. It can, however, be used to provide broad evidence concerning pre or postjunctional receptors, to identify transmitters and their actions or to determine the activities of modulators of the transmission process. It
was in these contexts that mechanical recordings were used during this current study. Results of these experiments showed that both NO and histamine depress mechanical responses of the mouse vas deferens by mechanisms which include postjunctional effects.

**Recordings of nerve evoked contractions provided the first evidence that ATP and noradrenaline are cotransmitters in the rodent vas deferens.**

Contractions of the rodent vas deferens evoked by electrical stimulation are biphasic with an initial rapid contraction (eg. Swedin 1971, Birmingham & Freeman, 1976 Fedan, 1981). This was originally termed the twitch response (Swedin & Åstrand 1985) although later referred to by others as phase I (Stjärne & Åstrand 1985). After a brief relaxation a second phase (phase II) appears which is a sustained contraction. The two phases are dependent on the frequency of electrical stimulation of the nerves with phase I being most clearly seen at 10Hz while the amplitude of phase II is larger than I at low frequencies. The two phases are also species dependent being more clearly seen in the rat than the guinea pig for example (Birmingham, et al 1976).

Phase I of nerve evoked contractions exhibits a pharmacology consistent with activation of P2x receptors and is resistant to α-adrenoceptor antagonism (Ambache & Zar, 1971, Ambache, Dunk, Verney & Zar, 1972). It is inhibited by antagonists of P2x receptors arylazido aminopropionyl-ATP (ANAPP3) in the guinea pig (Fedan, et al, 1981 ) and αβ-met.ATP in Guinea pig and mouse (Meldrum & Burnstock, 1983). Both phases of these contractions are sensitive to bretylium and guanethidine suggesting that they result from sympathetic nerve activation.

This and other pharmacological evidence supports the early conclusion that, "The assumption of two different transmitter processes in this organ ...... cannot be excluded" (Swedin, 1971). The evidence to date supports the view that in the rodent vas deferens noradrenaline and ATP are co-transmitters released from sympathetic nerves (see White, 1988).

The actions of both transmitters is different. The ATP evoked contractile response is triggered by a smooth muscle action potential (Blakeley, Brown, Cunnnane, French, McGrath & Scott, 1981) while noradrenaline activates the mechanical mechanism with no alteration to
muscle membrane potential. The mechanisms activated by the two transmitters also differ, this being partly due to the functions of the receptors at which ATP and noradrenaline act. At this point it is appropriate to discuss some of the current understanding of receptors for ATP and noradrenaline in the rodent vas deferens and their activities.

Receptors for neurotransmitters in the mouse vas deferens.

1. Purinoceptors

Purinoceptors have been identified in many tissues. They were classified as P1 and P2 types by their relative agonist potency orders. P1 purinoceptors having the agonist potency order adenosine > adenosine monophosphate (AMP) > adenosine diphosphate (ADP) > adenosine 5'-trisphosphate (ATP) (for a review of this see Hoyle & Burnstock 1991). P2 receptors have the reverse order of potency. P1 purinoceptors have been further subdivided into A1, A2 (see Burnstock & Buckley, 1985) and A3 (Ribeiro & Sebastiao 1986).

P2 receptors were further subdivided into P2X and P2Y following the determination of their rank order of potency for response to substitute analogues of ATP (Burnstock & Kennedy 1985). At contracting P2X receptors the characteristic rank order of potencies is Methyl-ATP > ATP > 2-Methyl-thio-ATP. At the relaxing P2Y receptors the order is reversed. Another feature of P2X receptors is their response to α.β.met-ATP. In many tissues including the vas deferens, an initial contraction evoked by this agonist is rapidly followed by desensitisation (Meldrum & Burnstock 1985). The P2Y receptor is not desensitised by α.β.met-ATP. Both receptor subtypes have been identified in the mouse vas deferens (see Boland, Himpens, Vincent, Gillis & Casteels 1992).

2. Adrenoceptors

Adrenoceptors are the recognition sites through which catecholamines exert their biological actions. These receptors have been classified according to their agonist potency orders. α receptors have the order noradrenaline > adrenaline > isoprenaline while for β receptors the reverse order applies.

Both α1- and α2-adrenoceptors are present in the rodent vas deferens (see Stjärne & Åstrand 1985). The α2 subtype is present on nerve terminals (see Starke, 1981) while α1 are present on the smooth
muscle membrane. The two types can be distinguished by consistent variations in agonist potency (for a review of these see Wilson, Brown & McGrath 1991). The mechanisms by which \( \alpha_1 \) and \( \alpha_2 \) receptors act are also different. \( \alpha_1 \) receptor activation stimulates phospholipid degradation. The dual products of diacyl glycerol and inositol 1,4,5-trisphosphate influencing intracellular \( \text{Ca}^{2+} \) binding and leading to the opening of \( \text{Ca}^{2+} \) channels. \( \alpha_2 \) receptor activation causes \( \text{Ca}^{2+} \) entry into the nerve terminal by a mechanism which does not necessarily involve adenylate cyclase activation (for a review see Wilson et al 1991).

A further subdivision of \( \alpha_1 \) receptors into \( \alpha_{1a} \) and \( \alpha_{1b} \) has been postulated in arteriolar smooth muscle. These two subtypes were proposed to differ in their sites with one type being 'junctional' and producing depolarisations of the smooth muscle membrane. The other type having the traditional pharmacological characteristics of \( \alpha \) receptors being 'extrajunctional' (Hirst & Neild 1980) This proposal has also been made for these receptor subtypes in the rat vas deferens (Mallard, Marshall, Sithers & Spriggs, 1992) (For a further discussion of these mechanisms see Brock, 1988). This is supported by the finding that \( \alpha_{1a} \) receptors in smooth muscles of rat vas deferens do not mobilise inositol 1,4,5-trisphosphate but activate contractions which require the entry of extracellular \( \text{Ca}^{2+} \) through dihydropyridine sensitive channels (Han, Abel & Minneman, 1987).

Recently prejunctional \( \beta_2 \) receptors have been demonstrated in the guinea pig vas deferens. The activation of these receptors increasing the overflow of \( [\text{H}] \)noradrenaline while at the same time decreasing the neural release of ATP (Driessen, Bultmann, Concalves & Stark, 1996).

**Electrophysiological methods provide a more sophisticated means of investigating the electrical responses of the smooth muscle membrane.**

The electrical properties of skeletal muscle make the mechanisms of neurotransmitter release relatively easy to investigate. Each muscle cell is electrically isolated from its neighbours and in mammals these receive a single branch of a motor neurone. As a consequence of this electrical activity recorded by a high resistance intracellular electrode can be attributed to transmitter released from a single nerve terminal. The situation at the autonomic neuroeffector junction is more complex.
Smooth muscle cells are electrically coupled by gap junctions to form a functional syncytium (see section 'The vas deferens and its nerve supply'). Each cell also receives a multiple innervation from varicosities arising from different axons. The release of transmitter following the stimulation of the sympathetic nerve supply will activate a response of the smooth muscle to produce a summed effect of all these aspects (for more details see section Smooth muscle physiology).

**Studies of transmitter release from postganglionic sympathetic nerves**

The application of intracellular recording techniques to record both postjunctional and postsynaptic potentials has provided unequivocal evidence for chemical transmission both in the peripheral and central nervous systems (see Kuffler, Nichols, & Martin, 1984). Burnstock and Holman (1961) were the first to use intracellular recording techniques to investigate electrical activity at the sympathetic neuroeffector junction using the outer longitudinal muscle layer guinea pig vas deferens. They showed that stimulation of the hypogastric nerves caused transient depolarisations of the smooth muscle membrane. When cells were penetrated with high resistance micro-electrodes and the excitatory nerves innervating the preparation electrically stimulated, a transient depolarisation is recorded which has been called the excitatory junction potential (EJP). EJPs are graded in amplitude with stimulus intensity and increase in amplitude in response to the first few pulses in a train of stimuli. This characteristic increase in EJP amplitude is frequency dependent and is known as facilitation. In the absence of stimulation, spontaneous excitatory junction potentials (SEJPs) were recorded (Burnstock & Holman, 1961, 1962a). The presence of SEJPs suggests that transmitter release at the sympathetic neuroeffector junction is quantal. However, unlike the miniature end plate potential (MEPP) and end plate potential (EPP) at the vertebrate skeletal neuromuscular junction which have closely similar time courses, the time course of the EJP in the vas deferens lasts 4-5 times longer than that of the SEJP. This difference in time course is caused by the smooth muscle cells being electrically coupled (see Bennett, 1972). When a single packet of transmitter is released spontaneously there is an increase in membrane conductance at the site of release. As the current generated at this site spreads rapidly into neighbouring cells, the charge at the point source will be rapidly dissipated and the time
course of the potential change (i.e. the SEJP) brief. When the nerves are stimulated transmitter is released at a number of different points throughout the muscle. Current spreading from each point source results in 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.

Blakeley and Cunnane (1979) noted that the rising phase of EJPs occasionally displayed discontinuities. They found that by electronically differentiating the rising phase of the EJP the discontinuities appeared as peaks in the rate of depolarisation. These were termed ‘discrete events’. The discrete events were found to be stimulus-locked at one or more fixed latencies but more importantly they were intermittent. The conclusion was made that the discrete event represented a measure of the release of transmitter from one or a few release sites in close proximity to the recording electrode. This was the first electrophysiological evidence to suggest that the release of transmitter from sympathetic nerves is intermittent.

ATP as a neurotransmitter at the sympathetic neuroeffector junction

The evidence supporting a role for ATP as a neurotransmitter at the sympathetic neuroeffector junction has been reviewed elsewhere (e.g. White, 1988, Brock & Cunnane, 1992a). However, it is useful to give a brief account of the evidence supporting the idea that ATP is the neurotransmitter responsible for the EJP in the rodent vas deferens and muscular arteries and arterioles. The key points are (1) noradrenaline and ATP are co-localised in vesicles isolated from sympathetic nerve terminals (see Klein & Lagercrantz, 1981) and are each secreted in a calcium-dependent manner following electrical stimulation (e.g. Lew & White, 1987; Kirkpatrick & Burnstock, 1987); (2) EJPs are abolished by adrenergic neurone blocking agents (e.g. bretylium and guanethidine) and are absent in tissues pretreated with 6-hydroxydopamine which destroys sympathetic nerve terminals; (3) EJPs can be recorded from tissues in which the noradrenaline has been depleted by pretreatment with reserpine (Burnstock & Holman, 1962b; Brock et al., 1990) which suggests that the transmitter generating the EJP is not noradrenaline; (4) EJPs are either unaltered or increased in amplitude by α-adrenoceptor antagonists and unaffected by β-adrenoceptor antagonists (see Brock &
Cunnane 1992a); (5) EJPs are markedly reduced in amplitude by the P2X-purinoceptor antagonists ANAPP3 (Sneddon, Westfall & Fedan, 1982) and suramin (Nally & Muir, 1992; Sneddon, 1992). EJPs are also abolished after P2X-purinoceptors have been desensitised by application of the stable ATP analogue α,β methylene ATP (Sneddon & Burnstock, 1984a,b); (6) brief focal application of ATP to either the rodent vas deferens or the rabbit ear artery produces a depolarisation which resembles the stimulation-evoked EJP (Sneddon & Westfall, 1984; Cunnane & Manchanda, 1988; Suzuki, 1985). In these tissues focal application of noradrenaline produces no detectable alteration in membrane potential.

The method of extracellular recording using focal electrodes was a significant step forward in the study of transmitter release and the electrical responses of the smooth muscle membrane (Brock & Cunnane, 1987 & 1992). For the first time currents flowing through the membrane following the activation of cell membrane receptor-linked ion channels could be recorded. Following the placement of a glass electrode on the smooth muscle surface a slight suction was applied to give a low resistance seal with the membrane surface (<1MΩ). This permitted recordings to be made of the activities of the smooth muscle and its innervation enclosed within the area covered by the electrode tip. Spontaneous negative going currents were recorded which were TTX insensitive. These were spontaneous junction currents (SJC) which arose following the spontaneous release of transmitter. Excitatory junction currents (EJC) were also recorded following nerve stimulation. These were TTX sensitive. Like EJPs, EJC recorded from rodent vas deferens were also intermittent. This intermittence has also been reported following the use of fluorescent dyes to stain the varicosities (Lavidis & Bennett, 1992).

What are the causes of the intermittence of transmitter release?

Two possible causes for this intermittence have been suggested: a) that conduction of action potentials failure may occur. b) That the action potential always invades the varicosity but failure occurs because of a low probability of release.

Failure of conduction may occur at either axon branches or at the level of the varicosity. At these junctions the membrane area increases and this may interfere with conduction by reducing the input impedance.
of the membrane. This would result in some area of highly branching networks never being invaded. Figure 1 illustrates this and shows that if the probability of conduction through each of two branches is only 0.5, the probability of an impulse reaching the terminal branches of a highly branched network would approach zero.

![Diagram](image)

Figure 1. A cartoon representation of the conduction of an impulse down a branched nerve fibre. Failure at axon branches was originally believed to contribute to the intermittence of transmitter release from sympathetic nerves supplying rodent vas deferens. This figure shows a cartoon representation of the conduction of an impulse down a branched nerve fibre where the probability of conduction along each branch is 0.5. In this example where there are only 6 branches, the probability of an impulses arriving at B from A would be: \( p = 0.0156 \). In a more highly branched network the probability of conduction would approach zero.

The development of the extracellular method of recording excitatory junction currents permitted the demonstration, using the guinea pig vas deferens, that the action potential always invades the varicosity even during a failure in the release mechanism (Brock & Cunnane, 1987 & 1992). Failures in the release mechanism were attributed to a low probability to release transmitter in agreement with an earlier proposal (Blakeley et al 1979).

However, it was still argued that failure of the action potential to invade the varicosity may occur under some circumstances (Cheung,
1990). This difference in interpretation arose partly because a correction was not made for the latency jitter of the response and latency walking of the nerve terminal impulse. More importantly Cheung stimulated his preparations preganglionically. Ganglionic failures were therefore, included in his data (Brock et al 1987).

In the mouse vas deferens, action potentials invading the varicosities during nerve stimulation are of very small amplitude and they are not usually above the level of the noise. For this reason they can usually only be observed in averaged recordings taken from several responses and cannot be used to determine failures in conduction. Where action potentials were recorded during this current study, they were more likely to belong to a nerve bundle within the recording electrode tip diameter rather than single fibres and as such are not necessarily related to the release events which may follow it. It was not possible, therefore, to use the responses recorded during the experiments described in this thesis to determine whether failure of conduction occurs in the mouse.

"Facilitation cannot fully overcome the effects of a-auto-inhibition at physiological Ca^{2+} concentrations." (Alberts, Bartfai & Stjärne, 1981). It was also proposed that the observed intermittence of transmitter release in this system may be caused by upstream permissive factors. These may be the sites of action of prejunctional agonists which modulate transmitter release (Stjärne, Msghina & Stjärne, 1990).

The visualisation of varicosities and use of small tip diameter recording electrodes, showed that single varicosities release single quanta but that the probability of release from different varicosities along a single fibre is a variable feature (Lavidis et al 1992).

Like the EJPs recorded by intracellular electrode, EJC's recorded by extracellular electrode are sensitive to αβ-met.ATP when recorded from rodent vas deferens. This shows that they are also evoked by ATP acting at P_{2X} postjunctional receptors. This supports the earlier conclusion that ATP evokes electrical activity of the postjunctional membrane.

Extracellular recording is also important as a method for the study of agents which may modulate the release of ATP. For this reason it was extensively used in this current study and results showed that both NO
and histamine depress the secretion of ATP by prejunctional mechanisms.

Recordings of evoked mechanical responses and electrical responses of the postjunctional membrane depend on mechanisms within the smooth muscle. It is, therefore, useful at this point to review the current knowledge of smooth muscle structure and functions

**Smooth muscle physiology: the need for inhibitory innervations and local regulators**

Smooth muscles are composed of discrete cells which differentiate during development and maturation and do not form fused fibres like those of skeletal muscles. They are spindle shaped structures which in the guinea pig vas deferens are 450 μm long with a thickened nuclear zone in the middle one third of the cell (Merrillees, 1968). Electrical continuity between neighbouring cells is maintained by gap junctions which permits groups of cells to function as electrical syncitia.

The innervation of smooth muscles is supplied by autonomic fibres. These are highly branched and varicose and release transmitter at many points of this terminal arborisation.

**The controls of smooth muscle activities**

Many tubular structures depend on layers of smooth muscle for the control of their functions. These include changes in length or diameter or both which may propel the contents of the lumen as in the digestive tract and vas deferens or alter the flow of fluids such as blood flow in the vascular system. The control of contractions may be produced by the antagonistic activities of the sympathetic and parasympathetic nervous systems but in the absence of a joint innervation local factors may oppose the activity of the nerves.

The concept of local controls which modulate both postjunctional target tissues and the activities of its nerve supply have been known for some time (Marrazzi, 1939. Brown & Gillespie, 1957). However, discoveries which include the modulatory effects of nitric oxide on smooth muscle/autonomic nerve systems represented a major advance in the understanding of how some of these effects may occur.

In the absence of local controls on smooth muscle function a tubular structure would be fully dilated. The only effect which could occur
under these conditions would be contraction. If this were the situation in blood vessels for example, constriction would offer the only controls to blood flow. Under normal physiological conditions, however, most blood vessels are neither fully dilated nor fully constricted or some way between. This state is maintained by the spontaneous development of tone by the smooth muscle in the vessel wall which provides a basal state which can be either relaxed or increased to provide a means of altering mechanical functions.

**Characteristics of smooth muscle tone**

The development of tone depends on the free calcium concentration surrounding contractile proteins and this depends on several factors including processes for releasing stored calcium and its uptake from outside the cell (Hiraoka, Yamagushi, & Sano, 1968).

Tonic contractions are maintained for long periods without apparent fatigue and are economical in terms of energy expenditure. Such contractions are slow and there is a strong relationship between shortening velocity and the energy economy of muscles of many species of vertebrates and invertebrates with the greatest energy economy being found in the byssus retractor and tonic adductors of bivalves (Baguet & Gillis, 1967 & 1968). Differences in muscle activation mechanisms and their determination of shortening velocity make some muscle types more tonic than others (Hoyle, 1957) but there are also differences in contractile proteins and their interactions. Examples of these include a slow rate of ATPase activity and therefore, cross bridge recycling (Murphy, Bohr, & Newman, 1969). Muscles with slow contractile mechanisms may also possess slow activation processes (membrane depolarisation and Ca\(^{2+}\) release or excitation-contraction coupling) (Holman, 1957. Burnstock, Holman & Prosser, 1963).

**Activities which produce smooth muscle tone**

Intermittent depolarisations related to the development of smooth muscle tone have been demonstrated in many vertebrate and invertebrate species (Holman, 1957. Prosser, 1960. Burnstock, 1969). The stimulus for this kind of tonic contraction depends on the muscle type and may be caused by the activity of nerves or of a pace maker within the muscle. In both types, the intermittent depolarisations of the muscle membrane causes Ca\(^{2+}\) release from intramuscular stores which triggers a contraction in one or a few smooth muscle cells.
Nerve dependent tone depends on nerve activity and is abolished by denervation (Bozler, 1948, Burnstock, 1969). An example of this is seen in vas deferens (Burnstock, 1969). Myogenic tone is produced by the spontaneous activation of muscle action potentials within pacemaker regions of the smooth muscle. This is independent of nerve activity (Bozler, 1948). In intestines, for example, smooth muscles exhibit slow continuous spike discharges. The frequency of this is directly related to the degree of tension development and is proportional to the muscle membrane depolarisation (Bozler, 1938). This was confirmed in guinea pig taenia coli (Bülbring, 1955).

The release of Ca$^{2+}$ without a muscle action potential may also occur. Stretch activated tone is an example of this which is important in the control of blood vessels (Bülbring, 1953).

Types of smooth muscle

The original classification of smooth muscle described multiunit and unitary types (Bozler, 1941) but intermediate types are also now known to exist. Smooth muscle of mouse and rat vas deferens and cat ciliary muscle are of the multiunit type in which all cells receive close contacts of 200Å with nerve varicosities (Burnstock, 1970). All or non muscle action potentials do not spread through the tissue and the tissue is not spontaneously active. Unitary smooth muscle is composed of three different types of muscle cells which are of different structure and function. 1. A few directly innervated cells. 2. Electrically coupled cells and 3. non-innervated cells which are activated by the spread of action potentials from the other two types. Unitary smooth muscle is often spontaneously active and exhibits slow, graded, local changes in tension. Examples of these are found in the longitudinal muscle coat of the intestine (Bülbring, 1960) and most vascular smooth muscle (Holman, M. E. 1969). Intermediate smooth muscle is found in guinea pig vas deferens, cat nictitating membrane, constrictor pupillae, urinary bladder, dog retractor penis circular intestinal muscle and seminal vesicles. There are low resistance connections between most cells and less than 50% of all cells have close neuromuscular junctions (200-500Å) (Merrillees, 1968). Only about 1% have close contacts with nerve varicosities of 200Å (Merrillees, Burnstock & Holman, 1963). Cells of this type have stable resting potentials which exhibit action potentials only when stimulated. Only directly innervated cells respond directly to
neurotransmitter release while remaining indirectly innervated cells are excited by electrotonic coupling with them.

**Agents which modulate transmission in smooth muscle**

Our present understanding of the modulatory mechanisms controlling smooth muscle contraction arises from studies largely based on the mechanical activities of blood vessels. Consequently more is known about the behaviour of vascular than non-vascular smooth muscle. The activities of many different modulating agents have been investigated but those of nitric oxide and histamine have stimulated great interest in recent years. A short résumé of the history of these findings is included below.

**Agents mediating smooth muscle relaxation: the roles of nitric oxide and histamine**

'A local mechanism independent of the centres in the medulla and spinal cord by which the dilation of blood vessels is varied according with the requirements of the tissue' (Roy & Brown, 1879-80).

This was proposed following studies of reactive hyperaemia in frog web. It was the first acknowledgement that the accumulation of local products would probably cause vessels to dilate. Also at this time it was proposed that working muscle in blood vessels are dilated by CO₂ and by other products released by them including lactic acid (Gaskell, 1880).

**Early experiments implicated histamine as the agent responsible for blood vessel relaxation**

Early this century ideas progressed to include controls over the production of local agents by target tissues. It was proposed that the rate of release of a dilating agent should be determined by the requirement of tissues for blood. The 'H substance' was suggested to be one such physiological dilator and it was thought that this might be histamine (Lewis, 1927).

Many experiments designed to investigate a role for histamine in the control of blood vessel dilation were carried out around this time but results were inconclusive because of the lack of sensitivity of the recording methods. Different types of local controls were discovered for example pulsatile pressure was shown to be important in the
maintenance of arterial tone (Bayliss, 1902). This was an effect distinct from standard reactive hyperaemia.

A method for the continuous recording of the rate of blood flow was not designed until 1948 (Folkow, Haeger & Kahlson, 1948). This was an important development which marked the beginning of studies of circulation. This used a Gaddum outflow recorder (Gaddum, 1929) and a Fleisch Ordiatenschreiber (Fleisch, 1930) adapted for use in the laboratory. Important observations were made during this time which confirmed the earlier discoveries by Bayliss (Folkow et al 1948 & 1949). However, these experiments showed that blood borne histamine was not the agent involved in reactive hyperaemia.

**EDRF: a diffusible molecule which opposes smooth muscle tone**

The adaptive dilation of arteries in response to changes in blood pressure has been recognised for many years as a rapidly activated process (Schretzenmayr, 1933. Hilton, 1959). Experiments carried out around the middle of this century gave conflicting results and showed that it was not always possible to correlate blood pressure with blood flow (Green, Lewis, Nickerson & Heller, 1944. Folkow, 1952). In addition, to obtain clear cut experimental conditions the tissue was often 'drastically interfered with' and was no longer intact (Folkow, 1952).

Other experiments investigated the effects of various agents as mediators of the control of blood flow. Acetylcholine caused both contractions or relaxation in the same vessels and at first the reasons for this were unclear. The discovery was eventually made using rabbit descending aorta that an intact endothelium was absolutely required for exogenous acetylcholine to relax vascular smooth muscle (Furchgott & Zawadzki, 1980). This lead to the concept of a diffusible endothelium derived relaxing factor (EDRF). This was formed in endothelial cells but exerted its affects on the smooth muscle of the blood vessels. A large amount of evidence was found from other work to support this (Furchgott, 1984. Griffith, Edwards, Lewis, Newby & Henderson, 1984. Rubanyi, Lorenz & Vanhoutte, 1985. Cocks, 1985. Gryglewski, Moncada, & Palmer, 1986).

The nature of EDRF was at first unknown but work on pre-constricted strips of rabbit aorta suggested that it was an unstable compound with a half life of 6.3 ± 0.6s. This was originally thought to
Possess a carbonyl group at or near its active site because of its inactivation by phenylhydrazine and KBH$_2$. It was not thought to be a lipoxygenase derivative or a free radical at this time (Griffith, Edwards, Lewis, Newby, & Henderson, 1984).

**Changes in the concentration of intracellular cGMP affect smooth muscle relaxation**

Cyclic GMP is associated with smooth muscle relaxation for example in bovine tracheal preparations (Katsuki, Murad, 1977) but the role of this cyclic nucleotide in the control of smooth muscle tone was unknown. The effects of exogenous 8-bromo derivatives of cGMP varied with tissue type. In the rat vas deferens for example there was no effect on smooth muscle basal tone although the contractile response of this tissue to exogenous noradrenaline was depressed. Conversely rat and rabbit aortic strips preconstricted with noradrenaline relaxed after a short time lag (Schultz, Bohme, & Kreye & Schultz, 1979). This indicated an involvement of cGMP in the tone of vascular smooth muscle.

Nitro-glycerine, a potent smooth muscle relaxing agent elevated the intracellular concentration of cGMP in arterial and other tissues (Diamond, Holmes, 1975. Diamond & Blisard, 1976). In several tissues including the rat vas deferens sodium-nitroprusside similarly evoked an increase in intracellular cGMP but a mechanism for the activation of guanylate cyclase was not suggested (Böhme, Graf & Schultz, 1978).

The important link between EDRF and smooth muscle relaxation was found when endothelium dependent relaxation was shown to involve the elevation of the intracellular concentration of cGMP in rat thoracic aorta (Rapoport & Murad, 1983) and this was confirmed in the same tissue in the rabbit (Furchgott, & Jothianandan, 1983).

**Is nitric oxide EDRF?**

The active principle responsible for vasodilatation had been proposed as being prostacyclin (PGI$_2$) (Pace-Asciak, 1976) but this was disputed when the actions of EDRF were later shown to be resistant to cyclo-oxygenase which inhibit prostacyclin activity (Holtz, Forstermann, Pohl, Giesler, & Bessinge, 1984).

A transient transmembrane signalling molecule should be lipophytic and unstable. Nitric oxide (NO), liberated by many commonly used vasodilators, possesses these characteristics and the idea that EDRF
might be NO was proposed when it was realised that both EDRF and compounds which liberate NO all stimulate guanylate cyclase (Kimura, Mittal, & Murad, 1975a & b. Katsuki, Arnold, Mittal, & Murad. 1977. Furchgott et al 1983. Rapoport et al. 1983). Basing their argument on the pharmacological behavioural similarities between the two molecules, two groups suggested that the identity of EDRF was NO (Furchgott, 1988. Ignarro, Byrnes & Wood, 1988). Further experiments verified that NO and EDRF were indistinguishable in various biological actions and chemical stability (Palmer, Ferrige & Moncada, 1987. Radomski, Palmer & Moncada, 1987a. Moncada, Palmer & Higgs, 1988).

If NO and EDRF are the same compound, then their characteristics should be identical. However, some discrepancies exist which suggest that some actions of EDRF are not entirely accounted for by NO. For example the EDRF released from bovine pulmonary arterial endothelium relaxes rabbit and rat blood vessels but not guinea pig tracheal or uterine smooth muscle or rabbit taenia-coli. NO on the other hand relaxes vascular and gastrointestinal but not tracheal or uterine smooth muscle (Shikano, Ohlstein & Berkowitz, 1987). In addition, EDRF binds to anionic exchange columns but NO does not (Cocks et al 1985. Long, Shikano & Berkowitz, 1987).

How is nitric oxide synthesised?

In 1988 L-arginine was shown to be the precursor of NO and extracellular L-arginine was needed for its production (Palmer, Ashton & Moncada, 1988a. Palmer, & Moncada, 1989). Mass spectrometry using $^{15}$N)L-arginine then showed that $^{15}$NO is derived from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer et al 1988a). This also demonstrated the specificity of the synthetic mechanism for the L-enantiomer because D-arginine did not increase the production of NO.

$^{3}$H citrulline was also shown to be a by-product of the reaction when $^{3}$H L-arginine was used. An increase in cGMP was also demonstrated to occur which was dependent on the concentration of L-arginine used (Moncada & Palmer, 1990).

How are the effects of nitric oxide mediated?

The signal transduction mechanism mediated by NO is dependent on the haem prosthetic group of guanylate cyclase (Ignarro, Degnan, Baricos, kadowitz & Wolin, 1982). Nitric oxide activates guanylate
cyclase by pulling the haem Fe$^{2+}$ away from the enzyme protein which alters the conformation of the protein and its function (Ignarro, Wood & Wolin 1984).

Smooth muscle relaxation is induced by cGMP in vascular, airway and intestinal smooth muscle (Katsuki, Murad, 1977. Katsuki, Arnold, Mittal, & Murad, 1977). Other work has shown that cGMP may produce its relaxant effect on smooth muscle by lowered cytosolic free Ca$^{2+}$ (Adams, Barakeh, Laskey & Van Breemen, 1989). The reduction in intracellular Ca$^{2+}$ may then stimulate the dephosphorylation of the myosin light chain (Draznin, Rapaport & Murad, 1983a & b) which reduces the interactions between actin and myosin during the contractile response.

Does nitric oxide satisfy the criteria of a biological mediator defined by Sir Henry Dale?

If NO is to be accepted as a biological mediator its characteristics should fulfil the criteria originally defined by Dale in 1933 which are:

i) the substance should be released in appropriate amounts during the response.

ii) the substance should produce the effect specified in vivo and in vitro.

iii) The enzymes necessary for synthesis should be present at the appropriate site.

iv) a mechanism for terminating the actions of the substance should exist.

v) Drugs which interfere with the action or the synthesis of the substance should produce a predicted alteration to the response.

vi) Receptors for the substance should be demonstrable on or in relevant cells.

vii) Experimental techniques or clinical conditions which result in deficiencies of the substance or of the synthesising or metabolising enzymes should result in an appropriate increase or decrease in the response.
Is nitric oxide released in appropriate amounts during the response?

Using the chemiluminescence produced by NO during its reaction with ozone it was shown that stimulation of cultured porcine aortic endothelial cells by Bradykinin induced a concentration dependent release of NO (Palmer, Ferrige & Moncada, 1987). This was sufficient to account for the relaxation of strips of vascular tissue. There was also a strong correlation between the amounts of NO detected by chemiluminescence and those measured by bioassay in other experiments (Radomski, Palmer & Moncada, 1987b & c).

Can nitric oxide produce the specified effects in vivo and in vitro?

L-NMMA induced an endothelium dependent constriction in rabbit aorta rings by inhibiting the synthesis of NO. This effect was reversed by L-arginine which indicated a role for NO in the correct maintenance of tone in this tissue (Palmer, Ashton & Moncada, 1988b).

Are the enzymes necessary for synthesis present at appropriate sites?

A family of NO synthases is now known to exist which are found in many different cell types including neuronal cells in the brain, smooth muscle, endothelial cells of blood vessels and macrophages (Palmer, Ashton & Moncada, 1988. Knowles, Palacios, Palmer & Moncada, 1989. Ozaki, Blondfield, Hori, Publicover, Kato & Sanders, 1992). Nitric oxide synthases are cytosolic but may be either (i) constitutively active, (ii) inducible or (iii) endothelial.

(i) constitutively active

These are present in a wide variety of tissues. (for a review of these see Moncada, Palmer & Higgs, 1991). They are Ca²⁺/calmodulin dependent, release small amounts of NO for short periods of time in response to receptor or physical stimulation and are down regulated by cytokines (Nishida, Harrison, Navas, Fisher, Dockerty, Uematsu, Nerem, Alexander & Murphy, 1992).
(ii) inducible

These enzymes are Ca\(^{2+}\) independent and are stimulated by cytokines and endotoxin in macrophages and endothelial cells for example to produce larger amounts of NO for long periods of time (Joulou-Schaeffer, Flemming, Schott, Parrat & Stoclet, 1990).

III) endothelial

These are found in endothelial cells. They are structurally different from the other two forms and possess an N terminal site sensitive to acyl transferase which permits its attachment to membranes. Bradykinin and other agonists activation results in phosphorylation of this site and the subsequent translocation of the enzyme into the cytosolic compartment which may alter its activity (Michel, Li & Busconi, 1993).

NOSs of the same type may co-exist within different tissues of the same organ. For example in large cerebral arteries constitutive forms have been demonstrated in endothelium cells and perivascular nerves (Cosentino, Sill & Katusic, 1993. Katusic, 1991. Nozaki, Moskowitz, Maynard, koketsu, Dawson, Bredt & Snyder, 1993). Conversely different forms may reside within a single organ and the NO produced by them may have different effects. An example of this is seen in vascular smooth muscle during basal function and during activity. NO is also produced by a constitutively active synthase in canine basilar arteries under basal conditions (Cosentino, Sill & Katusic, 1993). The function of the inducible form of the enzyme in the same tissue, however, contributes to the attenuated responsiveness to constrictor agonists during septic shock (Nishida, Harrison, Navas, Fisher, Dockerty, Uematsu, Nerem, Alexander & Murphy, 1992).

Does a mechanism exist for terminating the actions of nitric oxide?

Nitric oxide has one unpaired electron and is therefore, a highly reactive free radical with a very short half life. This offers a very simple and effective mechanism for terminating its actions.
Do drugs which interfere with nitric oxide synthesis produce a predicted alteration to the response

The discovery that L-arginine is the natural substrate for the synthesis in vivo and in vitro of nitric oxide lead to the production of synthetic non-hydrolizable analogous of the amino acid. These could then be used as competitive inhibitors of the postulated enzyme to block the synthetic process. Several compounds were produced including \( N^G \)-monomethyl-L-arginine (L-NMMA) which was first used in 1989 (Palmer & Moncada, 1989). This compound was not reported to have effects on any other arginine metabolising enzymes and has been shown to have no effect on arginase and arginine carboxylase (Granger, Hibbs, Perfect & Durack, 1990). The inhibition by L-NMMA also reduced the amount of cGMP and citrulline produced during the reaction. Ca\(^{2+}\) chelators were also shown to inhibit the production of these products which suggested that the enzyme involved in the synthesis of NO in endothelial cells is calcium dependent (Moncada & Palmer, 1990).

Is it possible to demonstrate receptors for nitric oxide on or in relevant cells?

In experiments described in this thesis the smooth muscle relaxing agent sodium nitroprusside was used as a donor of NO. Results which will be described later showed field stimulated contractions were increased in amplitude in the presence of this drug. This suggested that receptors for nitric oxide may be present in the vas deferens.

Do techniques or clinical conditions resulting in deficiencies of the substance or of the synthesising or metabolising enzymes result in an appropriate increase or decrease in the response?

During other experiments described in this current study L-arginine methyl ester (L-AME) was used to inhibit the synthesis of NO in the mouse vas deferens. Nerve evoked contractions of this tissue increased in amplitude and the quantal content of transmitter release also increased in the presence of this inhibitor. Those results were interpreted as indicating that NO is involved in the control of contractions in this tissue and the inhibition of its synthesis resulted in increased nerve evoked responses.
The roles of nitric oxide and histamine as transmitters and modulators of nerve and smooth muscle activity: the discovery that nitric oxide is a transmitter

The original division of the autonomic nervous system into sympathetic and parasympathetic systems was originally based on anatomical grounds supported by differences in the transmitters released by them (Bayliss & Starling, 1899. see also: Langley, 1921. The drug atropine which blocks the actions of acetylcholine at muscarinic receptors distinguished parasympathetic from sympathetic transmission (Yamamura & Snyder, 1974 Hulme, Birdsall, Burgen & Metcha, 1978). However, the effects of atropine on parasympathetic transmission could not always be predicted. One example of this was the ability of the drug to abolish sweating even though the innervation is anatomically sympathetic. It was later discovered that the innervation to these glands liberates acetylcholine.

Atropine resistance in which the drug fails to antagonise the response may also occur. The existence of a 'barrier' at sites of acetylcholine release was proposed to explain this phenomenon (Dale & Gaddum, 1930) in which only the effects of transmitter released outside the barrier could be blocked by atropine. It was subsequently proposed that the anomalous effects of atropine on bladder contractility were caused by transmitters other than acetylcholine being liberated from some parasympathetic nerves (Henderson & Roepke, 1934). Later the development of specific adrenergic antagonists showed that some sympathetic nerves also liberate transmitters other than noradrenaline (Burnstock, Campbell, Bennett, Holman, 1963). This supported the development of the concept of non adrenergic non cholinergic (NANC) transmission.

The bovine retractor penis (BRP) and the penile arteries of cat and cattle (Klinge & Sjostrand, 1974. Bowman, & Gillespie, 1983) as well as the rat annococcygeus (Gillespie, 1972) are innervated by nerves which arise in the sacral and lumbar spinal cord (Langley, & Anderson, 1895). They are therefore, mixed parasympathetic and sympathetic innervations. Nerve stimulation of the rat annococcygeus produces a rapid contractile response. This is sensitive to phentolamine a non-specific adrenoceptor antagonist and guanethidine which depletes adrenergic nerves of noradrenaline. However, if the adrenergic
component is blocked by these drugs in the presence of increased tone, a rapid TTX sensitive relaxation occurs in response to field stimulation. Treatment of the tissues with 6-hydroxydopamine destroyed the adrenergic nerves but left the nerves responsible for the novel relaxation intact. These were identified as non-adrenergic, non-cholinergic (NANC) nerves (Gibson & Gillespie, 1973). Many agonist candidates were investigated at this time in an attempt to identify the nature of the inhibitory transmitter. Haemoglobin which binds nitric oxide opposed NANC transmission but had no effect on the putative agonists used such as VIP and Bradykinin which cause relaxation in some other tissues (Keilin & Hartree, 1937. Gillespie & Sheng, 1989).

The NANC induced relaxation in BRP and rat annococcygeus was suggested to be mediated by NO. This may be released during stimulation of these nerves (Gillespie, & Sheng, 1989. Gillespie, Liu & Martin, 1989). This was confirmed in the mouse annococcygeus (Gibson, Mirzazadeh, Hobbs & Moore, 1990).

Other inhibitory nerves were identified which form part of the enteric supply to the digestive tract. These do not release acetyl-choline or noradrenaline. The release of vaso-active intestinal polypeptide (VIP) from these enteric nerves causes the inhibitory response of smooth muscles and although nitric oxide synthase (NOS) is present within these neurones the neuronal production of NO does not act as a transmitter but modulates the release of VIP (Grider & Makhlouf, 1988, Jin, & Grider, 1993). NO may, however, be the transmitter liberated at the canine ileo-colonic junction (Boeckxstaens, Pelckmans, Bult, De man, Herman & Van Maercke, 1990).

During adaptive relaxation in the pig stomach a dilation of the fundus occurs in response to small increases in gastric pressure (Paton & Vane, 1963). This has been shown to be under the control of the Vagus nerve and is abolished by both hexamethonium and TTX in the rat (Takahashi & Owyang, 1995).

VIP immunoreactivity is present in neurones to the stomach (Larson, Fahrkrug, Schaffalitzky, Sundler, Hakanson, & Rehfeld, 1976), and this transmitter is released during nerve stimulation (Grider & Makhlouf, 1987). However, VIP antagonists failed to prevent NANC induced relaxation in the cat gastric fundus (D'Amato, Beurme & Lefebvre, 1988) and it was demonstrated in the rat that this relaxation
could be mimicked by the application of sodium nitroprusside (Boeckxstaens, Pelckmans, Ruytjens, Bult, De man, Herman & Van Maercke, 1991). The presence of NOS has been identified in the myenteric plexus which implicates NO as the inhibitory transmitter involved in this effect (Bredt, Hwang & Snyder, 1990. Costa, Furness, Pompolo, Brookes, Bornstein, Bredt & Snyder, 1992).

Adaptive relaxation in the guinea pig stomach results from the stimulation of NANC nerves which are constituents of two different neuronal pathways (Desai, Sessa & Vane 1991). One of these is controlled by the CNS but the other is a local reflex arc. However, the opposition by L-NMMA indicates that NO is released by both pathways.

**Histamine: as a neurotransmitter**


Histaminergic nerves are similar to those of noradrenaline and 5-HT. These are localised in the hypothalamus with long descending and ascending tracts within the brain and spinal cord. This has been identified in the rat and mouse brain (Watenabe, Taguchi, Hayashi, Tanaka, Shiosaka, Tohyama, Kubota, Terano & Wada, 1983. Watenabe, Taguchi, Shiosaka, Tanaka, Kubota, Terano, Tohyama, Wada, 1984. Pollard & Schwartz, 1987. Airaksinen & Panula, 1988). The role of histamine in the CNS is speculative but has been implicated in several functions including arousal, temperature regulation and neuro-endocrine mechanisms (Shaw, 1971. Kalivas, 1982. Donoso & Alvarez, 1984).

In the periphery the histaminergic supply to the rat vas deferens has recently been proposed to function as a local reflex loop for the modulation of sympathetic transmission (Campos & Briceño, 1992).

**Histamine: as a neuromodulator**

The neuromodulatory roles of histamine includes effects on sympathetic transmission in medulla oblongata of guinea pig and the mouse uterus (Hey, del Prado, Egan, Kreutner & Chapman, 1992. Montesino, Villar, Vega & Rudolph, 1995). Histamine may also regulate
the activities of the parasympathetic system for example in guinea pig ileum and trachealis (Tamura, Palmer & Wood, 1988. Barnes & Ichinose, 1989). In addition, the contraction elicited by the myenteric plexus can be blocked by histamine acting at H$_3$ receptors. This is observed in the guinea pig ileum, pig trachea and human bronchi where H$_3$ receptors modulate transmission at Postganglionic nerve endings (Trzeciakowski, 1987).

A potent prejunctional cross modulation between histamine and other transmitters has also been demonstrated, for example, muscarinic and $\alpha_2$-adrenoceptors regulate the release of histamine in mammalian brain (Gulat-Marnay, Lafitte, Arrang & Schwartz, 1989). H$_2$ receptors are also involved in the modulation of ganglionic transmission in guinea pig superior cervical ganglia (Christian & Weinreich, 1992).

**Histamine receptors**

Three types of histamine receptors were identified from studies of isolated peripheral and brain slice preparations. The term H$_1$ receptor was initially introduced to describe a class of histamine receptors sensitive to low levels of classical antihistamines such as promethazine and mepyramine.

Responses to histamine including acid secretion from gastric mucosa. These were resistant to the classical antihistamines and were later suggested to be mediated by H$_2$ receptors identified by the use of burimamide (Black, Duncan, Durant, Ganellin & Parsons 1972). The third receptor type (H$_3$) was proposed to be involved in the control of histamine release from rat cortical brain slices (Arrang, Garbarg & Schwartz, 1983). This was confirmed by the use of the highly selective agonist R-a-methyl histamine which was used as a radioactive probe in that tissue (Arrang, Garbarg & Schwartz, 1987).

(For a review of histamine receptors see Hill, 1990)

**H$_1$ receptors**

The activation of these receptors includes the contraction of many smooth muscles of the viscera including uterine smooth muscle and longitudinal smooth muscle of the ileum (Dews & Graham, 1946. Ash & Schild, 1966).
Their role in membrane hydrolysis and the production of phospholipids.

H$_1$ receptors are linked to various second messenger systems depending on the subtype(s) of receptor(s) present. The activities of these receptors mediates the hydrolysis of membrane inositol phospholipids in both brain and peripheral tissues (Plevin & Boarder, 1988. Hall, Donaldson & Hill, 1989). The largest histamine evoked inositol phosphate response found to date is in guinea pig cerebellum which is selectively inhibited by antagonists of histamine at H$_1$ receptors (Daum, Downes, & Young, 1984). This stimulation of intracellular inositol production has different temperature and lithium dependencies from that evoked by other transmitters (Whitworth & Kendal, 1988).

Their role in evoking changes in intracellular concentrations of Ca$^{2+}$

The production of inositol phosphates causes the release of Ca$^{2+}$ from internal stores and the subsequent influx of Ca$^{2+}$ from outside the cell and the refilling of the intracellular stores (Irvine & Moore, 1986. Berridge, 1987. Berridge & Irvine, 1989). This, may provide an indirect link between histamine acting at H$_1$ receptors and the release of Ca$^{2+}$ from intracellular stores. This may offer a means by which histamine could modulate some cellular activities. Histamine induced Ca$^{2+}$ release from intracellular stores has been demonstrated in several tissues including in human umbilical vein endothelial cells and airway smooth muscle (Kotlikoff, Murray & Reynolds, 1987. Pollock, Wreggett & Irvine, 1988) and in most cases there is also an appreciable influx of Ca$^{2+}$ from outside the cell.

H$_2$ receptors

These have been identified in several tissues including the rat vas deferens and guinea pig lung (Foreman, Rising & Webber, 1985. Poli, Todorov, Pozzoli, & Bertaccini, 1994) and also in the mammalian central nervous system including guinea pig hippocampus and rabbit cerebral cortex (Palacios, Garbarg, Barbin & Schwartz, 1978. Al-Gadi & Hill, 1985. Hill, 1987).
Intracellular changes in cAMP and Ca\textsuperscript{2+} concentrations evoked by H\textsubscript{2} receptor activation

Histamine acting at H\textsubscript{2} receptors activates adenylate cyclase in many cells (Johnson, 1982). This activity is also associated with increased membrane phospholipid methylation and the production of phosphatidyl choline in rat brain for example (Ozawa, Nomura, & Segawa, 1987. Ozawa & Segawa 1988). In erythrocyte ghosts, rat brain synaptosomes and rat mast cells this occurs on the inside of the membrane. Subsequently these changes are translocated to the outer surface to increase the microviscosity of the membrane in the region of the H\textsubscript{2} receptor. (Tolone, Bonsera & Potieri, 1982). An increase in intracellular cAMP is also associated with the H\textsubscript{2} stimulated release of gastric HCl (Soll & Berglindh, 1987). This may also be associated with the release of Ca\textsuperscript{2+} from internal stores. In cardiac muscle a positive inotropic effect produced by H\textsubscript{2} activation is similar to the cAMP mediated effects produced by the action of adrenaline at \(\beta_1\) adrenoceptors (Bristow, Cubicciotti, Ginsburg, Stinson & Johnson, 1982).

H\textsubscript{3} receptors

These are the most recently identified and are the most sensitive to histamine but their activities have been less well defined. However, this is an active area of research and evidence is accumulating which will permit their characterisation and functional roles.

Their roles in the modulation of transmitter release

H\textsubscript{3} receptors appear to play a role in the prejunctional modulation of transmitter release. The interactions of histamine with prejunctional these receptors may prove to be complex and it is already known to involve two pharmacologically distinct subtypes H\textsubscript{3A} and H\textsubscript{3B} (West & Zweig, Shih, Egan & Clark, 1990). The prejunctional modulation of transmitter release including the auto inhibition of histamine release by activation of H\textsubscript{3} receptors has been identified in rat and human brains (Arrang, Garbarg & Schwartz, 1983. Arrang, Defontaine & Schwartz, 1988. Arrang, Devaux, Chokiewcz & Schwartz, 1988. Schlicker, Betz & Gothert, 1988 & 1990). There is evidence from rat cerebral cortical slices, that histamine may cause its inhibition of transmitter release by reducing the influx of Ca\textsuperscript{2+} influx following H\textsubscript{3} activation (Hill & Straw
1988). This may suggest that a mechanism by which prejunctional H₃ activation inhibits transmitter release in other tissues.

H₃ receptors have also been implicated in the prejunctional inhibition of sympathetic hypertensive responses in guinea pig (Hey, del Prado, Egan, Kreutner & Chapman, 1992). They have also been identified in guinea pig ileum and trachea and human tracheal smooth muscle where they may regulate the activity of the parasympathetic innervation to those tissues (Trzeciakowski, 1987. Barnes & Ichinose, 1989. Ichinose & Barnes, 1989. Hew, Hodgkinson & Hill, 1990). H₃ receptors are also present at nicotinic synapses in enteric ganglia (Tamura, Palmer & Wood, 1988) and have also been identified on NANC nerves in the guinea pig ileum where they modulate transmission (Lees & Steel, 1990).

The modulation of transmission in non-vascular smooth muscle: relative roles of nitric oxide and histamine in the controls exerted on the activities of the vas deferens

The modulatory effects of nitric oxide on the smooth muscle and innervation of the vas deferens is unknown although experiments described in a later chapter of this thesis showed that this messenger molecule does inhibit nerve evoked contractions of this tissue in the mouse. Such modulation would represent a local mechanism which would modify the control of transmission and tissue function.

A role for nitric oxide as a modulator of sympathetic transmission in the mouse vas deferens has not previously been identified. The present study was originally designed to investigate this. However, a histaminergic nerve supply has been identified in the rat vas deferens (Campos, 1988. Campos & Briceño, 1992. Campos & Dominguez, 1994). This may represent another local mechanism for the modulation of sympathetic transmission which may also be present in the mouse. In view of this other experiments were included. These were designed to determine if histamine mediates some relaxant effects of the longitudinal smooth muscle of the mouse vas deferens which might otherwise be attributable to nitric oxide.

Nitric oxide and histamine may present dual mechanisms by which transmission is modulated in this tissue. This would provide a means of varying the dominance of central control and permit the tissue to respond to some local demands.
The hypothesis

Little is known about the mechanisms and effects of local messengers on the release of transmitters in the rodent vas deferens. Trans-synaptic messengers of pre or post junctional origin may include nitric oxide or histamine or both. These may modulate the release of transmitter from sympathetic nerves in the vas deferens by prejunctional effects but may also have post junctional effects on the smooth muscle which they supply. By that means the controls exerted by the CNS may be modulated to suite local conditions.

Experiments were designed which would address questions arising from this hypothesis. These are:

1. Is the mouse vas deferens sensitive to nitric oxide?

2. Does the mouse vas deferens possess a form of nitric oxide synthase and does the addition of L-arginine cause nitric oxide release?


4. Can Histamine account for some of the effects attributed to nitric oxide in this tissue?

5. Does nitric oxide or histamine (or both) modulate the release of transmitters by more a prejunctional mechanism?
Methodology

Male BKW mice between 5 and 7 weeks old were anaesthetised with chloroform and decapitated. The abdominal body wall was opened by a single central incision to expose the reproductive and urinary organs. Both vasa deferentia were tied at prostatic and epididymal ends by silk threads, rapidly removed and placed in gassed Krebs saline. The abdominal cavity and excised tissues were bathed in Krebs saline at all times.

Protocol for stimulation and recording of:

Mechanical responses

The Krebs saline within the bath was maintained at 36°C by water circulating between the outer and inner glass skins of the hollow glass jacket. Krebs saline stored in a reservoir above the level of the organ bath was gassed (95% O₂, 5% CO₂) to pH 7.4. The Krebs saline in the organ bath was stirred by bubbles of the same gas (see Figure 2).

The preparation was suspended in the organ bath between parallel platinum wire stimulating electrode with its epididymal end uppermost. The prostatic end was within the electrical field to ensure maximal nerve stimulation. The tissue was attached to the transducer and hook assembly by the silk threads (see figure 3).
Drugs applied in surface of Krebs' saline

Overflow

Hollow bath jacket permits circulation of water at 36°C from water bath.

From water bath

Drugs applied in surface of Krebs' saline

Krebs saline

Water returns to water bath

Outlet for gases. 95% O₂ 5% CO₂

Inlet for gas

Inlet for Krebs saline from reservoir

Clamps

Outlet to drain saline from bath

Figure 2. Diagram of the organ bath used in mechanical response experiments
Evoking contractions

Field stimulation

Field stimulation was produced by 'Square One' stimulator. Train length, frequency, duration of pulses, interval between trains and intensity were varied and delivered to the tissue by parallel platinum stimulating electrodes in the organ bath. Contractions were recorded by Linseis penchart recorder (model number: L6522-2).

Exogenous agonists

The tissue was suspended in the organ bath as previously described. Agonists were applied under the surface of the Krebs saline in the organ bath and stirred by gas bubbles.
**Recording evoked contractions**

All contractions were recorded using an isotonic transducer. A resting load of 0.9g was applied to the preparation.

**Determination of parameters for field stimulation**

Field stimulated nerve evoked contractions

Preliminary experiments were designed which would evoke measurable and reproducible responses.

Trains of 10 pulses at 5Hz with a pulse duration of 0.6 ms gave a measurable maintained response and a rest interval of 10 minutes between trains permitted full recovery. These parameters were used for all recordings of contractions. In some experiments only a single stimulus intensity was used.

Stimuli at intensities of 0 - 50 V. were given in random order and intensities greater than 50V were only recorded singly or as the final stimulation in an experiment.

A series of stimuli delivered at the beginning of an experiment were referred to as Set 1. Subsequent repeated stimuli were referred to as Sets 2 or 3.

Table 1 summarises the stimulus parameters used during recordings of extracellular responses in most experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hz</th>
<th>train length</th>
<th>V</th>
<th>Pulse duration (ms.)</th>
<th>Rest interval (Minutes)</th>
<th>trains per stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vas deferens</td>
<td>5</td>
<td>10</td>
<td>10-50</td>
<td>0.6</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

**Correction for the effect of ageing of the preparation**

Nerve evoked contractions increased in amplitude with time. Responses recorded as Set 2 in the presence of a drug, were always compared with those recorded from other preparations as Set 2 in the absence of drug treatment. This allowed for changes caused by the drug
treatment to be corrected for effects caused by the ageing of the tissue. This protocol was also applied to comparisons of responses recorded as Set 3 in the presence or absence of a drug.

Figure 4 illustrates the general protocol adopted for field stimulation of the isolated preparations.

Protocol to determine that contractions were not evoked by direct stimulation of the smooth muscle

Contractions evoked by stimuli of 5-60V were recorded in the presence of tetrodotoxin (TTX). All responses including those evoked by stimuli up to and including 50V were abolished by TTX (4.6x10^{-5}M). A small residual response evoked by a stimulus of 60V intensity remained after this drug treatment which may have represented the direct stimulation of the smooth muscle or may have indicated that the concentration of TTX was not sufficient.

Normalising the responses and collection of data for analysis

Figure 4. General protocol for field stimulation in which trains of 10 pulses at 5Hz with 10 minute intervals. Trains were delivered to the tissue in random order of stimulus intensity from 0-50V. Responses were normalised to the response to 50V. Each experiment was designed to be 2 hours in total to avoid non-specific effects of tissue ageing to complicate results. The bath was washed out (w) and replaced with fresh Krebs saline after each response.
Responses recorded during the first 30 minutes following the dissection were not analysed. All responses were normalised to the maximum response of the preparation evoked by a stimulus of 50V unless otherwise stated in specific experimental protocols to be described later. Experiments were limited to a maximum duration of 2 hours.

**Addition of drugs.**

**Agonists**

All drug were applied to the Krebs saline in the organ bath. Agonists were washed out immediately after the peak of the contraction. This was especially important when using αβ-methylATP (αβ-met.ATP) which rapidly desensitises P2X receptors if left in contact with the tissue. A minimum rest interval of 15 minutes between applications was required for responses to recover following the exogenous application of agonists. This helped to minimise the risk of purinergic receptor desensitisation by αβ-met.ATP. Figure 5 illustrates the general protocol used; variations of this will be described as required in later chapters.

![Diagram](https://example.com/diagram.png)

Figure 5. Protocol for evoking responses by applying exogenous agonists to the isolated mouse vas deferens. Agonists were added to the Krebs saline. Following the peak of the response, the bath was washed out (w) and replaced with fresh saline three times. A 15 minute rest interval was allowed between drug applications to permit full recovery of responses. Responses were normalised to the responses which evoked the maximum responses of the tissue. Experiments did not exceed 2 hours duration.
Other drugs

These were either administered immediately after a washout and allowed to remain in the bath until field stimulation occurred or were present in the reservoir of Krebs saline. Protocols for this will be described as appropriate in the results.

Light was excluded at all times during the preparation and use of all solutions containing sodium nitroprusside.

Electrical responses of the smooth muscle membrane

Focal extracellular recording of nerve evoked junction currents

![Diagram of the isolated vas deferens positioned in the organ bath. The preparation was pinned securely to the Sylgard base of the organ bath at resting length taking care to avoid stretching. A section of the epymysium was carefully torn away to reveal the surface of the underlying longitudinal smooth muscle.](image)

Figure 6. Diagram of the isolated vas deferens positioned in the organ bath. The preparation was pinned securely to the Sylgard base of the organ bath at resting length taking care to avoid stretching. A section of the epymysium was carefully torn away to reveal the surface of the underlying longitudinal smooth muscle.
The isolated preparation was transferred to a 3ml organ bath which was perfused at a rate of 1 ml min⁻¹. The preparation was pinned through the connective tissue sheath onto the Sylgard covering the base of the bath at resting length (see figure 6). Care was taken to avoid stretching the preparation. The prostatic end of the tissue was drawn into a stimulating electrode composed of two parallel silver rings 2mm apart mounted in 3mm diameter polythene tubing. (figure 7).

![Diagram](image)

Figure 7. Diagram showing the cut prostatic end of the vas deferens drawn into the stimulating electrode.

The tissue was field stimulated by parallel silver wires 2mm apart.

**Parameters for stimulation**

A frequency of stimulation of 0.91Hz was used to avoid the production of contractions and to allow averaging to be used where appropriate to remove the 50 cycle interference. The pulse duration was maintained at 0.06 ms in all experiments. Preliminary experiments showed that responses evoked by trains of more than 30 pulses with a rest interval of less than 1 minute became increasingly intermittent. To prevent this responses for analysis from most experiments were
recorded as trains of 20 with a rest interval of 2 minutes. One experiment (The effect of L-AME present throughout the entire procedure) involved trains of 30 pulses with a one minute rest between trains. The results of this were compared with responses recorded from other preparations stimulated in the same way in the absence of the inhibitor.

**Terms explained**

In each experiment 10 trains of control responses were recorded. These will be referred to as Set 1 in the absence of drug treatment. A rest interval of 5 minutes was permitted before further responses were recorded. Drug additions were made during this interval. Subsequent responses will be referred to as Set 2 in the absence or presence of drug treatment.

The stimulus intensity was always raised from zero volts up to the threshold for the excitatory response and then increased by a further 20-25% to avoid loss of responses. This never exceeded a maximum of 5V.

Table 2 summarises the stimulus parameters used during recordings of extracellular responses in most experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Frequency (Hz)</th>
<th>Pulses in each train</th>
<th>Intensity (V)</th>
<th>Pulse duration (ms.)</th>
<th>Rest interval (Minutes)</th>
<th>Number of trains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vas deferens</td>
<td>0.91</td>
<td>20</td>
<td>1 - 5</td>
<td>0.06</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

**Recording responses by extracellular focal electrode**

2 mm diameter glass tubing without a filament was drawn out to produce a fine tip by 'Pull-one' electrode puller. The tip of each electrode was then carefully broken back to produce a diameter of approximately 50 µm.

The recording electrode was filled with Krebs saline by injection and the silver wire electrode and perfusion tube were inserted into it close to but not obstructing, the tip (see figure 8). The glass electrode
was then inserted into the holder attached to the NEUROLOG AC
preamplifier (NL 104). The tip was perfused by gravity from its own
reservoir of gassed Krebs saline at rate of approximately 0.05 ml min⁻¹.
A silver/silver chloride pellet in the bath was used as a reference.

The tip of the recording electrode was placed carefully onto the
exposed surface of the smooth muscle of the isolated preparation taking
care to make contact with the surface without depressing it deeply (see
figure 9).

Figure 8. Diagram of enlarged view of recording electrode. The silver wire recording electrode and nylon
perfusion tube were inserted into the glass electrode close to the tip without obstructing it.
Figure 9. Diagram of isolated mid vas deferens enlarged to show recording electrode positioned over nerve supply to the longitudinal smooth muscle.

Currents were amplified by NEUROLOG NL120 AC (between 0.1Hz and 5KHz). Signals were viewed on a fluorescent screen and passed to IBM hard disc.

Analysis and storage was carried out using the SCAN program by written by John Dempster from the Department of Pharmacology, Strathclyde University.

The absolute size of records depended on several factors including the diameter of the recording electrode tip and its seal resistance with the tissue. Absolute comparisons between different recording sites have little meaning. Comparisons of amplitudes are only valid when records from the same placement are examined.

The Krebs saline was gassed by 95% O2/5% CO₂ to pH 7.4 at 40°C. This prevented the formation of gas bubbles in the organ bath which was maintained at 35-37°C and was enclosed in a atmosphere of 95% O2/5% CO₂ and perfused at a rate of 1ml min⁻¹.
Addition of drugs:

To the bath

Drugs were added to the reservoir of Krebs saline and were thoroughly mixed. An interval of 20 minutes was allowed to permit the drug to reach the organ bath before further responses were recorded.

To the focal recording electrode.

Drugs were added to the reservoir which supplied the recording electrode tip. A rest interval of 5 minutes was permitted after the addition of drugs to this perfusate to allow them to reach the exposed surface of the smooth muscle. This time interval was chosen after observation of the time taken for a solution of Evan Blue to transverse the same path.

Protocol for the long term effects of nitric oxide synthase inhibition by L-AME

L-AME present throughout the entire procedure was added to all salines including the bathing saline used during the dissection.

Correction for the effect of ageing of the preparation

To determine the effect of ageing on junction currents, responses were recorded in the absence of drugs. These were evoked by field stimulation of the tissue described in the previous section and will be referred to as Set 1 in the absence of drug treatment. After a rest interval of 5 minutes, further responses were recorded. These were recorded as Set 2 in the absence of drug treatment. Where necessary a further third Set of control responses was recorded using the same protocol. These will be referred to as Set 3 in the absence of drug treatment.

Individual varicosities have been visualised (Lavidis. & Bennett, 1992 & 1993). This method was not used in experiments described in this thesis and so responses could not be related to individual varicosities. A single release site was defined in experiments described in this current study as occurring within a narrow latency band and was assumed to be releases from one or several varicosities on a single nerve.
fibre. Different nerves with the same conduction velocity within the recording electrode tip diameter cannot be excluded.

Figure 10. Highly stylised cartoon showing the area covered by focal extracellular recording electrode. Varicosities are not drawn to scale. Two different nerves are shown crossing the recording area. In this example the nerves are illustrated as having different diameters and therefore, different conduction velocities. Two release latencies would be present in recordings made from this area. Each nerve possesses varicosities within the area covered by the focal recording electrode tip. Negative going excitatory junction currents (EJCs) are produced by releases from varicosities situated within the area covered by the recording electrode tip (Brock & Cunnane, 1987). t1 and t2 represent the conduction time for nerve impulses in the two different neurones from site of field stimulation to site of recording electrode. The neurone with conduction time of t1 is of smaller diameter and slower conduction time than the neurone labelled as t2.

Methods of analysis

Figure 11 shows an example of an excitatory current recorded by focal extracellular electrode. The onset and amplitude of each response was determined and measured in ms and μV using the cursor in the SCAN program. All measurements were relative to a pre-Set zero reference area which was arbitrarily chosen and marked using the cursor (Z). Measurements were routinely made of the amplitude of the responses and the rise time and time taken for the response to decay to half amplitude was calculated.
Figure 11. An excitatory junction current evoked by field stimulation of the isolated vas deferens. Tissues were field stimulated with trains of pulses at 0.91Hz. Stimulus intensity was 20-25% above the threshold for excitatory responses and the stimulus duration was 0.06ms. Responses were recorded from the surface of the smooth muscle of mouse vas deferens by focal extracellular electrode and their onset and amplitudes defined and measured in ms and μV. All measurements were relative to a pre-set zero reference area which was arbitrarily chosen and marked using the cursor (Z). Measurements were routinely made of the amplitude of the response. The rise time and time taken for the response to decay to half amplitude were calculated.
Measuring responses

Latencies of release sites were measured using the SCAN program in the following way:

1. An area (Z in figures 11) between the stimulus artefact and the onset of the response was marked using the cursor. This was several ms long and was used by the SCAN program to give a zero reference for all amplitude measurements. The latency (ms) and amplitude (µV) of the responses were identified and measured relative to the zero reference area.

Construction of histograms:

To define release site latencies

The data from latency measurements was used to construct histograms. Two examples are illustrated in figure 12.

![Histograms](image)

Figure 12. An example of release site latencies. These were measured and the data used to produce latency frequency histograms. Bin widths were chosen by a process of trial and error to give the best definition of release site latencies for the data. (a) Responses in which a single site was recorded: Bin widths: 0.48 ms. (b) Responses from a different tissue from that in which (a) was recorded. Two sites were assumed to be present in these recordings. Bin widths: 0.44 ms. The peaks of the two sites were separated by 1.32 ms.
Release sites were observed to group together. Release of transmitter is subject to jitter (Katz & Miledi, 1965). This results in releases from single sites varying in latency and can be caused by any processes which may affect the mechanisms of transmitter secretion. The response illustrated in figure 12a was from one tissue in which one site predominated. No more than 2 ms. was allowed as jitter. In other recordings more than one site were apparently present (Figure 12b as an example of this).

In the example shown in figure 12a, events between 15.6 - 17.0 ms. were assumed to be from a single site. All responses recorded outside these limits were considered to belong to different release sites. Bin widths were chosen during the construction of the histogram which would allow sites of different latencies to be best distinguished. In the example chosen in figure 12a, these were 0.48 ms.

A few recordings were very difficult to analyse and included responses in which the peaks were not distinctly separate. Where more than one site was evident with peaks distinctly separated by the criteria previously defined, only the site with shortest latency was accepted for further analysis.

During the measurement of responses, only onset and peak were measured. Some responses may have been composed of multiple releases from sites of more than one latency but the individual contribution to the total response was difficult to assess. Figure 13 shows an example in which two release latencies were present. In this example there was 3.63 ms between the onset of each response, in other recordings the sites were not so well separated. For this reason, only responses with shortest latency were included in the analysis. This method permitted the maximum number of releases from a given site to be included in the analysis.
Figure 13. A response at two distinct latencies. Tissues were stimulated with trains of pulses at 0.91 ms. and a pulses duration of 0.06ms. The interval between trains was 2 minutes. EJC's were recorded from the surface of the longitudinal smooth muscle of the mid vas deferens by extracellular focal electrode. These sometimes contain more than one release site. In this example two release sites were at latencies (1) 7.6ms and (2) 16.3ms. The action potential (AP) may be associated with the release from the earliest release site.

**To determine response amplitudes**

Following the selection of a suitable release site, amplitudes of all responses from that site were measured. This data was then used to construct a histogram for amplitude of which Figure 14 is an example. To construct the histogram it was necessary to select suitable bin widths for the data. This varied with different tissues but was chosen to best reveal the distribution of the peaks of preferred values. The choice was made by trial and error. If the peaks were equidistant this was taken as evidence that a single released site was being observed. The point midway between peaks was found and the windows marked as in figure 14. The number of events in each window were then counted. The interval between \( n = 0 \) and \( n = 1 \) was not always the same as that between \( n = 1 \) and \( n = 2 \). This was caused by difficulties in defining the exact zero of the responses.
Fig. 14. An example of how the frequencies of amplitude group together. The tissue was field stimulated with 10 trains of 20 pulses at 0.91Hz with a 1 minute rest interval between trains. Stimulus intensity was 20--25% above the threshold for excitatory responses and the pulse duration was 0.06ms. In the example illustrated here, chosen bin widths were 3.5 μV. 5 bins separated each peak in this analysis. Amplitudes of responses recorded from this tissue ranged from 10 to 86.5 μV.

The data was observed to group around equi-spaced values assumed to correspond to transmitter releases of varying quantal content. In Figure 14 the peaks of each preferred value are marked by arrows. This shows that amplitude frequencies group together into preferred values.

Protocol for determining the number of release events and their quantal content

1. The peaks of each preferred value were identified and marked.

2. The window defining each preferred value was identified with its peak as the central marker.

3. The numbers of individual responses occurring between the two limits of each quantal content were counted.
Analysis of response amplitudes gives information about pre and Postjunctional effects

Two types of changes can occur in these responses following drug treatment of the tissue: These are defined by quantal analysis and can involve changes in:

Quantal effect

This is a measure of the post junctional response to liberated transmitter. This is distinguished by comparing the peaks and limits of each preferred value as defined in the controls. This may be altered during post junctional effects and shifts the peaks relative to those of the controls. Such a change indicates a change in the amplitude of the Postjunctional response in the short term but changes may also occur more slowly following prejunctional modifications. An example of this would include alterations in the size of each quantum due to changes in the synthesis of transmitter.

Quantal size

This is a measure of the number of transmitter molecules in each packet or quantum.

Quantal content

This is the number of packets of transmitter liberated from each release site which was initially shown by a change in the number of peaks with no change occurring in the spacing between them.

Fitting the responses to a Poisson probability distribution

When n, the number of individual events in a process is small and it is difficult to distinguish between a Poisson or binomial process the two distributions are similar.

The intermittent nature of responses recorded during experiments described in this thesis resulted in a small number of events being recorded from single release sites. In view of this it was considered appropriate to fit the data to a Poisson distribution. The numbers of each
preferred value were counted from the frequency distribution histogram (see figure 14) and fitted using the equation:

\[ P_X = \frac{(m^x/x!)}{e^{-m}} \]

where \( P_X \) is the probability of the an event of \( n \) quanta in magnitude occurring. \( m \) is the mean quantal content of all releases from an individual release site and \( x \) is the quantal content of responses from that site.

The advantage of using this equation is that only the mean quantal content is needed.

**Fitting releases from the mouse vas deferens to the Poisson distribution**

The EJC data was fitted to a Poisson distribution to provide a means of analysing changes caused by various drug treatments. This process would enable pre and post junctional changes to be determined which would help to identify underlying mechanisms.

![Figure 15](image.png)

Figure 15. An example showing how observed frequencies of events of each quantal size were used to predict a distribution using Poisson theorem \((P_X = \frac{(m^x/x!)}{e^{m}})\). The smallest quantal size and the failures did not fit a Poisson distribution but the fit for larger quantal sizes was more accurate. Hatched columns: observed data. J calculated probabilities. The value of \( m \) in this example was 1.3
The plotted results obtained from equation (i) were superimposed on a plot of observed probabilities for each value of quantal content (see figure 15). This showed that the observed data did not fit a Poisson probability distribution; there were too many failures and a deficit of the smallest quantal size.

It was proposed that the release of excitatory transmitter was determined by two processes (Blakeley et al 1982). These are:

1. The secretion of transmitter. This could be described by Poisson statistics.

2. Another process which was assumed to precede the secretion of transmitter. This produced failures in responses which contaminated the data and spoiled the fit to a Poisson distribution.

It was necessary, therefore to remove these failures from the analysis. This was done by fitting responses with quantal content greater than zero from the data and varying the assumed number of failures by the following process.

**How the data was fitted to a Poisson distribution**

A spreadsheet was designed using EXCEL which would accelerate the process. Table 3 is an example of the spreadsheet including the uncorrected raw data used shown plotted in figure 14 which did not fit a Poisson distribution.
Table 3. A copy of Poisson Fitter from Excel showing uncorrected raw data. The observed column contains raw EJC data, number of quanta were calculated by multiplying observed events by their individual quantal content (n). The Poisson distribution was calculated using $P_x = \frac{m^x}{x!}e^{-m}$ and using iterative changes to n = 0 and n = 1. The error^2 was calculated using the difference between calculated and observed values of 'n'. The best fit of the calculated data to this distribution was found when the total error^2 was at a minimum.

<table>
<thead>
<tr>
<th>N /Stimuli</th>
<th>Observed (O)</th>
<th>Quanta (n)</th>
<th>Calculated (from $P_x = \frac{m^x}{x!}e^{-m}$)</th>
<th>Error^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>102</td>
<td>0</td>
<td>55</td>
<td>2256</td>
</tr>
<tr>
<td>200</td>
<td>16</td>
<td>16</td>
<td>71</td>
<td>3009</td>
</tr>
<tr>
<td>300</td>
<td>30</td>
<td>60</td>
<td>46</td>
<td>258</td>
</tr>
<tr>
<td>300</td>
<td>29</td>
<td>87</td>
<td>20</td>
<td>82</td>
</tr>
<tr>
<td>300</td>
<td>18</td>
<td>72</td>
<td>6</td>
<td>133</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>25</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>200</td>
<td>260</td>
<td>200</td>
<td>5748</td>
</tr>
<tr>
<td>Mean (m)</td>
<td>1.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP Zero's</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The spreadsheet in table 1 was used to calculated a best fit of the observed data to a Poisson distribution in the following way:

1. The 'observed' column

Raw data was entered into this and the mean quantal content (m) was calculated from the observed data by:

Total quanta/(total number of events - non-Poisson failures)
in which the non-Poisson failures were initially set at zero.

2. The 'calculated' column

The number of events for each value of quantal content were calculated using:

(i) \( \frac{m^x}{x!} e^{-m} \)

and assuming that all failures were part of a Poisson process.

3. The 'Error' column

\((\text{observed-expected})^2\)

The number of events predicted by equation (i) was subtracted from the number of observed events and the squared difference was minimised to produce a best fit.

Why the data did not fit a Poisson distribution

Failures

The predicted number of failures at this stage was far lower than the number of observed failures. This was one factor which prevented the data from fitting a Poisson distribution.

The first correction

At this stage the number of non-Poisson failures is revealed. The number \( n = 0 \) and the number of non-Poisson failures were adjusted iteratively until the total error was at a minimum. Responses of some tissues were found to contain no non-Poisson failures but these were unusual. Ten tissues were used to determine the effects of endogenous nitric oxide removal by methaemoglobin for example. Of these only one set of control responses did not contain failures attributable to the non-Poisson process.

The numbers of events in both the corrected and calculated columns were converted into probabilities

(iii) \( p = \frac{\text{corrected number of events}}{\text{total number of quanta}} \).
The corrected and calculated data were plotted together

The best fit after this alteration still contained a deficit of responses of smallest quantal content $n = 1$ (see figure 16).

![Figure 16](image)

Figure 16. A further example of how observed frequencies of each quantal size were used to predict a distribution using Poisson theorem ($\text{Px} = (\frac{m^x}{x!})e^{-m}$). The smallest quantal size and the failures did not fit the predicted distribution although the fit for larger quantal sizes was more accurate. The probability of failure was corrected by fitting the data to a distribution based on the fit of the values for larger quantal content. This revealed the number of non-Poisson failures. Following this correction the data still did not fit a Poisson probability distribution. Observed events hatched columns: Observed probabilities. J: calculated probabilities.

Some of the small responses were mistakenly identified as failures

Figure 16 shows that altering the number of observed failures improved the fit but there was still a deficit of responses of smallest quantal content. One explanation for this was that some of these responses were small enough to have been lost in the level of the noise and therefore, recorded as failures. In the example chosen in figure 13, the level of noise was about 10 µV. The smallest responses measured were between 10 and 13.5 µV. Some responses may have been between 10 and 6.5 µV but these were not clearly distinguishable. To correct for this, the observed cell for $n = 1$ was adjusted according to the value shown in the equivalent calculated cell.
How the number of smallest responses was calculated

Another best fit of the data for releases greater than \( n = 1 \) was made by iterations of the values of both \( n = 1 \) and \( n = 0 \). This predicted the number of events of smallest quantal content by reducing the number of Poisson failures. The results of this are shown in table 4.

Table 4: Predicted values for \( n = 0 \) and \( n = 1 \) compared with observed values. A close fit of the data to a Poisson distribution was achieved by correcting both the number of failures and of the smallest quantal content.

<table>
<thead>
<tr>
<th>Quantal content</th>
<th>observed</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>102</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 17. A third example of how observed frequencies of each quantal size were used to predict a distribution using Poisson theorem \((P_x = \frac{m^x}{x!}e^{-m})\). The smallest quantal size and the failures did not fit such a distribution although the fit for larger quantal sizes was more accurate. Frequencies of events were converted into probabilities before plotting. Hatched columns: observed events. J predicted probabilities. The number of failures were corrected following the fit of the data to a distribution based on the larger releases. The observed number of failures was increased to match the number predicted by this fit. Following this correction the data still did not fit the predicted Poisson probability distribution because of a deficit of \(n = 1\). It was therefore, necessary to increase the value of observed numbers of \(n = 1\). This change was again made by using the predicted value. Following these corrections the data fitted the predicted Poisson distribution closely.

Probabilities for all observed and predicted values of quantal content can be calculated by:

\[
\text{number of events for each value of } n / \text{total number of quanta (See Table 5)}. 
\]
Table 5. Predicted events and probabilities of quantal releases of transmitter.

<table>
<thead>
<tr>
<th>Quantal content</th>
<th>Predicted frequency of events</th>
<th>Predicted probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>0.007</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>0.004</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Explaining the corrections made to the observed data

Events of larger quantal content \( (n \geq 2) \) have a lower signal to noise ratio than events of low quantal content \( (n = 1) \). This feature allows identification and measurement of larger responses to be made unequivocally while this is more difficult with small responses. In the initial attempt the larger responses fitted a Poisson distribution very closely while there was an excess of failures and a deficit of \( n = 1 \). The fit of the larger responses was therefore, used to extrapolate the remaining part of the distribution.

The physical basis of the generation of a Poisson process

The deficit in the number of the smallest quantal content \( (n = 1) \) can be explained by the inadequacies of measurements raw data. Some of the smallest events were inevitably lost among the noise level and mistaken for failures. This would also explain some of the initial excess of failures. However, all of the excess of failures cannot be explained by an equivalent deficit of single quanta. Another explanation must be
found if the corrected fit to a Poisson distribution is to be accepted. These inconsistencies may be explained by suggesting that there are two superimposed processes which influence the release of excitatory transmitter from sympathetic nerves in this tissue. These are:

**The process which can be described by Poisson statistics**

Controls the release mechanisms operating within the varicosity. This result in transmitter release and relates to mechanisms within the varicosity.

**Another process which cannot be described by Poisson parameters which may precede the secretion of transmitter**

This may interfere with the release mechanism and decreases the probability of release of excitatory transmitter. This process has not been identified but may be caused by factors preceding the secretion of transmitter but not immediately related to it. These may relate, for example to conductance changes along the axon.

The analysis of changes in these two types of processes can give information concerning effects occurring within the varicosity or elsewhere.

**How the fit a Poisson distribution was used in the analysis of experimental results**

**Prejunctional effects**

Changes in mean quantal release per pulse, mean quantal content \((m)\) and failure rates per pulse attributed to processes preceding the secretion of transmitter are prejunctional effects. These were used to assess the effects of various drug treatments on transmitter secretion from nerves in this tissue.

**Changes in mean quantal content**

Changes in the mean quantal content \((m)\) were interpreted as being either:

i) a change in the number of varicosities involved in the response or that:
ii) the total number of quanta released from individual release sites had changed.

It was not possible to distinguish between these two alternative because of the limitation of the recording method used in these experiments.

Changes in failures

It was expected that an increase in mean quantal content ($m$) might be associated with a decrease in the number of failures. This may involve only the failures of the process described by Poisson parameters or those evoked by the unidentified process assumed to precede the secretion of transmitter. If these processes are not related, then changes in $m$ should only involve changes in the secretion of transmitter and not the mechanisms which precede it.

Analysis of the failures relating to processes assumed to precede the secretion of transmitter may also give information concerning interactions between the two processes.

Recordings of membrane potentials

Membrane potentials were recorded from cells at or near the surface of the tissue by intracellular glass micro-electrodes (resistances 40-70 MΩ filled with KCl 3M). Cell penetration was only accepted if:

a. The change in membrane potential was abrupt.

b. The resting membrane potential sealed to a value more negative than the initial plateau.

c. Spontaneous excitatory junction potentials were recorded.

Signals recorded by d.c. preamplifier (NEUROLOG NL 125) and filtered at 5KHz. Signals were viewed on a fluorescent screen.

Test of significance

Tests of significance were carried out by paired and unpaired Students t test. Confidence limits for differences in means were also calculated where appropriate.
Drugs and solutions

The modified Krebs saline

<table>
<thead>
<tr>
<th>Concentration</th>
<th>(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.40</td>
</tr>
<tr>
<td>KCl</td>
<td>4.70</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.40</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.30</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.0</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1.13</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.10</td>
</tr>
</tbody>
</table>

The solution was gassed for 30 minutes with 95% O₂ and 5% CO₂ to a pH of 7.4. All constituents were supplied by Sigma.

Drugs used

The final concentration is given where appropriate. Stock solutions of all drugs were made up in 0.9% NaCl solution, these were finally diluted with gassed Krebs solution and used immediately.

- Noradrenaline hydrochloride, L-arginine methyl ester 2.3x10⁻⁴M, αβ-met, ATP
- RB₂ (Cibacron blue 3GA) 5x10⁻⁵M, Prazosin 1x10⁻⁷M, Tetrodotoxin 4.6x10⁻⁵M,
- Histamine dihydrochloride 1x10⁻⁵M, Sodium nitroprusside 4x10⁻⁶M,
- Ranitidine hydrochloride 1x10⁻⁴M, L-arginine 2.5x10⁻⁵M, 3x10⁻⁵M & 3.4x10⁻⁴M

(All supplied by Sigma Pharmaceuticals)

- Thioperamide maleate 1.3x10⁻⁷M (supplied by RBI).
Structure of L-arginine and the competitive antagonist of nitric oxide synthase L-arginine methyl ester

![Structure of L-arginine and L-arginine methyl ester](image)
Results

Mechanical responses.

Responses of the vas deferens from any species can be evoked by either stimulation of the nerves or by exogenous agonist application.

General features of nerve evoked contractions in the rodent vas deferens have been well characterised in many studies (eg. Swedin 1971, Birmingham & Freeman 1976 Fedan, 1981, Swedin & Astrand 1985). When stimulated by single trains, contractions vary both with the intensity of the stimulus and its frequency. The total response is observed to be composed of two phases described as a rapid twitch (Swedin & Astrand 1985) followed by a slower and more sustained contraction. These were later described as phase I and phase II (Stjärne & Astrand 1985). Although these phases are sometimes observed in recordings of contraction of the mouse vas deferens, they are more pronounced in other rodent species (e.g. guinea pig : Birmingham, et al 1976). The two phases of this response are also frequency dependent with phase I being most clearly seen at 10Hz while the amplitude of phase II is larger than I at low frequencies.

Examples of nerve evoked contractions of the isolated mouse vas deferens are shown in figure 18.

Figure 18. The time course of nerve evoked contractions of the mouse vas deferens. The examples shown were recorded from two different preparations of vas deferens in the absence of drugs. The isolated preparations were field stimulated by single trains of 10 pulses at 5Hz at a stimulus intensity of 60V with 0.6ms pulse duration. Responses were recorded at a pen chart speed of 20mm sec⁻¹ in the absence of drugs.
Nerve evoked contractions are assumed to contain both pre and postjunctional responses. These are evoked by ATP and noradrenaline (Stjärne & Åstrand, 1985; Von Kügelgen, Schöffel & Starke, 1989) but other transmitters may also be involved. Contractions evoked by low stimulus intensity are extremely prazosin sensitive. The effect of this adrenoceptor antagonist on the amplitude of the contraction becomes reduced as stimulus intensity increases (Singh 1991). This may suggest that contractions are mediated by purinoceptor activities under conditions of high intensity stimulation (>40V). However, other recordings made during this current study showed that responses evoked by very high stimulus intensity (60V) are sensitive to prazosin (1 x 10^-7M). Noradrenaline secretion is, therefore, involved in the response to high intensity nerve stimulation and is important in the production of phase II, the sustained phase of the contraction. An example recorded during an experiment carried out as part of this is shown below.

Figure 19. The time course of nerve evoked contractions of the mouse vas deferens in the presence and absence of prazosin (1 x 10^-7M). The examples shown were recorded from a single preparation of mouse vas deferens. The isolated tissue preparation was field stimulated by trains of 10 pulses at 5Hz at an intensity of 60V with 0.6ms pulse duration. Responses were recorded at a pen chart speed of 20mm sec^-1 (a) in the absence and subsequently (b) in the presence of prazosin (1 x 10^-7M). An incubation time of 20 minutes was allowed after adding prazosin to the bath before recording the response in its presence.

In addition to nerve stimulation, contractions can also be evoked by the exogenous application of agonist. These responses were used during this current study to give evidence concerning effects on post junctional components of the transmission process.

Recordings of mechanical responses were extensively used in experiments carried out as part of this thesis and were used to address several general key questions.
1. Are nitric oxide sensitive receptors present in the mouse vas deferens?

In view of the large volume of evidence concerning the actions of nitric oxide in many tissues it was thought extremely probable that nitric oxide may have complex actions. Examples of tissues affected by NO include vascular smooth muscle (Rees, Palmer & Moncada, 1989), non vascular smooth muscle (Katsuki, Arnold, Murad, 1977; Katsuki, Arnold, Mittal, Murad, 1977), neuronal tissues in the CNS (Knowles, Palacios, Palmer & Moncada, 1989; Costa, Trainer, Besser, Grossman, 1993) and macrophages (Hibbs, Vavrin, & Taintor, 1987).

Sodium nitroprusside (NaNP) is a potent smooth muscle relaxing agent which has a particularly pronounced effect on vascular tissue (Kreye, Baron, Luth & Schmidt-Gayk, 1975). To investigate the effects of NO on nerve evoked contractions of the mouse vas deferens NaNP was used as a NO donor.

NaNP also evolves cyanide when illuminated. This potential toxicity might also cause a depression of smooth muscle responses which would complicate the interpretation of results. To avoid this, light was excluded from all solutions containing NaNP used during experiments described in this section.

The use of NaNP was restricted to preliminary experiments because of the difficulties of excluding light during other experiments.
The effect of Sodium nitroprusside on neurogenic contractions of the mouse vas deferens.

Figure 20a. The effect of Sodium nitroprusside (4x10^{-6}M) on responses of the isolated mouse vas deferens. Preparations of isolated vas deferens were field stimulated with trains of 10 pulses at 5Hz with 10 minute rest intervals between trains and a pulse duration of 0.6 ms. Stimulus intensity was varied in random order with a 10 minute rest interval between trains. All responses were normalised to the response evoked by a stimulus intensity of 30V in the absence of sodium nitroprusside and expressed as a percentage of this. Two consecutive sets of responses were recorded from 5 preparations in the absence of drug treatment. These were compared to determine the effect of ageing of the preparation in the absence of drug treatment. This showed that contractions became larger with time especially in response to higher stimulus intensities. The preparations used to record set 1 were subsequently treated with sodium nitroprusside (4x10^{-6}M) following a rest interval of 20 minutes. Set 1 in the absence of sodium nitroprusside \( \bullet \) Set 2 in the absence of sodium nitroprusside \( \circ \) Set 2 in the presence of sodium nitroprusside O

NaNP depressed nerve evoked contractions. This effect which is illustrated in Figure 20a & b, was greater on responses to higher intensity stimulations. Contractions evoked by low intensity stimuli were \( 117.2\pm16.8\% \), \( n=5 \) in the absence of NaNP and \( 74.6\pm12.9\% \), \( n=5 \) in its presence. This difference of 36.0% was not significant (\( P>0.05 \), d.f.= 8).

Responses evoked by high intensity stimuli were significantly depressed by 63% from \( 495.0\pm86.0\% \), \( n=5 \) to \( 185.0\pm22.7\% \), \( n=5 \) (\( P<0.05 \), d.f.=9).
Contractions evoked by low and high intensity stimuli were compared. In the absence of NaNP responses were 39.0% greater when evoked by the high intensity stimulus compared with that evoked by the low intensity stimulus. NaNP, however, depressed responses evoked by the low intensity stimulus by 33.0% while at the high stimulus responses were depressed by 62.0%. This showed that NaNP exerted a significantly greater depressive effect on responses evoked by high intensity stimulus (P<0.05, d.f=8).

Figure 20b. The effect of Sodium nitroprusside (4x10^{-6}M) on responses of the isolated mouse vas deferens. Preparations of isolated vas deferens were field stimulated with trains of 10 pulses at 5Hz with 10 minute rest intervals between trains and a pulse duration of 0.6 ms. Stimulus intensity was varied in random order with a 10 minute rest interval between trains. All responses were normalised to the response evoked by a stimulus intensity of 30V in the absence of sodium nitroprusside and expressed as a percentage of this. The mean responses evoked by two stimulus intensities (30V and 50V) were compared in the presence and absence of sodium nitroprusside. The drug depressed all contractions but had a greater effect on responses evoked by the higher stimulus intensity.

Conclusions

Nerve evoked contractions were depressed by NaNP suggesting that receptors for nitric oxide are present in this tissue. The effect of NaNP
was greatest on responses evoked by high intensity stimulation. This may suggest that NO may selectively depress the secretion of ATP or post junctional responses mediated by purinoceptors.

2. Does the mouse vas deferens possess a nitric oxide synthase and does the addition of L-arginine stimulate the production of nitric oxide in this tissue?

Results show that NO sensitive receptors may be present in the mouse vas deferens. L-arginine is the natural substrate of the enzyme nitric oxide synthase (NOS) (Knowles, Palacios, Palmer & Moncada, 1989; Palmer, Ashton & Moncada, 1988 (a) 1988 (b)). This amino acid has no known toxicity. If a nitric oxide synthase is present in this tissue exogenous L-arginine applied to preparations of mouse vas deferens may evoke an increase in the intracellular concentration of NO. This might be expected to produce a depressive effect similar to that evoked by NaNP. Uptake into cells may be required for this to occur.

Other results also suggested that NO may selectively depress either purinergic nerve activities or postjunctional purinoceptor mediated responses.

To determine this the effect of L-arginine (1.5 $\times 10^{-5}$M and 3$x10^{-5}$M) on nerve evoke contractions in the presence of prazosin (1$x10^{-7}$M) was investigated. Results show that in the presence of $\alpha_2$ antagonism by prazosin (1$x10^{-7}$M), nerve evoked contractions were depressed. The remaining response was presumed to be evoked by ATP acting at postjunctional purinoceptors. When L-arginine was applied to preparations pretreated with prazosin (1$x10^{-7}$M) an elevation of nerve evoked contractions was observed. This suggested that the effects of the amino acid may have been complex. These may have included an increase in the synthesis of NO or may have involved another, previously unidentified action of L-arginine.

The possibility that the exogenous application of L-arginine may act through another agent distinct from NO was also investigated. When applied in high concentration L-arginine as single residues causes the degranulation of mast cells (Giraldo, Zappellini, Muscara, De luca, Hslop, Cirino, Zatz, Nucci & Atunes, 1994). To investigate a role for histamine in the elevation of responses observed in the presence of L-arginine with prazosin, a further experiment was designed. Nerve evoked responses
were recorded in the presence and absence of L-arginine (1.5x10^-5M) with prazosin (1x10^-7M). These were then repeated in the additional presence of the histamine H3 receptor antagonist thioperamide (1.3x10^-7M).

**Experimental protocol**

Preliminary experiments showed that the time required for recovery between pulses increased as stimulus intensity increased. The lower intensity of 30V was used in these experiments to avoid additional unidentified inhibitory effects. A 15 minute rest interval was chosen to ensure full recovery of the preparation between trains.

Responses were recorded in the presence or absence of L-arginine (3x10^-5 and 1.5x10^-5M). Preparations were field stimulated by repeated trains at 30V. All normalised responses were compared with the normalised fourth response recorded in the absence of L-arginine and expressed as a percentage change from this. Figure 21 illustrates the protocol used in these experiments.

![Figure 21](image_url)

Figure 21. Stimulation protocol for isolated preparation of mouse vas deferens in the absence and presence of L-arginine (1.5x10^-5M or 3x10^-5M). Preparations of isolated mouse vas deferens were field stimulated with repeated trains of 10 pulses at 30V. Pulse duration was 0.6 ms and a 15 minute rest interval was allowed between trains. All contractions were normalised to the fourth response and percentage changes were calculated from this. Additions of L-arginine were made to the organ bath to some preparations immediately after the fourth response. The organ bath was washed out and the saline and amino acid replaced after each response. Comparisons were made with responses recorded from other preparations stimulated in the absence of L-arginine.
The effect of L-arginine (1.5 x10^{-5} and 3 x10^{-5}M) on nerve evoked contractions

L-arginine (1.5 x10^{-5} and 3 x10^{-5}M) produced a depression of nerve evoked contractions which was greatest 30 minutes after its addition to the bath perfusate (see Figure 22). This was a significant depression of 25.1% (P<0.01, d.f=10).

Figure 22. The effect of L-arginine on nerve evoked contractions of isolated mouse vas deferens. Preparations were field stimulated with trains of 10 pulses at 5 Hz and pulse duration of 0.6 ms with 15 minute rest intervals between trains. Stimulus intensity was 30 Volts. Responses were normalised to the fourth control response following the dissection. a) Responses of different preparations were recorded in the presence and absence of L-arginine (1.5x10^{-5}M and 3x10^{-5}M). All responses were expressed as a percentage change compared with the fourth untreated response. The time at 40 minutes refers to the time at which L-arginine was applied to the bath perfusate.
Figure 23. A comparison of responses evoked by the same stimulus intensity (30V) between sodium nitroprusside (4x10^{-6}M) treated preparations and the maximum depression observed in L-arginine (3x10^{-5}M) treated preparations of mouse vas deferens. Responses recorded in the presence of sodium nitroprusside (4x10^{-6}M) (n=5) or L-arginine (3x10^{-5}M) (n=5) were compared with those recorded in the absence of drug treatment (n=7) and expressed as a depression from the 100% control contraction.

L-arginine was added to the organ bath immediately after recording the response at 40 minutes after the dissection. All subsequent responses were expressed as a percentage of this fourth response. The depression observed 30 minutes after L-arginine was added to the organ bath perfusate was comparable with that observed with sodium nitroprusside (4x10^{-6}M) recorded from other preparations stimulated by the same stimulus parameters. (See Figure 23). These results suggested that L-arginine may have evoked an increase in NO synthesis.
The effect of Prazosin (1 x10^{-7}M) on nerve evoked contractions

Figure 24 shows that the antagonism of α_2 receptors by prazosin (1x10^{-7}M) caused a significant depression of nerve evoked contractions (unpaired t test: P<0.01 d.f=9). This effect was maximal 15 minutes after the addition of prazosin to the Krebs saline. The mean response recorded in the absence of prazosin increased by 9.2% while in its presence it was depressed by 35.9%. These results show that part of the response was evoked by noradrenaline. The remaining prazosin insensitive response was assumed to be evoked by ATP acting at postjunctional purinoceptors.

The depression caused by L-arginine (1.5 x10^{-5}M) became maximal 30 minutes after the addition of the amino acid to the saline. The mean response was then depressed by 29.8%. This was also significantly different from the controls (P<0.01,d.f=14). Figure 24 shows that this depression was similar to that evoked by prazosin.
The effect of L-arginine (1.5x10^{-5}M) on nerve evoked contractions recorded from preparations of mouse vas deferens pretreated with prazosin (1x10^{-7}M)

Figures 22, 24 and 25 show that the addition of L-arginine (1.5x10^{-5}M) to the Krebs saline perfusing the organ bath depressed all control responses. This depression was maximal 30 minutes after the addition of L-arginine to the Krebs saline. The mean response recorded in the absence of drug treatment had increased by 2.5%. However, in the presence of L-arginine (1.5x10^{-5}M) 30 minutes after its addition the mean response was depressed by 19.8% compared with the fourth mean control response. This was a significant depression (P<0.01, d.f = 9).

In the presence of both L-arginine (1.5x10^{-5}M) and prazosin, however, (1x10^{-7}M) the mean response was elevated by 33.3% compared with the fourth mean response. Figure 25 shows that 60 minutes after the dissection the effect of L-arginine (1.5x10^{-5}M) and prazosin (1x10^{-7}M) applied together produced a significantly different effect on the mean response of this tissue compared with the effect of the amino acid alone (P<0.05, d.f=11).

The amino acid may have evoked an increase in the intracellular concentration of NO. These results show that the effect of this modulator may have one or more effect: it may elevate the secretion of ATP; may oppose the activity of inhibitory P_{2Y} receptors; may increase the activity of P_{2X} excitatory receptors or may evoke other effects not attributable to NO.
Figure 25. The effect of L-arginine in the presence and absence of Prazosin (1x10^-7M) on nerve stimulated contractions of isolated mouse vas deferens. Preparations were field stimulated with trains of 10 pulses at 5 Hz and pulse duration of 0.6 ms with 15 minute rest intervals between trains. Stimulus intensity was 30V. Responses were normalised to the fourth control response. Responses of 7 different preparations were recorded in the presence and absence of L-arginine (1.5x10^-5M). Responses were also recorded from 6 different preparations in the presence of both L-arginine and Prazosin and from 7 different preparations in the absence of drug treatment. All responses were normalised to the fourth response and expressed as the change in arb. units compared with the fourth control response.

The effect of the antagonism of histamine H3 receptors on the elevation of nerve evoked contractions caused by L-arginine in the presence of prazosin

The elevation evoked by exogenous L-arginine (1.5x10^-5M) with prazosin (1x10^-7M) was abolished by bath application of the histamine H3 receptor antagonist thioperamide (1.3x10^-7) (see Figure 26).

The mean response recorded 30 minutes after bath application of L-arginine (1.5x10^-5M) with prazosin (1x10^-7M) increased by 17.3% compared with controls responses recorded at the same time interval. In other tissues 30 minutes after the addition of thioperamide (1.3x10^-7) with L-arginine (1.5x10^-5M) and prazosin (1x10^-7M), the mean response was reduced by 14.3%. This was a significant depression compared
with mean responses recorded in the absence of thioperamide (P<0.01 d.f=9).

Conclusions

Results show that nerve evoked contractions are depressed by exogenous L-arginine in a concentration dependent manner. The synthesis of NO may have increased within the tissue but the effect evoked by L-arginine in the presence of prazosin (1x10^-7M) was reversed by the H₃ antagonist thioperamide (1.3x10^-7M). This suggested that histamine may be released endogenously following the exogenous application of L-arginine. This amine may have caused the elevation of the prazosin insensitive component of these contractions.

![Diagram](image.png)

Figure 26. The effect of Thioperamide (1.3x10^-7) L-arginine (1.5x10^-5M) with Prazosin (1x10^-7M) on nerve stimulated contractions of isolated mouse vas deferens. Preparations were field stimulated with trains of 10 pulses at 5 Hz and pulse duration of 0.6 ms with 15 minute rest intervals between trains. Stimulus intensity was 30V. Responses were normalised to the fourth control response. Responses (n=9) were recorded in the presence and absence of L-arginine (1.5x10^-5M), in the presence of both L-arginine and Prazosin from 6 different preparations and in the absence of drug treatment from 7 different preparations. Responses were recorded from 4 different preparations in the presence of Thioperamide (1.3x10^-7) with L-arginine and prazosin. All responses were normalised to the fourth response and expressed as the percentage from this.
3. Does the use of a nitric oxide synthase inhibitor reveal a normal synthesis of nitric oxide?

Results have suggested that the mouse vas deferens may be sensitive to nitric oxide. This tissue may also possess a nitric oxide synthase. L-arginine is the natural precursor for NO (Knowles, Palacios, Palmer & Moncada, 1989; Palmer, Ashton & Moncada, 1988 (a) 1988 (b)) but some results have suggested that this amino acid may have other effects. L-arginine methyl ester (L-AME) which is a competitive inhibitor of NOS was used throughout this present study to determine the possibility that NO is normally synthesised in this tissue and to identify its effects on transmission.

To determine the presence of a nitric oxide synthase (NOS) in this tissue L-AME (2.3x10^-4M) was used during the recording of nerve and exogenous agonist evoked contractions. Preliminary experiments, not described here, showed that a 60 minute incubation period of the tissue with L-AME was needed before its effects became established but the reason for this was unclear.

Other results have shown that nerve evoked contractions of this tissue are depressed by prazosin (1x10^-7M). The prazosin insensitive component of these contractions being assumed to be mediated by ATP acting at postjunctural P2X purinoceptors. Postjunctional responses mediated by nerve evoked ATP secretion may be selectively depressed by NO.

The possibility that responses evoked by noradrenaline may not be depressed by NO was also investigated. An experiment was designed in which L-AME was applied to isolated preparations of mouse vas deferens. Nerve evoked contractions were recorded in the presence and absence of P2X receptor desensitisation by αβ-met.ATP (5x10^-5M). After desensitisation the remaining response was assumed to be evoked by the secretion of noradrenaline.

The effects of nitric oxide on postjunctival responses of the mouse vas deferens have not previously been reported. To determine this, contractions evoked by exogenous agonists αβ-met.ATP and noradrenaline were recorded in the presence and absence of L-AME.
Experimental protocol

Nerve evoked contractions were field stimulated at low intensity (20V) by trains of 2, 5 and 10 pulses at 1 Hz with 15 minute intervals between trains (see figure 27). Other stimulus parameters were described in 'Methods and materials. Where L-AME (2.3x10^{-4}M) was applied to preparations which had previously been desensitised by bath application of αβ-met ATP, three different frequencies were also included (1,5 and 10 Hz).

Figure 27. Protocol for recording nerve evoked contractions from the isolated mouse vas deferens in the presence and absence of L-AME (2.3x10^{-4}M). all tissues were field stimulated at low intensity (20V) by trains of 2.5 and 10 pulses with a pulse duration of 0.6 ms at a frequency of 1 Hz with 10 minute rest intervals between trains. Contractions were recorded in the absence of drug treatment (Set 1 in the absence of drug treatment n=7). L-AME was then applied to the Krebs saline perfusing the organ bath. An incubation period of 60 minutes was permitted before a second Set of responses (Set 2 in the presence of L-AME n = 1). A second Set of responses was also recorded from other preparations in the absence of drug treatment (Set 2 in the absence of L-AME n=2).

Nerve evoked contractions were recorded from seven control preparations. These will be referred to as Set 1 in the absence of L-AME. Further control responses were recorded after a rest interval of 15 minutes from two of these preparations. These will be referred to as Set
2 in the absence of L-AME. This permitted a correction for the effects of ageing on the isolated preparation to be made. L-AME (2.3x10^{-4}M) was applied to the Krebs saline perfusing one preparation. This will be referred to as Set 2 in the presence of L-AME.

To determine the effect of L-AME on nerve evoked responses recorded from preparations in the presence and absence of P_{2X} desensitisation. Nerve evoked responses to a stimulus intensity of 20V were recorded from two preparations of isolated mouse vas deferens in the absence of drug treatment. Trains of 1, 2 and 10 pulses at 1, 5 and 10 Hz were delivered to the preparations in random order. This were referred to as Set 1 in the absence of L-AME. αβ-met.ATP (5x10^{-5}M) was then applied to one of the preparations and was not washed out for 2 minutes to allow desensitisation of P_{2X} purinoceptors. The other preparation remained untreated. Both preparations were then field stimulated as before. These were referred to as Set 2 in the presence or absence of αβ-met.ATP. The treated preparation was challenged every 20 minutes with concentrations of αβ-met.ATP (5x10^{-5}M). This ensured that desensitisation was maintenance for the duration of the experiment. L-AME (2.3x10^{-4}M) was applied to the Krebs saline perfusing both preparations and after an incubation period of 60 minutes, the preparations were stimulated again. These will be referred to as Set 3 in the presence of L-AME and Set 3 in the presence of L-AME with αβ-met.ATP.

The effect of inhibition of nitric oxide synthesis on nerve evoked contractions

Figure 28 shows the amplitude of nerve evoked contractions were elevated in the presence of L-AME (2.3x10^{-4}M) compared with controls recorded as Set 2 in the absence of the inhibitor. The L-AME treated response to a train of 10 pulses was 6.3 (arb. units) in the presence of L-AME (n = 1). This was 7 fold greater than the mean response evoked by the same train length recorded as Set 2 in the absence of L-AME which was 0.9 ± 0.2 (arb. units) n=2. The confidence limits for responses recorded in the absence of L-AME include a change of 1.3 (arb. units). The elevation of the response in the presence of L-AME (2.3x10^{-4}M) was greater than this confidence limit. This suggests that the inhibition of NOS caused a significant elevation of the nerve evoked response.
Figure 28. The effect of L-AME (2.3x10^{-6}M) on nerve stimulated responses of the isolated mouse vas deferens. Preparations were stimulated with trains of 2, 5 and 10 pulses at 5Hz with a pulse duration of 0.6 ms and a 10 minute rest intervals between trains. Trains of different lengths were delivered in random order. Responses were recorded from 7 preparations in the absence of L-AME (2.3x10^{-6}M) and again from 2 of these preparations after a rest interval of 60 minutes (Set 1 and Set 2 in the absence of L-AME). L-AME was applied to the Krebs saline perfusing the organ bath of one preparation and an incubation period of 60 minutes was permitted before responses were recorded from this preparation (Set 2 in the presence of L-AME (2.3x10^{-6}M)).

The effects of inhibition of nitric oxide synthase on nerve evoked contractions in the presence of P2X purinergic receptor desensitisation by αβ-met.ATP (5x10^{-5}M)

The inhibition of nitric oxide synthesis by L-AME increased the maximum amplitude of contractions. This elevation did not occur when L-AME was applied in the presence of purinoceptor desensitisation by αβ-met.ATP (see figure 29a-c). The limited nature of this data made statistical analysis inappropriate but these results support the suggestion that nitric oxide may selectively modulate purinoceptor mediated responses.
(a) Responses at 1 Hz (20 Volts)

Set 2 in the absence of drug treatment
+ e8-metATP (3x10^{-5}M) set 2
+ L-AME (2.3x10^{-4}M) set 3
+ e8-metATP (5x10^{-5}M) set 3
+ L-AME (2.3x10^{-4}M) set 3

(b) Responses to 5 Hz (20 Volts)

Train length

Response (arb. units)
Figure 29. The effect of the inhibition of nitric oxide synthesis by L-AME (2.3x10^{-4}M) in the presence and absence of desensitisation of purinergic receptors by αβ-met.ATP (5x10^{-5}M). Two preparations were field stimulated at 20 volts with trains of 1, 2, 5 and 10 pulses with 0.6 ms pulse duration with 10 minute rest intervals between trains. These were delivered at 5Hz, 1Hz and 10 Hz in random order. Responses were recorded in the absence of drug treatment (Set 1 in the absence of drug treatment). αβ-met.ATP (5x10^{-5}M) was exogenously applied to one preparation and allowed to remain for 2 minutes. A second Set of responses was then recorded in the presence or absence of purinergic desensitisation (n=1). L-AME (2.3x10^{-4}M) was added to the Krebs saline perfusing both organ baths and a 60 minute incubation period was allowed. A final Set of responses was then recorded from both preparations (Set 3 in the presence of L-AME ± αβ-met.ATP n=1). To ensure maintenance of desensitisation the treated preparation was challenged every 20 minutes with concentrations of αβ-met.ATP (5x10^{-5}M). Responses were normalised to the maximum at each frequency from the first Set of responses recorded in the absence of drug treatment from each preparation. All comparisons were made between responses recorded in the presence of L-AME and/or αβ-met.ATP with those recorded as Set 2 in the absence of drug treatment at (a) 1Hz, (b) 5Hz and (c) 10 Hz.

**The effect of nitric oxide synthesis on postjunctional responses evoked by exogenous noradrenaline**

The EC_{50} for noradrenaline in this tissue was shifted to the left in the presence of L-AME (2.3x10^{-4}M) from 3.8x10^{-3}M to 1.8x10^{-3}M (see figure 30). The maximum response was significantly elevated from 1.0 ± 0.2, n=5 to 1.6 ± 0.1 (arb. units) n=2. (unpaired t test: p < 0.01, d.f.= 5).
These results show that nitric oxide normally modulates post synaptic adrenoceptor mediated responses. This may also include an effect on contractile elements of the smooth muscle cells.

The effect of nitric oxide synthesis on postjunctional responses evoked by exogenous αβ-met.ATP

The EC50 for αβ-met.ATP in this tissue was shifted to the left from $1.05 \times 10^{-5} \text{M} \pm 2.6 \times 10^{-6}$ $n=12$ to $2.6 \times 10^{-6} \text{M} \pm 4 \times 10^{-6}$ $n=1$ in the presence of nitric oxide synthase blockade by L-AME (see figure 31). The maximum response was significantly elevated compared with the controls (Change in R.max: $n=14$, $P<0.05$).

Conclusions

These results show that NO is normally synthesised in the isolated vas deferens where it is a physiological modulator of contractions. The effects of NO were to depress nerve evoked contractions and...
postjunctional responses evoked by both adreno- and purinoceptors. NO may also modulate the activities of contractile elements within the smooth muscle cell this was suggested by the elevation of the maximum response evoked by the agonists noradrenaline and αβ-met.ATP.

![Figure 31. The effect of the inhibition of nitric oxide synthesis on responses evoked by the exogenous application of αβ-met.ATP. Responses to exogenously applied αβ-met.ATP were recorded in the presence + (n=1) and absence O (n=12) of L-AME (2.3x10⁻⁴M). All concentrations were applied in random order and responses were normalised to R.max. recorded in the absence of drug treatment. Arrows show EC50 values calculated by ALLFIT.](image)

4. Histamine may account for some of the effects attributed to nitric oxide in the mouse vas deferens. What effects does it have and are histamine receptors present in this tissue?

L-arginine is the natural substrate for nitric oxide synthase (Knowles, Palacios, Palmer & Moncada, 1989; Palmer, Ashton & Moncada, 1988 (a) 1988 (b)). However, results of experiments designed to investigate the effects of exogenous L-arginine on contractions of the mouse vas deferens showed them to be complex. Nerve evoked contractions were depressed in the presence of L-arginine compared with the controls. This mimicked the action of the NO donor NaNP and suggested that the amino acid had caused an intracellular elevation of NO. However, when preparations were pre-treated with prazosin the amino acid caused these contractions to become elevated. Several
explanations were suggested to account for this, one of which was that some of the effects of L-arginine were not attributable to NO.

When applied in high concentration L-arginine as single residues causes the degranulation of mast cells (Giraldo, Zappellini, Muscara, De luca, Hslop, Cirino, Zatz, Nucci & Atunes, 1994). Thioperamide is a highly specific antagonist of the actions of histamine at H3 receptors (Hill, 1992). This antagonist reversed the elevation of nerve evoked contractions caused by L-arginine in the presence of prazosin. This suggested that histamine may have been involved this action of the amino acid.

Experiments were, therefore, designed to investigate the possibility that histamine may account for some of the actions of L-arginine in this tissue. These addressed the following questions:

- Does exogenous histamine affect nerve evoked responses?
- Are histamine receptors present in this tissue?
- Do nerves of the mouse vas deferens release histamine during field stimulation?
- Does histamine affect the postjunctional responses of this tissue evoked by exogenous agonists?

Three different histamine receptors have been identified to date. These are H1, H2 and H3 (for a review of these see: Hill 1990). If histamine is normally released by this tissue during nerve evoked activity, it may act at one or more of these receptors which may be either pre or postjunctional or both.

Experimental protocol

Control nerve evoked contractions in response to different stimulus intensities were recorded. These were referred to as set 1 in the absence of thioperamide. After a rest interval of 10 minutes control responses were again recorded from some preparations. These will be referred to as set 2 in the absence of thioperamide. Responses were also recorded from other preparations in the presence of thioperamide. These will be referred to as set 2 in the presence of thioperamide (6x10^{-6}).
All comparisons were made between set 2 in the presence and absence of thioperamide to correct for the effects of ageing of the preparation.

The effect of exogenously applied histamine on nerve evoked responses

Exogenous histamine applied to the vas deferens does not evoke a recordable response. However, results of a preliminary experiment showed that pre-treatment of the tissue with histamine caused a depression of nerve evoked contractions. A further experiment was, therefore, designed to determine the concentration response of the tissue to exogenous histamine.

A determination of the concentration response to histamine during nerve evoked contractions

This was carried out during recordings of nerve evoked contractions. In separate experiments contractions were evoked by a low stimulus intensity (20V) or a high stimulus intensity (45V).

Figure 32 shows that histamine caused a depression of nerve evoked responses when the tissue was stimulated at both low (20V) and high (45V) intensities.

The maximum inhibition imposed by histamine on contractions evoked by low intensity stimulation (20V) was 100%. Contractions evoked by a higher stimulus intensity (45V) were inhibited by only 60%. This was a significant difference (P< 0.01, d.f. 5).

The IC_{50} (determined by ALLFIT) for histamine in this tissue at 20V was 2.2 x 10^{-6} \text{M} \pm 4 \times 10^{-6}. n=5. The plot of the data was not constrained to the IC_{50} determined for histamine on responses evoked by 20V stimuli. No shift in the IC_{50} value was observed at 45V (n=2).

When histamine (1.3 \times 10^{-4} \text{M}) was applied to the tissue, a long lasting depression of the responses resulted. The maximum inhibition of responses evoked by stimulation at an intensity of 45V was achieved by a concentration of 1 \times 10^{-5} \text{M}. This concentration was, therefore, used in all further experiments to produce a measurable inhibition which was not long lasting.
Figure 32. The effect of exogenously applied histamine (1.3x10^{-7}M) on the nerve evoked response to stimuli of 20V and 45V. Preparations were field stimulated at i) 20V (+) (n = 5) ii) 45V (O) (n=2) in the presence of increasing concentrations of exogenously applied histamine. Each stimulation was of trains of 10 pulses at 5Hz with 10 minute rest intervals between trains and a pulse duration was 0.6 ms. The plot of responses evoked by stimuli of 20V intensity were constrained to 100% inhibition. The plot of those evoked by stimuli of 45V were not constrained. Comparisons were made between responses recorded in the presence and absence of Histamine (1.3x10^{-7}M) and expressed as percentage inhibition. The arrow marks the IC_{50} for histamine at 20 and 45V which was determined by ALLFIT.

The effect of exogenous histamine (1.5x10^{-5}M) on nerve evoked responses

Figure 33 shows that the exogenous application of histamine (1.5x10^{-5}M) depressed responses evoked by all stimulus intensities.
Figure 33. The effect of histamine on nerve evoked responses. Preparations were field stimulated with trains of 10 pulses at 5Hz with 10 minute rest intervals between trains. Stimulus duration was 0.6 ms. Stimuli of different intensities were delivered in random order. Responses were recorded in the absence of drug treatment (set 1 in the absence of drug treatment n=23). Following a rest period of 20 minutes a further set of responses was recorded in the presence (n = 2) or absence (n =10) of Histamine (1.5x10^-5M) (set 2 in the presence or absence of histamine). All responses were normalised to the maximum response of set 1 in the absence of drug treatment.

Does exogenous histamine act at receptors to depress nerve stimulated responses?

The effect of exogenous histamine may have involved its action at specific receptors in this tissue. An experiment was, therefore, designed to determine a role for receptors in the mediation of the histamine induced depression. Nerve evoked responses of this tissue were recorded in the presence of exogenous histamine and additionally in the presence of ranitidine 1x10^-4M. Ranitidine is a highly selective antagonist of H2 receptors (Hill, 1992). Figure 34 shows that ranitidine (1x10^-4M) reversed the depressive effect of exogenous histamine on responses evoked by stimulations at an intensity of 20V.

The IC50 for histamine was 2.2x10^-6M. This was shifted to the right in the presence of ranitidine to 5.2x10^-4M. The maximum inhibition may
not have been affected but the data collected from this experiment was not sufficient to determine this.

These results indicate that the inhibition of nerve evoked responses caused by exogenous histamine was mediated in part by $H_2$ receptors.

Figure 34. The effect of ranitidine (1x10^{-4}M) on the inhibition exerted by histamine on nerve evoked responses of the isolated mouse vas deferens. Preparations were field stimulated by trains of 10 pulses at 5Hz with a pulse duration of 0.6 ms and rest interval between trains of 10 minutes in the presence of histamine in increasing concentration (+) ($n = 3$). Responses were then recorded as set 2 in the additional presence of ranitidine (1x10^{-4}M) with histamine ($n = 2$) or in the presence of histamine alone ($n = 1$) to determine the effects of ageing on the preparation (O). Plots of all data for responses evoked in the presence of histamine and histamine with ranitidine (1x10^{-4}M) were constrained to 100%. Arrows mark IC_{50}s for histamine and histamine with ranitidine. These were determined by ALLFIT.

Are other histamine receptors present in this tissue and do they mediate the effects of endogenous histamine? Does histamine act at $H_1$ receptors in this tissue?

The specific antagonist of $H_1$ receptors trans-triprolidine hydrochloride did not effect nerve evoked contractions. This suggested that $H_1$ receptors are not present in the mouse vas deferens.
Does histamine act at $\text{H}_3$ receptors in this tissue?

Thioperamide, a highly selective antagonists of histamine at $\text{H}_3$ receptors (Hill, 1992) was used during recordings of nerve evoked responses.

The amplitude of nerve evoked contractions were elevated in the presence of thioperamide ($6 \times 10^{-6} \text{M}$) (see figure 35). Only one preparation was treated with thioperamide and so statistical analysis could not be performed.

This result showed that histamine is released by the isolated vas deferens during nerve evoked contractions.

![Figure 35. The effect of thioperamide ($6 \times 10^{-6} \text{M}$) on nerve evoked responses of the isolated mouse vas deferens. Preparations were field stimulated with trains of 10 pulses at 5 Hz. with 10 minute rest intervals between trains and a pulse duration of 0.6 ms. Responses were recorded in the absence of drug treatment (set 1 in the absence of drug treatment $n=23$). Responses were then recorded in the absence of drug treatment (set 2 in the absence of drug treatment $n=10$) and also in the presence of thioperamide ($6 \times 10^{-6} \text{M}$) ($X \ n=1$).]
The postjunctional effects of histamine (1.5x10^{-5}M):

The effect of histamine on responses mediated by postjunctional purinoceptors

Figures 36 shows that histamine (1.5x10^{-5}M) had no detectable effect on the responses of this tissue evoked by exogenous αβ-met.ATP (see also table 6)

![Figure 36. The effect of Histamine on responses evoked by exogenous application of increasing concentrations of αβ-met.ATP.](image)

Figure 36. The effect of Histamine on responses evoked by exogenous application of increasing concentrations of αβ-met.ATP. Concentrations of αβ-met.ATP were exogenously applied to the isolated mouse vas deferens in random order of concentration. Responses were recorded in the presence (n = 1) and absence of histamine (1.5x10^{-5}M) (n = 12). Response to αβ-met.ATP (+), Response to αβ-met.ATP in the presence of histamine (1.5x10^{-5}M) (O). The best fit of the concentration response curve was calculated by ALLFIT.

The effect of histamine on responses mediated by postjunctional adrenoceptors

Figure 37 shows that histamine (1.5x10^{-5}M) caused a significant shift in the EC_{50} value for noradrenaline to the right from 3.8x10^{-3}M to 1.7x10^{-4}M and a small but significant decrease in R. max. from 1.0 ± 0.2, n=2, to 0.7 ±0.2, n=4. This indicated that exogenous histamine depressed postjunctional adrenoceptor mediated responses (see also table 6).
Figure 37. The effect of Histamine (1x10^{-5}M) on responses evoked by the exogenous application of noradrenaline to isolated preparations of mouse vas deferens. Noradrenaline was exogenously applied to preparations of isolated mouse vas deferens. Responses were recorded in the presence (n = 2) (O) and absence of histamine (1.5x10^{-5}M) (n = 4) (+). The best fit of the concentration response curve, R MAX, and the EC50 for noradrenaline were calculated by ALLFIT.

Table 6. Effects of histamine 1.5x10^{-5}M on the EC50 for αβ-met. ATP and noradrenaline in the isolated mouse vas deferens.

<table>
<thead>
<tr>
<th></th>
<th>αβ-met. ATP</th>
<th>noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control n=12</td>
<td>Control n=5</td>
</tr>
<tr>
<td><strong>R max.</strong></td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td><strong>EC50</strong></td>
<td>1.05 ± 2.6x10^{-6} x 10^{-5} M</td>
<td>1.05 ± 2.6x10^{-6} x 10^{-5} M</td>
</tr>
</tbody>
</table>
Conclusions

Results have shown that histamine is released during nerve evoked responses of the mouse vas deferens. The release of this amine causes a depression of postjunctional responses mediated by noradrenaline and nerve evoked contractions. \( \text{H}_2 \) and \( \text{H}_3 \) receptors are present in this tissue and both of these mediate the depressive action of histamine.

The depressive effects of NO and histamine are similar in this tissue. These results suggest, therefore, that the exogenous application of L-arginine may have caused the release of histamine from mast cells. This amine may, therefore, account for some of the actions previously attributed to nitric oxide.
Electrical responses of the smooth muscle membrane of mouse vas deferens

Extracellular recordings

The activity of a small population of nerve varicosities is recorded by focal recording electrode with a tip diameter of approximately 50µm. In the absence of stimulation negative going deflections are recorded which reflect the spontaneous release of packets of ATP from sympathetic nerves. During experiments carried out as part of this thesis, the presence of spontaneous junction currents (SEJC) was used to verify the presence of varicosities enclosed within the tip of the recording electrode. SEJC vary in their frequency of occurrence, shape and amplitude. These characteristics depend on the number of release sites within the tip of the recording electrode and the seal resistance between the smooth muscle membrane and the glass of the electrode (Lavidis & Bennett, 1992). Nerve stimulation evokes stimulus locked excitatory junction currents (EJC) which resemble SEJC in both time course and amplitude. When evoked within the recording electrode tip EJC are negative going deflections which may be referred to as EJC<sub>i</sub>'s. When evoked outside the area covered by the recording electrode they are positive going deflections which are referred to as EJC<sub>o</sub>'s (Stjärne, Mshgina & Stjärne, 1990). EJC are TTX sensitive and dependent on calcium and represent the conductance activated by the action of ATP at P<sub>2X</sub> receptors (Brock & Cunnane, 1988). In the guinea pig EJC are always preceded by an action potential. However, when recorded in the mouse the amplitude of the action potential is small and is obscured within the level of the noise of the recording. EJC are evoked intermittently. The reason for this is not known but evidence from the guinea pig has shown that it is not caused by a failure of the action potential to invade the varicosity (Brock & Cunnane, 1987 & 1992).

In recordings made from varicosities visualised by the use of a fluorescent dye, the amplitude of EJC were shown to vary as a function of the tip diameter. Smaller tip diameters (<10µm) gave recordings of 59-67µV while those recorded by electrodes with larger tip diameters (= 50µm) gave 25-29µV (Lavidis & Bennett, 1991).
Recordings of control EJCs made in this current study used electrodes with tip diameters in the region of 50μm; EJC amplitudes were 27-36μV in amplitude. These were similar to amplitudes recorded by Lavidis et al (1991) which gave a strong indication that the method of extracellular recording was being carried out correctly in this study.

It should be noted that the amplitude of junction currents should be referred to in units of current flow (μA). It is not possible to measure the resistance of the smooth muscle cell membrane, however, and so it is conventional to use the units μV.

Effects of NO and histamine on EJCs have not previously been reported in the mouse vas deferens but a histaminergic innervation has been identified in the rat (Campos, 1988. Campos & Briceno, 1992). This may also be present in the mouse.

Results of experiments which recorded the mechanical responses of this tissue confirmed that NO is synthesised within the mouse vas deferens. These results also showed that NO is an important physiological modulator. Postjunctional responses evoked by the agonists noradrenaline and αβ-met.ATP were elevated following the inhibition of nitric oxide synthesis. This may have been caused by effects exerted on purino- or adrenoceptors alone but elements of the contractile mechanism may also have been effected. Other experiments determined the effects of nitric oxide synthesis inhibition by L-AME on nerve evoked contractions. These responses became elevated in the presence of L-AME. This contrasted with contractions recorded from a preparation in which purinoceptors had been desensitised by αβ-met.ATP. In this preparation the inhibition of NO synthesis did not cause an elevation of the amplitude of these contractions. This suggested that where contractions are evoked by neuronally secreted noradrenaline, a reduction of the intracellular NO concentration may have no effect. This supported the conclusion which was made following the results of an experiment in which NaNP was used as a donor of NO during recordings of nerve evoked contractions. In that experiment NaNP had a greater effect on responses evoked by high intensity nerve stimulation. These contractions are less sensitive to prazosin than those recorded in response to lower stimulus intensities (Singh, 1991) and were assumed to be evoked by the neuronal secretion of ATP. The greater effect of NaNP on contractions evoked by high stimulus intensity
had suggested that NO may selectively depress purinergic components of these contractions.

To determine a role for NO as a modulator of transmitter secretion, EJCs were recorded in the presence and absence of L-AME (2.3x10^{-4}M) an inhibitor of NOS and/or L-arginine (3.4x10^{-4}M) which is the natural substrate of the synthase (Palmer, Ashton & Moncada, 1988. Knowles, Palacios, Palmer & Moncada, 1989).

Quantal analysis of EJCs is a powerful tool which distinguishes between pre and post junctional effects. Post junctional effects are manifest in changes in the quantal effect of transmitter secretion. This is represented by the interval between preferred values of amplitude distribution peaks and quantifies the quantal response of the tissue (described in 'Methods and Materials'). Prejunctional changes affect failures or the quantal content of transmitter release.

The inhibition of nitric oxide synthase was expected to reduce the concentration of NO within the tissue. To determine early prejunctional effects of this, recordings of EJCs were made five minutes after the application of L-AME to the Krebs perfusate.

Preliminary experiments in which nerve evoked contractions were recorded indicated that a 60 minute incubation period was needed for the full effects of L-AME to be observed. To ensure a full effect of antagonism of nitric oxide on EJCs, L-AME (2.3x10^{-4}M) was applied to isolated preparations in all salines and perfusates including the Krebs saline used to bathe the preparation during the dissection. EJCs recorded in the presence of L-AME were then compared with responses recorded in its absence from different tissues. Under these conditions the inhibitor was present in the salines bathing the tissue for not less than 60 minutes and its full effects could be expected to be recordable.

As L-arginine is the natural substrate of nitric oxide synthase (Palmer et al 1988. Knowles et al 1989), its exogenous application might be expected to increase the concentration of NO within the tissue. This might be expected to cause a prejunctional depression of ATP secretion. The amino acid produced an inhibition of nerve evoked contractions which reached a maximum 15 and 30 minutes after its addition to the Krebs perfusate. The interpretation of those responses was limited because they are assumed to contain both pre and post junctional
components and prejunctional effects cannot be interpreted by analysing them. In addition, changes which may have preceded the L-arginine depression of contractions could also not be determined by this method. This was partly due to the mixed nature of these responses and also because of the requirement for long rest intervals between trains of stimuli. To determine changes in transmitter release, therefore, other experiments were designed in which junction currents were recorded in the presence and absence of L-arginine (3.4x10^{-4}M).

L-arginine may cause an increase in the concentration of NO in the mouse vas deferens but it is not known to exert any other effects on this tissue. However, when used at high concentration this amino acid is a potent degranulator of mast cells (Giraldo, Zappellini, Muscara, De luca, Hslop, Cirino, Zatz, Nucci & Atunes, 1994). Histamine released into the tissue in this way may be taken up and either secreted as a false transmitter or may augment its normal secretion.

The effects of endogenous histamine have not previously been demonstrated in the mouse vas deferens. Similarly, receptors for this amine have not previously been identified in this tissue. An experiment which was previously described showed that L-arginine elevated the prazosin insensitive component of nerve evoked contractions. This was reversed by thioperamide, a highly specific antagonist of histamine at H_3 receptors. This suggested that some effects of L-arginine may be attributable to histamine and also identified the presence of those receptors in the mouse vas deferens.

Results of experiments carried out as part of this current study show that exogenous histamine depresses nerve evoked contractions and those evoked by exogenous noradrenaline. Post junctional responses evoked by exogenous application of αβ-met.ATP were not affected.

H_2 and H_3 receptors were identified in this tissue. This indicated that the roles of histamine may be complex. The release of endogenous histamine evoked during nerve stimulation might be expected to exert specific local effects while its exogenous application may have evoked a more complex tissue response.

Preliminary experiments were designed to investigate the effect of exogenous histamine on EJCs.
The effects of inhibiting nitric oxide synthase with L-AME (2.3x10^{-4}M): A1. The short term effects

EJCs were recorded from the same preparations in the presence and absence of L-AME (2.3x10^{-4}M). The analysis of all extracellular responses includes all failures in the transmission process. Records of mean amplitudes of EJCs quoted in the following experimental results is, therefore, lower than those quoted earlier which excluded failures.

Effect on the mean amplitude of responses

Figure 38 (overleaf) shows that the mean amplitude of EJCs recorded from 4 previously untreated preparations significantly increased by 37.1% in the presence of L-AME (2.3x10^{-4}M) from 12.4±0.5μV n=800 to 17.0±0.6μV n=800 (P<0.001, d.f=1598).
Figure 38. The effect of L-AME (2.3×10^{-4} M) on the mean quantal effect of responses from 4 isolated preparations of mouse vas deferens in the short term. Preparations were field stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between trains. EJCs were recorded by focal extracellular electrode from the surface of the mid vas deferens in the absence and from the same preparations in the presence of L-AME (2.3×10^{-4} M). The stimulus intensity was 20-25% above the threshold for the response. A five minute rest interval separated the collection of EJCs recorded in the absence of L-AME and EJCs recorded in the presence of L-AME (2.3×10^{-4} M). EJC amplitudes were normalised to the individual quantal effect determined from each preparation before constructing each histogram. (a) The peaks of the amplitude distribution was determined visually for EJCs recorded in the absence of L-AME (2.3×10^{-4} M). (b) The constant distance between peaks also visually fits the distribution observed in the presence of L-AME (2.3×10^{-4} M). In this example the mean quantal effect was 9.2±1.6 μV, n=4 in the absence of L-AME and also 9.2±1.6 μV, n=4 in its absence. The probability of total failures from these preparations recorded in the absence of L-AME were 0.5 and 0.4 in its presence.

The quantal effect of secreted ATP was visually determined from the EJC amplitude distribution in the absence of L-AME. Figure 38 shows that this distribution of preferred values also visually fits those observed in the presence of L-AME having the same separation between the peaks as is seen in the controls. This suggests that the L-AME treatment did not cause a change in the quantal effect of neuronally secreted ATP in the 4 preparations tested. In the absence of L-AME the mean quantal effect was 9.2±1.6 μV, n=4. This suggested that a reduction of the intracellular concentration of NO may not affect the postjunctional response of this tissue to secreted ATP in the short term. However, the
95% confidence limits calculated for this quantal effect includes a change of 37.1%. The possibility that a post junctional change in the response of the tissue had occurred cannot, therefore, be excluded from by this data. Additional experiments, should be carried out in order to clarify this possibility.

The effect of inhibiting nitric oxide synthase on the mean quantal content of release events

Figure 39 shows that the mean quantal content ($M$) of EJCs increased in all 4 preparations following their treatment with L-AME ($2.3 \times 10^{-4} \text{M}$) (paired t test $p = 0.08$, d.f. 3) from $1.8 \pm 0.4$, $n=4$ in the absence of L-AME to $2.6 \pm 0.6$, $n=4$ in its presence. This was a mean increase of 44.5% ($p=0.08$, d.f. 6). These results suggest that the increase in mean amplitude was caused by an increase in quantal content of release events.
Figure 39. The effect of L-AME (2.3x10^{-4} M) on the mean quantal content calculated for responses of previously untreated preparations of isolated vas deferens. The preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were first recorded in the absence of L-AME and then in the presence of L-AME (2.3x10^{-4} M) which was added to the recording electrode perfusate. This figure shows the changes in mean quantal content in all response which occurred following the application of L-AME to the perfusate of the recording electrode tip. The paired data points from 4 different preparations represent the mean quantal content calculated for responses from a single release site in each preparation.
The effect on the probability of failures attributable to processes preceding transmitter release

Figure 40. The effect of L-AME (2.3x10^{-4}M) applied to the recording electrode tip perfusate on the probability of failures attributable to the process preceding transmitter release. Preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were first recorded in the absence and then in the presence of L-AME (2.3x10^{-4}M) within the recording electrode perfusate. Failures attributable to the process preceding transmitter release are referred to as Non-Poisson failures in this figure. The paired data points from 4 preparations represent the probability of failures calculated to occur from a single release site in each preparation.

Failures which are not described by Poisson statistics are attributed to processes preceding the release of transmitter. Figure 40 shows that the mean probability of these failures did not change in the presence of L-AME (2.3x10^{-4}M) in the preparations tested. In the absence of L-AME the mean was 0.5±0.1, n=4. In the presence of LAME (2.3x10^{-4}M) this was also 0.5±0.1, n=4.
The data showed that there was a significant increase in the mean amplitude of EJCs. This may have been caused by a change in the postjunctional response of this tissue and more experiments should be carried out to determine this.

Quantal analysis showed that an increase in the quantal content of EJCs had occurred in the presence of L-AME. This may represent the start of an incomplete change. These results may, therefore, suggest that nitric oxide normally depresses the quantal content of ATP secretion by a prejunctional mechanism. However, this may require a longer exposure to L-AME for this effect to become fully established. No changes occurred in the probability of failure attributable to processes preceding the transmitter secretion mechanism in the short term (See Table 7).

Table 7. Summary of changes in excitatory responses of isolated mouse vas deferens recorded by focal extracellular electrode following treatment of four preparations with L-arginine methyl ester (2.3x10^{-4}M).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>L-AME (2.3x10^{-4}M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean amplitude of responses (µV)</td>
<td>12.4 ± 0.5 (n=1600)</td>
<td>17.0 ± 0.6 (n=1600)</td>
</tr>
<tr>
<td>Mean quantal content</td>
<td>1.8 ± 0.4 (n=4)</td>
<td>2.6 ± 0.6 (n=4)</td>
</tr>
<tr>
<td>Mean probability of failures attributable to processes preceding transmitter release</td>
<td>0.5± 0.1 (n=4)</td>
<td>0.5 ± 0.1 (n=4)</td>
</tr>
</tbody>
</table>

A2. The longer term effects of L-AME (2.3x10^{-4}M)

To investigate the possibility that the inhibition of NOS may be slow to establish, a further experiment was designed. During this experiment 4 preparations of mouse vas deferens were exposed to L-AME (2.3x10^{-4}M) at all times. This inhibitor was also included in the bathing saline used during the dissection. Comparisons were made between the...
responses recorded from these preparations and 4 others which were not exposed to L-AME.

**Effect on mean amplitude of EJCs**

EJCs were recorded from different preparations in the presence and absence of L-AME (2.3x10^{-4}M). Different focal recording electrodes were also used to record EJCs from each preparation. The mean amplitude of responses is partly a function of the diameter of the recording electrode and its seal resistance with membranes of the tissue (Lavidis, N. A. & Bennett, M. R. 1992) so that electrodes with small tip diameters record larger responses with greater variance. The mean amplitude recorded from 4 preparations in the absence of L-AME was 20.2 ± 0.7μV n=1200 while that recorded from 4 preparations in the constant presence of L-AME (2.3x10^{-4}M) was 25.8 ± 0.5μV n=1200. This was a significant increase of 27.1% (P<0.001 d.f. 2398). However, because different focal electrodes were used to record EJCs from each preparation, the difference in amplitude may have related to the variation in tip diameter rather than to the drug treatment.

**The effect of L-AME (2.3x10^{-4}M) on the quantal effect of secreted ATP**

Figure 41 shows the quantal effect of secreted ATP which was visually determined from the EJC amplitude distribution in the absence of L-AME. This distribution of peaks also visually fits those observed in the presence of L-AME having the same separation between them as is seen in the controls. When the quantal effect of secreted ATP was determined for individual preparations it was found to be 17.1±3.1 n=4 in the absence of L-AME but 11.0±1.2 n=4 μV in its presence. This was an increase of 34.8% but which was not significant (P>0.05, d.f. 6). The mean quantal effect recorded for all preparations in the presence and absence of L-AME was 14.0±2.0μV (n=8). The 95% confidence limits calculated from this mean quantal effect includes a change of 30.5%. This was greater than the increase in EJC amplitude observed in the presence of L-AME compared with its absence. Further experiments should be carried out to determine the possibility that the inhibition of NOS by L-AME (2.3x10^{-4}M) may cause a change in the postjunctional response of this tissue to secreted ATP.
Figure 41. The effect of L-AME (2.3x10^{-4} M) on the mean quantal effect of responses from 4 isolated preparations of mouse vas deferens in the long term. Preparations were field stimulated by trains of 30 pulses at 0.91 Hz and a pulse width of 0.06 ms with 1 minute rest intervals between trains. EJCs were recorded by focal extracellular electrode from the surface of the mid vas deferens in the absence and presence of L-AME (2.3x10^{-4} M). The stimulus intensity was 20-25% above the threshold for the response. EJC amplitudes were normalised to the individual quantal effect determined from each preparation before constructing each histogram. (a) The peaks of the amplitude distribution were determined visually for EJCs recorded in the absence of L-AME. (b) The constant distance between peaks also visually fits the distribution observed in the presence of L-AME recorded from preparations not previously exposed to L-AME. The mean quantal effect was 14.0±2.0 µV, n=4 in the absence of L-AME and 14.0±2.0 µV, n=4 in its presence. The probability of total failures from the preparations recorded in the absence of L-AME was 0.5 and 0.2 in its presence.

To investigate the possibility that the inhibition of NOS by L-AME may also have caused a prejunctional effect on the secretion of ATP, quantal analysis was performed.

The Effects on the mean quantal content of EJCs

Figure 42 shows that the mean quantal content calculated for responses recorded from preparations in the presence of L-AME was 2.4 ± 0.2, n=4 but 1.2 ± 0.1, n=4 in its absence. This was a significant difference of 100% (P<0.001, d.f. 6).
Figure 42. The effect of constant exposure to L-AME (2.3x10^-4M) on the mean quantal content of nerve stimulated responses. Preparations were field stimulated with 10 trains of 30 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 1 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. The mean quantal content of responses were compared in the absence of L-AME and in different preparations in the presence of L-AME (2.3x10^-4M) which was added to all Krebs perfusates throughout the entire procedure including the dissection. This figure shows the difference in mean quantal content which were calculated for treated and untreated preparations. Data points show values calculated from different preparations and represent the mean quantal content calculated for responses from single release sites in those preparations.

Effects on the probability of failures preceding transmitter release

There was no significant difference between the probability of these failures in the presence or absence of L-AME. The mean probability in the absence of L-AME was 0.4 ± 0.1, n=4 while in its presence it was 0.5 ± 0.1, n=4. This was not a significant difference (P>0.1, d.f. 6) (see figure 43).
Figure 43. The effect of constant exposure of isolated preparations of mouse vas deferens to L-AME (2.3x10^{-4}M) on the probability of failures attributable to the process preceding transmitter release. The preparations were field stimulated with 10 trains of 30 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 1 minute. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were recorded in the absence and presence of L-AME (2.3x10^{-4}M) from different preparations. Failures attributable to the process preceding transmitter release are referred to as Non-Poisson failures in this figure. The data points (B) represent the probability of these failures at a single release site in each preparation.
Table 8 summarises the differences which were observed to occur in the presence and absence of L-AME.

Table 8. Summary of differences in excitatory responses of isolated mouse vas deferens recorded by focal extracellular electrode from different preparations in the presence or absence of L-arginine methyl ester (2.3x10^{-4}M).

<table>
<thead>
<tr>
<th></th>
<th>Untreated (n=4)</th>
<th>L-AME (2.3x10^{-4}M) (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean amplitude of responses (µV)</td>
<td>20.2 ± 0.7</td>
<td>25.8 ± 0.5</td>
</tr>
<tr>
<td>Mean quantal content</td>
<td>1.2 ± 0.1</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Probability of failures attributable to processes preceding transmitter release</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

These results show that there was no effect on the probability of failures attributable to processes which precede the secretion of transmitter. The longer exposure time of the tissue to L-AME in this experiment resulted in a greater change in the quantal content of transmitter release events than was observed to occur in the short term. This suggested that the longer incubation time of the tissue with L-AME may be necessary for the full effects of reducing the concentration of NO to become established. These may also include postjunctional effects.

**Does L-arginine (3.4x10^{-4}M) reverse the effects of the inhibition of NOS by L-AME (2.3x10^{-4}M)?**

The effect of L-arginine (3.4x10^{-4}M) on the mean amplitude of responses recorded from preparations previously treated with L-AME (2.3x10^{-4}M)

The mean amplitude of EJCs recorded from four preparations was significantly depressed by 58.5% (P<0.01, d.f.1598) in the presence of
both L-AME and L-arginine compared with L-AME alone from 13.5±0.8μV n=800 to 7.9±0.5μV n=800.

Figure 44. The effect of L-arginine (3.4x10⁻⁴M) on the mean quantal effect of responses recorded from 4 isolated preparations of mouse vas deferens previously treated with L-AME (2.3x10⁻⁴M). Preparations were field stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between trains. EJCs were recorded by focal extracellular electrode from the surface of the mid vas deferens. The stimulus intensity was 20-25% above the threshold for the response. A five minute rest interval separated the collection of EJCs recorded in the presence of L-AME alone and EJCs recorded in the presence of L-AME (2.3x10⁻⁴M) with L-arginine (3.4x10⁻⁴M). EJC amplitudes were normalised to the individual quantal effect determined from each preparation before constructing each histogram. (a) The peaks of the amplitude distribution was determined visually for EJCs recorded in the presence of L-AME (2.3x10⁻⁴M) alone. (b) The constant distance between peaks also visually fits the distribution observed in the presence of L-AME (2.3x10⁻⁴M) with L-arginine (3.4x10⁻⁴M). The mean quantal effect was 11.4±2.2μV, n=4 in the presence of L-AME and 11.4±2.2μV, n=4 in the presence of L-AME with L-arginine. The probability of total failures from the preparations recorded in the presence of L-AME alone was 0.5 and 0.4 in the presence of both L-AME with L-arginine.

The quantal effect of secreted ATP was visually determined from the EJC amplitude distribution in the presence of L-AME (2.3x10⁻⁴M) alone. This was found to be 11.4±2.2 μV n=4. When the preferred values were compared visually with those in the presence of L-AME (2.3x10⁻⁴M) with L-arginine (3.4x10⁻⁴M) there was no apparent difference. This comparison can be seen in figure 44 The 95% confidence limits calculated from this mean quantal effect includes a change of
41.2%. This was less than the depression of EJC amplitude observed in the presence of L-arginine with L-AME compared with L-AME alone. This suggests that a postjunctional change in the response of this tissue to secreted ATP may not account for the depression of EJC mean amplitude caused by L-arginine.

The effect on the mean quantal content

![Graph showing the effect of L-arginine (3.4x10^{-4}M) on the mean quantal content of responses recorded from preparations previously treated with L-AME (2.3x10^{-4}M). Preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were recorded in the presence of L-AME (2.3x10^{-4}M) and then in the presence of both L-arginine (3.4x10^{-4}M) and L-AME (2.3x10^{-4}M). L-arginine and L-AME were applied to the perfusate of the recording electrode tip. The paired data points represent the mean quantal content calculated for responses from a single release site in each preparation.]

The mean quantal content recorded from all 4 preparations was 2.4±0.4 n=4 in the presence of L-AME (2.3x10^{-4}M) alone and 1.4±0.3 n=4 in the presence of L-arginine (3.4 x10^{-4}M) with L-AME (2.3x10^{-4}M). This was a reduction of 42.7% which was not significant (paired and
unpaired t test: P>0.1 df. 3 and 6). However, figure 45 shows that in 3 out of 4 preparations the mean quantal content decreased significantly in the presence of L-arginine (3.4x10^{-4}M) with L-AME (2.3x10^{-4}M) compared with L-AME alone. The mean value of $M$ from these 3 preparations was significantly reduced by 54.2% from 2.4±0.4, n=3 in the presence of L-AME to 1.1±0.2, n=3 in the presence of L-AME with L-arginine (paired t test: p=0.8, df.3. Unpaired t test: p=0.05, df. 6).

The effect on the probability of failures attributable to the processes preceding transmitter release

![Figure 46. The effect of L-arginine (3.4x10^{-4}M) on the probability of failures attributable to processes which precede the secretion of transmitter recorded from preparations previously treated with L-AME (2.3x10^{-4}M). The preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were recorded in the presence of L-AME (2.3x10^{-4}M) and in the presence of both L-arginine (3.4x10^{-4}M) with L-AME (2.3x10^{-4}M). Failures attributable to processes preceding the secretion of transmitter are referred to as Non-Poisson failures in this figure. The paired data points show these failures from 4 preparations. They represent the probabilities of these failures from a single release site in each preparation.](image-url)
The mean probability of failures recorded from these preparations in the presence of L-AME was 0.6 ± 0.1 n=4 while in the presence of both L-AME and L-arginine it was 0.5 ± 0.1, n=4 (see figure 46). This was a decrease of 16.7% which was not significant (paired and unpaired t test: P>0.1, d.f. 3 and 6). These results which are summarised in table 9 suggest that L-arginine when applied in the continued presence of L-AME, reversed the effects of NOS inhibition by L-AME on the quantal secretion of ATP.

Table 9. Summary of changes in electrical responses of isolated mouse vas deferens previously treated with L-AME (2.3x10^{-4}M) following the application of L-arginine (3.4x10^{-4}M) to the recording electrode tip perfusate in the continued presence of L-AME.

<table>
<thead>
<tr>
<th></th>
<th>L-AME (2.3x10^{-4}M) n=4</th>
<th>L-AME(2.3x10^{-4}M)+ L-arginine (3.4x10^{-4}M) n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean amplitude of responses (µV)</td>
<td>13.5 ± 0.8</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>Mean quantal content</td>
<td>2.4 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>non-Poisson failures</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

B. What effect does L-arginine (3.4x10^{-4}M) have in the absence of NOS inhibition?

L-arginine (3.4x10^{-4}M) rapidly reversed the effects of NOS inhibition by L-AME (2.3x10^{-4}M) on the quantal secretion of ATP. This amino acid may also increase the synthesis of NO in the tissue in the absence of L-AME. A further experiment was, therefore, designed to investigate this.

**The effect on the mean amplitude of responses**

The mean amplitude of responses was 10.8±0.7µV n = 500 in the absence of L-arginine and 11.0±0.8µV n = 500 in its presence. This change was a change of 1.75 which was not significant (P>0.1, d.f= 998).

Figure 47 shows the quantal effect of secreted ATP which was visually determined from the EJC amplitude distribution in the absence of L-arginine (3.4x10^{-4}M). This distribution of peaks also visually fits...
those observed in the presence of L-arginine (3.4x10^{-4}M) having the same separation between them as is seen in its absence. When the quantal effect of secreted ATP was determined for individual preparations it was found to be 8.2±0.7 μV in the absence of L-arginine and also 8.2±0.7 μV in its presence. The 95% confidence limits calculated from this mean quantal effect includes a change of 18.2%. This data does not exclude the possibility that a postjunctional change in the response of this tissue to secreted transmitter may have occurred.

Figure 47. The effect of L-arginine (3.4x10^{-4}M) on the mean quantal effect of responses recorded from 4 previously untreated isolated preparations of mouse vas deferens. Preparations were field stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between trains. EJC's were recorded by focal extracellular electrode from the surface of the mid vas deferens. The stimulus intensity was 20-25% above the threshold for the response. A 5 minute rest interval separated the recording of EJC's in the absence of L-arginine from those recorded in its presence. EJC amplitudes were normalised to the individual quantal effect determined from each preparation before constructing each histogram. (a) The peaks of the amplitude distribution was determined visually for EJC's recorded in the absence of L-arginine (3.4x10^{-4}M). (b) The constant distance between peaks also visually fits the distribution observed in the presence of L-arginine (3.4x10^{-4}M). The mean quantal effect was 8.2±0.7μV, n=4 in the absence of L-arginine and 8.2±0.7μV, n=4 in its presence. The probability of total failures from the preparations recorded in the presence of L-AME alone was 0.6 and 0.5 in the presence of both L-AME with L-arginine.
The effect on the mean quantal content of transmitter release

Figure 48 shows that the mean quantal content recorded from four preparations increased by 47.3% in the presence of L-arginine (3.4x10^{-4}M) from 1.3±0.2, n=4 to 1.9±0.2, n=4. This was not a significant increase (paired and unpaired t tests: P>0.1, d.f= 3 and 6).

Figure 48. The effect of L-arginine on the mean quantal content of responses recorded from isolated preparations of mouse vas deferens. Preparations were field stimulated with sets of 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were recorded in the presence and absence of L-arginine (3.4x10^{-4}M) which had been added to the recording electrode perfusate. This figure shows the changes in mean quantal content in all response which occurred following the application of L-arginine to the perfusate of the recording electrode tip. The paired data points from four preparations represent the mean quantal content calculated for responses from a single release site in each preparation.
The effect on the probability of failures attributable to the process preceding transmitter release

The probability of failures attributable to processes which precede the secretion of transmitter increased by 57.2% from 0.5±0.2, n=4 in the absence of L-arginine to 0.7 ± 0.1, n=4 in its presence. This was not a significant increase (P> 0.1, d.f. 6) (see figure 49).

![Figure 49. The effect of L-arginine (3.4x10^-4M) applied to the recording electrode tip perfusate on the probability of failures attributable to the process preceding transmitter release. Preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were first recorded in the absence of L-arginine and then in its presence (L-arginine 3.4x10^-4M) added to the recording electrode perfusate. The paired data points from 4 preparations represent the probability of these failures occurring from a single release site in each preparation.

These results suggested that L-arginine did not effect transmission in this tissue in the short term. The uptake of the amino acid was not in
question since experiments described in chapter 2 clearly demonstrated that nerve evoked responses were affected following its exogenous application. These results may suggest that exogenous L-arginine did not increase the concentration of nitric oxide in the tissue since there was no effect on prejunctional elements involved in the secretion of ATP. However, additional experiments should be carried out to further determine if the amino acid causes a change in the postjunctional response of this tissue to secreted transmitter. Table 10 summarises the results of this experiment.

Table 10. Summary of changes in the mean amplitude of EJCs, mean quantal content and the failures per pulse assumed to be attributable to processes which precede the secretion of transmitter observed in isolated preparations of mouse vas deferens following treatment with L-arginine (3.4x10^{-4}M).

<table>
<thead>
<tr>
<th></th>
<th>Untreated (n=4)</th>
<th>L-arginine (3.4x10^{-4}M) (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean amplitude of</td>
<td>10.8 ± 0.7</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td>responses (µV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean quantal content</td>
<td>1.3 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Mean probability of</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>failures attributable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>to processes which</td>
<td></td>
<td></td>
</tr>
<tr>
<td>precede the release of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>transmitter.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Histamine**

**Effects of exogenous histamine (1x10^{-6}M) on excitatory junction currents**

**Experimental protocol**

The data obtained during experiments designed to determine the effects of histamine was limited because only one preparation was treated in this way. A comparison was made between the changes which occurred following the application of histamine and those which
occurred with ageing. For this EJCs were recorded as set 1 from 6 preparations. A second set of these responses was recorded from these preparations. Histamine was added to the perfusate of the focal recording electrode used for one of these preparations while the other 5 were kept as controls.

**Effects of histamine on the mean amplitude of EJCs**

EJCs recorded from one preparation were significantly reduced in amplitude from 18.7 ± 1.5 μV n=200 in the absence of exogenous histamine to 6.2±0.8μV n=200 in its presence (P<0.001, d.f. 398). This was a depression of 67.0%.

**Effects of ageing of the preparation on the amplitude of EJCs**

An increase in the mean amplitude of EJCs of 5.9% occurred during the ageing of the preparation. Mean of set 1 untreated 13.6±0.6 μV n=1,000. Mean of set 2 untreated 14.4±0.6 μV n=1,000. This change was not significant(P>0.1, d.f. 1,998).

**The effects of histamine on the quantal effect of transmitter release**

A comparison of the preferred values in both sets of data can be seen in figure 50. The quantal effect of secreted ATP which was visually determined in the controls was 10.1μV (n=1). A comparison with the distribution of preferred values in the presence of histamine (1x10⁻⁶M ) showed that there was no apparent difference. This suggested that histamine did not cause a change in the postjuncional effect of secreted transmitter in this tissue. The limited nature of this data prevented further statistical analysis from being performed.
Figure 50. The effect of histamine (1x10^{-7}M) on the quantal effect of secreted ATP in a single preparation of mouse vas deferens. The preparation was field stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between trains. Excitatory junction currents were recorded by focal extracellular electrode from the surface of the mid vas deferens of mouse in the absence of drug treatment. The stimulus intensity was 20-25% above the threshold for the response. (a) Responses were recorded in the absence of histamine and (b) from the same preparation in the presence of histamine (1x10^{-7}M). The quantal effect shown in (a) and (b) was 10.1\mu V. The probability of total failures (a) in the absence of histamine 0.4 (b) in the presence of histamine 0.7(1x10^{-7}M).

The effects of ageing of the isolated preparation on the quantal effect of transmitter release

Figure 51 shows the quantal effect of secreted ATP which was visually determined from the EJC amplitude distribution in the controls. This distribution with an identical distance separating preferred values also visually fits those of a further set of EJCs recorded from the same preparations also in the absence of drug treatment. The quantal effect of secreted ATP in the controls was 16.2±4.6\mu V n=5. The 95% confidence limits calculated from this mean quantal effect includes a change of 60.6%. In view of this large confidence limit further experiments should be carried out to determine if ageing processes within the isolated preparation cause a change in the postjunctional response of this tissue to secreted ATP.
Figure 51. The effect of ageing processes within isolated preparations of mouse vas deferens on the quantal effect of secreted ATP. Preparations were field stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between trains. Excitatory junction currents were recorded by focal extracellular electrode from the surface of the mid vas deferens of mouse in the absence of drug treatment. The stimulus intensity was 20-25% above the threshold for the response. A five minute rest interval separated the collection of (a) Responses recorded in the absence of drug treatment and (b) a further set of EJCs were recorded from the same preparations also in the absence of drug treatment. EJC amplitudes were normalised to the individual quantal effect determined from each preparation before constructing each histogram. The quantal effect shown in (a) and (b) was 10.1μV. The probability of total failures (a) in the absence of histamine 0.4 (b) in the presence of histamine 0.7.

To investigate the possibility that histamine may effect transmission in this tissue by a prejunctional mechanism, quantal analysis was carried out.
Effects on the mean quantal content calculated by the fit to a Poisson distribution

The effect of histamine

Quantal analysis showed that there was a decrease of 64.0% in the mean quantal content of release events. This decrease from 2.5 in the absence of histamine to 0.9 in its presence can be seen in figure 52a.

The effect of ageing of the preparation

Figure 52b shows the mean quantal content recorded in the controls was 1.2 ± 0.3 n=5. This increased by 41.7% to 1.7±0.3 in Set 2 but this increase was not significant (paired and unpaired t test P>0.1).

Figure 52. The change in mean quantal content evoked by (a) exogenous histamine and (b) ageing processes of isolated preparations of mouse vas deferens. Preparations were field stimulated with 10 trains of 20 pulses at 0.91Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. (a) Responses were first recorded in the absence of histamine and then in its presence (1x10^{-6}M) which was added to the recording electrode perfusate. (b) A comparison with changes which were evoked by ageing of the preparation. The paired data points represent the mean quantal content calculated for responses from a single release sites in each tissue.
These results suggested histamine may depress the quantal content of ATP secretion.

The effect on the probability of failures attributable to processes preceding the release of transmitter:

The effect of histamine

These probability of these failures increased in the presence of histamine from 0.28 to 0.35 in its absence.

The effect of ageing of the preparation

Figure 53. The change evoked by exogenous histamine ($1 \times 10^{-6}$M) in the probability of failures attributable to processes preceding the release of transmitter. The preparations were field stimulated with 10 trains of 20 pulses at 0.91Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Failures assumed to be attributable to processes preceding the release of transmitter are referred to as Non-Poisson failures in this figure. (a) Responses were first recorded in the absence of histamine and then in its presence ($1 \times 10^{-6}$M) which was added to the recording electrode perfusate. (b) Changes which were evoked by ageing of the preparation. The paired data points represent the probability of failures calculated for a single release site.

Figure 53 shows that in the 5 preparations kept as controls, there was no significant change in the probability of these failures which
might have been attributable to the ageing of the preparations (paired and unpaired t tests: \( P > 0.1 \) d.f. 4 and 8). In set 1 the probability of failures was \( 0.3 \pm 0.1, n=5 \) while in set 2 this was \( 0.4 \pm 0.1, n=5 \).

These results suggested that histamine may exert prejunctional effects to depress the quantal secretion of ATP. This conclusion was made with caution because of the limitations of the histamine data. The failures attributable to processes preceding the secretory mechanism may not have been affected by histamine.

Summary of results

Table 11 summarises the results described in this chapter.

Table 11. Showing the effects of nitric oxide synthase inhibition by L-AME (3.4x10^{-4}M), its reversal by L-arginine (2.3x10^{-4}M) and the effects of the separate addition of L-arginine (2.3x10^{-5}M) and of histamine (1x10^{-6}M) compared with the effects of ageing of isolated preparations of mouse vas deferens.

<table>
<thead>
<tr>
<th></th>
<th>Change in mean amplitude of EJC's (%)</th>
<th>Change in mean quantal content (%)</th>
<th>Change in Non-Poisson failures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-AME</td>
<td>+37.1±0.7, n=4</td>
<td>+44.5±11.2, n=4</td>
<td>none detected</td>
</tr>
<tr>
<td>L-arginine with L-AME</td>
<td>-58.5±0.2, n=4</td>
<td>-55.2±10.2, n=4</td>
<td>none detected</td>
</tr>
<tr>
<td>L-arginine</td>
<td>none detected</td>
<td>none detected</td>
<td>none detected</td>
</tr>
<tr>
<td>Histamine</td>
<td>-67.0</td>
<td>-64.0</td>
<td>none detected</td>
</tr>
<tr>
<td>Effects of ageing</td>
<td>none detected</td>
<td>none detected</td>
<td>none detected</td>
</tr>
</tbody>
</table>

Conclusions

A nitric oxide synthase is present in the mouse vas deferens. This was first demonstrated when the inhibition of NOS by L-AME caused an elevation of nerve evoked contractions. This inhibition also depressed postjunctional responses. This was demonstrated by results which showed that the EC50 for both \( \alpha \beta \)-met.ATP and noradrenaline applied exogenously were shifted to the left in the presence of L-AME. The maximum response of this tissue to both agonists was also elevated.
Those results also suggest that the contractile mechanism may be sensitive to NO.

A physiological role for NO in transmission processes in this tissue is also strongly suggested. This was demonstrated to include the prejunctional modulation of the secretion of ATP but may also include a postjunctional effect in the longer term.

L-arginine did not cause the intracellular concentration of NO to increase in the absence of NOS inhibition in the short term. This was shown by its lack of effect on quantal content of release events. However, this amino acid did reverse the effect of L-AME in the short term by depressing the quantal content of release events (refer to table 4).

Exogenous histamine may also affect transmission in the short term by a prejunctional effect on the secretion of ATP. This effect may be greater than that of NO. The data used to form this conclusion was limited and more experiments should be designed to investigate the role of histamine on transmission processes in the mouse vas deferens.

The long term effect of L-arginine on junction currents

L-arginine may have complex effects in the mouse vas deferens. This amino acid is the natural substrate for nitric oxide synthase (NOS) (Palmer et al 1989). Results have shown that L-arginine significantly depressed nerve evoked contractions by 25.1%, n=5. The inhibition of NOS by L-AME elevated nerve evoked contractions and those evoked by both exogenous αβ-met.ATP and noradrenaline. These results suggest a physiological role for the enzyme in this tissue.

The mean amplitude of EJC's recorded by focal extracellular recording electrode was also elevated in the presence of L-AME. Quantal analysis showed that this was a prejunctional effect causing an increase in the quantal content of transmitter secretion. No change in quantal effect was observed and this showed that the effects of reducing the intracellular concentration of NO were prejunctional in the short term. L-arginine rapidly reversed the inhibition of NOS in the continued presence of L-AME.

Other experiments to investigate the possibility that L-arginine causes prejunctional changes in transmitter secretion in the absence of
NOS inhibition. However, results showed that the addition of L-arginine to the perfusate of the focal extracellular recording electrode had no observable effect on control EJCs in the short term.

L-arginine may have an effect on junction currents in the longer term which might explain the observed depression of nerve evoked responses. Results of experiments designed to determine this showed that with continued exposure to L-arginine (3.4x10^{-4}M), a new response was revealed. This was a junction current of opposite sign compared with EJCs recorded from the same patch.

Field stimulation of the ergotamine contracted rabbit portal vein produces nerve evoke relaxations (Reilly, Saville & Burnstock, 1987). This was attributed to P_{2Y} receptor activation. These relaxations were abolished by RB_{2} when used at a concentration range of between 1 and 5 x10^{-5}M. Within this range RB_{2} has no effect on postjunctional P_{2X} or adrenoceptor mediated contractions. The conclusion drawn from this evidence was, that although this drug had other effects at higher concentrations, when used between 1-5 x10^{-5}M it is a specific non-competitive antagonist of P_{2Y} receptors in the rabbit portal vein (Reilly et al 1987).

Inhibitory P_{2Y} postjunctional receptors are also present in the mouse vas deferens. These were identified when RB_{2} (5x10^{-5}M) elevated the maximum response of this tissue to exogenous αβ-met.ATP (see Blakeley, Brockbank, Kelly & Petersen, 1991). The assumption was also made that because RB_{2} (5x10^{-5}M) did not shift the EC_{50} of the concentration response curve to αβ-met.ATP that P_{2X} receptors in this tissue were not affected (Blakeley et al 1991).

Others have shown that a prejunctional purinoceptor subtype similar to postjunctional P_{2Y} receptors may also exist in the mouse vas deferens. These are resistant to RB_{2} when used at a concentration of 1x10^{-5}M (von Kügelgen, Schöffel & Starke, 1989). However, RB_{2} is thought to retain its specificity towards postjunctional P_{2Y} receptors only within a narrow concentration range (Reilly et al 1987). At the higher concentration of 5x10^{-5}M, RB_{2} may also antagonise prejunctional P_{2Y} receptors.

The development of the new current revealed by L-arginine, therefore, provoked other questions:
1. Are \( \text{P}_2Y \) receptors involved in the production of the new \( \text{L-arginine evoked current} \)?

To address this question the effect of \( \text{RB}_2 (5 \times 10^{-5} \text{M}) \) on the \( \text{L-arginine evoked current} \) was investigated. This was compared with the effect of this drug on control EJCs recorded from the same preparations. \( \text{P}_2Y \) receptors in other tissues are not directly linked to ion channels but activate second messenger mediated responses which evoke responses with time courses which are slow. If \( \text{P}_2Y \) receptor activation is involved in the production of the new current it would be expected to have a delayed latency of onset.

2. Are these new currents evoked within the area covered by the recording electrode tip?

\( \text{TTX} \) is a potent inhibitor of neuronal \( \text{Na}^+ \) channels which abolishes nerve evoked responses. To determine if the new response revealed by \( \text{L-arginine evoked current} \) was evoked within the area covered by the recording electrode tip, \( \text{TTX} \) was applied to the perfusate of the focal recording electrode.

**Experimental protocol**

In preparations where the new response which had been revealed by \( \text{L-arginine evoked current} \) had become fully established and the EJC had disappeared, \( \text{RB}_2 (5 \times 10^{-5} \text{M}) \) was added to either: i) The perfusate of the recording electrode tip. Or ii) The organ bath perfusate. Or iii) Both recording electrode and organ bath perfusates.

In one preparation, when \( \text{L-arginine evoked current} \) was present for more than 20 minutes and the response above the threshold for the original EJC had become a complex mixture of positive and negative components, \( \text{TTX} (7.5 \times 10^{-6} \text{M}) \) was added to the perfusate of the recording electrode. Responses were again recorded after an incubation period of 5 minutes.

**The longer term effect of \( \text{L-arginine evoked current} \)**

When \( \text{L-arginine (3.4} \times 10^{-4} \text{M)} \) was present in the focal recording electrode for about 20 minutes a new response was revealed. This was a current of opposite sign and lower threshold compared with EJCs recorded from the same site on the surface of the vas deferens. Figure 54 shows examples recorded from three different preparations. In this
figure the new current evoked by L-arginine was recorded below the threshold for the EJC recorded from the same patch in each preparation.

![Graph showing control and L-arginine effects](image)

**Figure 54.** Examples of the new response revealed below the threshold for EJCs by application of L-arginine (3.4x10^-4 M) to the recording electrode tip perfusate in 3 different preparations. Isolated preparations of vas deferens were stimulated by 10 trains of pulses at 0.91 Hz with a pulse duration of 0.06 ms and a 2 minute rest interval between trains. Responses were recorded at a stimulus intensity 20-25% above the threshold for the excitatory response (EJC). Following this L-arginine (3.4x10^-4 M) was added to the Krebs saline perfusing the recording electrode tip and after approximately 20 minutes a new response was revealed. This was a current of opposite sign and was evoked at a lower threshold compared with EJCs recorded from the same patch of the smooth muscle surface. In the examples shown in this figure a, b and c show responses recorded from 3 different preparations. EJCs (left hand figures) and new currents revealed below the threshold for EJCs by L-arginine (right hand figures) were recorded from a single patch of membrane in each preparation without moving the focal recording electrode tip.

The new response was revealed below the threshold for EJCs recorded from the same patch of smooth muscle membrane. At first this new response was absent from recordings which were made above the threshold for EJCs. At this time the amplitude of EJCs did not change. However, about 5 minutes after the new response had been revealed by L-arginine, it appeared amongst recordings of EJCs. Some responses were a complex mixture of negative and positive currents while others were...
of positive currents alone where the EJC had failed. At this stage the amplitude of EJCs began to become reduced. Eventually EJCs disappeared completely from the recordings leaving only positive currents. Increasing the stimulus intensity did not evoke a return of the EJC. This showed that their disappearance was not caused by an elevation of their threshold. Elevating the stimulus intensity increased the amplitude of the new response suggesting that a larger population of nerves was being recruited.

The characteristics of this new response were different from those of the EJCs recorded from the same patch, being of opposite sign, shorter latency of onset, with slower rise and decay times.

The decline of the EJC was too rapid to permit the quantal analysis of the mechanism of decay. Examples of these features recorded from one preparation are illustrated in figure 55.

Figure 55. The effect L-arginine (3.4x10⁻⁴M) on nerve evoked junction currents recorded above the threshold for EJCs. The isolated vas deferens was stimulated by 10 trains of pulses at 0.91 Hz with a pulse duration of 0.06 ms and a 2 minute rest interval between trains. Responses were recorded at a stimulus intensity 20-25% above the threshold for the excitatory response in the absence of the amino acid. L-arginine (3.4x10⁻⁴M) was then applied to the Krebs saline perfusing the focal recording electrode tip and the stimulation was repeated. (a) Excitatory junction current recorded at 20-25% above its threshold in the absence of L-arginine. (b) The response above the threshold for the EJC became a complex mixture of excitatory and superimposed inhibitory currents in the presence of L-arginine. (c) The EJC eventually disappeared from the higher threshold response leaving only the current of positive sign.

**Differences in threshold of the new current compared with the EJC**

The thresholds of all except one of the new current revealed by L-arginine were below that for the excitatory response recorded from the same area of each preparation. Table 12 shows examples of
these differences. Thresholds of excitatory and inhibitory responses in these examples were significantly different (paired t test: P<0.01, d.f= 8).

Table 12. Thresholds (V) for the activation of EJCs and new L-arginine evoked currents recorded from the same area of the surface of isolated preparations of mouse vas deferens from 10 different preparations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Threshold (V)</th>
<th>EJC (L-arginine absent)</th>
<th>New currents +L-arginine (3.4x10^-4M)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2</td>
<td>2.4</td>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>4.1</td>
<td></td>
<td>18.0</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>1.7</td>
<td></td>
<td>15.0</td>
</tr>
<tr>
<td>4</td>
<td>2.9</td>
<td>2.9</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>3.4</td>
<td>3.0</td>
<td></td>
<td>13.3</td>
</tr>
<tr>
<td>6</td>
<td>2.7</td>
<td>1.7</td>
<td></td>
<td>37.0</td>
</tr>
<tr>
<td>7</td>
<td>3.3</td>
<td>2.5</td>
<td></td>
<td>24.0</td>
</tr>
<tr>
<td>8</td>
<td>3.0</td>
<td>2.2</td>
<td></td>
<td>27.0</td>
</tr>
<tr>
<td>9</td>
<td>3.9</td>
<td>2.9</td>
<td></td>
<td>25.6</td>
</tr>
<tr>
<td>10</td>
<td>2.6</td>
<td>2.2</td>
<td></td>
<td>30.8</td>
</tr>
<tr>
<td>mean difference</td>
<td></td>
<td></td>
<td></td>
<td>21.6 ± 3.1 (n=10)</td>
</tr>
</tbody>
</table>

Differences in the latencies of responses

The new current was of significantly shorter latency of onset compared with EJC recorded from the same site on each preparation. (paired and unpaired t test: P<0.05 d.f=2 and 4). Table 13 compares these differences from three different preparations.
Table 13. A comparison of release sites of shortest latency for EJCs and new L-arginine evoked currents recorded from the same area of the surface of isolated different preparations of mouse vas deferens. Latencies of new current were measurable from only three preparations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Latency (ms)</th>
<th>New currents +L-arginine (3.4x10^{-6}M)</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EJCs (L-arginine absent)</td>
<td>13.2</td>
<td>9.6</td>
<td>72.7%</td>
</tr>
<tr>
<td>2</td>
<td>16.8</td>
<td>10.6</td>
<td>63.1%</td>
</tr>
<tr>
<td>3</td>
<td>19.1</td>
<td>12.6</td>
<td>66.0%</td>
</tr>
<tr>
<td>mean difference</td>
<td></td>
<td>67 ± 2.8%</td>
<td></td>
</tr>
</tbody>
</table>

Differences in the rise times of responses

EJCs had a significantly faster mean rise time than those of the new current. The mean rise time for EJCs was 6.4 ± 0.4 ms n=50, while that of the new current was 13.8 ± 1.6 ms n=50 (P<0.01, d.f=98). (See table 14 for comparisons of rise times from 3 preparations).
Table 14. A comparison between the mean rise time of EJCs and new L-arginine evoked currents recorded from the same three preparations of mouse vas deferens used in Table 13.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Rise time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EJC (L-arginine absent)</td>
</tr>
<tr>
<td>n=50</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>7.2 ± 0.1</td>
</tr>
</tbody>
</table>

Overall mean difference

13.8 ± 1.6 n=300

Differences in the decay to 50% of the maximum for each type of response

New currents revealed by L-arginine (n=50) and EJCs recorded from the same area of one preparation in the absence of the amino acid (n=50) were compared. The mean time taken for the new currents to decay to 50% of their maxima was longer than that calculated for the EJCs.

The mean time to decay to 50% of the peak for EJCs was 35.0 ± 0.5 ms (n=50). In the new response this was 48.7 ± 1.0 ms (n=50). This was a significant difference of 13.8 ± 1.8 ms n=100 (P<0.001, d.f. =98)
Table 15 compares the mean difference in the decay and rise times of the EJCs and new responses.

Table 15. A comparison of the mean time taken to decay to 50% of the maximum of EJCs and new L-arginine evoked currents recorded from the same patch of a single preparation of isolated mouse vas deferens.

<table>
<thead>
<tr>
<th></th>
<th>L-arginine (3.4x10^-4M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>absent</td>
</tr>
<tr>
<td>EJC (μV)</td>
<td></td>
</tr>
<tr>
<td>New currents (μV)</td>
<td></td>
</tr>
</tbody>
</table>
| Mean time to decay to 50% of maximum (ms)
| n = 50           | 35.0 ± 0.5 | 48.7 ± 1.0 |
|                  | n = 50 |         |
| Mean rise time (ms)
| n = 50           | 6.4 ± 0.4 | 13.8 ± 1.6 |
|                  | n = 50 |         |

The effect of RB₂ on the new current which had been revealed by L-arginine

RB₂ did not abolish the new response revealed by L-arginine when applied to either the recording electrode tip or the organ bath perfusates alone. However, when added to the recording electrode tip in its additional presence in the organ bath, the response of positive sign immediately became reduced in amplitude above the threshold for the original EJC and rapidly disappeared. EJCs simultaneously reappeared. Figure 56 illustrates examples of these stages recorded from one preparation. For the positive current to be abolished, RB₂ had to be present within the recording electrode tip perfusate. This suggested that the positive current had been evoked within the area covered by the tip.
Figure 56. An example of the effect of RB$_2$ (5x10^{-5}M) on the new response revealed by L-arginine recorded from a single patch of membrane on the surface of the isolated mouse vas deferens. The preparation was stimulated by 10 trains of pulses at 0.91 Hz with a pulse duration of 0.06 ms and a 2 minute rest interval between trains. Responses were recorded at a stimulus intensity 20-25% above the threshold for the excitatory response in the absence of drug treatment. L-arginine (3.4x10^{-4}M) was then applied to the Krebs saline perfusing the recording electrode tip and following a 5 minute incubation period nerve evoked responses were again recorded. (a) Excitatory junction current recorded at 20-25% above its threshold in the absence of L-arginine. (b) With exposure to L-arginine of longer than 20 minutes the response above the threshold for the EJC became a complex mixture of excitatory and superimposed inhibitory currents. (c) In this preparation the EJC disappeared from the high threshold response leaving only the positive current. (d) Treatment of the preparation with RB$_2$ caused the abolition of the positive current above the threshold for excitatory responses and the return of EJCs.

The EJC had reappeared following the abolition of the new current. This was present in recordings for only a few minutes before it abruptly disappeared again.

The new response may have been evoked within the area covered by the recording electrode tip. Why then was it necessary for RB$_2$ (5x10^{-5}M) to be present in the bath as well as the tip perfusates in order to abolish it?

The necessity for RB$_2$ to be present in both the bath and tip perfusates may suggest that leakage had occurred between the two compartments. Further experiments were designed to investigate the effects of RB$_2$ on transmission processes in this tissue. These were performed in the absence of L-arginine.
The effect of RB₂ (5x10⁻⁵M) on the mean amplitude of EJCs

The mean amplitude of control EJCs was 17.4±0.7μV, n=400 compared with 15.9±0.7μV n=400 in the presence of RB₂ (5x10⁻⁵M). This was a change of 8.7% which was not significant (p=0.1, d.f= 798).

Figure 57. The effect of RB₂ (5x10⁻⁵M) present in the focal recording electrode on the mean quantal effect of responses from isolated preparations of mouse vas deferens. Preparations were field stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between trains. EJCs were recorded by focal extracellular electrode from the surface of the mid vas deferens in the absence and presence of RB₂ (5x10⁻⁵M). The stimulus intensity was 20-25% above the threshold for the response. EJC amplitudes were normalised to the individual quantal effect determined from each preparation before constructing each histogram. A five minute rest interval separated the collection of EJCs recorded in the absence of RB₂ and EJCs recorded in the presence of RB₂ (5x10⁻⁵M) (a) The peaks of the amplitude distribution were determined visually for EJCs recorded in the absence of RB₂. (b) The constant distance between peaks also visually fits the distribution observed in the presence of RB₂ (5x10⁻⁵M) recorded from the same preparations. The mean quantal effect was 12.4±2.7μV, n=4 in the absence of RB₂ (5x10⁻⁵M) and 12.4±2.7μV, n=4 in its presence. The probability of total failures from the preparations recorded in the absence of RB₂ (5x10⁻⁵M) was 0.3 and 0.3 in its presence.

Figure 57 shows the quantal effect of secreted ATP which was visually determined from the EJC amplitude distribution in the absence of RB₂. This distribution of preferred values also visually fits those observed in the presence of RB₂ (5x10⁻⁵M) having the same separation.
between them as is seen in the controls. This suggests that the RB2 treatment did not cause a change in the quantal effect of neuronally secreted ATP in the 4 preparations tested. The mean quantal effect was 12.4±2.7µV, n=4 and in its presence it was also 12.4±2.7µV, n=4. The 95% confidence limits calculated for this quantal effect includes a change of 46.5%. In view of these large confidence limits further experiments should be carried out to determine if this antagonist does cause a postjunctional change in the response of this tissue to secreted ATP.

The effect of RB2 on the mean quantal content of EJCs

Figure 58 shows that there was no significant change in the mean quantal content of release events following the addition of RB2 (5x10^-5M) to the focal recording electrode tip perfusate (p=0.3, d.f= 3 and 6). In the controls this was 1.4±0.1 n=4 while in the presence of RB2 (5x10^-5M) it was 1.5±0.2 n=4.

![Figure 58](image)

Figure 58. The effect of RB2 (5x10^-5M) on the mean quantal content of responses. Preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were recorded in the absence of RB2 (5x10^-5M) and then in its presence in the focal recording electrode perfusate only. The paired data points represent the mean quantal content calculated for responses from a single release site in each preparation.
The effect of RB₂ on the failures attributable to processes which precede the secretion of transmitter

The probability of failures attributable to processes which precede the secretion of transmitter was 0.1±0.1 n=4 in the controls. In the presence of RB₂ (5×10⁻⁵M) this was also 0.1±0.1 n=4. This suggests that RB₂ (5×10⁻⁵M) when present in the recording electrode tip perfusate alone, does not affect the processes which precede the secretion of ATP in this tissue.

These results may also suggest that RB₂, when present only in the focal recording electrode perfusate, does not affect pre or postsynaptic elements of transmission in the mouse vas deferens. A further experiment was, therefore, designed to investigate the possibility that it may be necessary for RB₂ to be present in both focal recording electrode and bath perfusates in order to affect transmission in this tissue.

The effect of RB₂ (5×10⁻⁵M) on EJCs when applied to the perfusates of both the bath and the focal recording electrode:

The effect on the mean amplitudes of EJCs

The mean amplitude of EJCs was significantly reduced by 51.1% (P<0.001, d.f=398) from 18.4±0.8 μV (n=3) in the controls to 9.0±0.8 μV (n=3) in the presence of RB₂ (5×10⁻⁵M) in both focal recording electrode and organ bath perfusates.

Figure 59 shows the quantal effect of secreted ATP which was visually determined from the EJC amplitude distribution in the absence of RB₂. This distribution of preferred values also visually fits those observed in the presence of RB₂ (5×10⁻⁵M) having the same separation between them as is seen in the controls. Therefore, there was no apparent difference in the preferred values in the presence and absence of RB₂. This suggested that the RB₂ treatment did not cause a change in the quantal effect of neuronally secreted ATP in the 3 preparations tested. In the absence of RB₂ the mean quantal effect was 14.1±3.2 μV. The 95% confidence limits calculated for this quantal effect includes a change of 48.4%. This was less than the depression of the mean amplitude of EJCs observed in the presence of RB₂ (5×10⁻⁵M).
postjunctional change in the response of the tissue may not, therefore
have been responsible for this depressive effect of RB₂. Additional
experiments would improve this data and would further determine this.

Figure 59. The effect of RB₂ (5x10⁻⁵M) present in the focal recording electrode and organ bath on the
mean quantal effect of responses from isolated preparations of mouse vas deferens. Preparations were field
stimulated by trains of 20 pulses at 0.91 Hz and a pulse width of 0.06 ms with 2 minute rest intervals between
trains. EJCs were recorded by focal extracellular electrode from the surface of the mid vas deferens in the absence
and presence of RB₂ (5x10⁻⁵M). The stimulus intensity was 20-25% above the threshold for the response. EJC
amplitudes were normalised to the individual quantal effect determined from each preparation before constructing
each histogram. A five minute rest interval separated the collection of EJCs recorded in the absence of RB₂ and
EJC recorded in the presence of RB₂ (5x10⁻⁵M) (a) The peaks of the amplitude distribution were determined
visually for EJCs recorded in the absence of RB₂. (b) The constant distance between peaks also visually fits the
distribution observed in the presence of RB₂ (5x10⁻⁵M) recorded from the same preparations. The mean quantal
effect was 16.0±2.1 μV, n=4 in the absence of RB₂ (5x10⁻⁵M) and 16.0±4.1 μV, n=4 in its presence. The
probability of total failures from the preparations recorded in the absence of RB₂ (5x10⁻⁵M) was 0.6 and 0.8 in
its presence.

The effect of RB₂ on the mean quantal content of EJCs

Figure 60 shows that RB₂ (5x10⁻⁵M) caused a 53.3% reduction in the
mean quantal content of secreted ATP from 1.4±0.1 (n=3) in the controls
to 0.7±0.5 (n=3) in the presence of RB₂. This was not a significant
decrease (p= 0.2, df.4).
In view of the low significance of the data and the large confidence interval, further experiments should be carried out. However, a decrease in quantal content would seem to be the most plausible explanation to account for the decrease in mean amplitude of EJCs which occurred in the presence of RB₂ in both tip and bath perfusates.

![Graph](Image)

Figure 60. The effect of RB₂ (5x10⁻⁵M) present in focal electrode and organ bath perfusates on the mean quantal content of responses. Preparations were field stimulated with 10 trains of 20 pulses at 0.91 Hz with a pulse duration of 0.06 ms and a rest interval between trains of 2 minutes. The stimulus intensity was 20 to 25% above the threshold for the response. Responses were recorded in the absence of RB₂ (5x10⁻⁵M) and then in its presence in the focal recording electrode and organ bath perfusates. The paired data points represent the mean quantal content calculated for responses from a single release site in each preparation.

**The effect of RB₂ on the failures attributed to processes which precede the secretion of transmitter**

In one of the preparations there were no failures attributed to processes which precede the secretion of transmitter in either the controls or in the presence RB₂ (5x10⁻⁵M). In a second preparation the probability of these failures increased by 100% from 0.2 to 0.4. In a third preparation the site of shortest latency ceased to be active in the presence of RB₂. It was, therefore, not possible to assess the effect of RB₂ on these failures from this data.
These results suggest that RB₂ affect transmission in this tissue but for this to occur it must be present in both focal recording electrode and bath perfusates. This seems to be a prejunctional effect on the quantal secretion of ATP. Further experiments should be carried out to determine whether there is an effect on the failures attributable to processes which precede the secretory mechanism.

Are the new currents which were revealed by L-arginine evoked within the area covered by the recording electrode tip?

The effect of TTX applied to the perfusate of the recording electrode tip

TTX (7.5x10⁻⁶M) did not abolish the L-arginine evoked positive component of the response above the threshold for the original EJC. Neither was the new response below the threshold for the EJC abolished.

Intracellular recording

When a smooth muscle cell is penetrated by an intracellular electrode, transient depolarisations are recorded in response to nerve stimulation. These are excitatory junction potentials (EJPs). When recorded from the mouse vas deferens EJPs facilitate strongly reaching a plateau after 4-6 stimuli in a train at 0.9Hz. This is believed to reflect a pulse by pulse increase in neurotransmitter release. In the absence of stimulation spontaneous excitatory junction potentials (SEJPs) are recorded. These represent the spontaneous release of transmitter from the nerves and can be used as evidence of successful cell penetration.

This method of recording the electrical responses of smooth muscle cells is also used to record changes or differences in membrane potentials.

It is known that NO hyperpolarises the smooth muscle membrane of rabbit mesenteric arteries by activating ATP sensitive potassium channels (Murphy & Brayden, 1995). L-arginine may elevate the concentration of NO in the mouse vas deferens. If this does occur, the resting membrane potential of smooth muscle cells in this tissue may also become hyperpolarised. To investigate this, experiments were carried out in which membrane potentials were recorded by intracellular electrode.
Was a change in membrane potential responsible for the disappearance of the EJC?

The mean membrane potential recorded from 186 penetrated cells in the controls was -67.07 ± 0.87 mV, n=186, while from 165 cells penetrated the presence of L-arginine (where the amino acid was present for longer than 20 minute) this was -65.04±1.03mV, n=165. This was not a significant difference (P>0.1, d.f. 349). This result showed that the effects of the amino acid did not include a change in the resting membrane potential of penetrated smooth muscle cells.

Conclusions

The new current revealed by L-arginine may have been mediated by ATP acting at P₂Y receptors. This was suggested by its abolition by RB₂. This effect of RB₂ on the new current may have been postjunctional. However, P₂Y receptors are known to activate second messenger mediated responses in other cells (e.g. Schwann cells: Lyons, Morell & McCarthy, 1994). These would be expected to possess delayed onset latencies. It was unlikely that the new current revealed by L-arginine involved the activities of P₂Y receptors because the latency for onset of the new response was short.

When present in either the bath or recording electrode tip perfusates, RB₂ did not affect the new current which had been revealed by L-arginine. To abolish this new current, RB₂ had to be present in the focal recording electrode tip perfusate but also had to be present in the bath. When these criteria were met, the new current disappeared. This strongly suggested that leakage from the bath into the tip may have occurred. This would have caused a dilution of the drug within the tip. When present in both perfusions the correct concentration would have been expected to exist within the tip.

RB₂ is now known to affect potassium channels. This may have caused the observed effect on the new current revealed by L-arginine.

The effects of TTX were inconclusive. The results of the RB₂ experiments, however, strongly suggested that leakage of Krebs saline from the bath into the tip may have commonly occurred during these experiments. This would be expected to dilute drugs within the tip perfusate. TTX may, therefore, have failed to completely abolish
currents evoked within the tip because it had been diluted by saline from the bath.

Discussion

The aims of this current study

NO has been shown to modulate the release of Vasoactive Intestinal Polypeptide (VIP) from NANC nerves of the enteric system (Grider & Makhlouf, 1988, Jin & Grider 1993) but the role of NO as a modulator of the release of autonomic transmitters in the vas deferens has not been determined. This current study was, therefore, undertaken to investigate the role of NO on sympathetic transmission using the longitudinal smooth muscle of the mouse vas deferens as a model system.

The known roles of nitric oxide as a modulator of tissue function

The role of Nitric oxide (NO) in the control of vascular smooth muscle tone has evoked great interest since the discovery was made that its activities account for most of the actions of endothelium derived relaxing factor (EDRF) (Palmer, Ferrige & Moncada, 1987). NO is now known to be an important modulator of cell function with actions which include the relaxation of vascular and non-vascular smooth muscles. The ways in which NO does this are currently being investigate by many groups but one important mechanism involves the activation of guanylate cyclase which results in an increase in cGMP synthesis. This has been demonstrated in many tissues (Katsuki, Arnold, Mittal & Murad. 1977) and is an important factor in the relaxation of smooth muscle for example in bovine tracheal preparations (Katsuki, Murad, 1977). However, alternative mechanisms may involve other actions of NO. In rabbit mesenteric arteries for example NO relaxes the tissue by activating ATP sensitive potassium channels which results in a hyperpolarisation of the smooth muscle membrane. The membrane potential controls the entry of Ca^{2+} into the smooth muscle cells of this tissue and therefore, modulates tone and vessel diameter (Murphy & Brayden, 1995). The roles of NO as a modulator of tissue function may therefore, be many and varied and may depend on the tissue in question and the site of NO synthesis.

The modulatory effects of NO on non-vascular smooth muscle have not been as widely considered as that seen in their vascular
counterparts and the effects of nitric oxide on neuronal tissues are even less well documented. NO synthesis has, however, been demonstrated in the rat forebrain (Knowles, Palacios, Palmer & Moncada, 1989) and neurones with immunoreactivity for nitric oxide synthase have been identified in the guinea pig small intestine (Costa, Furness, Pompolo, Brookes, Bornstein, Bredt & Snyder, 1992). NO is released following the stimulation of NANC nerves of the canine ileo-colonic junction (Boecxkstaens, Pelckmans, Ruytjens, Bult, De man, Herman & Van Maercke, 1991) and it is also involved in the reflex relaxation of the stomach to accommodate food or fluid (Desai, Sessa & Vane 1991).

**Is nitric oxide a modulator of transmission in the mouse vas deferens?**

Results of experiments described in this thesis showed that NO is a modulator of nerve and smooth muscle activity in the mouse vas deferens which depresses both pre and post junctional responses. However, this appears to constitute only part of a complex control system.

**Which other agents are involved in the modulation of transmission in this tissue?**

The controls of transmission in the mouse vas deferens appear to be complex and experiments described in this thesis have shown that, in addition to NO, they also include histamine.

Histamine was shown to modulate both nerve and exogenous agonist evoked contractions in this tissue with effects mediated by H$_2$ and H$_3$ receptors to produce relaxation. The exogenous application of histamine did not evoke a contraction which meant that the activities of these receptors could not be recorded directly but their activities may have involved the modulation of the responses to either noradrenaline or ATP or both. As a result of these interactions histamine may control the balance of the activities of different innervations and the post junctional effects of their transmitters.

**How were the actions of nitric oxide in the mouse vas deferens identified?**

Experiments were designed which would address the following questions:
1. Are there receptors for nitric oxide that modulate autonomic transmission in the mouse vas deferens?

2. Where are these receptors?

3. Is there an endogenous source of nitric oxide acting on these receptors?

4. What are the effects of the nitric oxide which is synthesised in the vas deferens?

Is the mouse vas deferens sensitive to nitric oxide?

Sodium nitroprusside (NaNP) is a powerful smooth muscle relaxing agent with an especially pronounced effect on vascular tissue (Kreye, Baron, Luth & Schmidt-Gayk, 1975). This drug stimulates guanylate cyclase to increase the intracellular concentration of cGMP and this increase has been shown to be associated with smooth muscle relaxation for example in bovine tracheal preparations (Katsuki, Murad, 1977). NaNP has also been shown to elevate the intracellular concentration of cGMP in the rat vas deferens (Schultz, Schultz, Schultz, 1977) and this may also occur in the mouse.

During experiments carried out as part of this current thesis in which nerve evoked contractions were recorded, the NO donor sodium nitroprusside (NaNP) was applied to isolated preparations of mouse vas deferens. All contractions were depressed in the presence of this drug compared with the controls but this effect was greatest on responses evoked by high stimulus intensity. These contractions were significantly depressed from 495 ± 86% n=5 to 185 ± 22.7% n=5 and this suggested that a NO receptor system is present in this tissue.

NaNP, which was used as a donor of NO also evolves cyanide if illuminated. Every effort was made to ensure that its effects were not mediated by this potential toxicity but it was decided to confirm the preliminary results by another method.

Is a nitric oxide synthase present in the mouse vas deferens?

The enzyme NADPH diaphorase is synonymous with NOS in rat brain (Dawson, Bredt, Fotuhi, Hwang & Snyder, 1991. Hope, Michael, Knigge & Vincent 1991). This may also be the case in smooth muscle of the vas deferens.
NADPH diapherase was identified in rat brain by staining with nitro blue tetrazolium (NTB) for light microscopy (Dawson et al 1991b & Hope et al 1991) and for electron microscopy 2-(2-Benzothiazolyl)-5-styryl-3-(4-phthalhydrazidyl) tetrazolium chloride (BPST) (Wolf, Wurdig & Schunzel, 1992). These methods applied to the mouse vas deferens showed that this enzyme is associated with mitochondrial membranes of the smooth muscle (Al-Ayadhi, unpublished work).

Positive staining for NADPH diapherase suggested that a NOS may also be present in this tissue. If NOS is present, it may be possible to:

(a) increase its activity by applying its natural substrate (L-arginine) to the tissue. This would be expected to result in a depression of responses.

(b) reduce the concentration of nitric oxide in the tissue by inhibiting the synthase. This would be expected to elevate nerve evoked contractions if nitric oxide is normally synthesised in the isolated tissue.

Are nerve evoked contractions depressed in the presence of the natural precursor of nitric oxide?

L-arginine is the natural precursor of nitric oxide (Palmer, Ashton & Moncada, 1988). When this amino acid was applied to isolated preparations of mouse vas deferens during experiments carried out as part of this current thesis, nerve evoked contractions were significantly depressed by 25.1 ± 7.2% n=8. This depression developed with time and became maximal 70 minutes after the amino acid was applied to the tissue.

These results confirmed the conclusion of the previous experiment in which NaNP was used that there are receptors for nitric oxide in this tissue.

Are nerve evoked contractions elevated in the presence of nitric oxide synthase inhibition?

If the previous conclusions are correct inhibiting the production of NO should cause an elevation of the nerve evoked contractions.

L-arginine methyl ester (L-AME) was used to inhibit NOS. This is a competitive non-hydrolyzable inhibitor of the enzyme which reduces the synthesis of product (NO) by competing with the natural substrate for
binding at the active site. Such inhibition may be expected to elevate nerve evoked contractions by reducing the intracellular concentration of nitric oxide.

Preliminary experiments showed that the effects of L-AME on nerve evoked contractions were observed to occur only after a delay of about 60 minutes. This observation was also confirmed by other work (Al - Ayadhi, 1994 unpublished). Results of an experiment described in this thesis in which the isolated tissue had been incubated with L-AME for this length of time showed that nerve evoked responses were 7 times larger in amplitude in the presence of L-AME compared with the controls.

These results further confirmed the conclusion that receptors for nitric oxide are present in the mouse vas deferens and that NO plays an important physiological role in this tissue.

Can the elevation of nerve evoked contractions which was caused by the inhibition of nitric oxide synthesis be reversed by L-arginine the natural substrate of the synthase?

In view of the competitive nature of the inhibitor it should be possible to reverse its effects by applying L-arginine in a concentration which exceeds that of the L-AME. However, the 60 minute delay in the development of the elevation of nerve evoked responses in the presence of L-AME extended the length of experiments beyond acceptable time limits and any depression of contractions might be attributed to the effects of ageing of the preparation. In view of this it was decided not to attempt to reverse the inhibition of NOS during further recordings of nerve evoked responses.

Where are the nitric oxide sensitive systems situated in this tissue?

Nerve evoked contractions of the mouse vas deferens are the product of many elements. This includes the release of transmitters, their actions at pre and postjunctional receptors and the response of all smooth muscles (longitudinal and circular) including excitation-contraction coupling. In view of this complexity it was not possible to determine the site of nitric oxide sensitive receptors using those responses.
Are post junctional nitric oxide sensitive sites present in the mouse vas deferens?

The application of the exogenous agonists (noradrenaline or αβ-met ATP) causes the smooth muscles of the mouse vas deferens to contract. The analysis of these responses gives direct information about post junctional agonist/receptor interactions.

If nitric oxide normally depresses the responses evoked by post junctional receptors, an inhibition of NOS might be expected to produce an enhancement of the effects mediated by those receptors.

The effect of the NOS inhibition by L-AME, on these contractions, was to produce a shift to the left of the concentration response curve of this tissue for both noradrenaline and αβ-met ATP. The maximum response of these tissue to each agonists were also elevated. For noradrenaline there was an elevation of 24.4 ± 0.8% n=2 compared with the fourth mean response recorded from 5 control preparations. The elevation of the maximum seen in the response to exogenous αβ-met.ATP was 19% greater in 1 tissue treated with L-AME compared with the maximum response of 12 control preparations.

These results indicate that post junctional sites are present in this tissue which are sensitive to nitric oxide.

Does nitric oxide modulate the secretion of ATP?

EJCs recorded by focal extracellular electrode are elicited by the action of secreted ATP at postjunctional P2X receptors on the smooth muscle membrane (Cunnane & Manchanda, 1988. Åstrand, Brock & Cunnane, 1988). Quantal analysis of these currents can give information about mechanisms involved in the release of transmitter and its postjunctional effects.

Which method of statistical analysis was used to determine effects exerted on transmitter release?

EJCs recorded from mouse vas deferens are intermittent. When the number of individual events (n) in a process is small, it is difficult to distinguish between a Poisson or binomial process. Under this condition the two distributions are similar. The release of ATP in this tissue is not thought to affect subsequent release events. This aspect of the release
mechanism in this tissue is typical of a random or stochastic process. In such a process the probability of individual events occurring over an appreciable length of time is constant and is not affected by any other factor such as a previous release of transmitter. In view of these features it was decided to fit the data from EJC amplitude histograms to a Poisson distribution.

Transmitter release from the skeletal neuromuscular junction has been fitted to a Poisson distribution (Del Castillo & Katz, 1954. Boyd & Martin, 1956. Liley, 1956) but transmission at the autonomic neuromuscular junction of rodent vas deferens includes a larger number of failures than can be predicted by Poisson statistics. Some of these are attributable to processes preceding the secretion of transmitter and when these are excluded from the analysis the release process can be described by Poisson statistics and quantal analysis can be performed.

How does quantal analysis permit pre and post junctional effects of various treatments to be assessed?

The definition of the discrete event during intracellular recordings of excitatory junction potentials (EJPs) from the mouse vas deferens was a significant advance which was presumed to allow individual release sites to be analysed. This was the first time that the analysis of transmitter release in this tissue local to a penetrated cell could be performed (Blakeley, Cunnane & Petersen, 1982). The development of the extracellular method of recording excitatory junction currents (EJCs) provided another way of doing this since these currents represent the response of purinoceptors on the post junctional membrane to locally secreted ATP (Cunnane & Manchanda, 1988).

Measurements of EJCs reveal that their amplitudes fall into a few preferred values which are assumed to correlate in amplitude with the amount of transmitter released from the nerves during each response. This, therefore, provides a means of calculating the number (n) of quanta released, the mean quantal content (m) and the post junctional effect (q) of transmitter release.

The size of quanta released during transmitter secretion is assumed to remain constant provided that the pool of transmitter does not become depleted. Experiments described in this thesis did not exceed
two hours in duration and nerve stimulation protocols were designed to avoid depletion or exhaustion of the pool.

The quantal effect remained constant throughout control experiments with a duration not exceeding 2 hours. Intracellular changes attributable to the experimental procedure may have occurred but these should have been minimised by the experimental design.

Failures in transmitter release occur and these may be caused by effects on the release mechanism within the varicosity or on sites which precede this. Evidence from the work of others suggest that transmitter secretion is not only dependent on factors local to the release machinery of the varicosity but may also be controlled by other factors affecting sites which are assumed to precede the release of transmitter (Stjärne, Mshgina & Stjärne, 1990. Stjärne, Bao, Ganon, Mshgina & Stjärne, 1993). These may include K+ or other channels situated on the axon membrane which may be subject to various forms of modulation. Other mechanisms by which NO modulates prejunctional ion channels have not been fully identified but in rabbit mesenteric arteries post junctional ATP-sensitive K+ channels are activated by NO and this results in the hyperpolarisation of the vascular smooth muscle (Murphy & Brayden, 1995).

Various treatments may have pre or postjunctional sites of action. These may be seen as changes in quantal content of release events or in failures which are assumed to precede this. Control experiments carried out as part of this current study showed that these failures may decrease with time. These may also vary with different drug treatments and give information about prejunctional effects. Changes in the quantal effect of released transmitter reflect postjunctional changes in receptor activities. Quantal analysis is, therefore, a powerful tool which can separately determine both pre and post junctional effects.

It was not possible to determine the number of varicosities contributing to the EJCs recorded during experiments described in this thesis but the assumption was made that usually each response involved transmitter released from several varicosities. Therefore, it was not possible to determine the quantal release from individual varicosities and the term single release site referred to the activity recorded at a single latency within the area covered by the recording electrode tip.
Does the inhibition of NOS by L-AME affect EJCs and does this indicate the presence of prejunctional sites sensitive to nitric oxide?

The effect of L-AME recorded during experiment A1 was to increase EJC amplitude by about 37.1% during the 15 minute period following the addition of the inhibitor to the perfusate of the focal recording electrode. This was caused by an increase in mean quantal content from 1.8 ± 0.4 to 2.6 ± 0.6 n=4 (see experiment A1). This confirmed a physiological role for NO in the depression of ATP secretion which was a prejunctional effect exerted on the secretory mechanism. There was no significant change in the number of failures attributable to processes which precede the secretory mechanism. These results indicated that there is a prejunctional sensitivity to nitric oxide in this tissue and that this has effects on aspects of transmitter secretion processes. It was not possible to determine the identity of these sites but they may include ion channels, second messenger generating systems, phosphorylation mechanisms or cytoskeletal elements for example.

No change occurred in the quantal effect of ATP secretion during the first 15 minutes following the inhibition of NOS by L-AME. This indicated that in the short term a reduction in the intracellular concentration of nitric oxide did not affect postjunctional purinoceptors.

The effects of L-AME on NO synthesis were slower to establish during recordings of nerve evoked contractions compared with those of EJCs. Mechanical responses involve both receptor and contractile machinery effects in which the latter may play the predominant role. EJCs represent only a local receptor effect. The conclusion from this was that reducing the intracellular concentration of NO caused effects on excitation-contraction coupling which are longer lasting than either those on the post junctional receptor activities or on prejunctional changes in the ATP secretory mechanisms.

If the tissue is exposed to L-AME for a longer period of time does the inhibition of NOS evoke a greater increase in the mean quantal content of transmitter release?

The inhibition of NOS may increase with time. To determine this, some preparations were exposed to L-AME throughout the entire experiment (see experiment A2 ). The inhibitor was present in all salines including that used to bathe the tissue during the dissection. This exposure time exceeded 60 minutes in all preparations tested. For
comparision the mean quantal content was calculated from other untreated preparations. This was $1.2 \pm 0.1$, $n=4$ which was similar to the value recorded from controls in other experiments $1.4 \pm 0.1$, $n=24$. In the extended presence of L-AME it was $2.4 \pm 0.2$, $n=4$. This was similar to that recorded from preparations exposed to L-AME for only a short period of time. These results indicated that the effects of reducing the intraneuronal concentration of NO establish quickly following the inhibition of the synthase and also suggest that no further effect on the release of transmitter occurs even if the synthase is inhibited for longer periods of time. The conclusion that the effects of NO on the processes involved in the quantal release of transmitter was also confirmed.

**Can the inhibition of nitric oxide by L-AME be overcome by applying the natural substrate?**

It was discussed previously that the addition of the natural substrate should overcome the competitive inhibition of an enzyme. L-arginine is the natural substrate of NOS (Palmer, Ashton & Moncada, 1988). Since this tissue possesses a form of NOS, it might be possible to overcome the inhibition of NO synthesis by L-AME endogenously by the exogenous application of L-arginine. No other effects of supplying this amino acid to preparations of mouse vas deferens have been reported.

The inhibition of NO synthesis by L-AME evoked an increase in the mean amplitude of EJCs in the short term. This was caused by a significant increase in the mean quantal content of transmitter release from $1.8 \pm 0.4$ to $2.6 \pm 0.6$, $n=4$. L-arginine applied to other preparations in the continued presence of this inhibitor reversed this effect within 15 minutes and reduced the mean amplitude of EJCs. This was caused by a significant reduction in the mean quantal content of transmitter release from $2.4 \pm 0.4$ to $1.4 \pm 0.3$, $n=4$. No other effects were evoked during this reversal by L-arginine in the short term. The reversal of the inhibition of NOS returned EJC amplitudes to values which approximated to those observed in controls. These effects were prejunctional and may suggest that nitric oxide normally modulates ATP release by limiting the mean secretion to between 1.3 and 1.4 quanta per nerve impulse.

These results confirm the earlier conclusion that there are prejunctional sites in the mouse vas deferens which are sensitive to nitric oxide. The synthesis of NO decreases the secretion of ATP and this is a direct effect on the secretion mechanism.
Does the exogenous application of L-arginine increase the production of nitric oxide in the absence of synthase inhibition?

When L-arginine reversed the antagonism of NO synthesis by L-AME the quantal content of EJCs returned to levels which approximated those of the controls. This reversal was effected within 15 minutes following the application of the amino acid to the perfusate of the recording electrode tip. However, when applied to the tissue in the absence of established inhibition of NOS, L-arginine failed to alter the secretion of ATP in the short term (see experiment B on page 121) but did affect junction currents with longer exposure to the tissue.

Why did exogenous L-arginine fail to alter ATP secretion in the absence of inhibition of nitric oxide synthase?

Two suggestions may be proposed to explain this:

1. The exogenous application of this amino acid may have elevated its intracellular concentration above normal physiological levels but this may have failed to increase the rate of synthesis of NO in this tissue. The reasons for this were not clear but the production of NO may proceed at a maximal rate (i.e., NOS may be saturated) in the absence of extracellular L-arginine for the duration of the experiment. This suggestion was supported by the observation that the mean quantal content of control EJCs was approximately the same as that observed during the reversal of the NOS inhibition by L-arginine. This may also suggest that in the isolated vas deferens the intracellular concentration of that amino acid remains at normal physiological levels for the duration of the experiment and maintains maximal NO synthesis. The concentration of NO therefore, may remain constant during this time to maintain the mean quantal secretion of ATP at a minimal level.

2. More than one NOS may co-exist in this tissue. One type may be constitutive and may use intracellular L-arginine to produce a constant basal level of NO. This source of nitric oxide may be responsible for the effects on ATP secretion which were indicated by the inhibition imposed by L-AME. The other type may be inducible and may produce a higher concentration of NO. This would be expected to involve a slower time for its activation since the induction of protein synthesis is involved. This would explain the delay observed before the onset of the effects of the amino acid. This type of NOS has been demonstrated in macrophages.
where its activity is dependent on extracellular L-arginine (Baydoun, Bogle, Pearson & Man, 1993)

**What effects were observed when L-arginine was applied to the tissue?**

The effects of applying L-arginine to the perfusate of the extracellular recording electrode tip followed two distinct phases.

1) In the short term following its application to the tissue there were no recordable effects. The amplitude of EJCs were unaltered which was supported by the observations that the mean quantal content and the failures assumed to be attributable to processes which precede transmitter secretion were unaltered.

There were also no changes in the quantal effect of secreted ATP which indicated that L-arginine also had no effect on post junctional purinergic receptors or on the conductance activated by them in the short term. These results may suggest that extracellular L-arginine was not used to increase the production of NO in the short term.

2) After a minimum exposure to L-arginine of 20 minutes a new current was revealed below the threshold for the EJC. This was a current of opposite sign which possessed other characteristics. This further distinguished it from EJCs recorded from the same site. With increased time of exposure to the amino acid, the new current became present above the threshold for the original EJC. Following this, EJCs rapidly decreased in amplitude and eventually disappeared leaving only the new current to be evoked by all stimulus intensities.

These results may suggest that in the longer term, extracellular L-arginine may increase nitric oxide concentration and this increase may evoke effects which are not observed during the basal production of NO using intracellular L-arginine.

**What were the other characteristics which distinguished the new current from EJCs recorded from the same site**

1. A difference in shape compared with that of the EJC. The rise time of the currents revealed by L-arginine was about 50% slower than that of EJCs and their time to decay to 50% of the maximum was also
slower than that of EJCs recorded from the same site (see tables 14 and 15).

2. A lower threshold than the EJC. The new current was always found to have thresholds below that of the EJCs and this was often as much as 30% lower (see table 12).

3. A shorter latency than the EJC. The onset of the new current was difficult to determine accurately. The reason for this was caused by the slow rise time which allowed the onset to be obscured by the background noise of the recording. However, estimates of the onset from 3 different preparations which were measurable showed that the latency of the new current was between 6.6 and 3.6 ms. faster than that of the EJC (see table 13).

Characteristics 2 and 3 are consistent with the new current being the product of a population of nerves with a faster rate of conduction than that which produces EJCs.

Are these new currents EJCs evoked outside the area covered by the recording electrode tip?

EJCs are elicited by the action of secreted ATP at P2X receptors on the smooth muscle surface (Cunnane & Manchanda, 1988. Åstrand, Brock & Cunnane, 1988). These represent a flow of negatively charged ions across the membrane resulting in its depolarisation. When evoked within the area covered by the focal extracellular recording electrode these are of negative polarity with charge flowing away into the cell (Brock & Cunnane, 1987 &1988). Responses with positive polarity are evoked outside the area covered by the recording electrode tip and represent currents flowing out of the cell (Åstrand, Brock & Cunnane, 1988. Brock & Cunnane, 1987 &1988).

When TTX is applied to the perfusate of the extracellular focal recording electrode all currents evoked within the area covered by the tip are abolished but currents evoked outside the tip are unaffected (Stjärne, Mshgina & Stjärne, 1990).

Outward going currents were observed in the absence of L-arginine during experiments carried out as part of this thesis. These had identical thresholds, shapes and latencies as those of EJCs. It was assumed that
these were EJCs generated outside the area covered by the recording electrode tip.

Other currents with positive polarity were recorded in the absence of L-arginine but it was not possible to evoke EJCs from these sites by increasing the stimulus intensity. These positive polarity currents did not resemble inverted EJCs because their shapes were different and may not have been EJCs generated outside the recording electrode tip. No currents of positive polarity were present in recordings below the threshold for an EJC in the absence of L-arginine. This suggested that the new currents revealed by L-arginine were not EJCs evoked outside the recording electrode tip.

**Did the new currents develop below the threshold of the EJC with increasing age of the tissue?**

After 200 EJCs had been recorded in the absence of L-arginine, the stimulus intensity was reduced below the threshold of the EJC to investigate the possibility that the new current may have developed with time. This removed all responses and no other response was present. L-arginine was then applied to the perfusate of the recording electrode tip and a further 200 EJCs were recorded. After 20 minutes the stimulus intensity was again reduced and now the new response was present below the threshold for EJCs in most preparations. In some a longer time delay occurred. This showed that the new response did not develop as a result of ageing processes within the preparation but was revealed by L-arginine.

**Were the new currents evoked within the area covered by the recording electrode?**

The new response revealed by L-arginine did not resemble an EJC. Their rise and decay times were slower. Their latency of onset was shorter and they were evoked at lower thresholds than EJCs recorded from the same patch. These differences suggested that the new response was not an EJC evoked outside the area covered by the recording electrode tip. The lower thresholds of the new current also suggested that it was the product of a population of nerves with a faster rate of conduction than those which produced the EJC.
Does TTX abolish the new response?

TTX is a toxin which binds to sites on the sodium channels of nerves and prevents impulse conduction. Its application to the vas deferens within the extracellular recording electrode tip perfusate is used to identify responses to nerve activity evoked within the area covered by the electrode tip. If the new response is evoked within this area, TTX should abolish it. If the new response is simply an EJC evoked outside the tip it will remain unchanged. When TTX was added to the perfusate of the focal recording electrode tip the new L-arginine evoked current was not abolished. However, the EJCs were not completely abolished either. This may suggest that the full effect of TTX was not produced. Reasons for this were not clear but may have been caused by a dilution of the drug by leakage of saline from the bath into the tip perfusate. This may have resulted in an inadequate concentration of TTX reaching the surface of the tissue.

The application of drugs to the perfusate of the focal recording electrode restricts their action to regions of the tissue surface enclosed by the electrode tip which are local to the recorded sites of transmitter release. This method of application depends on the development of a good seal between the glass of the electrode and the membranes of the tissue. In the absence of such a seal the bath and tip perfusates may mix which would result in a dilution of the contents of the tip perfusate. This would reduce the effectiveness of drugs applied to the perfusate of the recording electrode.

When recording the electrical responses of the smooth muscle by focal recording electrode in the presence of suction, the tip of the recording electrode makes a better seal with the surface of the smooth muscle membrane and prevents leakage between the perfusates of the bath and the recording electrode tip. This, therefore permits the analysis of compartmentalised responses internal and external to the recording electrode tip (Stjärne et al 1990). However, electrical responses described in this current thesis were recorded in the absence of suction and so a perfect seal between the glass of the electrode and the membranes of the smooth muscle may not have developed.

The failure of TTX to abolish the junction currents did not suggest that the new current was evoked outside the area covered by the
recording electrode tip. Further experiments should be carried out to clarify this.

Was there any other evidence which supported the idea that leakage could occur between the bath and tip perfusates?

Inhibitory P\textsubscript{2}\text{Y} postjunctional receptors are present in the mouse vas deferens. This was demonstrated when RB\textsubscript{2} elevated the maximum response of this tissue to exogenous αβ-met-ATP (Blakeley et al 1991). It was assumed that because RB\textsubscript{2} (5x10^{-3}M) did not shift the concentration response curve that, at the concentration used, this drug had not affected P\textsubscript{2x} receptors. Other effects can be observed at higher concentrations but Blakeley et al had shown that at 5x10^{-5}M the effects of RB\textsubscript{2} are those of a specific P\textsubscript{2Y} antagonist.

Experiments were, therefore, designed in this current study to investigate a role for P\textsubscript{2Y} receptors in the production of the L-arginine evoked new current. RB\textsubscript{2}, when applied to the saline perfusing the recording electrode tip had no effect on the new current. This may have suggested that the new current was really an EJC evoked outside the area covered by the recording electrode tip. When present in the saline perfusing the bath there was also no effect on the new current. This may have been taken as confirmation that RB\textsubscript{2} had no effect on the new current. However, when RB\textsubscript{2} was applied to both the bath and recording electrode tip perfusions the new current was rapidly abolished and the EJC became re-established. These results showed that the new current is a purinergic response evoked by ATP acting at P\textsubscript{2Y} receptors.

The failure of RB\textsubscript{2} to abolish the new current when present in the recording electrode tip perfusate alone may be explained by the leakage of saline from the bath. However, when the bath saline also contained RB\textsubscript{2}, inward leakage would not cause a dilution and the correct concentration would be present inside the area covered by the recording electrode tip.
How might the nerves responsible for the EJC and the new current interact?

Does the activity of excitatory supply inhibit the low threshold nerves?

At most sites on the surface of the tissue, the new current was revealed below the threshold of the EJC but only when the preparation had been exposed to L-arginine for at least 20 minutes. During the next 5 to 10 minutes the new current was not present in recordings above the threshold of EJCs recorded from the same site but after this time they became evident when EJCs failed. These observations suggested that in the absence of L-arginine, the new current was inhibited by the activity of excitatory nerves responsible for the EJC. Activities of L-arginine may disrupt this inhibitory effect and allow the new current to be revealed.

The inhibition of the new current by the nerves which evoked the EJC appeared to be very long lasting. During the initial stages of each experiment preparations were repeatedly stimulated in attempts to find an excitable recording site. It was assumed that the inhibition of the low threshold supply occurred at that time.

Currents resembling the L-arginine evoked new currents were recorded from some areas of the smooth muscle surface from which EJCs were absent but only when the amino acid was not present. This suggests that in areas of the tissue where excitatory nerves are not active the low threshold supply is not inhibited.

Following the disinhibition of low threshold nerves does their activity inhibit the production of the EJC?

After the new current had begun to appear in recordings above the EJC threshold, recordings became complex and consisted of a short latency inward depolarising component followed by a later outward deflection. This stage progressed rapidly and during this time the amplitude of the EJCs began to decrease. This may have been caused either by an inhibitory effect of the nerves responsible for the new currents or by a delayed direct depressive effect of L-arginine exerted on the excitatory supply.
As the amplitude of the EJC became reduced that of the hyperpolarising component increased. Eventually only the new current was present in recordings above the threshold for the original EJC. This suggested that the nerves responsible for the new current were now inhibiting the activity of the excitatory nerves. However, other effects evoked by L-arginine on the EJC cannot be excluded and these include:

- A direct inhibitory effect of the amino acid. This was unlikely because no direct depressive effects have been reported by others in this or any other tissue.

- A delayed increase in the synthesis of NO which may have depressed ATP secretion. If this had occurred, its effects may be assumed to have been selective towards the excitatory supply and not on the low threshold nerves.

- A third nerve population had been activated or had begun to release more of its transmitter. This activity disrupted the interactions between the other two.

Was nitric oxide involved in these changes?

The effect of L-AME had established within 15 minutes to evoke an elevation of the mean amplitude of EJCs. This demonstrated that a decrease in the intracellular concentration of NO evoked rapid changes in ATP secretion. This also confirmed the assumption that as NO is a small, highly reactive molecule with a short half life, it would initiate rapidly established actions. This would be assumed to be the nature of a messenger possessing the activity of a biological on/off switch.

When L-arginine was applied to the perfusate in the recording electrode tip, there was no change in the secretion of ATP in the first 20 minutes. This slower time course for the effect of L-arginine suggested that the amino acid may not have caused an increase in the synthesis of NO.

Suggestions for other causes of the slower time course for the effects of L-arginine may have been:

- That the natural amino acid was transported more slowly into cells than L-AME. This was unlikely because L-arginine had
reversed the inhibition of NOS as rapidly as L-AME had established its inhibition.

- The effects may have resulted from a large increase in the production of NO following the activation of a second, inducible NOS by extracellular L-arginine. The decrease in EJC amplitude following the application of L-arginine only occurred after the establishment of the new current. This suggested that NO was unlikely to have been involved in the observed abolition of EJCs by a direct depressive effect on the excitatory supply. This suggests that an inducible NOS was not involved in the development of the new junction current and the disappearance of the EJC following the application of L-arginine.

- The amino acid had evoked some other action which was slow to establish.

**What other effects may L-arginine have produced: Are these effects prejunctional?**

L-arginine may have exerted effects on the secretion of other transmitters. Some of these may not produce electrical changes in the smooth muscle membrane and so their activities cannot be recorded by either extracellular or intracellular methods. Noradrenaline is electrically silent and its secretion may have been depressed by L-arginine. This transmitter may have been involved in either the initial inhibition of the low threshold nerves or in the later inhibitory effect exerted on the higher threshold excitatory population.

Changes in the smooth muscle membrane potential are evoked by ATP acting at purinergic P$_2X$ receptors (Cunnane & Manchanda, 1988). Recordings of junction currents would not, therefore, provide direct information about the action of noradrenaline. Changes in its secretion may, however, occur following alterations to that of ATP since it is known that the two transmitters have potent reciprocal prejunctional effects.

**How does ATP affect noradrenaline secretion?**

ATP and its degradation product adenosine inhibit the release of [H]$^3$noradrenaline in the mouse vas deferens by acting at prejunctional P$_1$ and P$_2Y$ receptors (von Kügelgen, Schöffel & Starke, 1989. Kurz, von Kügelgen & Starke, 1993).
How does noradrenaline affect ATP secretion?

Prejunctional $\alpha$-inhibition of transmitter release in the guinea pig vas deferens was first demonstrated by the use of yohimbine which is a specific $\alpha_2$ antagonist (Blakeley, Cunnane & Petersen, 1981). The extracellular recording method was later used to further investigate the action of prejunctional $\alpha$-receptors in the guinea pig vas deferens on an impulse to impulse basis. This showed that $\alpha_2$ receptor activation modulates subsequent ATP release from sympathetic nerves but may not act locally to inhibit secretion from recently activated varicosities (Brock & Cunnane, 1991).

How may noradrenaline be involved in the inhibitory effects observed in the presence and absence of L-arginine?

The low threshold nerves responsible for the new current posses prejunctional $\alpha_2$ receptors. This was shown by other work from this laboratory when yohimbine present in the perfusate of the focal recording electrode elevated the amplitude of the new currents which had been revealed by L-arginine (Al-Ayadhi unpublished work). In the absence of extracellular L-arginine prejunctional $\alpha_2$ receptors may be activated by neuronally secreted noradrenaline. L-arginine may depress the secretion of noradrenaline even though it may be without effect on ATP release in the short term. If noradrenaline secretion is reduced in the presence of this amino acid, $\alpha_2$ receptors would be expected to become inactive and their inhibitory effects would be relieved.

Noradrenaline secreted by the excitatory supply may be involved in the observed initial inhibition of the low threshold nerves. In the presence of L-arginine the new current may be revealed because the nerves which were responsible for it were no longer inhibited by the activities of $\alpha_2$ receptors.

Does L-arginine have post junctional effects?

NO hyperpolarises the smooth muscle membrane of rabbit mesenteric arteries by activating ATP sensitive potassium channels (Murphy & Brayden, 1995). If L-arginine does elevate the concentration of NO in the smooth muscle of the mouse vas deferens, a similar effect may occur.
The equilibrium potential for ions which influence the smooth muscle membrane potential is less negative than the resting membrane potential. Because of this the flux of ions through membrane channels depends on the membrane potential to supply a driving voltage. In this respect the flux evoked by the action of ATP at P2X receptors would be expected to be similarly affected. If the membrane became hyperpolarised for any reason, the driving force on ions crossing the membrane would increase and the mean amplitude of EJCs would be elevated. Conversely, a depolarisation of the membrane potential would reduce the driving force on ions crossing the membrane and currents would become reduced in amplitude.

To investigate this, experiments were carried out in which membrane potentials were recorded. The mean membrane potential recorded from 186 penetrated cells in the controls was $-67.07 \pm 0.87\text{mV}$ (n=186), while from 165 cells penetrated in the presence of L-arginine (where the amino acid was present for longer than 20 minute) this was $-65.04 \pm 1.03\text{mV}$ (n=165). This was not a significant difference (unpaired t test: P>0.1, d.f. 349) which showed that the effects of the amino acid did not involve a change in the post junctional membrane potential. This result showed that the disappearance of the EJC following the development of the complex response recorded by extracellular electrode was not caused by a change in the postjunctional membrane potential. Similarly the appearance of the new current could not be explained by this mechanism.

Do the effects of L-arginine involve the release of an unidentified agent which may be taken up and released as a false transmitter?

The ultimate effect of L-arginine on responses recorded by extracellular electrode was that the EJC, which had previously been recorded at intensities 20-25% above its threshold, disappeared. The new current remained and its amplitude recorded at this stimulus intensity steadily increased with time.

It was not possible to measure the latencies of new currents accurately because their onsets were often slow and hidden within the level of the noise of the recordings. This prevented quantal analysis from being carried out. It was not, therefore, possible to determine why the new current increased in amplitude. An effect of NO on noradrenergic nerves cannot be excluded. However, another possibility
is that L-arginine may have evoked the release of an unidentified agent within the tissue. Once released, this may have been increasingly taken up into some nerves by an uptake process. This may have been released as a false transmitter or may have augmented its normal secretion. This may have been the transmitter which evoked the new current, or it may have acted as an activator of the low threshold nerves.

The gradual increase in new current amplitude may be explained by suggesting that the uptake of the false transmitter and therefore its effects increased with time.

What is the identity of this false transmitter?

Compounds with a large positive charge cause the degranulation of mast cells. Poly-arginine is especially potent in this respect (Foreman & Lichtenstein, 1980). Cells of the vas deferens may possess an enzyme for the polymerisation of single amino acid residues but for this to occur L-arginine would need to be taken up by the cells. If this had occurred, the polymerised form of L-arginine would need to be packaged and re-exported by the Golgi apparatus before being available to act as a releasing agent of histamine from mast cells. Such processes may explain the delay which was observed before the new current was revealed. Why this tissue would pursue such processes which must be costly in intracellular energy expenditure is not clear.

When applied in high concentration, L-arginine as single residues also causes the degranulation of mast cells (Giraldo, Zappellini, Muscara, De Luca, Hslop, Cirino, Zatz, Nucci & Atunes, 1994). The concentration of the amino acid used in the experiments described in this thesis was within or just below the range used by Giraldo et al, which may have been high enough to cause mast cells to degranulate.

Histamine may, therefore, have been released from mast cells and taken up by nerves in this tissue for release as a false transmitter. This would suggest the possibility that the activities attributed to L-arginine were actually evoked by histamine.
Do mechanisms exist which control the degranulation of mast cells?

The concentration of L-arginine used in these experiments was close to the plasma fasting level for mammals (Williams, 1959). This suggests that in vivo the concentration of circulating L-arginine would be high enough to cause the degranulation of mast cells.

This effect on mast cells may only occur in the additional presence of other permissive factors. Mast cells possess P2Z receptors which are activated by ATP for example (Cockcroft & Gamberts, 1980). This suggests that mast cells may only be degranulated by circulating L-arginine if they are also activated by a high local concentration of extracellular ATP. In the mouse vas deferens ecto enzymes rapidly degrade extracellular ATP which suggests that for degranulation to occur mast cells would need to be innervated by a purinergic supply.

Other permissive factors may also be required. The vas deferens for example would normally be exposed to circulating sex hormones in vivo especially during mating, their absence in vitro may cause mast cells to be more susceptible to degranulation in the presence of L-arginine.

Do mechanisms exist which might mediate the uptake of histamine into nerves?

Acetylcholine is rapidly degraded by cholinesterases within the synaptic cleft. This represents a mechanism for the rapid inactivation of this transmitter. There is no equivalent extracellular mechanism for the inactivation of noradrenaline. Following its release noradrenaline can only be degraded enzymically following its uptake into nerves and other cells. The mechanisms for the uptake of noradrenaline are not specific for this transmitter but can carry other amines. For example, circulating adrenaline can also be taken up by these processes.

How were these mechanisms first identified?

Two amine uptake mechanisms were originally identified in the heart. These differ in kinetic properties and also have different substrate and inhibitor specificity. Uptake 1 is neuronal. This has a high affinity for noradrenaline but a relatively low maximum rate of uptake. Uptake 2 is present in non-neuronal cells, has a lower affinity for noradrenaline but a higher maximum rate of uptake. Both mechanisms will permit the uptake of other amines (Iversen, 1976).
Do sympathetic nerves possess specific mechanism for the uptake of histamine?

The uptake of $[^3]$H histamine into sympathetic nerves was demonstrated in rabbit iris. A subjective comparison was made between the radioactivity recorded following the uptake of $[^3]$H histamine and $[^3]$H noradrenaline in vivo and in vitro in separate preparations of rabbit iris. The conclusion was made from this that the uptake of histamine was less efficient than that of noradrenaline. Sympathetic denervation of the iris by excision of the homolateral cervical sympathetic chain demonstrated that the uptake of $[^3]$H histamine was by sympathetic neurones. (Ehinger, 1973). The less efficient uptake of $[^3]$H histamine compared with that of $[^3]$H noradrenaline may suggest an involvement of the Uptake I mechanism.

Are histaminergic nerves present in the vas deferens?

The idea that peripheral histaminergic fibres may exist is not new. Early observations identified histamine in nerves (Osborne & Vincent, 1900) and later various nerve preparations (peripheral, ganglia, spinal cord and brain) from human war fatalities and many other species were found to contain 'relatively large amounts of histamine' following traumatic shock (Kwiatkowski, 1943).

Histaminergic nerves have been identified more recently in the rat vas deferens. This was demonstrated by various surgical methods to disrupt nervous pathways and cause the degeneration of selected nerve pathways to occur. Subsequent fluorometric measurements of histamine and noradrenaline demonstrated that histaminergic pathways are adjacent to those of the sympathetic system (Campos, 1988. Campos & Briceno, 1992). An interaction may also occur between noradrenergic and histaminergic neurones in that tissue. This was proposed following preganglionic stimulation of the hypogastric nerve which caused a marked increase in histidine carboxylase activity. This was assumed to correlate with the activation of the histaminergic nerves (Campos & Dominguez, 1995)
What evidence was found during this current study to suggest that histaminergic nerves are present in the mouse vas deferens?

Results described in this thesis showed that nerve evoked histamine secretion occurs in the absence of exogenous L-arginine in this tissue. This amine acts at H\textsubscript{3} receptors to depress nerve evoked contractions. This was shown by the use of thioperamide, a highly specific antagonist of histamine at H\textsubscript{3} receptors which elevated these contractions. These results indicated that histamine normally modulates sympathetic transmission in this tissue and also suggested that, as in the rat, there is a histaminergic supply to the mouse vas deferens.

Might the histaminergic nerves which are postulated to be present in this tissue possess an uptake mechanism specific for histamine?

Results obtained during this current study of the mouse vas deferens do not provide evidence to suggest that extraneuronal histamine was taken specifically into histaminergic nerves. However, the presence of histaminergic nerves was postulated when it was found that the antagonism of H\textsubscript{3} receptors by thioperamide elevated nerve evoked contractions. Exogenous histamine or histamine from mast cells may be taken up by this supply and released to augment transmission from those nerves. The possibility that uptake of histamine into noradrenergic or other nerves may occur, should not be excluded from this discussion. These nerves may then transmit histamine as a false transmitter.

Histamine affects nerve evoked responses of this tissue. Which receptors mediate this effect?

What is the effect of exogenous histamine?

Nerve evoked contractions of the mouse vas deferens involve both pre and post junctional events. Exogenously applied histamine depressed those responses but this amine did not act as an agonist to evoke a contraction. This suggested that the effect of histamine on nerve evoked responses involved either the modulation of the release of excitatory transmitter/s or an alteration of their interactions at either pre or postjunctional sites. The depressive effect of histamine on these responses was opposed by ranitidine, a highly selective antagonist at H\textsubscript{2}
receptors which demonstrated an involvement of these receptors in this depressive effect.

In other work using rat vas deferens (Zamfirova, Todarov & Konstantinova, 1994), histamine was shown to inhibit the uptake of noradrenaline into nerve terminals. This was demonstrated by incubating preparations of this tissue with $^3$H noradrenaline in the presence and absence of histamine ($10^{-4}$M). Antagonists of histamine at $H_1$ and $H_2$ receptors were also included in the presence of histamine. Both opposed the effect evoked by histamine but the antagonism of $H_1$ receptors elevated noradrenaline accumulation more than that of $H_2$ receptors. The possibility that histamine may have interacted non-specifically with nerve membranes was not exclude. However, the effectiveness of histamine receptor antagonism suggested that occupation of those receptors by the amine was involved (Zamfirova et al 1994). This may represent a common mechanism by which responses of smooth muscle/nerve preparations are depressed by histamine.

**What is the effect of endogenous histamine?**

Thioperamide ($6\times10^{-6}$M), which is a highly specific antagonist of histamine at $H_3$ receptors, elevated nerve evoked responses. This demonstrated that endogenous histamine depresses these contractions when released during nerve activity.

**Does histamine depress transmitter evoked responses by acting at pre or postjunctional receptors?**

EJCs recorded by the extracellular method are evoked following the neuronal secretion of ATP in this tissue (Cunnane & Manchanda, 1988. Astrand, Brock & Cunnane, 1988). Quantal analysis of these currents is a powerful tool which provides information about both pre and postjunctional effects exerted on the secretion of ATP.

Quantal analysis showed that in the short term histamine ($1\times10^{-7}$M) caused a reduction in the quantal content of ATP release and an increase in the number of failures attributable to processes which precede this. These are prejunctional effects. There was no change in the quantal effect of ATP secretion which suggested that histamine ($1\times10^{-7}$M) did not affect postjunctional responses mediated by purinoceptors in the short term. This was supported by observations made during the recording of agonist evoked contractions during which histamine had no
effect on post junctional responses mediated by exogenous αβ-met.ATP. These results also support conclusions made by others that histamine depresses the release of excitatory sympathetic transmitter in this tissue (Campos & Briceno, 1992).

**Prejunctional H₃ receptors are present in other tissues. What processes are activated by them?**

Prejunctional H₃ receptors have been identified in the guinea pig superior cervical ganglia (Christian & Weinreich 1992) and ileum (Lees & Steel, 1990). Other work has also shown that (R)-α methyl-histamine which is a highly specific agonist of H₃ receptors causes a depression of nerve evoked contractions of the guinea pig vas deferens. This was also opposed by thioperamide. These effects were not observed on contractions elicited by direct smooth muscle stimulation or in response to exogenously applied noradrenaline which suggested that prejunctional H₃ receptors were responsible for the observed depression. (Luo Xiao-Xing & Tan Yue-Hua 1993). H₃ stimulation may cause a reduction in Ca²⁺ influx needed for transmitter release (Hill & Straw 1988). This supports the conclusion made from this current study that histamine acts prejunctionally in the mouse vas deferens to depress ATP secretion.

**H₂ receptors may be pre or postjunctional in other tissues. What processes are activated by them?**

In the rat brain, H₂ activation is associated with an increase in cAMP accumulation (Ozawa, Nomura & Segawa, 1987. Ozawa & Segawa 1988). An increase in membrane microviscosity also occurs in the region of the receptor following phospholipid methylation. This has been demonstrated in erythrocyte ghosts, rat brain synaptosomes and rat mast cells (Tolone, Bonsera & Potieri, 1982). Under these conditions activation of prejunctional H₂ receptors might be expected to result in a decrease in transmitter release. Results described in this current thesis showed that H₂ activation was associated with a depression of nerve evoked responses of the mouse vas deferens. This may have been a prejunctional effect mediated by these receptors.

Postjunctional H₂ receptors are involved in adenylate cyclase activation in many other cell types (Johnson, 1982). This has not been
demonstrated in the vas deferens of any species to date but should not be excluded as a possibility.

**What is the location of histaminergic receptors in the mouse vas deferens?**

The presence of H$_2$ and H$_3$ receptors was demonstrated in the mouse vas deferens but their location within the tissue was not fully identified. Information was not available in the literature to help elucidate this matter. Further experiments should, therefore, be carried out. These should investigate the effects of H$_2$ and H$_3$ receptors on junction currents using the highly specific antagonist ranitidine and thioperamide. Quantal analysis of EJCs recorded in the presence and absence of these antagonists might be expected to give information on the pre or post junctional sites of these receptors.

**The new current was abolished by RB$_2$. Does this suggest the identity of the transmitter involved in its production?**

The nerves which evoked the new current in the presence of L-arginine were shown to possess a low threshold for their activation. The new current was abolished by RB$_2$ (5x10$^{-5}$M). This drug has no reported effects on adrenoceptors which suggests that noradrenaline did not evoke the new current.

When RB$_2$ is used at concentrations between 1-5 x10$^{-5}$M it is a specific non-competitive antagonist of inhibitory P$_{2Y}$ receptors. This was demonstrated during work using rabbit portal vein (Reilly, Saville & Burnstock, 1987). Inhibitory P$_{2Y}$ postjunctional receptors are present in the mouse vas deferens. These were identified when RB$_2$ (5x10$^{-5}$M) elevated the maximum response of this tissue to exogenous $\alpha$/$\beta$-met.ATP.(see Blakeley, Brockbank, Kelly & Petersen, 1991). Furthermore, the assumption was also made that P$_{2X}$ receptors were not affected because RB$_2$ (5x10$^{-5}$M) did not shift the EC$_{50}$ of the concentration response curve to $\alpha$/$\beta$-met.ATP in this tissue (see Blakeley et al 1991).

Relaxations of the mouse vas deferens pre-contracted with ergotamine are mediated by the activities of P$_{2Y}$ receptors (Boland, Himpens, Vincent, Gillis & Casteels 1992). This suggests a role for these receptors in the mediation of an inhibitory response of the smooth muscle membrane of this tissue.
Responses mediated by $\text{P}_{2Y}$ receptors activate second messenger systems in other cells and tissues. Such systems produce their effects more slowly than those linked to ion channels. If these receptors were involved in the production of the new current revealed by L-arginine, they would be expected to have a delayed latency of onset. However, its latency was not delayed but was shorter than that of EJC$\text{s}$ recorded from the same site. This strongly suggests that this current was not activated by $\text{P}_{2Y}$ receptors.

An alternative suggestion is that this new current was abolished by one of the non-specific actions of RB$_2$. This may have involve the inhibition of potassium channels for example. However, RB$_2$ (1 x10$^{-5}$M) causes an increase in the nerve evoked overflow of $[^3\text{H}]$ noradrenaline from this tissue (von Kügelgen, Schöffel & Starke, 1989). The postjunctional effects of noradrenaline do not include electrical changes across the smooth muscle membrane. Changes in noradrenaline secretion cannot, therefore, be recorded directly by the extracellular method. However, an increase in the secretion of noradrenaline may have occurred during experiments using RB$_2$. The reduction in mean quantal content of EJC$\text{s}$ may have been caused by the action of noradrenaline at $\alpha_2$ receptors on excitatory nerves.

Evidence from nerve evoked contractions of the rabbit vas deferens suggests that noradrenaline with ATP are derived from different vesicular origins (Trachte, Binder & Peach, 1989). Cotransmission of these two transmitters may occur in the mouse vas deferens or they may be transmitted from separate nerve populations. The possibility that more than one type of nerves may coexist in this tissue should not be excluded. These may secrete identical or different transmitters and they may also differ in their prejunctional receptors. The abolition of the new current by RB$_2$ showed that the low threshold nerves secreted ATP. EJC$\text{s}$ which are the product of the higher threshold supply are also evoked by ATP (Cunnane & Manchanda, 1988; Åstrand, Brock & Cunnane, 1988). Therefore, two nerve populations exist in the mouse vas deferens but it is not known if either or both of these populations cotransmit noradrenaline. Other work from this laboratory has shown that yohimbine when added to the perfusate of the recording electrode, elevates the amplitudes of both control EJC$\text{s}$ and the L-arginine evoked new current (Al-Ayadhi unpublished work). This suggests that both of these nerve populations possess $\alpha_2$ receptors. The secretion of
noradrenaline may have been elevated following the addition of RB$_2$ to the focal recording electrode perfusate. This may have been sufficient to activate prejunctional $\alpha_2$ receptors. In this current study the EJCs which had abruptly returned after the abolition of new currents by RB$_2$ subsequently also abruptly disappeared. This may have been caused by the activation of $\alpha_2$ receptors on the excitatory supply.

**Do different nerve populations interact to modulate the responses of this tissue?**

The analysis of EJCs and new currents showed that these currents were produced by the activities of different nerve populations. The EJCs were the product of excitatory nerves while new currents were produced by lower threshold nerves. There are, therefore, more than one type of nerve in this tissue which includes at least two sympathetic populations.

A histaminergic supply to the rat vas deferens has been identified (Campos et al 1988, 1992 and 1995). Nerve evoked contractions of the mouse vas deferens were elevated by thioperamide a highly specific histamine H$_3$ receptor antagonist. This demonstrated that histamine is normally secreted during these responses of this tissue and histaminergic nerves may also be present in this tissue as they are in the rat.

The possibility also exists that some nerves may co-release more than one type of transmitter. The activities of these various nerves may normally involve prejunctional inhibitory interactions. Under some conditions the balance of these activities may be altered. This may have been the cause of the changes reported in this current study but results of experiments described in this thesis did not permit a full description of these changes to be made. Histamine from degranulated mast cells may have been taken up by one or more nerve population in this tissue and released either to augment the normal release of this amine or as a false transmitter. This may have exaggerated the normal responses of the tissue to histamine. This effect may have been caused as a result of the protocol used in these experiments but the concentration of L-arginine used in these experiments was close to that found in mammals in vivo (Williams, 1959). This suggests that the new current may be a normal response of the smooth muscle of the mouse vas deferens which may represent a mechanism by which the modulation of
transmission is varied. Such a system may range from full excitation at one end of the spectrum to full inhibition at the other. Such variability of response may be important in the control of the transport functions of this tubular tissue.

Conclusions

The controls exerted on transmission in the mouse vas deferens appear to be complex and may include both histamine and nitric oxide as important modulators.

Nitric oxide (NO) has prejunctional actions where it inhibits transmitter release by effects on the mechanism of transmitter secretion. The use of quantal analysis showed that the inhibition of NOS by L-AME caused the quantal content of release events to increase.

NO also inhibits responses of the smooth muscle of this tissue evoked by both ATP and noradrenaline. No other attempt was made to identify other components of the contractile mechanism which might also be affected by this inhibition.

More than one sympathetic nerve supply is present in the mouse vas deferens. These are distinguishable by the thresholds for their activation. Prejunctional interactions between these two nerve populations was demonstrated. Some nerves may also secrete histamine.

Histamine depresses the activities of excitatory nerves and may control the balance between the activities of different nerve populations. This amine also depresses contractions mediated by noradrenaline and may influence other postjunctional activities by its receptor interactions.

An increase in histamine secretion may also be involved in the production of the new current.

A prejunctional interaction of exogenous histamine was demonstrated during experiments described in this current study which may have been a pharmacological effect. Conversely this interaction may have a physiological importance although specific receptors were not identified.

The modulatory actions of nitric oxide and histamine are complex and may permit a wide range of effects in the mouse vas deferens. These include the inhibition of excitatory nerves and the EJCs which are
a product of their activities. The modulatory effects of nitric oxide and
histamine may be wide ranging. Fine adjustments of these effects may
allow responses of the mouse vas deferens to be varied in a graded
manner between full excitation to full inhibition. This may be an
important feature in the functions of a tubular tissue which experiences
rapid and transitory changes in the demands for its transport
mechanisms.
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