Release of adenosine 5'-triphosphate as a cotransmitter with noradrenaline from sympathetic nerves innervating the rat and guinea-pig vas deferens.

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In all fields (of) endeavour there are those who have gone before: if we see more clearly it is because they lit the way.

Furness & Costa. 1987
Abstract

In this thesis, the release of adenosine 5'-triphosphate (ATP) and noradrenaline (NA) from sympathetic nerves innervating the vasa deferentia of the rat and guinea-pig has been studied, using various neurochemical and pharmacological methods. The endogenous release of ATP was assayed using the luciferin-luciferase technique whilst noradrenaline efflux was measured by [³H]-NA detection.

Field stimulation of the guinea-pig vas deferens caused a substantial increase in the release of ATP and a biphasic contraction. Reserpine, which depleted the vasa deferentia of measurable NA, inhibited neither the release of ATP nor the initial component of contraction, clearly demonstrating that the release of ATP is not secondary to the efflux of NA, nor is the twitch mediated by NA acting via \( \chi \)-receptors. Tetrodotoxin, 6-hydroxydopamine and guanethidine abolished or greatly reduced the neurogenic efflux of ATP and both phases of the mechanical response; suggesting that ATP is released from sympathetic nerves. Furthermore, whilst electrical stimulation in the presence of prazosin and \( \alpha,\beta \)-methylene ATP failed to elicit a contraction, the co-release of ATP, NA and neuropeptide Y was undiminished. The neurogenic release of ATP was inhibited by adenosine acting via \( P_1 \)-purinoceptors.

The indirect sympathomimetic tyramine was shown to release both ATP and NA from the vasa deferentia of the rat and guinea-pig; further substantiating the sympathetic
nerves as a source of ATP release. Certain anomalies shown to occur between the two species in their response to tyramine are discussed. In the rat vas deferens, the suggestion that much of the field stimulation-induced efflux of ATP is released secondary to the efflux of NA was investigated. Exogenous NA was shown to induce the release of ATP from the vasa deferentia of the rat and subsequently a variety of denervation techniques were applied in order to investigate the source of the NA-induced ATP efflux. The results obtained suggest that in sympathectomised rats sensory nerve fibres are a major source of the NA-evoked ATP release.
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ABSTRACT ................................................................................. 2
ACKNOWLEDGEMENTS .............................................................. 4
PUBLICATIONS ARISING .......................................................... 5
CONTENTS .................................................................................. 8
LIST OF FIGURES AND TABLES ..................................................... 12
LIST OF ABBREVIATIONS ......................................................... 18
CHAPTER 1 - HISTORICAL INTRODUCTION ............................... 20
  1.1 Discovery of the sympathetic nervous system .................. 21
  1.2 The concept of chemical neurotransmission ................... 22
  1.3 Basic organisation of the autonomic nervous system ........ 25
    1.3.1 Sympathetic ............................................................... 25
    1.3.2 Parasympathetic ......................................................... 26
    1.3.3 Enteric .................................................................... 27
  1.4 Innervation of the vas deferens ........................................ 28
  1.5 Autonomic neuroeffector junction .................................... 29
  1.6 Nonadrenergic, noncholinergic nerves ............................... 31
  1.7 ATP as a principle neurotransmitter ................................. 33
  1.8 Purinergic receptors .......................................................... 36
    1.8.1 Subdivision of the P1-purinoceptor ............................. 37
    1.8.2 Subdivision of the P2-purinoceptor ............................. 39
    1.8.3 P3-purinoceptors ....................................................... 43
  1.9 Cotransmission hypothesis ................................................ 45
  1.10 ATP and NA as cotransmitters in sympathetic nerves ........ 48
  1.11 Neuromodulation of sympathetic cotransmission .............. 54
CHAPTER 2 - GENERAL MATERIALS AND METHODS

2.1 General
2.2 Organ bath experiments
2.3 Electrophysiology
2.4 ATP assay
2.5 [3H]-NA detection
2.6 NPY assay
2.7 Evoked Transmitter release
   2.7.1 Field-stimulation evoked release
   2.7.2 Drug-evoked release of neurotransmitters
2.8 Chemical denervation
   2.8.1 6-OHDA pretreatment
   2.8.2 Reserpine pretreatment
   2.8.3 Chronic guanethidine pretreatment
   2.8.4 Capsaicin pretreatment
   2.8.5 Capsaicin + Guanethidine pretreatment
2.9 Catecholamine content assay
2.10 Statistical Analysis
2.11 List of drugs and chemicals used

CHAPTER 3 - SYMPATHETIC NERVE MEDIATED RELEASE OF ATP FROM THE GUINEA-PIG VAS DEFERENS IS UNAFFECTED BY RESERPINE

3.1 Summary
3.2 Introduction
3.3 Materials and Methods
3.4 Results
3.5 Discussion
CHAPTER 4 - DIRECT EVIDENCE FOR THE CONCOMITANT RELEASE OF NORADRENALINE, ADENOSINE 5'-TRIPHOSPHATE AND NEUROPEPTIDE Y FROM SYMPATHETIC NERVES SUPPLYING THE GUINEA-PIG VAS DEFERENS... 103

4.1 Summary................................................104
4.2 Introduction........................................105
4.3 Materials and Methods...............................107
4.4 Results...............................................108
4.5 Discussion..........................................112

CHAPTER 5 - EVIDENCE THAT THE INHIBITION OF ATP RELEASE FROM SYMPATHETIC NERVES BY ADENOSINE IS A PHYSIOLOGICAL MECHANISM............. 125

5.1 Summary..............................................126
5.2 Introduction........................................127
5.3 Materials and Methods..............................130
5.4 Results...............................................131
5.5 Discussion..........................................134

CHAPTER 6 - COMPARATIVE STUDY OF THE ACTIONS OF AP₄A AND α,β-METHYLENE ATP ON NONADRENERGIC, NON-CHOLINERGIC NEUROGENIC EXCITATION IN THE GUINEA-PIG VAS DEFERENS........ 147

6.1 Summary.............................................148
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2</td>
<td>Introduction</td>
<td>150</td>
</tr>
<tr>
<td>6.3</td>
<td>Materials &amp; Methods</td>
<td>152</td>
</tr>
<tr>
<td>6.4</td>
<td>Results</td>
<td>153</td>
</tr>
<tr>
<td>6.5</td>
<td>Discussion</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Table</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Figures</td>
<td>164</td>
</tr>
</tbody>
</table>

**CHAPTER 7 - RELEASE OF ENDOGENOUS ATP FROM THE VASA DEFERENTIA OF THE RAT AND GUINEA-PIG BY THE INDIRECT SYMPATHOMIMETIC TYRAMINE**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Summary</td>
</tr>
<tr>
<td>7.2</td>
<td>Introduction</td>
</tr>
<tr>
<td>7.3</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>7.4</td>
<td>Results</td>
</tr>
<tr>
<td>7.5</td>
<td>Discussion</td>
</tr>
<tr>
<td></td>
<td>Table</td>
</tr>
<tr>
<td></td>
<td>Figures</td>
</tr>
</tbody>
</table>

**CHAPTER 8 - SENSORY NERVES ARE A SOURCE OF ATP RELEASED BY NORADRENALINE IN THE RAT VAS DEFERENS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>Summary</td>
</tr>
<tr>
<td>8.2</td>
<td>Introduction</td>
</tr>
<tr>
<td>8.3</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>8.4</td>
<td>Results</td>
</tr>
<tr>
<td>8.5</td>
<td>Discussion</td>
</tr>
<tr>
<td></td>
<td>Tables</td>
</tr>
<tr>
<td></td>
<td>Figures</td>
</tr>
</tbody>
</table>

**CHAPTER 9 - GENERAL DISCUSSION**

REFERENCES | 243
LIST OF FIGURES AND TABLES.

CHAPTER 3. Release from the guinea-pig vas deferens.

TABLE 3.1 - Effect of frequency on ATP release from the guinea-pig vas deferens.................................95

TABLE 3.2 - Effect of denervation pretreatments on NA levels in the guinea-pig vas deferens..............96

FIGURE 3.1 - Release of endogenous ATP from control guinea-pig vasa deferentia.................................98

FIGURE 3.2 - Release of endogenous ATP from vasa deferentia of reserpinized guinea-pigs.................100

FIGURE 3.3 - Effects of guanethidine, 6-OHDA and TTX on ATP release from the guinea-pig vas deferens.....102
CHAPTER 4. Release from the guinea-pig vas deferens.

FIGURE 4.1 - Concomitant release of [³H]-NA, ATP and NPY from the field stimulated guinea-pig vas deferens. 116

FIGURE 4.2 - Effects of TTX on the release of [³H]-NA and ATP from the guinea-pig vas deferens. 118

FIGURE 4.3 - The effects of α,β-methylene ATP and prazosin on the release of [³H]-NA and ATP from the guinea-pig vas deferens. 120

FIGURE 4.4 - Effects of α,β-methylene ATP on [³H]-NA and ATP efflux from the guinea-pig vas deferens. 122

FIGURE 4.5 - The effects of prazosin on [³H]-NA and ATP release from the guinea-pig vas deferens. 124
CHAPTER 5. Release from the guinea-pig vas deferens.

FIGURE 5.1 - Release of endogenous ATP from the field stimulated guinea-pig vas deferens ......................... 138

FIGURE 5.2 - Dose-dependent inhibition of ATP release and twitch contraction of the guinea-pig vas deferens by adenosine ................................................... 140

FIGURE 5.3 - Effects of adenosine on the endogenous release of ATP from the guinea-pig vas deferens .... 142

FIGURE 5.4 - Antagonism of the inhibitory actions of adenosine on ATP release and twitch contraction of the guinea-pig vas deferens by 8-phenyltheophylline ............ 144

FIGURE 5.5 - Effects of dipyridamole on the release of ATP from the guinea-pig vas deferens ................. 146
CHAPTER 6.  Isolated guinea-pig vas deferens.

TABLE 6.1 - Comparison of the effects of α,β-methylene ATP and AP₄A on excitatory responses of the field stimulated guinea-pig vas deferens.......................162

FIGURE 6.1 - Desensitization of the guinea-pig vas deferens by α,β-methylene ATP and AP₄A.........................164

FIGURE 6.2 - The rate and extent of recovery of the neurogenic contractions of the guinea-pig vas deferens after desensitization by α,β-methylene ATP and AP₄A......166

FIGURE 6.3 - Effects of AP₄A on electrical responses recorded in the guinea-pig vas deferens.......................168

FIGURE 6.4 - The concentration-dependent inhibition of EJP amplitude in the guinea-pig vas deferens by AP₄A...170

FIGURE 6.5 - Effect of increasing concentrations of AP₄A on EJPs elicited by stimulation at 0.2 Hz and 0.75 Hz.................................................................172
CHAPTER 7. Release from rat vas deferens.

TABLE 7.1 - Comparison of the effects of tyramine on the release of $[^3H]$-NA and ATP from the guinea-pig and rat vas deferens..........................188

FIGURE 7.1 - Release of $[^3H]$-NA and ATP from the rat vas deferens by tyramine.........................190

FIGURE 7.2 - Effects of increasing concentrations of tyramine on $[^3H]$-NA and ATP release and the contractile response of the rat vas deferens..........................192

FIGURE 7.3 - The effects of tyramine on $[^3H]$-NA and ATP efflux from the prostatic and epididymal regions of the guinea-pig vas deferens.................................194

FIGURE 7.4 - Effects of $\alpha,\beta$-methylene ATP on the tyramine-evoked release of $[^3H]$-NA and ATP from the rat vas deferens.................................196

FIGURE 7.5 - The effects of prazosin on tyramine-evoked $[^3H]$-NA and ATP release in the rat vas deferens........198
CHAPTER 8. Release from the rat vas deferens.

TABLE 8.1 - The release of endogenous ATP by exogenous NA from the vasa deferentia of control and pretreated rats ...............................................................215

TABLE 8.2 - NA content of samples of vasa deferentia removed from control and pretreated rats ..................216

FIGURE 8.1 - The effects of superfusion with NA on ATP ATP release from vasa deferentia of saline-injected rats .............................................................218

FIGURE 8.2 - The effects of prazosin on the NA-evoked responses and ATP release from vasa deferentia of saline-injected rats ........................................220

FIGURE 8.3 - Shows the effects of superfusion with NA on ATP release in the vasa deferentia of guanethidine-pretreated rats .............................................222

FIGURE 8.4 - The effects of prazosin on NA-evoked responses and ATP release from vasa deferentia of guanethidine-pretreated rats ........................................224

FIGURE 8.5 - Shows the effects of superfusion with NA on ATP release from vasa deferentia of rats pretreated with capsaicin and guanethidine ........................................226

FIGURE 8.6 - The effects of prazosin on NA-evoked responses and ATP release from vasa deferentia of capsaicin and guanethidine pretreated rats ............228
List of Abbreviations

ACh  acetylcholine
ADP  adenosine monophosphate
AMP  adenosine diphosphate
ANAPPa  arylazido aminopropionyl ATP
APxA  Pi,Pi-di-{adenosine-5'} tetraphosphate
APxA  Pi,Pi-di-{adenosine-5'} pentaphosphate
ATP  adenosine 5' triphosphate
cAMP  3':5'-cyclic monophosphate
CCh  carbachol
CGRP  calcitonin-gene related peptide
CGS21680  2-{p-[2-carboxyethylphenylethylamine]-5'-N-ethylcarboxamidine adenosine
CHA  N'-cyclohexylcarbox-amidoadenosine
CO2  carbon dioxide
CP66,713  4-amino-1-phenyl-[1,2,4] triazolo [4,3-a]-quinoxaline
CV 1808  2-phenyl-aminoadenosine
DHBA  dihydroxybenzylamine
DBH  dopamine β hydroxylase
DPCPX  1,3-diisopropyl-8-cyclopentylxanthine
FR  percentage fractional rate (%)
5HT  5-hydroxytryptamine
Hz  Hertz
EJPs  excitatory junction potentials
ELISA  enzyme-linked immunosorbent assay
IJPs  inhibitory junction potentials
HPLC  high pressure liquid chromatography
MAO monoamine oxidase
α,β-meATP α,β-methylene ATP
β. meATP β -methyleneATP
i.p. intra peritoneally (injection)
LDV large dense cored vesicles
NA noradrenaline
NANC nonadrenergic noncholinergic
NECA 5'-N-ethylcarboxamidoadenosine
NGF nerve growth factor
NPY neuropeptide Y
5-OHDA 5-hydroxydopamine
6-OHDA 6-hydroxydopamine
O2 oxygen
PBS phosphate buffered saline
8-PT 8-phenyltheophylline
R-PIA N*-R-1-phenyl-2-propyladenosine
s.c. subcutaneous (injection)
SDV small dense cored vesicles
S.E.M standard error of the mean
S-PIA N*-S-1-phenyl-2-propyladenosine
TTX tetrodotoxin
VIP vasoactive intestinal polypeptide
CHAPTER 1.

Historical Introduction
1.1 Discovery of the autonomic nervous system

The earliest recorded reference to the autonomic nervous system was made by Galen in the second century. From his detailed dissections of pigs, barbary apes and Roman gladiators (see Andrews, 1986) he described what we now know to be the paravertebral sympathetic chains with their superior and inferior cervical and semilunar ganglia (see Sheehan, 1936; White, 1952). The next major discovery was made some 1500 hundred years later by Eustachius, who was first to recognise that the vagi and the sympathetic trunks were anatomically and functionally distinct. By the eighteenth century, Winslow believed that 'sympathy' between the various organs of the body was maintained by the 'great intercostal nerves'; he renamed them 'sympathetic' to emphasise this point (see White, 1952). The two major subdivisions of the autonomic nervous system were first described by Gaskell but later expanded upon by Langley who, because he preferred his own terminology to that of Gaskell's, renamed the 'involuntary nervous system' the 'autonomic nervous system' (see White, 1952). In 1921, on a functional and anatomical basis, Langley then subdivided the autonomic nervous system into three subsystems namely: the sympathetic, the parasympathetic and the enteric system (Langley, 1921; see section 1.3).
1.2 The concept of chemical neurotransmission

The concept that transmission between nerve and muscle may be mediated by a powerful stimulatory substance was first expressed by Du Bois Reymond in 1877 (see Bacq, 1975). Elliot was more specific; he proposed that sympathetic neurotransmission was mediated by 'adrenalin or its immediate precursor' (Elliot, 1904; 1905; Von Euler, 1980). Inspired by Elliot's work, Dixon tried to isolate the parasympathetic neurotransmitter. After applying intensive vagal stimulation to dog hearts and examining the pharmacological properties of the perfusion fluid, he concluded that parasympathetic stimulation was mediated by a muscarine-like substance (Dixon, 1906), which Dale (1914) later suggested was acetylcholine. By the 1920's, although it was believed that chemical substances might mediate neurotransmission and that adrenaline and acetylcholine (ACh) might function as neurotransmitters, there was as yet, no direct evidence. Proof finally came in 1921, when Loewi in a series of ingenious but fairly straightforward experiments, demonstrated that the perfusion fluid collected from an electrically stimulated frog heart contained a substance, which when applied to a second heart, mimicked the effects of vagal stimulation; he called this substance 'vagusstoff' (vagus substance). By chance when he repeated these experiments in the spring, when the frog heart is more receptive to sympathetic stimulation (see Bacq, 1975), he was able to show that electrical
stimulation of the sympathetic nerves to the frog heart caused acceleration of the heart and liberated a substance which he called 'acceleransstoff'. In the same year, Cannon & Uridil (1921) demonstrated that stimulation of the hepatic nerves released a substance which could accelerate the denervated heart. This was followed by experiments which demonstrated that an adrenaline-like substance was, as Elliot predicted, a transmitter released by sympathetic nerves supplying the intestine (Finkleman, 1930) and heart (Cannon & Bacq, 1931), the latter named this substance 'sympathin'. The lack of identity between the action of adrenaline and the transmitter 'sympathin' was emphasised by Cannon & Rosenbleuth: they postulated that there were two sympathins, sympathin I and sympathin E formed, by the interaction of adrenalin with its effector tissues (Cannon & Rosenbleuth, 1933). Meanwhile, others proposed that noradrenaline (NA) might in fact be the transmitter released from sympathetic nerves (Greer et al., 1938; Mellville, 1937) as originally hinted at by Barger & Dale in 1910. Von Euler (1946) finally verified Elliot's strangely prophetic statement, when he demonstrated that sympathetic neuroeffector transmission was indeed chemically mediated by, 'adrenalin or its immediate precursor'; for, in most species, the transmitter is truly the immediate precursor or adrenaline, N-Ohne-Radikal (nor-) adrenaline.

The idea that neurotransmission was chemical did not go unchallenged (see Bacq, 1975) but by the 1950's it was
accepted by even is most ardent opponents (Eccles, 1970) as the major method of transmission at neuroeffector junctions.
1.3 Basic Organisation of the Autonomic Nervous System

1.3.1 Sympathetic

Preganglionic sympathetic nerves originate in the lateral horns of the thoracic and lumbar (thoracolumbar) segments of the spinal cord (Langley, 1921): they emerge as myelin-rich white rami communicantes, some of which synapse with the paravertebral ganglia. These ganglia lie on either side of the spinal cord and with their longitudinal connections, make up the left and right sympathetic chains (see Gabella, 1976). The sympathetic chains extend from the superior cervical ganglia in the neck (see Mitchell, 1953) to the coccyx where they converge to form a small ganglion impar. White rami communicantes can either synapse with the ganglion corresponding to the thoracolumbar segment from which they arise, or they can ascend or descend the sympathetic chain prior to terminating. Postganglionic fibres are unmyelinated. They emerge from the sympathetic chains either as gray rami communicantes which rejoin the spinal cord or as sympathetic nerve trunks or plexuses.

Not all of the white rami communicantes synapse in the sympathetic chains. Some axons traverse the paravertebral ganglia to synapse in the major or minor prevertebral ganglion cells which are located in closer proximity to the abdominal and pelvic viscera (Kuntz, 1949). Also, preganglionic fibres emerging from the tenth and eleventh thoracic segments run in the greater
splanchnic nerves to synapse in the chromaffin cells of the adrenal medulla.

1.3.2 Parasympathetic

Preganglionic parasympathetic neurons emerge from the cranial and sacral regions of the spinal cord to form synapses with ganglia in or close to the innervated organ (Kuntz, 1949). The cranial outflow consists of the third, seventh, ninth, tenth, and eleventh cranial nerves. Fibres in the third cranial nerve go to the iris, those in the seventh and ninth mainly innervate the salivary glands and the vessels of the tongue. The tenth cranial nerves (or vagi) innervate the heart, lungs, stomach, and intestine (see Kuntz, 1949; White, 1952). Parasympathetic fibres in the sacral division emerge as the third and fourth sacral nerves which synapse with scattered ganglia in the pelvic plexus innervating the colon, bladder, and genital organs. In contrast to the sympathetic nervous system, parasympathetic fibres are considered to give more precise control over limited regions of an organism (Campbell, 1970). When an organ is innervated by both the parasympathetic and the sympathetic nervous system, fibres from the two subsystems interweave in the terminal plexus and they may become mingled in a nerve trunk some distance away from the organ (Campbell, 1970).
1.3.3 Enteric

Although the enteric nervous system is ontogenetically, anatomically and physiologically related to the parasympathetic nervous system (Kuntz, 1949), it has several unique features which merit its classification as the third division of the autonomic nervous system (see Langley, 1921; Furness & Costa, 1987; Hoyle & Burnstock, 1989). The enteric nervous system is made up of the cell bodies and intrinsic nerve fibres of the gastrointestinal tract. The cell bodies are located within the wall of the gut and are grouped together to form the enteric ganglia; the enteric neurons are arranged in interconnecting plexuses. These enteric neurons may be either excitatory or inhibitory. Unlike the sympathetic or parasympathetic subdivisions, the enteric nervous system contains entire reflex pathways which permit greater intrinsic control of gastrointestinal function (see Furness & Costa, 1987; Hoyle & Burnstock, 1989).
1.4 Innervation of the vas deferens

The vas deferens is innervated by both the sympathetic and parasympathetic subdivisions of the autonomic nervous system. Sensory neurons accompany both types of innervation. Since the late nineteenth century, it has been accepted that the parasympathetic innervation of the vas deferens is derived from the visceral sacral outflow via the pelvic nerves; and that the sympathetic innervation is carried in the hypogastric nerves which arise mainly from the inferior mesenteric ganglia (Jänig & McLachlan: 1987). Unlike most other organs, the vas deferens is innervated chiefly by 'short adrenergic' neurons (Sjöstrand. 1962; Birmingham & Wilson. 1963; Kuriyama. 1963); that is to say, that the preganglionic hypogastric nerves and accompanying sensory neurons terminate in ganglia close to the vas deferens (Ferry. 1967).
1.5 Autonomic Neuroeffector Junction

The concept that the nervous system was a complex network of anatomosing neurons which fused with muscle cells, the 'reticular theory' was disregarded by Ramón y Cajal (see Gershon, 1970): who, by recognising synapses, provided further evidence for the discontinuity between nerve and muscle, and support for the so called 'neuron doctrine' (see Gershon, 1970). Neuronal individuality was finally confirmed with the advent of the electron microscope, which had sufficient resolution to demonstrate the gap existing between nerve and muscle.

The autonomic neuroeffector junction differs from the more classical synapses, such as those found at the skeletal neuromuscular junction or at ganglia, in several important ways (see Burnstock, 1986a). Firstly, the autonomic effector is a smooth muscle bundle rather than a single muscle cell. Electron microscopy has shown that the cells within each bundle are in close apposition with one another, the plasma membrane of some cells forming 'bridges' or 'intrusions' with neighbouring cells (Burnstock, 1970). These areas of close apposition known as 'gap junctions' or 'nexuses' may provide the morphological basis for electrotonic coupling and propagation of electrical activity through the smooth muscle bundle (Burnstock, 1970).

The autonomic nerves on reaching the effector tissue branch and become varicose in appearance, the varicosities which occur at approximately 5 μM intervals:
(Burnstock. 1970) are separated by intervaricose regions. Within the vicinity of the effector tissue the axons lose their Schwann cell coat on the muscle side, and the last few varicosities are naked (Merillees. 1968). Whilst there is occasionally some thickening of the pre-junctional membranes of the varicosities, there is little evidence of any postjunctional specifications (Richardson. 1962), except perhaps where nerve and muscle are closely apposed (Uehara & Burnstock. 1972). At the autonomic neuroeffector junction, transmitters are released en passage from varicosities during conduction of an impulse. It seems likely that only a small proportion of the varicosities release transmitter during a nerve impulse (Cunnane & Stjärne. 1984). Those muscle cells that are in close contact with the varicosities are directly affected by the transmitters released by them. Those further away rely on spread of activity through gap junctions or nexuses (Burnstock. 1970: 1986a). The width of the junctional cleft varies enormously in autonomically innervated organs; in densely innervated tissues such as the vas deferens the autonomic junction may be as narrow as 20 nm but in large elastic arteries the width of the junction may be as much as 2 μM (Burnstock. 1986a). With its variable cleft width, the autonomic neuroeffector junction is especially suited to neuromodulation by either pre- or postjunctionally acting neuromodulators (see section 1.12).
1.6 Nonadrenergic, noncholinergic nerves

The idea that autonomic nerves released only (nor)adrenaline or acetylcholine and therefore could be classified as either (nor)adrenergic or cholinergic (Dale, 1935) was initially challenged by Burnstock and colleagues in 1963: although anomalous observations which did not fit Dale's orderly classification had previously been reported.

For example, at the end of the last century, Langley and Anderson demonstrated that electrical stimulation of the parasympathetic pelvic nerves of the bladder could produce an excitatory response which was only partially reduced by the cholinergic antagonist, atropine. Furthermore, in 1898 Langley demonstrated that vagal stimulation to the stomach in the presence of atropine caused an inhibitory response. Further aberrant observations have been reported more recently (Ambache, 1951; Paton & Vane, 1963; McSwinney & Robson, 1969). However, the essential orthodoxy of cholinergic and (nor)adrenergic neurotransmission was preserved when these authors attributed their findings to the presence of some sympathetic noradrenaline-containing fibres accompanying parasympathetic nerves or to the inaccessibility of the neuromuscular junction to cholinergic antagonists (Paton & Vane, 1963).

In 1963, Burnstock and colleagues (Burnstock et al., 1963) demonstrated that stimulation of the intrinsic nerves of the guinea-pig taenia coli in the presence of
atropine and bretylium, produced a transient hyperpolarisation and relaxation. Since these hyperpolarisations were abolished by tetrodotoxin which inhibits action potential conduction in nerves, they concluded that these hyperpolarisations were inhibitory junction potentials (IJPs) produced by stimulation of nonadrenergic, noncholinergic (NANC) nerves (Burnstock et al., 1963). These inhibitory responses were not due to stimulation of the perivascular sympathetic nerves since the relaxations produced by stimulation of sympathetic neurons are readily abolished by low concentrations of adrenergic neuron blockers or α- and β-adrenoceptor antagonists (Burnstock, 1975). Moreover, IJPs and relaxations produced by stimulation of the guinea-pig colon were unaffected after sympathetic degeneration (Burnstock, 1972). Similar conclusions were reached by Martinson and Muren (1963) who had been investigating the relaxation of cat stomach by vagal stimulation in the presence of atropine. Since then, NANC responses have been reported in a variety of species and organs (Burnstock, 1972; 1975; Campbell, 1970; Furness & Costa, 1973). The existence of NANC nerves in the gastrointestinal tract and bladder is now well established (see Furness & Costa, 1987; Hoyle & Burnstock, 1989).
1.7 ATP as a principle neurotransmitter

Systematic studies were undertaken in an attempt to identify the transmitter utilised by the nonadrenergic, noncholinergic nerves of the gut and urinary bladder. There are several criteria which must be satisfied prior to establishing a substance as a neurotransmitter (Eccles, 1964; Barchas et al., 1978). Firstly, a putative transmitter must be synthesised and stored within the nerve terminals from which it is released; once released, it must interact with specific postjunctional receptors; the resultant nerve-mediated response must be mimicked by the exogenous application of the transmitter substance. Also, enzymes which inactivate the transmitter and/or uptake systems for the neurotransmitter or its derivatives must also be present; and finally, drugs which affect the nerve-mediated response must be shown to modify the response to exogenous transmitter in a similar manner.

Many substances were examined as putative transmitters in the NANC nerves of the gastrointestinal tract and bladder but the substance that best satisfied the above criteria was the purine nucleotide, ATP (Burnstock et al., 1970; Burnstock et al., 1972). Nerves utilising ATP as their principle transmitter were subsequently named 'purinergic' (Burnstock, 1971) and a tentative model of storage, release and inactivation of ATP for purinergic nerves was proposed (Burnstock, 1972). Since then a great deal of evidence has accumulated in support of the purinergic hypothesis, although there have also been
reports to oppose it (see Burnstock, 1975; Stone, 1981; Gillespie, 1982).

Initially, nonadrenergic, noncholinergic nerves were regarded as the 'third nervous system' but it now seems unlikely that they represent a single population of neurons utilising the same transmitter substance. In fact, electron microscopy has revealed that there are at least 9 ultrastructurally different types of axon profile in the enteric nervous system (Gabella, 1972; Cook & Burnstock, 1976) and that many of these nerve profiles contained a complex mixture of vesicles suggesting that they contain more than one transmitter substance. Furthermore, immunohistochemical studies have localised a wide variety of biologically active substances in autonomic nerves, for example: enkephalin, dynorphin, VIP, somatostatin, substance P, neurotensin and NPY (Furness & Costa, 1980; Hökfelt et al., 1980; Sundler et al., 1982; see Costa et al., 1986; Burnstock, 1986b; d: Morris & Gibbins, 1989).

More recently, ATP has been shown to act as a 'fast' excitatory transmitter at neuro-neuronal synapses in peripheral ganglia and in the CNS (see Burnstock, 1993). For example, studies in the coeliac ganglion (Evans et al., 1992) and in the medial habenula (Edwards et al., 1992) have demonstrated that excitatory junction currents evoked by either endogenous transmitter or exogenous ATP were antagonised by the P2 purinoceptor antagonist suramin. However, it is not as yet clear whether the
receptor involved is of the P2X or P2Y subtype since neither α,β-meATP nor 2-methylthio ATP were more potent than ATP although it is noteworthy that the receptors involved rapidly desensitize which is more typical of the P2X subtype (Kennedy & Burnstock, 1985; Iles and Nörenberg, 1993).

The physiological and pathological roles of purines in the nervous system, muscle, secretory, endocrine and immune cells, as well as in spermatocytes and tumour cells has recently been reviewed (Burnstock, 1993).
1.8 Purinergic receptors

Implicit in the purinergic hypothesis was the existence of purinergic receptors on the postjunctional membrane. Analysis of existing literature concerning the actions of purine nucleotides and nucleosides, in a wide variety of tissues led Burnstock (1978) to propose the subdivision of purinergic receptors. Based on several criteria (see Burnstock & Buckley, 1985), the following classification was proposed: $P_1$ purinoceptors are much more responsive to adenosine and AMP than to ADP and ATP: methylxanthines such as theophylline and caffeine are selective competitive antagonists at $P_1$ purinoceptors; the occupation of these receptors leads to changes in adenylate cyclase activity resulting in alterations in intracellular levels of cAMP. On the other hand, $P_2$ purinoceptors are more responsive to ATP and ADP than to AMP and adenosine; they are not antagonised by methylxanthines and their stimulation does not lead to a change in the intracellular concentration of cyclic AMP but it may increase the production of prostaglandins (see Burnstock, 1978; 1991; Kennedy, 1990). Further pharmacological, biochemical and receptor-binding studies have led to the subclassification of $P_1$ and $P_2$ purinoceptors.
1.8.1 Subdivision of the P₁-purinoceptor

It was several years after adenosine was first shown to interact with adenylate cyclase (Sattin & Rall, 1970) before P₁ purinoceptors were subdivided on the basis of whether their activation stimulated or inhibited this enzyme (Londos & Wolf, 1977; Van Calker et al., 1979; Londos et al., 1980). Londos and his colleagues termed the two subdivisions of the P₁-purinoceptor R₁ and R₂, the subscripts of which refer to the inhibition (i) and activation (a) of adenylate cyclase. Van Calker et al. (1979) named the same receptors A₁ and A₂ and it is the latter terminology that has been adopted (Burnstock & Buckley, 1985; Stone, 1985; Stiles, 1986). However, because it is was not always possible to demonstrate a clear link between the responses of tissues to adenosine and changes in adenylate cyclase activity (see Stone, 1985), P₁ purinoceptors were additionally classified according to the rank order of potencies of various adenosine analogues in eliciting responses (see Stone, 1985; Burnstock & Buckley, 1985). Thus, A₁-receptors mediate responses for which the order of potency is R-PIA = CHA ≥ 2-chloroadenosine ≥ adenosine = S-PIA while the rank order of potency at A₂ adenosine receptors is CGS 21680 ≥ NECA ≥ 2-chloroadenosine ≥ R-PIA = CHA ≥ Adenosine = S-PIA (see Burnstock & Buckley, 1985; Williams, 1989; Jacobson et al., 1992). Failure of the rank order of potencies to correspond completely in some tissues led Riberio & Sebastio (1986) to propose the existence of a
third type of P1-purinoceptor, the A3 receptor. The existence of this receptor has not received universal recognition and with the reported cloning, expression and functional characterization a new A3 receptor (Zhou et al., 1992) that is distinct from the first. It has even been suggested that the first A3 receptor should finally be laid to rest (Carruthers & Fozzard, 1993). Recently, binding studies with $[^3H]CV$ 1808 have led to the pharmacological identification of another adenosine receptor which has tentatively been designated as A3 (Cornfield et al., 1992). The A1 and A2 receptors from several species and the A3 subtype have recently been cloned (Maenhaut et al., 1990; Libert et al., 1991; Linden et al., 1991; Stehle et al., 1992; Zhou et al., 1993).

The A3 adenosine receptor has been further subdivided into high (A3a) and low affinity subtypes (A3b; see Abbracchio et al., 1993). A3a receptors have a rank order of potency of CGS 21680 ≥ NECA > CV 1808 > R-PIA: CGS 21680 (Jarvis et al., 1989) is the most selective agonist for A3a receptors to date while CP66 713 (Sarges et al., 1990) and KF 17837 (Suzuki et al., 1992; Kurakawa et al., 1994) are antagonists at this receptor. A3 adenosine receptors are antagonised by the highly selective (740-fold) xanthine derivative, DPCPX (Jacobson et al., 1992; Stiles, 1992; Abbracchio et al., 1993). APNEA is most potent agonist at A3 receptors where the rank order of potency is APNEA > R-PIA = NECA >> CGS 21680 (see Abbracchio et al., 1993) and $[^3H]CV$ 1808 is a radioligand
for $A_1$ receptors (Cornfield et al., 1992). Neither of the recently discovered adenosine receptors ($A_3$ and $A_4$) are antagonised by methyloxanthines (Zhou et al., 1992; Cornfield et al., 1992) and thus they appear pharmacologically distinct from $A_1$ and $A_2$ adenosine receptors.

Originally, both $A_1$ and $A_2$ receptors were believed to inhibit or stimulate adenylate cyclase, respectively (Van Calker et al., 1979; Londos et al., 1980) and while this may still be so for the $A_2$ subtype, $A_1$ receptors are now known to couple to a variety of effector mechanisms (see Stiles, 1992). The $A_3$ receptor appears to be linked to adenylate cyclase via a pertussis sensitive G-protein (Carruthers & Fozzard, 1993). In contrast, $A_4$ activation does not appear to involve G-proteins but may be mediated by potassium channel activation (Cornfield et al., 1992).

Finally, adenosine has been shown to interact with another recognition site, the function of this internal 'P-site' is still unknown (Williams, 1987).

1.8.2 Subdivision of the P$_2$-purinoceptor

The subclassification of P$_2$-purinoceptors was initially proposed by Burnstock and Kennedy (1985; for reviews see Burnstock & Kennedy, 1985; Kennedy, 1990; Burnstock, 1991) on the basis of criteria which included: the rank order of potencies of various metabolically stable derivatives of ATP in eliciting responses; the susceptibility to antagonism by ANAPP, and
desensitization of the receptor prior to exposure with \( \alpha,\beta\text{-meATP} \). At that time, the two main \( \text{P}_2 \) subtypes were the \( \text{P}_{2X} \) and \( \text{P}_{2Y} \) purinoceptors (Burnstock & Kennedy, 1985). It is generally accepted that \( \text{P}_{2X} \) purinoceptors mediate the excitatory responses of adenine nucleotides on smooth muscle wherever they are widely distributed e.g. the vas deferens, bladder and many blood vessels (see White, 1988; Burnstock, 1990); and they exhibit the following rank order of potency: \( \alpha,\beta\text{-meATP} \geq \gamma\text{-meATP} \geq \text{ATP} = 2 \text{ methylthio ATP} \). The first selective antagonist at \( \text{P}_{2X} \) purinoceptors was ANAPP3, which irreversibly combined with the receptor (Hogaboom et al., 1980; Fedan et al., 1985) however, technical difficulties in the use of this agent and its lack of specificity (Westfall et al., 1982; Frew & Lundy, 1986) meant that, until recently, selective \( \text{P}_2 \) purinoceptor blockade was preferentially achieved by receptor desensitization using the slowly degradeable analogue of ATP, \( \alpha,\beta\text{-meATP} \). \( \alpha,\beta\text{-meATP} \) was shown to be a more effective as a desensitizing agent than ATP (Kasakov & Burnstock, 1983). The desensitization produced by \( \alpha,\beta\text{-meATP} \) was specific for \( \text{P}_{2X} \)-purinoceptors since responses to NA and carbachol were not affected (Meldrum & Burnstock, 1983; MacKenzie et al., 1988). More recently, \( \text{P}_2 \) purinoceptor blockade has been achieved using the trypanocide, suramin (Dunn & Blakely, 1988; Sneddon, 1992) although it is not selective for \( \text{P}_{2X} \) purinoceptors, since it also antagonised \( \text{P}_{2X} \) mediated responses (Den Hertog et al., 1989; Hoyle et al., 1990). The \( \text{P}_{2X} \) purinoceptor has
recently been isolated from membranes of the rat vas deferens and has been shown to have a molecular mass of approximately 62,000 daltons (Bo et al., 1992).

The P2y purinoceptor has also been purified (Jeffs et al., 1991) and the further subclassification of this receptor into P2y1, P2y2 and P2y3 subtypes has recently been reviewed (see Barnard et al., 1994). Inhibitory P2y purinoceptors have the following rank order potency: 2-methylthio ATP > ATP > α,β-me ATP = β,γ-meATP. These receptors are only poorly antagonised by ANAPPs and they are not desensitised by α,β-meATP (Burnstock & Kennedy, 1985). P2y-purinoceptors mediate the inhibitory responses of ATP in the smooth muscle of the intestine: the longitudinal muscle of the rabbit portal vein and are found on vascular endothelial cells where they mediate the release of endothelium-derived relaxing factor (see White, 1988; Burnstock, 1991). They may also be present on the prejunctional membranes of postsynaptic sympathetic nerves where they are thought to impair transmitter release (Kurtz et al., 1993). P2y purinoceptors are selectively antagonised by the anthraquinone sulphonic acid derivative, reactive blue 2 (see Kennedy, 1990; Burnstock, 1991). Stimulation of P2y receptors frequently leads to the activation of phospholipase C which cleaves PLP2 to produce IP3 which then enhances the release of calcium from internal stores (see Kennedy, 1990).

P2x purinoceptors that are excitatory have been located on central and sensory neurones (Illes &
The excitatory P2X receptors found on the cell bodies of primary afferent neurons mediate their effects via two distinct transduction mechanisms. The neurons can either be depolarised directly by the opening of nonselective cation channels (Bean, 1992; Illes & Nörenberg, 1993) or indirectly via a G-protein linked mechanism that inhibits hyperpolarisation (Illes & Nörenberg, 1993). The inhibitory actions of ATP mediated via P2X purinoceptors in the intestine are thought to be associated with the selective opening of K+ channels (Hoyle & Burnstock, 1989).

There are now at least five P2 purinoceptor subtypes (see Abbracchio et al., 1993; Cusack, 1993) and they have been designated as P2X. P2Y (or P2Y2 see Barnard et al., 1994). P2X. P2Y and the P2X purinoceptors. In addition, a P2X receptor present on smooth muscle of guinea-pig ileum has also been proposed but not confirmed (Wiklund & Gustafsson, 1988). We now know that the P2X receptor originally described on platelets (Gordon, 1986) is actually an ADP receptor (Burnstock, 1991; Cusack, 1993) and that the agonist at the P2X subtype found on mast cells (Gordon, 1986), is the tetrabasic acid ATP4− (Tatham et al., 1988; Cusack, 1993). In the rabbit ear and basilar artery and in the rat tail and femoral artery the pyrimidine nucleotide, uridine triphosphate, produces a vasoconstriction that is mediated by the putative P2Y receptor (Von Kügelgen et al., 1987; Sajag et al., 1990; Von Kügelgen & Starke. 1990).
All of these P₂ purinoceptors, with the exception of the P₂X subtype, are believed to mediate their effects via second messenger production and they appear to be part of the growing G-protein linked receptor superfamily (Okajima et al., 1987; Kennedy, 1990; Barnard et al., 1994). In contrast, the effects of P₂X purinoceptors appear to be associated with the opening of nonselective ligand-gated ion channels permeable to Na⁺, K⁺ and Ca²⁺, resulting in a depolarization and the subsequent opening of voltage-dependent Ca²⁺ channels (Friel et al., 1988; Bean, 1992; Kennedy, 1993). In skeletal muscle the channel is also permeable to small anions (Thomas & Hume, 1990).

1.8.3 P₃ purinoceptors

It has been reported that the sympathetic nerves innervating the rat caudal artery and vas deferens possess prejunctional purinoceptors that mediate the inhibition of NA release and that these receptors exhibit characteristics which are different from P₁ and P₂ subtypes (Shinozuka et al., 1988; 1990; Forsyth et al., 1991). At these receptors, β.γ-meATP is more potent than ATP at inhibiting the evoked release of NA and the effects of both drugs were antagonised by the potent P₂X-receptor agonist, α.β-meATP: although α.β-meATP alone did not reduce the efflux of NA. Thus, these unique receptors appeared to possess some of the characteristics of a P₁-purinoceptor since they had a high affinity for 2-chloroadenosine and they were antagonised by methylxanthines and some of the characteristics normally
associated with the P₂-subtype including activation by adenine nucleotides such as β, γ-meATP and ATP (Shinozuka et al., 1988; 1990; Forsyth et al., 1991). Westfall and his colleagues proposed that this hybrid receptor be designated P₂. However, an alternative explanation might be as was initially proposed in the mouse vas deferens (Von Kügelgen et al., 1989) that the mixture of effects mediated by adenylnucleosides and nucleotides was not due to activation of one purinoceptor, the hybrid P₂-receptor, but rather due to stimulation of two distinct prejunctional receptors. Indeed, Von Kügelgen et al. (1989) demonstrated that the sympathetic nerves of the mouse vas deferens possessed both P₁- and P₂-purinoceptors and that both receptors inhibited the release of NA. Recent studies in the rat and mouse vas deferens indicate that the P₁-purinoceptor is of the A₁-subtype and that the P₂-receptor involved is probably P₂Y (Kurz et al., 1993; Von Kügelgen et al., 1993; 1994) and that these receptors together, mediate the effects of the putative P₃-purinoceptor.
1.9 Cotransmission hypothesis

For many years neuroscientists adhered to the view that single nerve fibres synthesised, stored and released only one transmitter, a concept which became known as 'Dale's Principle' (Eccles, 1964); and although the original belief expressed by Dale (1935) was that all branches of a neuron should release the same transmitter, 'Dale's principle' has become synonymous with the single transmitter concept (see Whittaker, 1983).

Abrahams et al. (1957) were among the first to suggest plurality of transmitters, when they reported that impulses conducted along the hypothalamiconeurophysiological neurosecretory fibres of the dog, liberated ACh which in turn, stimulated the release of vasopressin and oxytocin from the same terminals. Similarly, it was proposed, in what became known as the 'Burn & Rand Hypothesis' (Burn & Rand, 1959: 1965), that sympathetic nerve terminals contained in addition to NA, ACh and that nerve stimulation released the latter which in turn stimulated the efflux of NA from the same sympathetic nerve terminals.

Further examples of transmitter plurality were reported. As many as four different transmitter substances were shown to be released from the frog vagal-stomach preparation (Singh, 1964: Singh & Singh, 1966); and biologically active substances were shown to coexist in the nerves of some invertebrates (Brownstein et al., 1974: Cotterell, 1976) and in certain peripheral endocrine...
cells particularly those of the gut (see Pearse, 1969).

On the basis of these early findings and on information gleaned from comparative studies on the evolution of the autonomic nervous system, Burnstock (1976) questioned the single transmitter concept and proposed that neurons may synthesise, store and release more than one transmitter. Considerable evidence has now accumulated to support the concept of cotransmission (Burnstock, 1976; Cuello, 1982; Osborne, 1983; Chan-Palay, 1984; Burnstock, 1986; Campbell, 1987; Kaschube & Zetler, 1989; Burnstock, 1990; Von Kügelgen & Starke, 1991); and it may now be more unlikely to find a neuron with only one transmitter.

There are numerous examples of the coexistence of polypeptides with classical neurotransmitters. For example: somatostatin, enkephalin, VIP and NPY have been demonstrated in different populations of sympathetic neurones (Hökfelt et al., 1977; Schultzberg et al., 1979; Cuello, 1982; Lundberg et al., 1982: 1985) and the parasympathetic fibres innervating the cat salivary gland contain ACh and its cotransmitter, VIP (Lundberg et al., 1979: Bloom & Edwards, 1980: Lundberg, 1981).

Furthermore, studies on culture preparations have shown that, under certain conditions, single sympathetic neurons may be able to release both NA and ACh (Furshpan et al., 1976) and in addition, these same cultured neurons may also release a purinergic substance, possibly ATP (Potter et al., 1983). The coexistence of ATP and ACh in
both motor and parasympathetic nerves, in various different tissues has recently been reviewed (see Burnstock, 1986a).

There is also evidence that substance P (Gamse et al., 1980; Hagermark et al., 1978) and ATP (Holton, 1959; Jahr & Jessel, 1983; Fyffe & Perl, 1984) are transmitters in a population of primary afferent fibres and that they may be released as cotransmitters during 'axon reflex' (Burnstock, 1977).

To date, the most compelling evidence for the cotransmission hypothesis has come from studies on the corelease of ATP and NA from sympathetic nerves (Stjärne, 1989; Burnstock, 1990b; Von Kügelgen & Starke, 1991).
1.10 ATP and NA as cotransmitters in sympathetic nerves

The concept that ATP and NA may coexist is not a new one. The costorage of NA and ATP within chromaffin granules (Blaschko, 1956) and in storage vesicles isolated from sympathetic nerves (Schüman, 1958; Von Euler et al., 1963) has been recognised for more than 25 years.

Sympathetic nerves contain two types of storage vesicle: large dense cored vesicles (diameter 70–90 nm) and small dense cored vesicles (diameter 45–55 nm); both types store NA and ATP (Klein et al., 1982; Fried et al., 1984). The proportion of large and small dense cored vesicles varies according to species and tissue (Klein et al., 1982) but generally, the proportion of large dense cored vesicles (LDV) increases with animal size. Thus, 95% of vesicles in the guinea pig vas deferens are of the SDV type whereas in the ox 50% are LDV (Neuman et al., 1984). There is some debate over the molar ratio of NA to ATP stored within these vesicles. Early work on chromaffin granules and small dense cored vesicles (SDV) had suggested that the ratio of NA to ATP was around 4:1 (Schüman et al., 1958; Geffen & Livett, 1971) but later studies with better techniques for isolation and purification of vesicles suggest that this ratio is considerably higher: ranging from around 7:1 in LDV to 20-60:1 in SDV (Fried et al., 1981; Lagercrantz & Stjärne, 1974; Klein et al., 1982).

The first indication that ATP might be released from sympathetic nerves was the demonstration that stimulation
of the periarterial sympathetic nerves led to the release of tritium from taeni coli previously loaded with $^{3}H$-adenosine (Su et al., 1971) and later confirmed by Rutherford & Burnstock (1978) and White et al. (1981). Since then, ATP has been shown to act as a sympathetic cotransmitter in many tissues, including several blood vessels (see Burnstock, 1988; Burnstock, 1990b; Von Kügelgen & Starke, 1991). The contribution of ATP towards sympathetic transmission varies from preparation to preparation. For example, ATP is believed to be the major transmitter in the rabbit jejunal artery (Ramme et al., 1987), the noradrenergic component only being revealed at higher frequencies of stimulation or during longer trains of stimuli (Evans & Cunnane, 1992). ATP also plays a prominent role in both the rabbit saphenous artery (Burnstock & Warland, 1987; Warland & Burnstock, 1987) and the mesenteric artery of the rabbit and dog (Von Kügelgen & Starke, 1985; Muramatsu, 1986; 1987; Machaly et al., 1988; Muramatsu et al., 1989). On the other hand, it has a more minor role in sympathetic neurotransmission in the rabbit ear and rat tail artery (Sneddon & Burnstock, 1984b; Saville & Burnstock, 1988; Sedaa et al., 1986; Westfall et al., 1987). Sympathetic/purinergic cotransmission may also occur in the rabbit pulmonary and hepatic artery (Katsuragi & Su, 1980; 1982; Brizzolara & Burnstock, 1990) and the femoral artery of the rat (Åstrand & Stjärne, 1989).

Noradrenergic-purinergic cotransmission has also been
demonstrated *in vivo*. In preliminary experiments using the pithed rat model, \(\alpha.\beta\)-meATP attenuated the pressor response only after prior exposure to \(\alpha\)-adrenoceptor antagonists and even then its effects were small (Flavahan et al., 1985; Grant et al., 1985). However, more encouraging results were obtained with a modified desensitization schedule and in these experiments, also in the pithed rat, \(\alpha.\beta\)-meATP substantially reduced both the pressor response and twitch of the vas deferens, even in the absence of \(\alpha\)-antagonists (Bulloch & McGrath, 1988). The pressor response of the pithed rat is also reduced by the \(P_{z}\)-antagonist, suramin (Schlicker et al., 1989). \(\alpha.\beta\)-meATP also abolished the prazosin-resistant contraction in the autoperfused intestine of the anaesthetized cat (Taylor & Parsons, 1989). The role of \(P_{z}\)-purinoceptors in the cardiovascular system has recently been reviewed (Ralevic & Burnstock, 1991).

However, the most extensive evidence for sympathetic cotransmission has come from studies on the vas deferens (for recent reviews see Burnstock, 1990b; Von Kügelgen & Starke, 1991) where ATP is believed to be the neurotransmitter mediating the electrically evoked EJPs and the initial component of the subsequent contraction.

EJPs were first recorded in the smooth muscle of the vas deferens in the 1960's, when the fact that they were attenuated by sympathetic neurone blocking drugs such as guanethidine and bretylium led Burnstock & Holman (1960: 1964) to suggest that they were mediated by a transmitter
released from sympathetic nerves: since at that time NA was believed to be the only neurotransmitter utilized by these neurons, it was assumed that NA mediated the EJP. However, subsequent studies have shown that it is unlikely that EJPs are mediated by NA as they are not antagonised by α-adrenoceptor antagonists such as phentolamine or prazosin and they are unaffected by reserpine or cocaine (Sneddon et al., 1982). In fact, more recent evidence strongly suggests that EJPs are mediated by ATP released from sympathetic nerves. For example, EJPs are abolished by 6-OHDA (Alcorn et al., 1986); antagonized by the P2-purinoceptor antagonist, ANAPPs; blocked by desensitization of the P2-purinoceptor by α,β-meATP and mimicked by local pressure ejection of ATP (Stjärne & Astrand, 1984: Sneddon & Burnstock: 1984a: Sneddon et al., 1982; Alcorn et al., 1986). EJPs are also antagonised by the P2-receptor antagonist, suramin (Sneddon, 1992) and attenuated by the L-type calcium channel blocker, nifedipine (Blakeley et al., 1981). Furthermore, Stjärne and colleagues (Cunnane & Stjärne: 1984; Stjärne & Astrand, 1984) have shown that 'discrete events' recorded in the guinea-pig and mouse vas deferens, measure single quanta of ATP secreted from sympathetic nerves.

The neurogenic response of the rodent vas deferens is biphasic, a fast phasic component (the 'twitch') followed by a more tonic contraction. Only the second, maintained component of the mechanical response is mimicked by exogenous NA, enhanced by cocaine, abolished by α-
adrenoceptor antagonists and reserpine pretreatment and therefore believed to be mediated by NA (Ambache & Zar. 1971; Swedin. 1971; Sneddon & Westfall. 1984; Allcorn et al.. 1986). The twitch response is resistant to these drugs (Ambache & Zar. 1971; Sneddon & Westfall. 1984; Allcorn et al.. 1986) but is abolished by ANAPP₃ (Fedan et al.. 1981; Sneddon & Westfall. 1984) and suramin (Dunn & Blakeley. 1988) and by desensitization of the P₂ₓ-purinoceptor with α,β-meATP (Meldrum & Burnstock. 1983; Allcorn et al.. 1986) and ATP (MacKenzie et al.. 1988). The twitch response is also mimicked by exogenously applied ATP (Westfall et al.. 1978). Together these studies provide convincing evidence to support the view that ATP released as a cotransmitter from sympathetic nerves. Indeed, ATP has been shown to be released from the sympathetic nerves innervating the guinea-pig vas deferens (Westfall et al.. 1978; Fedan et al.. 1981; White et al.. 1981; Levitt et al.. 1984; Kirkpatrick & Burnstock. 1987: Lew & White. 1987; Kasakov et al.. 1988).

Yet, while the evidence to support the hypothesis that ATP is released from sympathetic nerve terminals during stimulation is strong, uncertainty remains over which storage vesicle(s) are involved in transmitter secretion. and it is not yet clear whether ATP and NA are released from the same or different vesicles. Initially, it was thought that only SDV were involved in transmitter secretion from sympathetic nerves (Basbaum & Heuser. 1979; Pollard et al.. 1982; Neuman et al.. 1984) but later
studies proposed that both SDV and LDV are involved in transmitter release (Thureson-Klein. 1983; Fried et al., 1984; Thureson-Klein, 1984). The existence of multiple storage/secretory sites may provide a morphological explanation for the recent demonstration of differential prejunctional modulation of the release of NA and ATP (Trachte. 1985; Trachte. 1988; Trachte et al., 1989; Ellis Burnstock. 1989a; 1989b; Hata et al., 1991; Donoso et al., 1992; Sperlàgh & Vizi. 1992). For example, in the vas deferens angiotensin III augmented the noradrenergic phase of the contraction while the twitch was depressed (Trachte. 1988). These effects were probably due to the fact that during low frequency stimulation angiotensin III reduces the release of ATP but enhances the efflux of [³H]-NA (Ellis & Burnstock, 1989a). Angiotensin III also increased the levels of [³H]-NA released with stimulation at 20 Hz but had no effect on ATP release (Ellis & Burnstock. 1989a). The converse was true with PGEs which inhibited the release of [³H]-NA at 2 Hz and 20 Hz but enhanced the efflux of ATP at 2 Hz (Ellis & Burnstock. 1989b).

The existence of multiple storage sites involved in transmitter secretion, whether they be different vesicle subtypes or subpopulations of sympathetic nerves (Burnstock. 1990b) is fundamental to the belief that the release of ATP occurs independently to that of NA.
1.11 Neuromodulation of sympathetic cotransmission

Any substance that modifies neurotransmission may be defined as a neuromodulator. A neuromodulator may exert its effects either at the prejunctional or postjunctional membrane. Once secreted from the sympathetic nerve terminal, NPY and ATP can act as potent prejunctional and postjunctional modulators of neurotransmission.

After its release from the sympathetic nerve terminal, ATP is rapidly dephosphorylated to adenosine by ectonucleotidases (Pearson, 1985; Gordon, 1986; Meghji, 1993) and subsequently most of the prejunctional effects are mediated via the metabolite adenosine. Adenosine is a potent neuromodulator of transmitter release in both the peripheral and the central nervous system (Fredholm & Dunwiddie, 1988; Williams, 1989). In the vas deferens, P1-purinoceptor agonists have been shown to inhibit the release of \( ^3\text{H}\)-NA in the rat (Clanachan et al., 1977; Taylor et al., 1983; Kurz et al., 1993). Guinea-pig (Hedqvist & Fredholm, 1976) and mouse vas deferens (Kurz et al., 1993). Adenosine also inhibits EJPs (Sneddon et al., 1984; Blakeley et al., 1988) and the twitch response of the vas deferens (Clanachan et al., 1977; Taylor et al., 1983). Recent studies suggest that the P1-purinoceptor subtype inhibiting the twitch and EJPs in the rat and mouse vas deferens closely resembles the A1-receptor (Blakeley et al., 1988; Hourani et al., 1993). The existence of prejunctional A2 adenosine receptors that enhance the release of \( ^3\text{H}\)-NA has recently been
proposed (Gonçaveles & Queiroz. 1993).

However, ATP itself has also been shown to inhibit the release of [³H]-NA from the vas deferens and caudal artery of the rat (Clanachan et al. 1977; Taylor et al. 1983; Shinozuka et al. 1988; 1990; Forsyth et al. 1991) and the mouse vas deferens (Von Kügelgen et al. 1993; 1994). These effects are now believed to be mediated via P₂₁ purinoreceptors (Von Kügelgen et al. 1989; Kurz et al. 1993; Von Kügelgen et al. 1993; 1994) and not P₃ as originally proposed (see section 1.8.3). Some of the prejunctional effects of ATP are thought to be due to adenosine (Clanachan et al. 1977).

NA is also a potent inhibitor of neurotransmitter release, inhibiting both its own release and that of ATP via interactions with prejunctional α₂-adrenoceptors (Starke 1977; 1987). In the vas deferens, α₂-receptor agonists reduce the amplitude of EJPs (Blakeley et al. 1981; Illes & Starke 1983; Brock et al. 1990) and have recently been shown to decrease the release of ATP from the mouse (Von Kügelgen et al. 1989; 1991) and guinea-pig vas deferens (Sperlagh & Vizi 1992; Dreissen et al. 1993). Presynaptic α₂-adrenoceptors have also been shown to modulate the release of NPY-like immunoreactivity (Dählof et al. 1986).

NPY also modulates transmitter secretion from sympathetic nerve terminals via, at least in the rat vas deferens, prejunctional Y₅ receptors (Wahlstedt 1990; Doods & Krause 1991) to reduce the release of NA and ATP.
(Lundberg & Stjärne, 1984; Lundberg et al., 1984; Stjärne & Lundberg, 1986; Stjärne et al., 1986; Donoso et al., 1988; Ellis & Burnstock, 1990). However, the neuromodulatory actions of adenosine, NA and NPY are not restricted to the prejunctional level; these substances also interact postjunctionally. For example, adenosine has been shown to potentiate the contractile actions of NA in the vas deferens of several species (Hedqvist & Fredholm, 1976; Holck & Marks, 1978; Long & Stone, 1986; Tsunoo et al., 1991; Witt et al., 1991) and in the guinea-pig, the effects of exogenous ATP are markedly enhanced by NA (Holck & Marks, 1978; Kazic & Milosavljevic, 1980; Kishi et al., 1990). This and the finding that adenosine could increase the rate of α-adrenoceptor resensitization in this tissue, led Holck & Marks (1978) to suggest that adenine compounds may be a prerequisite for normal α-adrenoceptor sensitivity. Adenosine also influences the functionality of α-adrenoceptors in the rat vas deferens although it is interesting to note that in this tissue there is a decrease in the rate of resensitization (Long & Stone, 1986). In rat vas deferens the effects of adenosine on resensitization are probably mediated via A2 adenosine receptors. Postjunctional A2 receptors may also inhibit the twitch response of the rat vas deferens (Hourani et al., 1993), the receptors concerned are probably of the A2B-subtype since the A2B selective agonist, CV 1808 (Bruns et al., 1986) has no effect in this tissue (Major et al., 1989).
This apparent synergism is not restricted to ATP and NA. Postjunctionally, NPY enhances the contractions produced by ATP and NA suggesting that the role of NPY is to amplify the effects of the 'true' cotransmitters NA and ATP (Stjärne et al., 1986). Since NPY is preferentially released by bursts of high frequency stimulation (Lundberg et al., 1985) its postjunctional actions would allow maximum contractile responses while its inhibition of transmitter release would serve to spare prejunctional ATP, NA and NPY stores.
Chapter 2.

General Methods
2.1 General

Male Dunkan Hartley guinea-pigs (300-400 g) and Wistar rats (180-230 g) were killed by cervical dislocation and then bled, (except in Chapters 7 & 8, where the rats were killed by increasing levels of carbon dioxide).

The vasa deferentia of both species were excised, stripped of connective tissue and then bisected, to give epididymal and prostatic portions. Unless stated otherwise, the prostatic half of the vas deferens was used. In Chapter 4, the preparations were additionally bisected along their length. The vasa deferentia, mounted on either tissue-holders or electrodes, were then attached by thread to a Grass FT03C force-displacement transducer and placed under an initial load of 1 g. Mechanical recordings were displayed on a Grass polygraph (Model 79 D). The vasa deferentia were continuously superfused using a Watson-Marlow pump, at a rate of 2.5 ml/min (or 1.2 ml/min in Chapter 1) with modified Krebs' of the following composition (mM): NaCl 133; KCl 4.7; NaHCO3 16.3; MgSO4 2.5; CaCl2 2.5 and glucose 7.7. The solution was bubbled with 95% O2 and 5% CO2 and the temperature maintained at 36-37 °C. The pH was 7.4. The vasa deferentia were allowed to equilibrate for 90 min prior to experimentation.
2.2 Organ bath experiments (see Chapter 6)

Guinea-pig vasa deferentia were excised and prepared as above. The isolated preparations were attached to electrodes and then mounted in 20 ml overflow organ baths. The vasa deferentia were placed under an initial load of 1 g and allowed to equilibrate for 90 min. Isometric tension was recorded and displayed as detailed above. Drugs were added directly to the bath.

The tissues were field stimulated for 30 s at either 5 or 20 min intervals with trains of pulses of varying frequency. supramaximal voltage. The pulse width was 0.5 ms. In addition, control responses to carbachol (1-10 μM) and ATP (100-150 μM) were obtained before and after the addition of prazosin (5 μM). The concentration of ATP and carbachol used was chosen so that the response obtained was of a similar magnitude to the neurogenic response of the tissue at 8 Hz. After these control responses had been established, prazosin (5 μM) was added to the bath for the duration of the experiment.
2.3 Electrophysiology (see Chapter 6)

The prostatic end of the vasa deferentia was excised and prepared as described in section 2.1, then placed in a perspex recording chamber, the base of which had been coated with sylgard (Dow & Corning). The preparation was immobilised by gently stretching and pinning out the connective tissue with steel pins. The recording chamber was continually perfused with Krebs' solution of the following composition (mM): NaCl 120; KCl 5.9; NaHCO₃ 15.4; MgCl₂ 1.2; NaH₂PO₄ 1.2; CaCl₂ 2.5 and glucose 11.5. The temperature in the bath was maintained at 36 °C by means of a local heating coil and feedback thermistor. Drugs were added to the continually flowing Krebs' solution.

Conventional intracellular recording techniques were used: electrical recordings (WP1 W707) were displayed on a storage oscilloscope and could be retained on an instrumentation tape recorder (Hewlett Packard) or chart recorder (Gould 2200S). Intracellular glass micro-electrodes (60–70 MΩ) were filled with 3 M KCl. Electrical field stimulation was achieved using trains of pulses (0.5 ms width) delivered from a Grass S44 stimulator and stimulus isolation unit via platinum plates at variable frequency (generally up to 3 Hz, but occasionally 10–20 Hz).

This procedure was performed by Dr. Ian MacKenzie.
2.4 ATP assay

The ATP contained in the superfusate samples was assayed using the luciferin-luciferase bioluminescence technique. The terms 'luciferin' and 'luciferase' were first introduced by DuBois in the late 19th century. From his studies on beetle and clam luminous organs, he discovered that the luminescence reaction was dependent on two components: one heat stable the other heat labile; he named these two components luciferin and luciferase. Bioluminescence may be defined as the emission of light due to oxidation of a substrate in the presence of an enzyme (Giese, 1962). In present terminology, luciferin refers to the substrate that undergoes oxidation catalysed by the enzyme, luciferase.

In his pioneering study, McElroy (1947) noted that firefly extracts, which contain the enzyme complex luciferin-luciferase, could act as a sensitive quantitative assay for ATP. This is because ATP is an essential cofactor in these events and the luciferin-luciferase enzyme is specific in its requirement of ATP (Lundin & Thore, 1975). (The firefly utilizes this unique transduction process for attracting a mate (Seliger et al., 1964.)

The actual sequence of reactions in firefly bioluminescence was originally summarized by McElroy & Selinger (1966) but briefly the reduced luciferin, in the presence of Mg$^{2+}$ reacts with ATP and the enzyme luciferase to form luciferyladenylate and an inorganic pyrophosphate.
The adenylate complex then reacts with molecular oxygen to form the excited intermediate which decays to the final product, emitting light in the process (for a detailed description of the chemical reactions catalysed by firefly luciferase see DeLuca & McElroy, 1974).

**Experimental Protocol**

The ability of luciferin-luciferase to detect very low concentrations of ATP (25 fmol) meant that all Krebs' containers, tubing and glassware had to be scrupulously clean. At all times, Krebs' was made up with freshly collected, distilled water and where necessary, the water was stored overnight at 4 °C. All superfusate samples were collected in ice-cold vials, directly placed on ice and assayed for ATP. [3H]-NA and NPY as described below.

The luciferin-luciferase was reconstituted in sterile water to give a final concentration of 13.3 mg/ml and gently inverted 3 times, to ensure proper mixing. 60 µl of luciferin-luciferase was then pipetted into borosilicate glass cuvettes (Kimble, 6 x 50 mm); after covering, the cuvettes were stored at 4 °C for 1-2 h, thereby enabling the background activity of the enzyme to exponentially decay to a steady basal level.

Prior to experimentation, bioluminescent intensities of various concentrations of ATP, ranging from 10⁻⁹ to 10⁻¹³ M, were measured and a standard calibration line prepared. For the assay, 200 µl of standard solution or tissue superfusate were added to cuvettes containing the enzyme and the bioluminescence measured on a Packard
pico! detector (Model AG100) for 30 s. The Krebs' solution which was assayed for background ATP prior to each period of stimulation and sampling acted as a blank and its value was subtracted from the other samples assayed. The amount of ATP present was read from the standard line and converted to either nmoles or pmoles/g of vas deferens.
2.5 $[^3]H$-NA detection

Isolated vasa deferentia were incubated for 60 min in oxygenated Krebs' containing 0.1 nmol/ml tritium-labelled noradrenaline ($[^3]H$-NA. New England Nuclear: specific activity 1624.3 GBq/mmol; two preparations in 5 ml) at 36-37 °C. After rinsing five times with cold Krebs'. the tissues were mounted on electrodes (Chapter 4) or tissue-holders (Chapter 6) and set up and superfused as described in section 2.1. The vasa deferentia were then allowed to equilibrate for 90 min prior to experimentation.

Experimental Protocol

2.5.1 $[^3]H$-NA release by nerve stimulation (see Chapter 4)

After equilibration, the superfusate was collected during 21 consecutive (3 min each) periods in ice-cold vials (fractions 7, 8, 10, 11, 12, 19, 20 were discarded). Aliquots for ATP (0.5 ml. see section 2.4) and NPY determination (0.5 ml. see section 2.6) were taken from each of the remaining fractions and assayed as detailed above and below.

During the course of the experiment, the vasa deferentia were field stimulated twice: at the beginning of the 4th and 16th collections (ie. the 9th and 45th min respectively) for 60 s, with trains of rectangular pulses (0.1 ms duration, 9 Hz, 50 V) delivered by Grass SD9 stimulators. The first stimulation ($S_1$) was used as a control: the second stimulation ($S_2$) was performed in the presence/absence of drugs. The changes in stimulation-
evoked release or spontaneous efflux of \(^{3}\text{H}\)-NA were
quantified as \(S_{2}/S_{1}\) ratio (the increase of \(^{3}\text{H}\)-NA release
over the basal level. in collections 16 and 17 versus the
level in collection numbers 4 and 5) or 10/3 ratio (the
basal level of \(^{3}\text{H}\)-NA radioactivity in collection 15
versus the level in sample 3 as described by Taube et al.

For \(^{3}\text{H}\)-NA detection. 2.5 ml aliquots of superfusate
were added to 15 ml Aquasol scintillation cocktail and \(^{3}\text{H}\-
radioactivity counted in a Beckman 7500 liquid
scintillation counter. The superfusion was stopped
immediately after the 21st collection. the preparations
were weighed then dissolved in 0.5 ml Soluene-350 tissue
solubilizer. The total tissue \(^{3}\text{H}\)-NA was counted as
above. The amount of \(^{3}\text{H}\)-NA released was expressed as a
fraction of the total radioactivity present at the time of
collection (mean ± S.E.M) as described by Alberts et al.
(1981). Previous investigations have shown that \(^{3}\text{H}\)-NA
is the major constituent of the total catecholamine
release evoked by electrical stimulation. while it is a
minor component of the spontaneous release of
radiolabelled catecholamines (Taube et al.. 1977: Beattie
et al.. 1986). Hence the evoked efflux of \(^{3}\text{H}\)-NA
reflected a quasi physiological release of NA
(Reichenbacher et al.. 1982).
2.5.2 [³H]-NA release evoked by tyramine (see Chapter 7)

The flow rate was 2.5 ml/min. Superfusate samples were collected for 65 consecutive (1 min each) periods in ice-cold polystyrene vials. The vasa deferentia were perfused 3 times with tyramine (10-300 µM, see section 2.7.2) at the beginning of the 3rd, 33rd and 63rd min. Fractions 6-10: 12-20: 22-30: 36-40: 42-50 and 52-60 were discarded. Aliquots from the remaining samples (0.5 ml) were removed and immediately assayed for ATP content (see section 2.4). The remaining 2 ml was added to 12 ml Aquasol and counted as detailed above. Again the vasa deferentia were solubilized and total radioactivity counted, results were calculated as above.
2.6 NPY assay

The NPY released from the guinea-pig vasa deferentia as described in Chapter 4, was measured in the following way. Samples (0.5 ml with 100 kallikrein inhibitory units/ml aprotinin added) were collected before during and after nerve stimulation as described above (see 2.5.1). NPY levels were quantified using an inhibition enzyme-linked immunosorbent assay (ELISA) in the following manner. For each incubation the wells of the Dynatech polystyrene microelisa plates contained 100 μl of solution. The plates were coated with 0.15 μg/ml NPY in 0.1 mol/l carbonate-bicarbonate buffer, pH 9.6 containing 0.02 % sodium azide by incubating at 18 h at 4 °C. The coated ELISA plates were washed 3 times in phosphate-buffered saline (PBS) containing 0.05 % Tween 20 (PBS-Tween) and incubated at 1 h at room temperature with PBS-Tween containing 0.1 % gelatine to prevent non-specific binding. The plates were emptied by inversion. 50 μl of sample or standard (prepared in the superfusion buffer) were added to the wells (each sample was assayed in triplicate) followed by 50 μl of antiserum raised in rabbits against synthetic porcine NPY diluted 1 in 12,500 in 0.2 % gelatine, 0.1 % Tween, 0.04 % sodium azide in PBS. The plates were covered and incubated for 6 days at 4 °C. The plates were washed 3 times in PBS-Tween. Goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase diluted in 1 in 500 in PBS-Tween containing 0.1 % gelatine was added to each well and plates were
incubated in a humidity chamber for 2 h at 37 °C. The plates were washed 3 times in PBS-Tween and once in 0.1 mol/l glycine buffer containing 1 mmol/l MgCl₂ and 1 mmol/l ZnCl₂ (pH 10.4). p-Nitrophenylphosphate (1 mg/ml in the above glycine buffer) was added to each well and left to develop colour at room temperature. The absorbance was read on a Titertek Multiscan spectrophotometer at 405 nm. The standard absorbance readings were plotted against NPY concentration (0-235 fmol/well) on logarithmic graph paper to give a straight line from which the sample NPY values were obtained. Results are expressed as pmol NPY/g tissue/3.6 ml sample (mean ± S.E.M.). Wells with all solutions except either anti-IgG-alkaline phosphatase, coating NPY or antiserum to NPY, respectively, were run through the assay and gave blank values. The intra-assay variability was 4%. The minimal detectable level of NPY was 0.49 fmol.

This assay was performed by Dr. Pamela Milner.
2.7 Evoked Transmitter Release

The release of $[^3H]$-NA, ATP and NPY from vasa deferentia of rats or guinea-pigs was evoked by two methods, either by electrical field stimulation or by superfusion with drugs (eg. tyramine or exogenous NA).

2.7.1 Field stimulation-evoked release

The prostatic portions of guinea-pig vasa deferentia were mounted between two platinum ring electrodes (2 mm diameter, 10-20 mm apart) attached to Grass SD 9 stimulators. The preparations were stimulated with a variety of stimulation parameters which are outlined in each experimental chapter (see Chapters 3, 4, & 5 for further details). Superfusate samples were collected before (pre-stimulation), during and after (post-stimulation) electrical stimulation and the samples collected for assay as detailed in the experimental chapters concerned and assayed for ATP, $[^3H]$-NA and NPY as discussed above.
2.7.2 Drug-evoked release of neurotransmitters

a) Tyramine

Tyramine was prepared daily as a 10^{-4} M stock solution in sterile water and diluted in Krebs' to obtain the concentration required.

The tyramine-induced release of ATP and [^{3}H]-NA from rat and guinea-pig vasa deferentia was measured as described above. The vasa deferentia of both species were superfused with Krebs' containing tyramine (10-300 µM) for periods of 1 min at 30 min intervals. The preparations were exposed to tyramine 3 times during the course of each experiment. The first period of superfusion with tyramine (S1) acted as a control; the second exposure to tyramine (S2) was carried out in the presence or absence of either prazosin (1 µM) or α,β-methylene ATP (1 µM). The last period of tyramine superfusion (S3) was used either as a control or recovery. Any changes in the tyramine-induced release of either ATP or [^{3}H]-NA were quantified as the S3/S1 ratio.

Tissue superfusates were collected for two min (1 min fractions) prior to, for 1 min during and for two min (1 min fractions) after superfusion with tyramine and assayed for their ATP and [^{3}H]-NA content as described in sections 2.4 and 2.5 above.
b) Noradrenaline

NA was prepared daily as a $10^{-1}$ M stock solution in $10^{-4}$ M ascorbic acid and diluted in Krebs' to the appropriate concentration.

The NA-induced release of ATP from the vasa deferentia of control or pretreated (see section 2.8) rats was examined using a protocol similar to that of tyramine. The preparations were perfused 3 times with Krebs' containing NA (50 μM) for 1 min at 30 min intervals. Superfusion samples were collected as described for tyramine in section 2.7.2a. The effects of prazosin (1 μM) on ATP release during the second period of NA superfusion were investigated.
2.8 Chemical Denervation

Various methods of chemical denervation were applied. The rationale behind such procedures and their known effects are given in greater detail in the experimental chapters concerned.

2.8.1 6-OHDA pretreatment (see Chapter 3)

6-OHDA was dissolved in 1 mg/ml ascorbic acid which had been gassed with oxygen-free nitrogen for at least 15 min; it was prepared immediately prior to use and kept under nitrogen, on ice to prevent oxidation. Guinea-pigs were pretreated with 6-OHDA using the following method: on day 1, they were injected intraperitoneally (i.p) with 100 mg/kg 6-OHDA. On the second day the dose was increased to 250 mg/kg. The guinea-pigs were killed on the third day.

2.8.2 Reserpine pretreatment (see Chapter 3)

Reserpine was dissolved in 200 mg/ml ascorbic acid immediately prior to use. Guinea-pigs were injected (i.p) with reserpine (5 mg/kg) 24 h prior to sacrifice.

2.8.3 Chronic guanethidine pretreatment (see Chapter 8)

Guanethidine monosulphate was obtained in ready prepared ampoules (1 mg/ml). 8 day old rats were injected subcutaneously (s.c) daily, for 6 weeks with 60 mg/kg guanethidine or saline (age-matched controls). The injections continued until the day before the rats were
sacrificed. The rats were killed when they were 7 weeks of age by carbon dioxide overdose to avoid any damage to the delicate cerebral vessels (which were required for another study).

2.8.4 Capsaicin pretreatment

Capsaicin (8-methyl N-vanillyl-6-nonenamide) was dissolved in the following vehicle: 80% saline/ 10% ethanol/ 10% Tween 80 (v/v/v). The capsaicin/vehicle solution was sonicated to ensure that the drug was properly dissolved. One day old rats, which had been anaesthetised by chilling in ice (until all movement had stopped), were then injected (s.c) with 50 mg/kg capsaicin or capsaicin vehicle. The procedure was repeated the following day. After injection, the rat pups were massaged to recovery and returned to their mother. The rats were sacrificed at 7 weeks of age by increasing levels of carbon dioxide.

2.8.5 Capsaicin + Guanethidine pretreatment

In an attempt to obtain a preparation which was devoid of both sensory and sympathetic neurons, 1 day old Wistar rats were initially pretreated with capsaicin or capsaicin vehicle as above and the procedure repeated on the second day. Then, on day eight, the rats were pretreated with guanethidine or saline for 6 weeks as detailed above. The rats were sacrificed at 7 weeks again.
by carbon dioxide overdose.
2.9 Catecholamine content assay

The extent of sympathetic denervation was quantified by measuring catecholamine content of small pieces of the prostatic portion of the vas deferens taken from the pretreated guinea-pigs (Chapter 3) and rats (Chapter 8). NA content was assayed using high pressure liquid chromatography (HPLC).

Experimental Protocol

The vasa deferentia were quickly dissected out, washed in cold Krebs', and then blotted on filter paper before being frozen and stored in liquid nitrogen. Prior to assay, the tissues, after being weighed, were homogenized (using a motor driven glass-glass homogenizer) in 500 μM of perchloric acid (0.1 M) containing 0.4 mM sodium bisulphite and 12.5 ng dihydroxybenzylamine (DHBA). Following low speed centrifugation, the supernatants were subjected to alumina extraction (Keller et al., 1976). NA and DHBA levels were measured using HPLC with electrochemical detection (Moyer & Jiang, 1978). Separation was achieved on a radial 10 μM Bondapak C-18 reverse-phase column (Waters Association) using a mobile phase of 0.1 M sodium hydrogen phosphate (pH 5.0) containing 5 mM heptane sulphonate, 0.1 mM ethylenediaminetetraacetic acid and 10% (v/v) methanol at a flow rate of 1.5 ml/min. Quantification was performed with a waxy carbon paste electrode set at a potential of +0.72 V. NA levels were corrected for recovery using the DHBA as an
internal standard. Tissue content of NA was calculated per mg wet weight tissue.

This procedure was performed by Mr. David Biundell.
2.10 Statistical Analysis

The results in this thesis are expressed as the mean ± standard error of the mean (S.E.M.). 'n' denotes the number of experiments. Statistical significance between two sets of data was tested either by Student's t-test or using a paired t-test where appropriate. When P, the level of probability was < 0.05, values were considered significantly different.
2.11 Drugs and Chemicals used:

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CHAPTER 3.

Sympathetic nerve-mediated release of ATP from the guinea-pig vas deferens is unaffected by reserpine.
3.1 Summary

The release of ATP from the guinea-pig vas deferens was measured using the luciferin-luciferase assay. The effects of reserpine, tetrodotoxin, 6-hydroxydopamine and guanethidine on this efflux were investigated. Reserpine, which produced extensive loss of NA (about 99%) and eliminated the second phase of the nerve mediated contraction, failed to impair either ATP release from the vas deferens or the concomitant 'twitch' contraction; in fact both were slightly increased. Therefore the neurotransmitter mediating the twitch cannot be NA, thus excluding the $\beta$-receptor hypothesis. Furthermore, since the release of ATP is unaffected by reserpine, it is unlikely that ATP is being released from smooth muscle as a consequence of the postjunctional actions of NA. Tetrodotoxin, 6-hydroxydopamine and guanethidine substantially reduced or abolished the release of ATP and both phases of the nerve mediated response. To conclude, in the guinea-pig vas deferens, ATP coreleased with NA from sympathetic nerves, mediates the twitch phase of contraction and its underlying electrical events.
3.2 Introduction

The neurogenic response of the guinea-pig vas deferens is biphasic, consisting of an initial twitch followed by a slower maintained contraction. It has been suggested that the first phase of contraction is nonadrenergic in origin (Ambache & Zar, 1971; Swedin, 1971; Wakade & Krusz, 1972). More recently, much evidence has been presented to indicate that ATP is the neurotransmitter mediating the twitch and its underlying electrical events (Fedan et al., 1981; Meldrum & Burnstock, 1983; Sneddon et al., 1982; Sneddon & Burnstock, 1984a; Sneddon & Westfall, 1984; Stjärne & Astrand, 1984; 1985). The innervation of this tissue is predominantly sympathetic (Burnstock, 1970). Since ATP and noradrenaline (NA) coexist within storage vesicles of sympathetic nerve terminals (Langercrantz, 1976; Smith, 1979), it seem feasible that ATP may be released from these neurons together with NA.

Su et al. (1971) were first to show that ATP and NA can be released together from sympathetic nerves. Since then the role of ATP as a possible cotransmitter has been extensively studied in a variety of tissues including the guinea-pig vas deferens (see Burnstock, 1986c).

Earlier studies using radiolabelled adenosine have shown that purines may be taken up by (Rowe et al., 1978) and released from (Westfall et al., 1978) the guinea-pig vas deferens and that purine efflux is induced by nerve stimulation. Similar results have been obtained for the
vasa deferentia of the mouse (Jenkins et al., 1977) and rat (Fredholm et al., 1982). The inherent difficulties with this type of experiment have been outlined in a recent review by Fredholm and Hedqvist (1980). The major problem in labelling tissues with precursors of ATP is the predominant loading of postsynaptic sites, mainly the smooth muscle cells (Fredholm and Hedqvist, 1980; Fredholm et al., 1982). It is hoped that, by using the ATP-specific enzyme luciferin-luciferase (Lundin and Thore, 1975) to measure the endogenous release of ATP, these difficulties have been avoided.

In this study, the endogenous efflux of ATP from guinea-pig vasa deferentia was studied during electrical stimulation. To test the hypothesis that ATP is released from sympathetic nerves, we examined the endogenous efflux of ATP from vasa deferentia which had been exposed to: 6-OHDA, which destroys sympathetic nerve terminals; TTX, which blocks nerve conduction and guanethidine, which prevents the release of sympathetic neurotransmitters. The effect of reserpine, which depletes NA from sympathetic nerves, is examined in particular, in view of the alternative hypothesis that the twitch response may be mediated by Y-receptors (Hirst and Neild, 1980; 1981).
3.3 Methods

The vasa deferentia were prepared and set up as described in Chapter 2. The preparations were field-stimulated three times, at 30 min intervals for periods of 1 min (pulse width 0.5 ms, 8 Hz, 20 V).

During investigations into the effects of frequency on ATP release, the vasa deferentia were stimulated with trains of 180 pulses at 4, 8 and 16 Hz (0.5 ms, 20 V).

Guinea-pigs were pretreated with 6-OHDA and reserpine as described in section 2.8 and the extent of sympathetic denervation quantified by measuring catecholamine content as described in section 2.9.

TTX (1.6 μM) was superfused for 10 min and guanethidine (5 μg/ml) for 20 min before stimulation and sampling. In some experiments, vasa deferentia removed from reserpinized guinea-pigs were superfused with prazosin (5 μM) for 10 min prior to stimulation and sampling.

All superfusate samples were collected and assayed for ATP content as detailed in Chapter 2. ATP release quantified as nmol/min/g of vas deferens.
3.4 Results

3.4.1 Endogenous ATP release

In 32 control experiments, field stimulation of the guinea-pig vasa deferentia significantly (P < 0.001) enhanced ATP release (Figure 3.1B). During stimulation ATP efflux increased approximately 50 times above spontaneous levels of release, from 0.001 ± 0.001 to 0.18 ± 0.003 nmol/min/g. Cessation of stimulation immediately reduced the amount of ATP released towards that of the spontaneous prestimulation level. There was no significant difference in spontaneous ATP efflux during pre- or postjunctional periods.

When 1 min periods of stimulation (at 8 Hz) were repeated at 30 min intervals, successive stimulation-induced effluxes diminished consistently (Figure 3.1B). In control tissues the S2/S1 ratio was 0.605 ± 0.034. Initially, there was no parallel decrease in 'twitch' height (Figure 3.1A). Since the amount of ATP released during the threshold period of stimulation is supra-threshold, it seems likely that far more ATP is secreted than is actually required to initiate a contraction. There was no significant change in spontaneous ATP levels with successive periods of stimulation. Increasing the frequency of stimulation of the vasa deferentia increased the release of ATP and the concomitant mechanical responses: ATP efflux appeared frequency-dependent (Table 3.1).
Field stimulation of the guinea-pig vas deferens evoked a biphasic mechanical response as shown in the Figure 3.1A. The endogenous NA content of control vasa deferentia is given in Table 3.2.

3.4.2 Effects of reserpine

Perfusates from the vasa deferentia of guinea-pigs pretreated with reserpine \( (n = 12) \) were collected and assayed as before. When electrically stimulated, the vasa deferentia of reserpinized animals released approximately 17% more ATP than did control tissues. This non-significant increase in evoked ATP efflux also occurred during the second and third periods of stimulation. Initially, the spontaneous efflux of ATP was unaffected by the reserpine pretreatment. However, as the experiment progressed the levels of spontaneous release rose. Following the second period of stimulation the increase in spontaneous ATP release was statistically significant \( (P \leq 0.01) \), although no changes in the mechanical activity of the vasa deferentia were observed (see Figure 3.2).

The effects of reserpine on endogenous NA levels in the vas deferens is summarized in Table 3.2. the NA content was reduced by about 99%. The effect of this substantial NA loss on the contractile response of the guinea-pig vas deferens to nerve stimulation is shown in Figure 3.2B. The second, more tonic phase of contraction, which is believed to be mediated by NA, was greatly reduced by reserpine. The twitch was not inhibited by this pretreatment regime but, on the contrary, it was
slightly increased.

3.4.3 Effects of prazosin

There was no significant difference (P > 0.1) in the amount of ATP released from the vasa deferentia of reserpinized guinea-pigs (0.219 ± 0.039 nmol/min per g) than from reserpine-pretreated tissues (n = 6) which had been perfused with prazosin (5 uM) 10 min prior to stimulation (0.124 ± 0.038 nmol/min per g). Whilst the twitch was unaffected by prazosin, any residual noradrenergic component of contraction was abolished.

3.4.4 Effects of TTX

The vasa deferentia were perfused with TTX (1.6 uM) for 10 min prior to stimulation and sampling. During sympathetic nerve stimulation, TTX significantly (P < 0.001) reduced the evoked ATP release by approximately 96 %, such that the ATP content of perfusates following field stimulation in TTX did not significantly differ from spontaneous release levels (Figure 3.3). In all seven experiments, TTX completely abolished the contractile response of the guinea-pig vas deferens, as shown in the upper panel of Figure 3.3, suggesting that the release of ATP and the concomitant contractile response are a consequence of nerve stimulation.
3.4.5 Effects of guanethidine

Guanethidine significantly (P < 0.001) reduced the amount of ATP released from the vasa deferentia (n = 7) in response to sympathetic nerve stimulation but had no effect on spontaneous efflux (Figure 3.3). The residual evoked efflux was accompanied by a diminished contraction which was abolished on perfusion with TTX (1.6 µM; not shown), thus indicating that the remaining contractile response was neurogenic in origin.

3.4.6 Effects of 6-OHDA

Pretreatment with 6-OHDA produced a significant (P < 0.001) decrease in the amount of ATP released from the guinea-pig vasa deferentia in response to sympathetic nerve stimulation (Figure 3.3). Evoked ATP levels were reduced by 93 % compared to control. The amount of ATP released in response to field stimulation was not significantly greater than spontaneous efflux levels. The evoked ATP release remaining after 6-OHDA pretreatment was accompanied by a greatly reduced mechanical response (Figure 3.3, upper panel). 6-OHDA pretreatment reduced endogenous NA content by 94 % (Table 3.2).
3.5 Discussion

In this study, field stimulation of the guinea-pig vas deferens enhanced the endogenous efflux of ATP by approximately 50-fold. This is consistent with the findings of Levitt et al. (1984). The stimulation-evoked efflux of ATP appears to be frequency-dependent, the amount of ATP released increased with increasing frequency and this is most obvious at lower frequencies of stimulation.

The evoked ATP release and the concomitant biphasic contraction were abolished by TTX. TTX did not inhibit the spontaneous efflux of ATP. These results are consistent with those of Westfall et al. (1978) who demonstrated that most of the release of tritium from tissues pretreated with [3H]-adenosine was TTX-sensitive. However, White et al. (1981) reported that the efflux of ATP from field stimulated guinea-pig vasa deferentia was unaffected by TTX and suggested that ATP was not released as a consequence of action potential conduction in nerves; but in that particular study the tissues were continuously exposed to the luciferin-luciferase, which may damage muscle and therefore may release ATP.

Since TTX abolished the biphasic mechanical response and the endogenous release of ATP from the guinea-pig vas deferens, this was taken as evidence to support the view that ATP is released from nerves. In the rat vasa deferentia, it has been proposed that purines may be released from postjunctional sites following contraction.
of the smooth muscle to NA (Fredholm & Hedqvist, 1980; Fredholm et al., 1982). However, in electrically stimulated guinea-pig vasa deferentia where contraction has been prevented by using a hypertonic bathing medium (Westfall et al., 1978) there is still considerable release of tritiated purines, suggesting that in this tissue a major component of ATP released is not from the smooth muscle. Furthermore, in the present study the release of ATP was not diminished following removal of NA with reserpine. It is unlikely that, after reserpinization, any residual NA could act postjunctionally to release ATP since, in reserpinized vasa deferentia, neither the efflux of ATP nor the concomitant twitch were reduced by prazosin, which is known to antagonise via α-adrenoceptors, the postjunctional effects of NA (Cavero & Roach, 1980). The inability of prazosin to impair the release of ATP from reserpinized vasa deferentia also suggests that newly synthesized NA (Duval et al., 1986) does not act postjunctionally to release ATP. Furthermore, NA in concentrations ranging from 0.1 to 10 μM failed to release ATP from control vasa deferentia (unpublished observations).

The hypothesis that ATP and NA are released as cotransmitters from sympathetic nerves in the guinea-pig vas deferens was tested by examining the efflux of ATP from vasa deferentia of animals exposed to substances known to disrupt sympathetic neurotransmission. Pretreatment with 6-OHDA depletes peripheral NA stores by
anatomically and functionally destroying sympathetic nerve terminals (Thoenen & Tranzer, 1968). In this study, 6-OHDA produced substantial loss of endogenous NA from the vasa deferentia, and this may be taken as evidence for the destruction of sympathetic nerve terminals. The amount of evoked ATP release from the vasa deferentia of 6-OHDA pretreated animals was negligible indicating that sympathetic nerves were the source of neuronally released ATP. The residual ATP release on nerve stimulation was accompanied by a greatly diminished mechanical response.

The sympathetic neuron-blocking drug guanethidine greatly diminished the evoked efflux of ATP and the subsequent neurogenic response in the guinea-pig vasa deferentia. Su et al. (1971) showed that release of both NA and ATP from sympathetic nerves supplying the guinea-pig taeni coli was prevented by guanethidine.

Unlike 6-OHDA, reserpine does not anatomically destroy adrenergic nerve terminals but depletes central and peripheral stores of NA by blocking the uptake mechanism in granular vesicles (Anden et al., 1969; Iversen, 1967). In the present study, pretreatment with reserpine substantially reduced the second, noradrenergic (Swedin, 1971) phase of contraction but slightly enhanced the initial contractile response. The twitch response of the vas deferens is dependent on the summation of excitatory junction potentials (EJPs) (Burnstock & Holman, 1961; Blakeley et al., 1981). Previous studies have shown that neither the EJPs nor the twitch are reduced by
reserpine (Ambache & Zar, 1971; Burnstock et al., 1964; Swedin, 1971; Sneddon & Westfall, 1984; Stjärne & Astrand, 1985). Since this pretreatment regime produced extensive loss of NA from the vasa deferentia (Wakade & Krusz, 1972; this study), the neurotransmitter mediating the EJP and the twitch cannot be NA. This therefore appears to exclude the ⍺-receptor hypothesis (Hirst & Neild, 1980: 1981). Furthermore, since pretreatment with reserpine failed to abolish the release of ATP from the stimulated vasa deferentia, we conclude that ATP is the neurotransmitter mediating the EJP and the consequent twitch response in this tissue. This conclusion is strongly supported by previous studies (Fedan et al., 1981; Meldrum & Burnstock, 1983; Sneddon et al., 1982; Sneddon & Burnstock, 1984a; Sneddon & Westfall, 1981).

The results presented here are consistent with the view that NA, via prejunctional α-adrenoceptors, can inhibit the twitch response in the guinea-pig vas deferens (Ambache & Zar, 1971; see Vizi, 1979). In this study, pretreatment with reserpine produced a substantial loss of endogenous NA from guinea-pig vasa deferentia and under these circumstances, the evoked release of ATP and the concomitant twitch were enhanced. A similar effect has been shown by Stjärne and Astrand (1985) in the guinea-pig (but not the mouse) vas deferens.

In summary, NA does not mediate via postjunctial ⍺-receptors the twitch response of the guinea-pig vas deferens, since severe NA loss after reserpine
pretreatment failed to impair the initial component of contraction. Furthermore, in reserpinized tissues, the twitch is not mediated by newly synthesized NA since the α-adrenoceptor antagonist, prazosin, did not significantly decrease the contractile response. Since the efflux of ATP was unaffected by reserpine and reserpine plus prazosin pretreatment, but decreased by pretreatments known to destroy sympathetic neurons, we conclude that, in the guinea-pig vas deferens, the twitch and its underlying EJPs are mediated by ATP coreleased with NA from sympathetic neurons.
Table 3.1  Effect of electrical stimulation at various frequencies (0.5 ms; 20 V; 480 pulses) on the release of ATP from guinea-pig vasa deferentia.

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>nmol ATP released/g of vas deferens</th>
<th>Number of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.042 ± 0.010</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>0.164 ± 0.019</td>
<td>56</td>
</tr>
<tr>
<td>16</td>
<td>0.186 ± 0.026</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 3.2  Effects of pretreatments on endogenous NA levels.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>NA levels (ug/g) mean ± S.E.M.</th>
<th>% of control</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.682 ± 1.223</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Reserpine</td>
<td>0.140 ± 0.059***</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>0.829 ± 0.030***</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

*** P ≤ 0.001.
Figure 3.1

Release of endogenous ATP from control vasa deferentia (n=32) during field stimulation at 8 Hz (pulse width 0.5 ms, 20 V). A. Shows the contractile responses of the guinea-pig vas deferens to field stimulation. The vasa deferentia were stimulated three times at 30 min intervals for 1 min. B. Shows the mean ± S.E.M. ATP release nmol/min /g of vas deferens.
nmoles ATP released/min/g of vas deferens
Figure 3.2
Release of endogenous ATP from vasa deferentia removed from guinea-pigs pretreated with reserpine. The preparations were stimulated three times at 30 min intervals for 1 min (8 Hz, 0.5 ms, 20 V). A. Shows the contractile responses, note that the second slow phase of the mechanical response has gone in reserpinized tissues. B. the effects of reserpinization on the release of ATP mean ± S.E.M. ATP release nmol/min per g. ** P < 0.01.
nmoles ATP released/min/g of vas deferens
Figure 3.3

Release of endogenous ATP from control (n = 32), 6-OHDA pretreated (n = 7), TTX (1.6 μM, n = 7) and guanethidine (GUAN) exposed (5 μg/ml, n = 7) guinea-pig vasa deferentia during field stimulation at 8 Hz (0.5 ms, 20 V). Preparations were electrically stimulated for 1 min. Vasa deferentia were perfused with guanethidine for 20 min and TTX for 10 min prior to stimulation and sampling. A. shows contractile responses to field stimulation in control and pretreated tissues. B. mean ± S.E.M. ATP release nmol/min per g of vas deferens. *** P < 0.001.
nmoles ATP released/min/g of vas deferens

![Graph showing ATP release](image)
Direct evidence for concomitant release of noradrenaline, ATP and NPY from sympathetic nerves supplying the guinea-pig vas deferens.
4.1 Summary

Concomitant release of $[^3H]^{-}$-NA: ATP (using the luciferin-luciferase firefly technique) and NPY (using the enzyme linked immunosorbant assay) during electrical stimulation of the guinea-pig vas deferens has been demonstrated. The release of $[^3H]^{-}$-NA and ATP is unaffected following selective desensitization of the $P_{X}$ purinoceptor by $\alpha,\beta$-meATP. Furthermore, complete block of the postjunctional responses in this tissue due to nerve stimulation by a combination of prazosin and $\alpha,\beta$-meATP does not reduce the release of $[^3H]^{-}$-NA and ATP. These results are consistent with the hypothesis that NA, ATP and NPY are released as cotransmitters or modulators in the sympathetic nerves supplying the guinea-pig vas deferens.
4.2 Introduction

Since the concept of cotransmission was first raised (Burnstock, 1976), evidence has been growing that most autonomic nerves store several substances with neurotransmitter properties. They are co-secreted upon nerve stimulation and utilised as cotransmitters (see Cuello, 1982; Osborne, 1983; Chan-Palay & Palay, 1984; Hökfelt et al., 1986). Cotransmission has been investigated extensively in the guinea-pig and rat vas deferens: these tissues have a predominantly sympathetic motor innervation (Swedin, 1971) and were initially considered as models of 'pure' noradrenergic neurotransmission (Hukovic, 1961). This was later challenged (Ambache & Zar, 1971) and it is now widely accepted that the neurogenic contraction in the guinea-pig and rat vas deferens is biphasic, involving at least two substances released as cotransmitters: ATP triggers the initial twitch phase of the response (Westfall et al., 1978; Fedan et al., 1981; Meldrum & Burnstock, 1983; Cunnane & Stjärne, 1984; Sneddon & Westfall, 1984; Stjärne & Astrand, 1985; Allcorn et al., 1986; Kirkpatrick & Burnstock, 1987; Lew & White, 1987), while NA is responsible for the slow second phase (Swedin, 1971; McGrath, 1978). A third substance implicated in sympathetic neurotransmission in vas deferens is NPY (Huidobro-Toro et al., 1985; Stjärne et al., 1986). Stjärne and Lundberg (1986) concluded that while the contractile response of the vas deferens results from the
participation of ATP and NA. NPY has a dual function, initially producing postsynaptic potentiation of ATP and NA mediated responses and later prejunctional inhibition of release of ATP and NA.

Despite numerous publications in this field, there has been no direct evidence for a concomitant release of ATP, NA and NPY upon excitation of the sympathetic nerves innervating the guinea-pig vas deferens. The aim of the present investigation therefore was to quantify the release of ATP, NA and NPY from guinea-pig vas deferens preparations during electrical field stimulation and to clarify the source from which these substances are released.
4.2 Materials and Methods

The guinea-pig vasa deferentia were prepared and loaded with $[^3H]-NA$ as described in Chapter 2. In this study, the prostatic portions of the vas deferens were additionally bisected along their length and they were superfused at a rate of 1.2 ml/min. During the experiment the preparations were electrically stimulated for 1 min (0.1 ms. 9 Hz. 50 V) at 30 min intervals. The vasa deferentia were stimulated twice at the beginning of the 4th and 16th collection (9 and 45 min respectively). The first stimulation ($S_1$) acted as a control: the second period of stimulation ($S_2$) was performed in the presence/absence of drug(s) under investigation. Superfusion of the preparation with Krebs' containing drug(s) began at 28 min until 63 min after the beginning of fraction collection. Superfusate was assayed for $[^3H]-NA$, ATP and NPY as described in the Materials and Methods chapter.
4.3 Results

Four parameters have been simultaneously monitored in this investigation: the mechanical activity, the efflux of \[^{3}H\]-NA and the endogenous release of ATP and NPY.

4.3.1 'No drugs' responses

The contractile response of the vas deferens during neurotropic electrical stimulation consisted of two clearly distinguishable phases: an initial twitch response which reached its maximum in 2-3 s then rapidly faded and a slow sustained phase which reached its maximum in 12-15 s and slowly decreased in amplitude lasting the duration of stimulation (Figure 4.1). The second period of stimulation, performed 36 min after the first, induced a similar contraction except that the first phase was slightly attenuated. The radioactivity of the superfusate collected during stimulation periods showed a substantial short-lasting increase in \[^{3}H\]-NA overflow (Figure 4.1). The overflow induced by the second stimulation was almost unchanged, the $S_2/S_1$ ratio was 0.956 ± 0.059 (n=8). The quantitation of ATP and NPY in the same superfusate showed a definite and short-lived increase over the basal levels (Figure 4.1) and in control experiments the $S_2/S_1$ ratio for ATP release was 0.814 ± 0.05 (n=8). However, the mean percent release of NPY over basal levels was higher during the second stimulation period (549 ± 117 %) than during the first stimulation period (210 ± 32 %, n=7, P < 0.02).
4.3.2 Effects of tetrodotoxin

The vasa deferentia were superfused with TTX (1 μM) for 18 min before the second stimulation period. TTX completely blocked the stimulation-induced contractions and substantially reduced the evoked efflux of \(^{3}H\)-NA and ATP. In the presence of TTX, the \(S_2/S_1\) ratio for \(^{3}H\)-NA efflux was reduced from 0.956 ± 0.059 in 'no drugs' experiments to 0.004 ± 0.001 (n=5, P < 0.001) and the \(S_2/S_1\) ratio for ATP decreased to 0.031 ± 0.013 (n=5, P < 0.001). NPY release during stimulation period with TTX was 3.2 ± 1.4 pmol/sample/g tissue. No drug experiments, while TTX did not significantly change the spontaneous outflow of \(^{3}H\)-NA (10/3 ratio was 0.786 ± 0.035, n=5). ATP (basal release was 1.00 ± 0.2 pmol/sample/g, n=5) or NPY (basal release with TTX was 4.3 ± 1.3 pmol/sample/g tissue, n=4).

4.3.3 Effect of \(\alpha,\beta\)-methylene ATP and prazosin

In order to block the contractile response postjunctionally whilst keeping the nerves intact, \(\alpha,\beta\)-methylene ATP (1 μM), a known \(P_{2x}\) purinoceptor desensitizing agent (Kasakov & Burnstock, 1983) that eliminates the purinergic component of the vas deferens contractile response (Kirkpatrick & Burnstock, 1987), and prazosin (1 μM), a selective \(\alpha_1\)-adrenoceptor antagonist (Cambridge et al., 1977) eliminating the noradrenergic component of the response (Reichenbacher et al., 1982; Kirkpatrick & Burnstock, 1987), have been applied together. As can be seen in Figure 4.3, this treatment completely inhibited
the stimulation-induced contraction. At the same time the release of \(^3\)H-NA was even slightly potentiated (\(S_2/S_1\) ratio was 1.136 ± 0.048, n=1) as was the release of ATP (in 2 out of 4 experiments the stimulation-evoked release of ATP was potentiated whilst in the other two it was the same as for \(S_1\)). The \(S_2/S_1\) ratio for ATP release was 0.941 ± 0.073 (n=4). In the same series of experiments a large increase in the spontaneous efflux of \(^3\)H-NA was found (10/3 ratio was 3.418 ± 0.091, n=4). There was no significant change in the spontaneous release of ATP.

4.3.4 Effect of \(\alpha,\beta\)-methylene ATP

The effect of \(\alpha,\beta\)-meATP on the mechanical activity and the release of \(^3\)H-NA and ATP is summarised in Figure 4.4. \(\alpha,\beta\)-meATP abolished the initial fast component of the contraction while the second noradrenergic component remained unchanged. The concomitant release of \(^3\)H-NA was not affected (\(S_2/S_1\) ratio was 1.066 ± 0.139, 10/3 ratio was 0.924 ± 0.131, n=4). \(\alpha,\beta\)-me ATP did not significantly affect either the evoked or basal release of ATP. the \(S_2/S_1\) ratio for ATP release was 0.786 ± 0.065 (n=1).

4.3.5 Effect of prazosin

As shown in Figure 4.5, prazosin (1 uM) was able to antagonise completely the slow second phase of the contraction, being ineffective on the first phase or on the basal tone of the preparations. However, the drug increased the spontaneous outflow of \(^3\)H-NA (10/3 ratio
was 2.453 ± 0.515 (n=4) and to a lesser extent the stimulation-evoked release of [³H]-NA as well (S₂/S₁ ratio was 1.325 ± 0.112. n=4). An extensive investigation into the prejunctional actions of prazosin is now in progress (Ellis et al., 1989). The evoked release of ATP was not significantly reduced by superfusion with prazosin. the S₂/S₁ ratio was 0.644 ± 0.058 (n=4).
This investigation provides the first direct evidence for the concomitant release of NA, ATP and NPY from the sympathetic nerves supplying the guinea-pig vas deferens. We have already shown using 6-OHDA pretreatment, that NA and ATP are released from sympathetic nerves in this tissue (Kirkpatrick & Burnstock, 1987) and others have shown that the tissue content of both NA and NPY in the guinea-pig vas deferens is substantially reduced after 6-OHDA treatment (Nagata et al., 1987). Thus, there is clear evidence that ATP, NA and NPY are contained in sympathetic nerves. There is no easy way to show whether all 3 substances come from single sympathetic nerves or from different populations of sympathetic nerves. However, it seems likely that they are all contained in individual fibres, albeit in variable proportions, in view of the localisation of NPY and NA within the same profiles as seen with electron microscopy and electron microscopic-immunohistochemistry (Fried et al., 1985; Stjärne et al., 1986; Fehér & Burnstock, 1987).

The finding that ATP, NA and NPY are released upon stimulation of the sympathetic nerves of the guinea-pig vas deferens satisfies one of the three criteria for cotransmission i.e. that the substances must be shown to be stored, released and act postjunctionally on their own receptors as neurotransmitters concomitantly (Burnstock, 1986 a,b). This study did not distinguish between transmitter or modulator roles for NPY.
It has been suggested that the main source of ATP released in response to nerve stimulation might be a postjunctional myogenic pool (Fredholm & Hedqvist, 1980; Fredholm et al., 1982). However, two findings in the present study argue against this possibility: (1) the release of ATP was completely blocked by TTX and (2) the release of ATP was not affected when the nerves remained intact, but the mechanical response was entirely blocked postjunctionally by α,β-meATP and prazosin. In addition, Kirkpatrick and Burnstock (1987) have reported that ATP release was not reduced following NA depletion with reserpine pretreatment, so it appears that the levels of ATP released are not influenced by postjunctional actions of NA. The effectiveness of α,β-meATP as a specific purinoceptor desensitizing agent (Kasakov & Burnstock, 1983) have been challenged (Byrne & Large, 1986; Amobi & Smith, 1987) and it was suggested that α,β-meATP may have a non-specific pre- or postjunctional inhibitory action. However, this seems unlikely, because in the present study α,β-meATP antagonised effectively only the fast purinergic component of the contractile response, being without effect on the second noradrenergic phase of the contraction. Furthermore, the drug had no significant effects on the release of [³H]-NA and ATP. In an attempt to explain the failure of adrenergic blocking agents to antagonise the neurogenic responses in some tissues with sympathetic motor innervation, Hirst and Neild (1980; 1981) suggested the existence of a third class of
adrenoceptor, the so called β-adrenoceptor. However, Kirkpatrick and Burnstock (1987) showed that the prazosin resistant component of the contractile response was unaffected or if anything slightly potentiated, following pretreatment with reserpine. In conclusion, the present investigation shows that NA, ATP and NPY are co-secreted upon electrical stimulation of the sympathetic nerves supplying the guinea-pig vas deferens and then might be utilised as cotransmitters and/or modulators.
Figure 4.1

'No drugs' contractile response of guinea-pig vas deferens induced by electrical stimulation (▲-on; ▼-off) and the corresponding fractional rate of [³H] NA, ATP and NPY release measured in the superfusate. Upper panel shows the effects of electrical stimulation of the prostatic portion of the guinea-pig vas deferens for 1 min (9 Hz, 50 V, 0.1 ms). Note the different time scales: the mechanical activity is recorded at 50 mm/min; each point in the [³H]-NA fractional rate graph represents the values in the superfusate of 11 consecutive 3 min collections. The columns represent the levels of ATP and NPY in the superfusate of the prestimulation, stimulation and poststimulation 3 min collections. S₁ and S₂ bars mark the duration of the stimulus (1 min). The stimulation-evoked increase of [³H]-NA fractional rate and ATP levels are significantly different from pre- and poststimulation values (P < 0.01, n=8). NPY levels are significantly higher during stimulation periods compared to prestimulation periods (paired t-test: S₁, P < 0.02, n=12; S₂, P < 0.05, n=7).
Figure 4.2

The effect of TTX on the electrically induced (same parameters as before) contractile response of the guinea-pig vas deferens and the corresponding fractional rate of \(^{3}\text{H}\)-NA and ATP release in the superfusate. For explanations see legend to Figure 4.1. The differences in the fractional rate of \(^{3}\text{H}\)-NA and ATP in the presence of TTX are significantly different from the 'no drugs' values (\(P < 0.001, n = 5\)).
Figure 4.3

The effect of α,β-meATP and prazosin on the electrically induced contractile responses of the guinea-pig vas deferens and the corresponding fractional rate of [3H]-NA and ATP release in the superfusate. For explanations see legend to Figure 4.1. The differences in the values of the basal outflow and the electrically evoked overflow of [3H]-NA in the presence of α,β-meATP and prazosin (10/3 ratio; S2/S1 ratio) are significantly different from the 'no drugs' values (P < 0.01, n=1). Neither the basal nor the evoked release of ATP were significantly increased in the presence of α,β-meATP and prazosin.
ATP pmol/sample

\[ \text{CO}_{3}^{2-} \text{MTTP (1mm) + Prazosin (1um)} \]

\[ \text{CO}_{3}^{2-} \text{MTTP (1mm) + Prazosin (1um)} \]

\[ \text{CO}_{3}^{2-} \text{MTTP (1mm) + Prazosin (1um)} \]

\[ \text{CO}_{3}^{2-} \text{MTTP (1mm) + Prazosin (1um)} \]

\[ \text{CO}_{3}^{2-} \text{MTTP (1mm) + Prazosin (1um)} \]

\[ \text{CO}_{3}^{2-} \text{MTTP (1mm) + Prazosin (1um)} \]
Figure 4.4

Effect of α,β-meATP on the electrically induced contractile responses of the guinea-pig vas deferens and the corresponding fractional rate of [³H]-NA and ATP release in the superfusate. For explanation see legend to Figure 4.1. α,β-meATP had no significant effects on the release of ATP.
Figure 4.5

Effect of prazosin on the electrically induced contractile responses of the guinea-pig vas deferens and the corresponding fractional rate of $[^3\text{H}]$-NA and ATP release in the superfusate. For explanations see legend to Figure 4.1. The differences in the values of the basal outflow and the electrically evoked release of $[^3\text{H}]$-NA in the presence of prazosin (10/3 ratio; $S_3/S_1$ ratio) are significantly different from 'no drugs' values ($P < 0.05$, $n=4$). Prazosin did not significantly reduced the evoked release of ATP.
Evidence that the inhibition of ATP release from sympathetic nerves by adenosine is a physiological mechanism.
5.1 Summary

Superfusion with the P1-purinoceptor agonist, adenosine (1-500 μM) greatly reduced the stimulation-induced release of ATP and the initial contractile phase of the response of the guinea-pig vas deferens to field stimulation. The inhibitory effects of adenosine (100 μM) were attenuated by the P1-purinoceptor antagonist, 8-phenyltheophylline (10 μM). 8-phenyltheophylline also enhanced the basal release of ATP. In contrast, the adenosine uptake inhibitor, dipyridamole (10 μM) inhibited the stimulation-evoked release of ATP from the guinea-pig vas deferens and reduced the initial component of contraction.

These results support the view that adenosine, resulting from ectoenzymatic breakdown of ATP released as a cotransmitter from sympathetic nerve terminals, acts as a physiological prejunctional regulator of transmitter release.
5.2 Introduction

The guinea-pig vas deferens has a rich supply of sympathetic nerves (Sjöstrand, 1962; Burnstock, 1970) arranged in a dense 'ground plexus' around the tissue (Hillarp, 1946). Electrical stimulation of these motor nerves elicits a biphasic mechanical response consisting of a rapid phasic contraction or twitch, with pharmacological properties characteristic of a non-adrenergic mediator (Ambache & Zar, 1971) and a slower tonic contraction which typifies a noradrenergic response (Swedin, 1971; McGrath, 1978). Much evidence has been presented to indicate that ATP is released as a cotransmitter with noradrenaline from the sympathetic nerves of the vas deferens and that ATP is the neurotransmitter mediating the twitch and its underlying electrical events (Fedan et al., 1981; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984a; Allcorn et al., 1986; Stjärne, 1989; Burnstock, 1990). Indeed, studies using the ATP-specific enzyme luciferin-luciferase (Lundin & Thore, 1975) have quantified the stimulus-evoked release of endogenous ATP from this tissue (White et al., 1981; Kirkpatrick & Burnstock, 1987; Lew & White, 1987; Kasakov et al., 1988; Drake & Petersen, 1992).

Extracellular ATP released either as a cotransmitter from sympathetic nerves (see above) or secondarily as a result of the action of noradrenaline in several tissues of the rat (Fredholm & Hedqvist, 1980; Fredholm et al., 1982; Vizi & Burnstock, 1988) is rapidly degraded to

Drury and Szent-Györgyi (1929) were the first to demonstrate the profound ability of adenosine to modify physiological processes. The findings of subsequent extensive studies concerning the extracellular role of adenosine have been reviewed elsewhere (see Hedqvist & Fredholm, 1976; Stone, 1981; White, 1988). Ginsborg & Hirst (1972) were also first to demonstrate that adenosine could modify the output of neurotransmitters, when they showed that adenosine could decrease the release of acetylcholine from rat phrenic nerve terminals. More recent studies have shown that adenosine is able to inhibit the release of neurotransmitters from nerve terminals innervating both the central and peripheral nervous systems (Fredholm & Dunwiddie, 1988; Williams, 1989).

In the guinea-pig vas deferens, Sneddon et al. (1984) demonstrated that adenosine and its more potent analogue, 2-chloroadenosine (Muller & Paton, 1979), inhibited the EJPs and the twitch response; an effect which was reversed by the more effective and more specific P1-purinoceptor antagonist 8-PT (Smellie et al., 1979; Bruns, 1981; Griffiths et al., 1981). Thus it would appear that, in the guinea-pig vas deferens, adenosine mediates inhibition of transmitter release via prejunctional P1-purinoceptors.

The adenosine uptake inhibitor dipyridamole (Baer &
Yriend, 1984) has been shown to potentiate the inhibitory action of adenosine on the twitch response of the vas deferens of the rat (Clanachan et al., 1977; Muller & Paton, 1979). If endogenous adenosine plays a physiological role in inhibiting ATP release in the vas deferens then one would expect dipyridamole, by increasing the concentration of adenosine at the prejunctional nerve terminal, to inhibit the endogenous release of ATP.

Thus the aim of this study was to investigate, by use of the ATP-sensitive enzyme luciferin-luciferase, that adenosine did inhibit the endogenous release of ATP in the guinea-pig vas deferens and to further investigate its physiological role in this tissue.
5.3 Methods

Guinea-pig vasa deferentia were prepared and set up as described in section 2.1. They were field stimulated at 30 min intervals for 1 min (8 Hz, 20 V, 0.5 ms) three times during the course of an experiment. The vasa deferentia were continuously perfused at a rate of 2.5 ml/min and allowed to equilibrate for 90 min prior to experimentation. All superfusate samples were collected in ice-cold vials and assayed for ATP immediately as described in section 2.1.

8-PT was dissolved in 80 % v/v methanol 20 % NaOH and diluted in fresh Krebs' to obtain the concentration required. The dipyridamole was purchased in ready to use ampoules.
5.4 Results

5.4.1 Electrical stimulation

Field stimulation of the guinea-pig vas deferens (8 Hz, 20 V, 0.5 ms) for 1 min resulted in a statistically significant (n=12, P < 0.001) increase in the endogenous release of ATP, from a prestimulation level of 0.005 ± 0.001 to 0.178 ± 0.029 nmol/min/g as shown in Figure 5.1. The dramatic increase in ATP release was accompanied by a biphasic response characteristic of this tissue (see Figure 5.1). On cessation of electrical stimulation, the evoked release of ATP rapidly declined towards prestimulation levels and the vasa deferentia relaxed.

When 1 min periods of stimulation (parameters as before) were repeated at 30 min intervals, successive stimulation-evoked effluxes decreased consistently (Figure 5.1). There was no change in the spontaneous release of ATP. In each of twelve experiments, the reduction in evoked ATP efflux during the second (S₂) period of stimulation was expressed as a ratio of the ATP released during the first stimulus (S₁). In control experiments, the mean S₂/S₁ ratio of released ATP was 0.667 ± 0.052. Similarly, the twitch height obtained during S₂ was expressed as a ratio of the S₁ twitch height. The S₂/S₁ ratio of twitch heights in control tissues was 0.931 ± 0.008 (Figure 5.2).
5.4.2 Effects of adenosine

Superfusion of the guinea-pig vas deferens with adenosine (1-500 μM) for 10 min prior to stimulation and sampling greatly reduced the evoked release of ATP; this inhibition was dose-dependent and statistically significant at concentrations greater than 10 μM (see Figure 5.2). Adenosine also inhibited the twitch component of the mechanical response. This inhibition was statistically significant at all concentrations investigated. Adenosine (100 μM) significantly (n=5, P < 0.005) reduced the S2/S1 ratio for ATP release from 0.667 ± 0.052 to 0.267 ± 0.08 and markedly diminished the initial component of the biphasic response (Figure 5.3). The mean S2/S1 ratio for twitch height was significantly (P < 0.001) reduced from 0.931 ± 0.008 to 0.386 ± 0.053.

It is interesting to note that, at higher concentrations (> 100 μM), adenosine also increased the basal release of ATP. Washing with normal Krebs solution for 30 min significantly (P < 0.05) increased the stimulus-evoked release of ATP and restored the twitch response (see Figure 5.3).

5.4.3 Effects of 8-phenyltheophylline

Adenosine (100 μM) in the presence of 8-PT (10 μM) failed to significantly (P > 0.05) decrease the evoked efflux of ATP from vasa deferentia (n=5) perfused with 8-PT (10 μM) for 10 min prior to electrical stimulation (see Figure 5.4). A comparison of the S2/S1 ratios of control
vasa and tissues stimulated in the presence of adenosine and 8-PT revealed that perfusion with Krebs' solution containing a cocktail of the latter increased the evoked release of ATP. The S2/S1 ratio for ATP release in the presence of adenosine and 8-PT was 0.805 ± 0.112 compared to a S2/S1 ratio of 0.007 ± 0.05 in control experiments. Furthermore, in the presence of adenosine and 8-PT, the spontaneous efflux of ATP appeared to be enhanced (P < 0.001). The contractile response appeared little affected in the presence of adenosine and 8-PT (Figure 5.4); the S2/S1 ratio for twitch heights was 0.91 ± 0.033.

5.4.4 Effects of dipyridamole

Perfusion with dipyridamole (10 uM) for 20 min prior to the second period of electrical stimulation appeared to reduce the stimulation-evoked release of ATP (Figure 5.5): the S2/S1 ratio was decreased from 0.667 ± 0.052 to 0.491 ± 0.05. Dipyridamole also significantly (P < 0.05) diminished the height of the twitch response. The mean S2/S1 ratio was reduced from 0.931 ± 0.008 to 0.893 ± 0.019. Occasionally, in the presence of dipyridamole, the second component of the contraction was enhanced (Figure 5.5).
5.5 Discussion

In agreement with previous studies, electrical stimulation of the guinea-pig vas deferens greatly enhanced the endogenous efflux of ATP and subsequently evoked a mechanical response which was biphasic in nature (Kirkpatrick & Burnstock, 1987; Kasakov et al., 1988; Lew & White, 1987). These findings are consistent with the view that ATP is released as a cotransmitter from the sympathetic nerves innervating this tissue (see Introduction).

Sneddon et al. (1984) reported that exposure to adenosine markedly reduced EJPs in the guinea-pig vas deferens. In the present study, we have confirmed the initial observations made by Sneddon et al. (1984), and by demonstrating that adenosine inhibits the endogenous release of ATP, we provide still further evidence to support the hypothesis that ATP mediates the twitch and its underlying electrical events in this tissue. Adenosine also inhibited the initial component of contraction, which is a finding consistent with previous studies (Clanachan et al., 1977; Muller & Paton, 1979; Taylor et al., 1983; Sneddon et al., 1984).

Theophylline has been shown to antagonize the presynaptic actions of adenosine (Clanachan et al., 1977; Muller & Paton, 1979; Taylor et al., 1983) and thus, it is hardly surprising that the more effective (Smellie et al., 1979; Bruns, 1981) and more specific (Griffiths et al., 1981) Pi-purinoceptor antagonist 8-PT is also capable of
Inhibiting the presynaptic effects of adenosine. In the guinea-pig vas deferens, Sneddon et al. (1984) demonstrated that 8-PT could reverse the reduction of EJP amplitude produced by adenosine. The fact that in the present study the inhibitory actions of adenosine were so readily reversed by 8-PT further suggests that, in the guinea-pig vas deferens, adenosine mediates its effects via prejunctional P1-purinoceptors. There are at least two subtypes of P1-purinoceptor (see Burnstock & Buckley, 1985; Kennedy, 1990; Burnstock, 1991; Jacobson et al., 1992). Since methylxanthines such as 8-PT act at both the A1 and A2 P1-purinoceptor subtypes, the results presented in this study provide no information as to which receptor subtype mediates the prejunctional inhibitory actions of adenosine in the guinea-pig vas deferens, although they are believed to be of the A1-subtype (Paton, 1981). The A1-receptor has been shown to inhibit EJPs and the twitch in the mouse vas deferens (Blakeley et al., 1988). Further studies with more specific antagonists (see Williams, 1987; Burnstock, 1991) are now required to investigate this in the guinea-pig vas deferens.

The precise mechanism underlying the prejunctional inhibitory action of adenosine on neurotransmitter release is as yet unknown but it seems likely that more than one subcellular transduction mechanism is involved (see Stiles, 1986; Fredholm & Dunwiddie, 1988).

To date, there has been little compelling evidence to suggest a physiological role for adenosine in the guinea-
pig vas deferens. Whilst in the rat vas deferens Muller & Paton (1979) and Taylor et al. (1983) have shown that adenosine uptake inhibitors can potentiate the inhibitory effects of exogenously applied adenosine. Any attempts to potentiate the effects of endogenous adenosine, by either increasing its concentration at the prejunctional membrane or by inhibition of P1-purinoceptors have met with mixed success (Katsuragi & Su, 1982; Sneddon et al., 1984). In the guinea-pig vas deferens, neither dipyridamole or 8-PT alone were capable of significantly enhancing the effects of endogenous adenosine on EJPs, although dipyridamole did occasionally reduce EJP magnitude (Sneddon et al., 1984). We have shown for the first time that the adenosine uptake inhibitor dipyridamole (Baer & Vriend, 1984), by increasing the concentration of adenosine at the prejunctional membrane, can inhibit the endogenous release of ATP from the guinea-pig vas deferens and subsequently reduce the initial component of contraction. This provides supporting evidence to suggest a physiological role for adenosine in this tissue.
Figure 5.1

The guinea-pig vasa deferentia were field stimulated three times (S1, S2, and S3) at 30 min intervals for 1 min periods at 8 Hz (pulse width 0.5 ms, 20 V). A. The characteristic biphasic mechanical response obtained upon electrical stimulation of this tissue. B. The mean ± S.E.M. efflux of ATP (nmol/min/g of vas deferens) released from the tissues 1 min before, during and after field stimulation. n = 12.
nmol ATP released/min/g of vas deferens

B

A
Figure 5.2

The effects of perfusion with increasing concentrations of adenosine (1-500 μM) for 10 min prior to stimulation of the guinea-pig vas deferens (same parameters as before) A. the evoked efflux of ATP (depicted as the S₂/S₁ ratio) and B. twitch height (given as S₂/S₁ ratio). For each point, n = 5. *, P < 0.05. **, P < 0.005. ***, P < 0.001. ****, P < 0.0005.
% Inhibition of ATP release

% Inhibition of twitch

log [Adenosine (M)]

A

B

log [Adenosine (M)]

-7

-6

-5

-4

-3

0

20

40

60

80

100

0

20

40

60

80

100

-7

-6

-5

-4

-3

0

20

40

60

80

100
The effect of adenosine (100 μM) on the mechanical response and endogenous release of ATP from the guinea-pig vas deferens during electrical stimulation (8 Hz, 0.5 ms, 20 V) for 1 min. Perfusion with adenosine (100 μM) for 10 min prior to the second period of electrical stimulation (S2) markedly reduced the first phase of the contraction. Similarly, the evoked release of ATP was significantly reduced (n=6; **, P < 0.005). The twitch and the release of ATP were enhanced after 30 min washing with fresh Krebs's solution.
The effects of superfusion with adenosine (100 μM) + 8-PT (10 μM) for 10 min prior to field stimulation (8 Hz, 20 V, 0.5 ms) on the mechanical response and release of ATP from the vasa deferentia of the guinea-pig. A. In the presence of 8-PT, adenosine failed to attenuate either the twitch response or B. the endogenous efflux of ATP. The spontaneous efflux of ATP appeared to be enhanced in the presence of 8-PT (10 μM: n=5).
nmol ATP released/min/g of vas deferens
Figure 5.5

A. The effects of superfusion with dipyridamole (10 μM) for 20 min prior to electrical stimulation of the guinea-pig vas deferens for 1 min (8 Hz, 0.5 ms, 20 V) were not always obvious but the S2/S1 ratio (for twitch heights) was significantly decreased (see text). B. The stimulation-evoked release of ATP appeared to be decreased (n=5) after perfusion with dipyridamole (10 μM).
Chapter 6.

Comparative study of the actions of ATP and α,β-methylene ATP on nonadrenergic, noncholinergic neurogenic excitation in the guinea-pig vas deferens.
6.1 Summary

The ability of two nucleotide analogues, P\textsuperscript{1},P\textsuperscript{8}-di-(adenosine-5') pentaphosphate (AP\textsubscript{8}A) and α,β-meATP to stimulate and desensitize P\textsubscript{2}-purinoceptors in the isolated guinea-pig vas deferens has been investigated.

In organ bath studies, both AP\textsubscript{8}A and α,β-meATP were approximately 100 times more potent than ATP in producing phasic contractions of the vas deferens smooth muscle. Repeated additions of either agonist (1-10 μM) produced desensitization to a subsequent addition of the test substance. AP\textsubscript{8}A and α,β-meATP were approximately equipotent in the production of desensitization. After desensitization had been produced in the vas deferens by AP\textsubscript{8}A or α,β-meATP, excitatory responses elicited by ATP (100-150 μM) and nonadrenergic field stimulation (2-20 Hz) were blocked, whereas those elicited by carbachol (1-10 μM) were augmented.

Intracellular electrical recordings demonstrated that AP\textsubscript{8}A and α,β-meATP produced similar effects on the membrane activity of the vas deferens. Concentration-dependent depolarizations were produced by both substances until the threshold for action potential discharge was attained; thereafter, action potential discharges were superimposed on the depolarization and an accompanying phasic contraction recorded. Upon restoration of the membrane potential to its control value (5-10 min after the addition of either AP\textsubscript{8}A or α,β-meATP), EJPs elicited by field stimulation (up to 3 Hz) and spontaneous EJPs
were reduced by AP₄A (>0.1 μM) in a concentration manner (as previously described for α,β-meATP). The antagonistic effects of AP₄A on mechanical responses elicited by field stimulation were more quickly reversed on washout of AP₄A than were the effects of α,β-meATP, this may suggest some dissimilarity in their mechanism of action at the receptor level.

The antagonistic effects of AP₄A on the nonadrenergic contractile responses of the vas deferens were not produced by the structurally related P¹, P'-α₁-(adenosine-5') tetraphosphate (AP₃A) even with cumulative concentrations up to 200 μM.
6.2 Introduction

The guinea-pig vas deferens receives an excitatory sympathetic innervation: the biphasic mechanical response elicited by nerve stimulation consists of a rapid phasic contraction which exhibits pharmacological properties characteristic of a nonadrenergic mediator (Ambache & Zar, 1971) and a slower tonic contraction which typifies a noradrenergic response (Swedin, 1971; McGrath, 1978).

Although there have been several hypotheses to reconcile the apparently anomalous properties of the primary phasic contraction within the framework of a purely noradrenergic system which mediates sympathetic excitation (Swedin, 1971; Hirst & Neild, 1980: 1981), the weight of direct experimental evidence at present lies with those experiments which utilize receptor-blocking agents as pharmacological tools. Two nucleotide derivatives have proven useful in recent years in the characterization of these components of sympathetic neuromuscular excitation. For example, the photoiyosed form of the P2-purinoceptor antagonist, ANAPPa blocked both the phasic nonadrenergic component of neurogenic excitation in the vas deferens and the phasic response elicited by ATP (Fedan et al., 1981) and α,β-meATP produced desensitization of its own action and a comparable antagonism of phasic contractions elicited by nerve stimulation and ATP (Meldrum & Burnstock, 1983). Similarly, intracellular recording techniques have demonstrated that both ANAPPa (Sneddon & Westfall, 1981)
and α,β-meATP (Sneddon & Burnstock, 1984a; Stjärne & Astrand, 1984; Allcorn et al., 1986) inhibit the EJP's which give rise to the action potential underlying nonadrenergic phasic contraction in the vas deferens. These data have led to a consensus of opinion that ATP and NA may be released as cotransmitters from sympathetic nerves supplying the vas deferens (Fedan et al., 1981; Meldrum & Burnstock, 1983; Sneddon & Westfall, 1984; Stjärne & Astrand, 1985; Kirkpatrick & Burnstock, 1987).

In the present experiments we have used intracellular recording and organ bath techniques to study the desensitization process induced by α,β-meATP in the guinea-pig vas deferens. We have examined whether this antagonistic activity was confined to the substituted nucleotide and have explored some of the structural requirements of the receptor for the desensitizing agonist. We have shown that the pharmacological profile of α,β-meATP is shared by a diadenosine nucleotide, AP4A, but not by a closely related compound, AP4A.
6.3 Materials and Methods

The vasa deferentia of the guinea-pig were removed and prepared as described in Chapter 2. In the organ bath studies, 'control' responses to nerve stimulation (0.5 ms, 2-20 Hz, supramaximal voltage, 30 s duration), ATP and carbachol were obtained, initially in the absence of but later in the presence of prazosin (5 μM). Prazosin remained in the organ bath at the onset and for the duration of the desensitization period.

After the control responses had been established, α,β-meATP and APsA were tested for their capacity to induce desensitization to their own excitatory actions and to the effects of nerve stimulation and exogenous ATP.

In the electrophysiological experiments, prostatic portions of vasa deferentia were removed and prepared as described in Chapter 2 (section 2.3). The preparation was immobilised by gently stretching and pinning out the connective tissue with steel pins. Conventional intracellular recording techniques were employed using microelectrodes (60 -70 MΩ) filled with 3 M KCl. Drugs were added to the continually flowing Krebs' solution.
6.4 Results

6.4.1 Organ bath experiments

In these experiments, neurogenic responses were established at variable frequency (a stimulation period of 30 s was routinely used) and supramaximal voltage: these excitatory responses were blocked by tetrodotoxin (1 mM). After supramaximal voltage was established, frequency-response relationships were examined (2-20 Hz) and the characteristic biphasic mechanical response of the intact vas deferens became apparent. Reproducible responses to exogenous ATP and carbachol were also established. A concentration of carbachol was then chosen (1-10 μM) that produced a response of similar magnitude to that of a nerve stimulation response (at 8 Hz) which, in turn, was approximately 75 % of the maximal response over the frequency range studied here (71.7 ± 1.8 %, n=33). A concentration of ATP was chosen (usually 100-150 μM) that elicited reproducible responses of a similar magnitude to those of the other two stimuli. After these control responses had been established, prazosin (5 μM) was routinely added for the duration of the experiment to exclude the contribution of NA to the neurogenic 'control' response.

Having established the various control responses in the absence and presence of prazosin, α,β-meATP was then tested for its capacity to induce desensitization to its own agonist (excitatory) action. α,β-meATP was usually
used in the concentration range 1-10 μM. Within this concentration range α,β-meATP elicited a phasic contraction upon its first addition to the organ bath: this phasic excitatory response declined within a minute or so and, when the muscle reattained its former atonic state, another addition of α,β-meATP was made (Figure 6.1). The amplitude of this excitatory response was progressively attenuated as the number of subsequent additions of α,β-meATP was increased, until a point was reached at which the addition of α,β-meATP elicited no further excitatory response. At this time, desensitization to the excitatory action of α,β-meATP was judged to be complete. At this point, the phasic contractions produced by neurogenic stimulation were substantially or completely blocked (n=14) and the responses to exogenous ATP were similarly antagonised: in contrast, the responses to carbachol were either unaffected or increased slightly (Table 6.1).

AP₅A and α,β-meATP (each at a concentration of 1 μM) both produced phasic excitatory responses on their initial exposure which were of similar magnitude (AP₅A = 3.2 ± 0.35 g; α,β-me ATP = 3.1 ± 0.27 g; n=10 in each case), but larger than those produced by a much higher concentration (100 μM) of ATP (ATP = 2.1 ± 0.19 g. n=20). AP₅A also produced desensitization to its own action (1-5 μM. n=12. Figure 6.1). A series of paired experiments followed in which we tried to quantify the ability of AP₅A and α,β-meATP to produce desensitization. It was found that AP₅A and α,β-meATP were similarly effective in the
production of desensitization and antagonism of the neurogenic responses (Figure 6.1, Table 6.1). However, one manner in which AP₄A and α,β-meATP differed in their action was in the way the neurogenic excitatory responses recovered following desensitization. In four experiments of the type shown in Figure 6.1 and Table 6.1, neurogenic excitatory responses were more quickly and more completely restored following the washout of AP₄A (after desensitization had been produced) in comparison with parallel responses in tissues when αβ-meATP had been used (Figure 6.2).

In a few other experiments (n=5) we also studied the action of AP₄A, an analogue of AP₄A which has one less phosphate group in the polyphosphate bridge linking the two adenosine moieties. AP₄A was a less potent agonist than either AP₄A or α,β-meATP (AP₄A at 1 μM = 0.53 ± 0.05 g; n=5) and there was no parallel desensitization induced against the neurogenic response, even with multiple (up to 20) additions of low agonist concentration (1 μM) or addition of higher agonist concentration (6-10 μM), yielding cumulative concentrations of up to 200 μM.

6.4.2 Electrophysiological experiments

The prostatic end of the vas deferens exhibits a prominent nonadrenergic innervation whose electrical correlate is a graded EJP (Burnstock & Holman, 1961). In the present experiments the resting membrane potential averaged -65 ± 0.7 mV (n=54). Spontaneous EJPs (usually
5-10 mV, but up to 28 mV) were superimposed on an otherwise stable membrane potential. After a satisfactory impalement had been obtained, electrical responses to field stimulation were elicited at several frequencies (routinely trains of 10 pulses at 0.2, 0.5, 0.75 and 1 Hz were studied in each cell, but these were sometimes studied at 2 or 3 Hz). EJPs exhibited a frequency-dependent facilitation before they attained a steady state value, with the ultimate firing of action potentials which completely depolarized the cell membrane potential to 0 mV, or by an additional 5-15 mV.

Agonists such as ATP or α,β-meATP (Burnstock et al., 1986) elicited electrical (and mechanical) responses which were similar to those produced by AP₄A. (Intervals of 12-20 min separated subsequent additions of agonist at a fixed concentration to ensure reproducibility; neurogenic responses were also periodically studied during recovery periods to provide an indication of any desensitization that might have arisen). Low concentrations of AP₄A produced a concentration-dependent membrane depolarization; higher concentrations of AP₄A upon reaching the electrical threshold for the activation of voltage-dependent Ca²⁺ channels (Burnstock et al., 1986), elicited a depolarization with a superimposed discharge of action potentials (Figure 6.3). These action potentials also attained 0 mV: they were accompanied by mechanical excitation.

The inhibitory effects of AP₄A (0.1-10 μM) on fully
facilitated EJPs (taken at the mean amplitude of the 6th-10th pulses in a train) were assessed in a series of experiments where control responses to nerve stimulation were obtained over a wide frequency range (0.2-1 or 2 Hz) in a single cell, and were then examined on progressively increasing concentrations of APbA. once the depolarization (and any action potential discharge) had waned and the membrane potential had attained its former (pre-drug) level. EJP amplitudes were significantly reduced by APbA (Figure 6.4) in a concentration-dependent manner: action potentials were not produced by these EJPs, since they no longer reached the electrical threshold for action potential initiation (Figure 6.5). As the concentration of APbA was increased, the frequency and amplitude of spontaneous EJPs was also reduced.
6.5 Discussion

A prominent component of the neurogenic excitatory response in the guinea-pig vas deferens is resistant to the action of adrenoceptor receptor antagonists, but is inhibited by two compounds (ANAPP and α,β-meATP) which are structurally related to ATP (Fedan et al., 1981; Kasakov & Burnstock, 1983; Meldrum & Burnstock, 1983). These observations support the hypothesis that ATP may act as a cotransmitter (with NA) in the vas deferens (Fedan et al., 1981). An analogous cotransmitter role for ATP in several vascular tissues has also been postulated on the basis of similar experimental observations (Sneddon & Burnstock, 1984b; Ishikawa, 1985; Von Kügelgen & Starke, 1985; Kennedy et al., 1986; Burnstock & Warland, 1987).

Of the two 'antagonists' of the nonadrenergic noncholinergic responses described so far, α,β-meATP is more potent than ANAPP (Fedan et al., 1981; Meldrum & Burnstock, 1983) and does not require prior photolysis by u.v. radiation. In the present experiments we have shown directly that a third nucleotide derivative, APβA, was approximately equipotent with α,β-meATP in its agonistic and antagonistic (or desensitization) effects in organ bath experiments. We have, furthermore, quantified the concentration-dependent antagonism of APβA on EJPs of the vas deferens in continuous recordings from single cells with intracellular microelectrodes. APβA (present work) and α,β-meATP (Sneddon & Burnstock, 1984a) have similar 'antagonistic' effects on spontaneous and evoked EJPs in
the vas deferens and a similar agonistic action on the membrane potential (Burnstock et al., 1986: present work). A postjunctional action at a restricted locus rather than a prejunctional site of action of APβA seems more likely, since spontaneous EJPs were inhibited and since contractile responses to carbachol were not reduced in parallel with those elicited by nerve stimulation and ATP. One striking difference between the action of APβA and αβ-meATP in organ bath studies was the faster (within 5 min) complete recovery of the transmission mechanism from desensitization induced by APβA. This infers some mechanistic difference in the actions of these two substances at their receptor (receptor, rather than receptors, since ATP is similarly inhibited by both compounds). Preliminary experiments have also shown that APβA inhibits atropine-resistant neurogenic contractions and phasic contractions to ATP in detrusor muscle strips from the guinea-pig (Mackenzie, Kirkpatrick & Burnstock, unpublished observations) in a similar manner to αβ-meATP, and suggests some common properties of the mechanism of the antagonism at the P2-purinoceptor.

The structure of adenine nucleotide derivatives contributes to changes in pharmacological potency at the P2-purinoceptor when compared to ATP in these and other tissues (Stone, 1981) which could derive from either their steric presentation at the receptor or at the active site of their metabolizing enzymes. The stereochemistry of ATP (stylistically represented by structural notation
AOPOPOP, where A = the adenosine group, O = anhydride oxygen, P = phosphate group), and APtA (AOPOPOPOPOPOA) are possibly quite similar - the dinucleotide lacks the longer C-P bond length and larger C bond angle exhibited by $\alpha,\beta$-meATP (AOPCPOP, where C is a methylene substituent: Maguire & Satchell, 1979a). At physiological pH, APtA and APtA exist in an extended (more linear) form (Kolodny et al., 1979). Furthermore, if one imagines a plane of symmetry passing through the central phosphoryl group in the polyphosphate 'bridge' of APtA, this yields two shorter fragments which are, essentially, equivalent to the presence of two ATP molecules in close proximity with the added advantage that there are no exposed terminal phosphates (at least in the unbound form) available for enzymatic degradation. The metabolic routes for dinucleotide degradation in smooth muscle (Pearson, 1985) may be similar for ATP and APtA (once the pentaphosphate has been cleaved at some point by whatever available mechanism: how this can be achieved is unclear) and may involve the production of di- and monophosphates. The length of the dinucleotide (and its products of metabolism) is certainly important - the deletion of a single phosphate moiety yields a compound (APtA, AOPOPOPOPOA) with little agonist and subsequent 'desensitizing' activity. The stability of $\alpha,\beta$-meATP is an important determinant of its activity but its metabolites would (by virtue of its methylene bridge) differ from those of APtA and ATP.
Another interesting property of APsA and AP4A is that they are naturally occurring. It is difficult, however, to explain how the well documented inhibition of myosin adenylate kinase by APsA (Lienhard & Secemski, 1973) might contribute to its antagonistic activity in the vas deferens, when nucleotides are thought to be incapable of entering the muscle cell membrane in their unmetabolized form. Information about the storage of AP4A is sparse and the assay techniques used can also cross react with ATP (Momsen, 1973; Ogilvie, 1981). We are also unaware of any literature to date, which describes the action of APsA in the whole animal; it might be a useful tool in experiments where rapid nucleotide degradation to adenosine (with the consequence of ATP action) would be an otherwise undesirable action. Long-term binding of the nucleotides APsA and α,β-meATP to their receptors, or long-term modification of associated receptor-operated second messenger systems might also be possible.

To date, therefore, the antagonism of excitatory nucleotide receptors requires their prior activation (and/or physical modification, e.g. photolysis and the antagonistic effects of ANAPPs) by synthetic or endogenous nucleotide analogues whose structural conformation and metabolic stability are important determinants of the expression of their biological activity.
Table 6.1

Comparison of the effects of repetitive additions of α,β-meATP (1 μM) or APsA (1 μM) on excitatory responses elicited by ATP (100 μM), carbachol (5 μM) and field stimulation (8 Hz for 30 s) in a series of paired experiments in the guinea-pig vas deferens (n=5).

<table>
<thead>
<tr>
<th>Desensitizing Agonist</th>
<th>α,β-meATP</th>
<th>APsA</th>
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<tbody>
<tr>
<td>Successive additions at 1 μM required for desensitization</td>
<td>5.6 ± 0.57</td>
<td>5.2 ± 0.42</td>
</tr>
<tr>
<td>% Change in magnitude of control response after desensitization</td>
<td>ATP*</td>
<td>-98 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>NSb</td>
<td>-95 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CChc</td>
<td>+21 ± 10.4</td>
</tr>
</tbody>
</table>

Prazosin (5 μM) was continually present.

* ATP (100 μM)

b nerve stimulation (8 Hz, 0.5 ms, supramaximal voltage)

c carbachol (5 μM)
Contractile responses to nerve stimulation (NS, 8 Hz for 30 s). ATP (100 μM) and carbachol (CCh, 5 μM) were obtained in two preparations from the same animal. In the upper panel, APsA elicited an initial contraction which was progressively smaller upon repeated additions (six additions, each at 1 μM). When the tissue was desensitized to APsA, responses to nerve stimulation and exogenous ATP were blocked, in contrast to that elicited by carbachol. In the lower panel, α,β-meATP was used as the desensitizing agonist in the contralateral vas deferens and produced a similar selective desensitization to that produced by APsA when used at the same concentration. Periods of exposure to the excitatory stimuli are denoted by horizontal bars. Prazosin (5 μM) was continually present in both these experiments.
Figure 6.2

The rate and extent of recovery of the neurogenic contractions from desensitization were followed in seven pairs of experiments. Data shown in this illustration came from four of those experiments described in Table 6.1 (and shown in Figure 6.1). Desensitization was produced by either agonist and responses to nerve stimulation. ATP and carbachol were then tested. λ-Pi-A (X) or α,β-meATP (Δ) was then washed out (time = 0) and responses to nerve stimulation were tested over a 2 h period at those times indicated on the graph. They were compared to their control (pre-desensitization) amplitudes. Neurogenic responses were more quickly and more completely restored in preparations which had been exposed to APi-A (statistically significant changes indicated by asterisks. *P < 0.05). Prazosin (5 μM) was continually present.
Percentage of control neurogenic response

Desensitisation

Time after washout of agonist (min)

Δ AR2A

Δ α,β-Me ATP
Figure 6.3

Electrical responses elicited by field stimulation (1 Hz, 10 pulses) and APsA in the vas deferens are shown here. The EJPs facilitated to reach the electrical threshold for the initiation of action potentials. APsA (1 μM) elicited an initial depolarization and then an action potential discharge. (The time course of the action potentials is shown with more clarity at the faster chart speed.) The action potentials became less frequent and then stopped before the depolarization fell off and the former (pre-drug) membrane potential was recorded again (even in the continual presence of APsA). EJPs elicited thereafter were antagonized by APsA in a concentration-dependent manner (Figure 6.4 and 6.5).
The concentration-dependent inhibition of EJP amplitude by APbA is shown here. The control response is the mean amplitude of a fully-facilitated EJP elicited at 0.75 Hz (mean of 6th to 10th pulse in a train) in a series of cells (number of cells indicated). APbA was added in progressively increasing concentrations and EJP amplitudes were redetermined in the same cells once drug-induced depolarization had subsided (Figure 6.5). Significant antagonism of EJP amplitude was observed over all the concentration-range shown here (indicated by asterisks: *P < 0.05).
Mean e.j.p. amplitude at 0.75Hz (mV)
Figure 6.5

Control EJPs elicited by stimulation at various frequencies (as shown) were progressively antagonized by increasing concentrations of APsA (up to 3 μM). The action potentials elicited by stimulation were absent once the EJPs failed to attain the electrical threshold for action potential initiation. EJPs shown here at 0.2 Hz were fully-facilitated (8th to 10th pulses in a train) and underwent facilitation at 0.75 Hz (first 5 pulses in a train).
0.2Hz

CONTROL

+0.1μM AP5A

+0.3μM

+0.6μM

+1μM

+3μM

0.75Hz

-70mV

-58

-68mV

5s
CHAPTER 7.

Release of endogenous ATP from the vasa deferentia of the rat and guinea-pig by the indirect sympathomimetic tyramine.
7.1 Summary

ATP as well as $[^3H]$-NA is released by perfusion of the vas deferens with the indirect sympathomimetic, tyramine (100 μM): this result is consistent with the concept of sympathetic cotransmission. While tyramine produced a strong contraction in the vas deferens of the rat, it had little mechanical action in the guinea-pig vas deferens. This appears to be largely because tyramine induces considerably lower levels of release of both ATP and NA from the guinea-pig compared to the rat vas deferens. Furthermore, NA released by tyramine appears to release ATP from a secondary pool in the rat, but not guinea-pig vas deferens since prazosin reduced the tyramine-induced release of ATP in the rat vas deferens.

α,β-meATP increased both the spontaneous release of ATP and the tyramine-evoked efflux of ATP and $[^3H]$-NA. The basal and tyramine-induced efflux of $[^3H]$-NA was also enhanced by the $\alpha_1$-adrenoceptor antagonist, prazosin, suggesting that prejunctional $\alpha_1$-adrenoceptors modulate neurotransmitter release.
7.2 Introduction

Indirect sympathomimetic amines produce their post-junctional effects in sympathetically innervated tissues by releasing intraneuronally stored NA into the junctional cleft (Burn & Rand, 1958). The exact mechanism whereby tyramine and other indirect sympathomimetic agents can evoke the efflux of highly polar NA from sympathetic nerve endings has yet to be fully elucidated (Trendelenberg et al., 1987), but it differs from the release mechanism utilised when sympathetic neurons are electrically stimulated (Chubb et al., 1972). Since the process does not involve extracellular calcium, a mechanism other than exocytosis is believed to be involved (Chubb et al., 1972). What is clear, is that tyramine enters nerve endings via uptake, initially displacing NA from the axoplasm and later mobilising the release of NA from vesicular stores (Trendelenberg et al., 1987).

Sympathetic nerve endings contain two types of NA-storage organelle which are normally distinguished according to their size and their vesicular content: they are referred to as the small (SDV) and large (LDV) dense cored vesicles (Fried et al., 1984). Both vesicle subtypes contain NA and ATP (Fried et al., 1984) whilst only the LDV contain the polypeptide NPY (Fried et al., 1985).

While the ability of tyramine to release NA from sympathetic nerve endings is well documented, more recent studies have shown tyramine capable of releasing NPY from
a variety of preparations including rabbit ileum (Cheng & Shen, 1986) and the rat vas deferens (Cheng & Shen, 1987). Furthermore, in the rabbit ileum, perfusion with tyramine evoked a triphasic mechanical response, the latter two components of which were blocked by substances known to interfere with noradrenergic and NPYergic responses (Cheng & Shen, 1986). On the other hand, the initial phasic component of the contraction was unaffected by any of these pretreatments. It is now known that ATP, as well as NA and NPY, are released concomitantly from the field stimulated guinea-pig vas deferens (Kasakov et al., 1988) and all three substances are known to coexist within the nerve terminals of the sympathetic neurons innervating this tissue in the rat (Fried et al., 1985).

The vasa deferentia of the rat and guinea-pig differ greatly in their response to tyramine. The guinea-pig vas deferens is insensitive to the actions of this drug (Ambache et al., 1972), while in the rat, tyramine evokes a large (Ambache et al., 1972; Pennefather et al., 1974) and frequently biphasic (Lucchelli et al., 1983) contraction. It is also known that the vasa deferentia of the two species differ in that almost all of the stimulus-evoked efflux of ATP from the vas deferens of the guinea-pig appears to occur directly from sympathetic nerves (Kirkpatrick & Burnstock, 1987; Kasakov et al., 1988), while nearly 80% of the ATP released from this tissue in the rat arises as a result of the actions of NA (Vizi & Burnstock, 1988).
Thus the main aim of this study is to investigate whether or not the indirect sympathomimetic, tyramine, can release ATP from the vasa deferentia of either the rat or the guinea-pig and to establish whether or not any fundamental differences exist between the two species in the mechanism of ATP release which might in turn explain the disparity in the response of these two tissues to tyramine.
7.3 Materials and Methods

The vasa deferentia of both species were prepared, labelled with \( ^{3}H \)-NA and mounted in tissue holders as described in Chapter 2. Both the epididymal and prostatic ends of the guinea-pig vas deferens were used in this study. The vasa deferentia were continuously perfused with Krebs' at a rate of 2.5 ml/min. The preparations were allowed to equilibrate for 90 min prior to experimentation.

The vasa deferentia of both species were superfused with Krebs' containing tyramine (10-300 \( \mu \)M) as described in section 2.7.2. Any changes in the basal or tyramine-evoked release of either ATP or \( ^{3}H \)-NA were quantified as either the \( b_2/b_1 \) or \( S_2/S_1 \) ratio, where the \( b_2 \) or \( b_1 \) value was the basal level of ATP or \( ^{3}H \)-NA release immediately prior to \( S_2 \) or \( S_1 \) period of stimulation.

All superfusate samples were collected in ice-cold polystyrene vials and directly placed on ice; they were assayed for ATP and \( ^{3}H \)-NA as described in Chapter 2.
7.4 Results

7.4.1 The effects of Tyramine

Perfusion of the prostatic region of the rat vas deferens with tyramine (100 μM, n=8) evoked a statistically significant increase in the release of ATP (P < 0.01) and \[^{3}H\]-NA (P < 0.001). The spontaneous release of ATP rose from 3.04 ± 1.29 to 38.77 ± 9.20 pmol/min/g (approximately 13-fold increase, see Table 7.1) whilst the fractional rate of \[^{3}H\]-NA release increased (almost 4-fold see Table 7.1) from a basal level of 0.112 ± 0.011 to 0.437 ± 0.050 (see Figure 7.1B & 7.1C). This increase in transmitter efflux was accompanied by a pronounced mechanical response which was frequently biphasic in nature (Figure 7.1A). In every experiment, the mechanical response increased and became more biphasic in appearance as the experiment progressed. Increasing concentrations of tyramine (10-300 μM) evoked a concentration-dependent rise in the release of both ATP and \[^{3}H\]-NA and subsequently produced an increased mechanical response (see Figure 7.2). Furthermore, increasing the concentration of tyramine prolonged the duration of the \[^{3}H\]-NA efflux (Kirkpatrick & Burnstock, unpublished observation).

The effects of tyramine were markedly different in the guinea-pig vas deferens. In this tissue, tyramine (100 μM) evoked only a two- to three-fold increase in the efflux of \[^{3}H\]-NA and ATP (see Table 7.1). While
perfusion of the same concentration of tyramine (100 μM) in the rat vas deferens induced a 4-fold increase in the overflow of [³H]-NA and a substantial 13-fold rise in the release of ATP (see Table 7.1). Furthermore, although perfusion of the prostatic end of the guinea-pig vas deferens with tyramine (100 μM) did release ATP and [³H]-NA, subsequent contractions were either very small or absent (see Figure 7.3). Even at concentrations as high as 1 mM, tyramine failed to elicit a contraction in this tissue (not shown). Similar experiments carried out on the epididymal region of the guinea-pig vas deferens, which is believed to be more sensitive to NA, produced analogous results. Once again, tyramine (100 μM) induced the release of ATP and [³H]-NA but failed to elicit a mechanical response (Figure 7.3). There was no statistically significant difference in the release of ATP or [³H]-NA from the prostatic or epididymal regions of the guinea-pig vas deferens (see Figure 7.3). Unlike the rat vas deferens (Pennefather et al., 1974), the epididymal region of the guinea-pig vas deferens did not appear to be more sensitive to tyramine.

7.4.2 The effects of α,β-meATP

Superfusion of the rat vas deferens with α,β-meATP (1 μM) gave rise to a substantial, phasic contraction (not shown). Perfusion with α,β-meATP for 10 min prior to superfusion with both tyramine (100 μM) and α,β-meATP (1 μM) produced a statistically significant rise (n=8, P <
in the tyramine-induced release of \[^3H\]-NA. the 
S/S ratio increased from 0.792 ± 0.051 in control to 
1.030 ± 0.045 in α,β-meATP perfused vasa deferentia (as 
indicated by the asterisk in Figure 7.1B, n=8). α,β-meATP 
had no significant effect on the basal efflux of \[^3H\]-NA 
or the maximum \[^3H\]-NA release (measured after stimulation 
with tyramine. Figure 7.1C). α,β-meATP also significantly 
(P < 0.05) enhanced the tyramine-evoked release of ATP, 
the S/S ratio increased from 0.619 ± 0.133 (n=8) in 
control tissues to 1.017 ± 0.126 (n=8) in the presence of 
α,β-meATP (1μM). Furthermore, the basal efflux of ATP was 
also significantly increased by α,β-meATP. the b\_2/b\_1 
ratio rose from 0.881 ± 0.14 (n=6) in control experiments 
to 2.726 ± 0.671 (n=7) when perfused with α,β-meATP 
(Figure 7.1B). The initial phasic component of contraction 
was greatly reduced after perfusion with α,β-meATP (see 
Figure 7.1A).

7.4.3 The effects of Prazosin

Similarly the effects of prazosin (1 μM) on 
tyramine-induced release of ATP and \[^3H\]-NA from the rat 
vas deferens were studied. Superfusion with prazosin 
(1 μM) for 10 min prior to perfusion with tyramine 
(100 μM) and prazosin (1 μM) caused a statistically 
significant rise in both the tyramine-evoked (n = 12; P < 
0.001) and basal (n = 12; P < 0.05) \[^3H\]-NA release (see 
Figure 7.5). Perfusion with prazosin increased the b\_2/b\_1 
ratio from 1.156 ± 0.104 in control (n=8) vasa deferentia 
to 1.654 ± 0.136 (n=12) and the S\_2/S\_1 ratio for evoked
release from 0.792 ± 0.051 to 1.185 ± 0.061 (as indicated by the asterisks. Figure 7.5C). Prazosin had no significant effect on the levels of [³H]-NA measured after stimulation by tyramine. In contrast, prazosin (1 µM) significantly decreased (P < 0.05) the evoked release of ATP, the S'/S₁ ratio decreased from 0.619 ± 0.133 to 0.426 ± 0.020 (n=8, see Figure 7.5B). The basal release of ATP was unaffected. The mechanical response of the rat vas deferens was usually almost totally abolished by prazosin although on two occasions, a small residual contraction remained.
7.5 Discussion

In recent years, much evidence has been presented to suggest that NA and ATP are released as cotransmitters from sympathetic neurons (Burnstock, 1990b; Von Kügelgen & Starke, 1991). The phenomenon of cotransmission has been extensively studied in the vas deferens (e.g., Stjärne & Astrand, 1985; Kirkpatrick & Burnstock, 1987; Guidobono-Toro & Parada, 1988; Kasakov et al., 1988; Kaschube & Zetler, 1989; Drake & Petersen, 1992; Vizi et al., 1992). The demonstration in this paper that the indirect sympathomimetic tyramine can release not only NA but also ATP from the sympathetic nerves of the guinea-pig and rat vas deferens, is consistent with the view that ATP and NA are released as cotransmitters from sympathetic nerves.

Tyramine has also been shown to evoke the release of NPY from the rat vas deferens and from synaptosomes prepared from this tissue (Cheng & Shen, 1987), since the release was inhibited by 6-OHDA and desipramine the authors concluded that it arose prejunctionally from sympathetic nerves via a mechanism involving the neuronal carrier (Cheng & Shen, 1987). Since ATP is known to be co-stored with NPY and NA in the LDVs (Fried et al., 1985) it seems feasible that tyramine may exert its effects there but it would be too speculative at this stage to suggest that all three substances may be released as some sort of complex.

A previous study by Teixeira et al. (1989) has shown that when high concentrations of NA are administered in vivo a metabolite of NA could mimic the effects of the neurotoxin
6-OHDA by causing an initial increase in the release of NA, and that this effect was centred around the LDV. It is interesting to note that tyramine and NA have metabolites in common.

The differences in the effects of tyramine on the vas deferens of the rat and guinea-pig may give further insights into the release mechanisms involved. It has become apparent that the pathway for ATP release differs significantly between the vasa deferentia of the two species. In the rat, a major part of the ATP efflux from the vas deferens arises secondarily as a result of the action of NA (Vizi & Burnstock, 1988). This coupled with the observations made in this study, namely that prazosin significantly reduced the tyramine-induced release of ATP, suggests that in the rat vas deferens, tyramine acts partly by potentiating the efflux of NA, with ATP release occurring largely as a result of the increased concentration of NA. Consequently, in the rat vas deferens, tyramine is able to evoke a large and occasionally biphasic mechanical response. In contrast, data presented in this study suggests that this pathway is less important in the guinea-pig vas deferens and consequently the effect of tyramine is much less. However, in two recent studies, Katsuragi et al. (1990) and Vizi et al. (1992) have shown that when the guinea-pig vas deferens is incubated in an organ bath, for substantially longer, with considerably higher concentrations of NA (100 µM), interaction of NA with α1-adrenoceptors (and not
muscle contraction per se) can enhance the release of ATP. The exact role of this \( \alpha \)-adrenoceptor mediated ATP release in neurotransmission in the vas deferens requires further study but it is possible that these receptors are prejunctional in origin (Vizi et al., 1992).

Alternative reasons for the apparent insensitivity of the guinea-pig vas deferens have been put forward. For example, the existence of tyramine-insensitive pools of NA (Takimoto et al., 1981). The present findings do not support such a theory, since in this study tyramine significantly enhanced the release of [\( ^{3} \text{H} \)]-NA. Although the tyramine-evoked efflux of [\( ^{3} \text{H} \)]-NA was significantly greater in the rat vas deferens, it would be unwise to draw any direct comparisons without first taking into account any differences in uptake and/or metabolism which might exist between the two species. Ambache et al. (1972) proposed that the guinea-pig vas deferens was insensitive to tyramine because NA was not the motor transmitter in this tissue. Again the results of the present study do not entirely support this view since tyramine significantly potentiated the release of ATP from both the rat and the guinea-pig vas deferens. ATP is believed to be the neurotransmitter mediating the initial contractile response in both of these tissues (e.g. Kirkpatrick & Burnstock, 1987; Kasakov et al., 1988; Huidobro-Toro & Parada, 1988; Vizi & Burnstock, 1988; Vizi et al., 1992).

\( \alpha, \beta \)-meATP has previously been shown to inhibit the nerve mediated twitch response of the rat vas deferens in
vitro, in both the epididymal (Amobi & Smith, 1987) and prostatic region (Huidobro-Toro & Parada, 1988; Vizi & Burnstock, 1988) and in vivo (Bulloch & McGrath, 1988). Similarly, in this study α,β-meATP appeared to reduce the initial phasic component of the tyramine-induced contraction. α,β-meATP may enhance the endogenous release of ATP by interacting with prejunctional P₂-purinoceptors on the sympathetic nerve terminal. The existence of prejunctional P₂-purinoceptors has already been proposed both in the portal vein (Kennedy & Burnstock, 1985) and in the guinea-pig vas deferens (Stiärne & Astrand, 1985). Stiärne and Astrand (1985) have proposed that ATP may mediate its own (and NA) release through prejunctional P₂-purinoceptors. Alternatively, α,β-meATP may act as an inhibitor of ATPase, preventing the enzymatic breakdown of ATP and thereby increasing the concentration of ATP released (Unsworth & Johnson, 1990).

In agreement with others (see Ellis et al., 1989) the release of [³H]-NA was substantially augmented by the specific α₁-antagonist prazosin. Similar results were obtained when the effects of prazosin on field stimulation induced [³H]-NA were studied (Kasakov et al., 1988; Vizi & Burnstock, 1988) and a growing number of studies have presented results inferring the existence of prejunctional α₁-adrenoceptors (see Starke, 1987). It is unlikely that prazosin enhances the release of NA by a reserpine-like action (Starke, 1987) since the enhancement of [³H]-NA release by prazosin was not blocked by the uptakei
inhibitor cocaine (Ellis et al., 1989). Thus it would appear that the specific \( \alpha_1 \)-antagonist prazosin mediates its effects on \(^{3}H\)-NA release by interacting with \( \alpha_1 \)-adrenoceptors present on the prejunctional membrane. Although it is generally accepted that prazosin, at the concentration used in this study (1 \( \mu \)M), acts selectively on \( \alpha_1 \)-adrenoceptors (Kahan & Hjemdahl, 1987) a more recent report by Yamatoto et al. (1988) claimed that prazosin (1 \( \mu \)M) potentiated the release of \(^{3}H\)-NA whilst much lower concentrations did not.

To conclude, the demonstration that the indirect sympathomimetic tyramine can release ATP and NA from the sympathetic nerves innervating the rat and guinea-pig vas deferens is consistent with the view that NA and ATP are cotransmitters in these tissues. The marked differences in effect of tyramine in the vas deferens of the rat and guinea-pig suggests that tyramine preferentially releases NA from sympathetic nerve terminals and that a significant component of its action on the rat vas deferens is due to release of ATP secondary to NA action.
Table 7.1. The effects of perfusion for 1 min with tyramine (100 μM) on the release of ATP and [3H]-NA from prostatic portions of vasa deferentia from the rat and guinea-pig. n = 8 in both species.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th></th>
<th>Guinea-pig</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal Evoked Increase</td>
<td></td>
<td>Basal Evoked Increase</td>
<td></td>
</tr>
<tr>
<td>ATP release (pmol/min/g)</td>
<td>3.04 ±1.3</td>
<td>38.77 ±9.2</td>
<td>x13.1</td>
<td>9.14 ±1.5</td>
</tr>
<tr>
<td>[3H]-NA efflux (%)</td>
<td>0.112 ±0.01</td>
<td>0.437 ±0.05</td>
<td>x3.9</td>
<td>0.110 ±0.3</td>
</tr>
</tbody>
</table>

Results show mean ± S.E.M. 'n' = number of observations.
**Figure 7.1**

Shows the effects of perfusion of the rat vas deferens with tyramine (100 μM) indicated by the bar, on release of ATP and $[^{3}H]$-NA. The vasa deferentia were perfused for 1 min with tyramine at 30 min intervals. A. The mechanical response of the rat vas deferens during perfusion with tyramine (100 μM), note how the contraction increases with repeated exposure to tyramine. B. Represents the release of endogenous ATP during perfusion with tyramine. C. Shows the tyramine-evoked increase in the fractional rate of $[^{3}H]$-NA release. Results show mean ± S.E.M., n = 8.
Figure 7.2
The effects of perfusing rat vasa deferentia with increasing concentrations of tyramine (10-300 μM) on the efflux of ATP and [3H]-NA. A. The release of endogenous ATP (pmoles/min/g of vasa deferens). B. The fractional rate of [3H]-NA efflux (%) and C. The mechanical response of rat vasa deferentia during perfusion with increasing concentrations of tyramine (10-300 μM). n > 5 for each concentration.
Tension (g)

Fractional rate of [3H]-NA release (%)

pmoles ATP released/min/g of vas deferens
Figure 7.3

Shows the effects of tyramine (100 µM) indicated by the bar. in the epididymal and prostatic regions of the guinea-pig vas deferens. A. & D. Show the mechanical response elicited by tyramine in these tissues. B. & E. The effects of tyramine perfusion on the release of endogenous ATP from these tissues. C. & F. Show the tyramine-induced increase in[^3]H]-NA release from the prostatic and epididymal regions of the guinea-pig vas deferens. Results show mean ± S.E.M., n = 8. Note that the amounts of ATP and[^3]H]-NA released are about half of that released in the rat vas deferens.
Fractional rate of [3H]-NA release (%)

pmoles ATP released/min/g of vas deferens

Epididymal

Fractional rate of [3H]-NA release (%)

pmoles ATP released/min/g of vas deferens

Prostatic
Figure 7.4

The effects of α,β-meATP (1 µM) on the responses of the rat vas deferens to tyramine (100 µM). Perfusion with tyramine (100 µM) indicated by bar. α,β-meATP was perfused for 10 min prior to perfusion with Krebs' containing tyramine (100 µM) and α,β-meATP (1 µM). A. The contractile response of the rat vas deferens evoked by tyramine (100 µM) before, during and after perfusion with α,β-meATP (1 µM). B. The effect of α,β-meATP (1 µM) on the tyramine-evoked endogenous release of ATP. C. Shows the fractional rate of [³H]-NA release before, during and after perfusion with α,β-meATP. Results show mean ± S.E.M., n = 12. * P < 0.05.
Fractional rate of [3H]NA release (%)

pmoles ATP released/min/g of vas deferens

< Diagrams and graphs showing data related to fractional rate of [3H]NA release and ATP release in vas deferens. 

A: Traces showing contractions induced by ATP.
B: Bar graph showing ATP released/min/g.
C: Time course of fractional rate release.

(μM ATP)
Figure 7.5

The effects of 10 min perfusion with prazosin (1 µM) on the responses of the rat vas deferens to tyramine (100 µM) indicated by bar. A. Tyramine induced contraction before, during and after perfusion with prazosin (1 µM).

B. The effect of prazosin on the tyramine-evoked release of endogenous ATP and C. Demonstrates the effect of prazosin on the fractional rate of [3H]-NA release from the rat vas deferens. Results show mean ± S.E.M., n = 12. * P<0.05, *** P<0.001.
Fractional rate of [3H]-NA release (%)

pmoles ATP released/min/g of vas deferens
CHAPTER 8.

Sensory nerves are a source of ATP released by NA in the sympathectomised rat vas deferens.
8.1 Summary

After chronic sympathectomy produced by pretreatment with guanethidine, the NA content of the vas deferens was substantially reduced as confirmed by HPLC. Superfusion of these sympathectomised tissues with NA (50 μM) produced a significant increase in the NA-evoked release of ATP compared to the appropriate control. In contrast, there was no such increase in the NA-evoked release of ATP in tissues taken from rats which had been pretreated with capsaicin prior to sympathectomy thereby preventing the proliferation of primary afferent sensory fibres that is known to occur with sympathectomy alone.

However, the levels of NA-evoked ATP released from the vasa deferentia of rats pretreated with capsaicin only were not significantly different from that of 'control' tissues. This suggests that under normal circumstances the NA-evoked release of ATP does not come from sensory nerves but from a non-neuronal source, probably the smooth muscle. In control tissues, the NA-evoked secretion of ATP was substantially reduced by prazosin and therefore was dependent on NA-stimulation of α1-adrenoceptors. This provides further evidence to support the view that part of the NA-evoked secretion of ATP from the rat vas deferens arises from the smooth muscle.

This study provides evidence for the release of ATP from smooth muscle and suggests that after sympathectomy, compensatory changes occur which make sensory nerves a major source of ATP release.
8.2 Introduction

There is considerable evidence to show that adenosine 5'-triphosphate (ATP) is released, along with noradrenaline (NA), during nerve stimulation of the rat vas deferens (French & Scott, 1983; Allcorn et al., 1986; Amobi & Smith, 1987; Huidobro-Toro & Montiel, 1987; Huidobro-Toro & Parada, 1988). Although the precise source of the ATP released from this tissue has always been contentious (Fredholm & Hedqvist, 1980; Fredholm et al., 1982). In a study by Vizi & Burnstock (1988) it was shown that, while some ATP was released as a cotransmitter from sympathetic nerves, almost 80% of the ATP released from the electrically stimulated rat vas deferens was released secondarily as a result of the actions of NA.

What remains unclear is precisely where NA exerts its ATP-releasing actions. It has been suggested that much of the ATP released from this tissue was released secondarily to the postjunctional actions of NA on the smooth muscle membrane (Fredholm & Hedqvist, 1980; Fredholm et al., 1982; Vizi & Burnstock, 1988) but it is also possible that NA acts prejunctionally to enhance the secretion of ATP. After all, NA has been shown to act like an indirect sympathomimetic and potentiate its own release from the sympathetic nerve terminals innervating the mouse vas deferens (Vizi et al., 1985; 1986) and we have recently shown that tyramine evokes the release of ATP from this tissue in the rat (see Chapter 7). However, it is unlikely that this is the main mechanism utilized by NA to enhance
the secretion of ATP from the vas deferens since the NA-evoked release of ATP does not appear to be reduced after sympathectomy (Vizi et al., 1992). In fact, after sympathectomy the NA-evoked release of ATP in the vas deferens is actually increased (Vizi et al., 1992).

There are other neuronal sites where NA might act to evoke the release of ATP. The rat vas deferens is innervated not only by sympathetic nerves, but also by parasympathetic cholinergic (Richardson, 1962; Dixon & Gosling, 1972) and sensory (Carvalho et al., 1986; Jänic & McClaughlin, 1987) fibres. Moreover, cholinergic and sensory neurons are known to have α-adrenoceptors on their nerve terminals (Starke, 1977; Vizi, 1979). Therefore, in order to investigate whether or not sensory neurons contribute to the NA-evoked release of ATP, we looked at the NA-stimulated secretion of ATP from vasa deferentia removed from rats pretreated with capsaicin at birth. A pretreatment regime that is known to cause massive degeneration of small diameter, unmyelinated primary afferent sensory neurons (Jancso et al., 1977; 1987).

Also of interest was the source of the increased release of ATP by NA seen in sympathectomised rats (Vizi et al., 1992). Since sensory fibres are known to store and release ATP (Holton & Holton, 1954; Jahr & Jessel, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985; Burnstock, 1987) and have been shown to proliferate after chronic sympathectomy (Yodlowski et al., 1984; Carvalho et al., 1986; Aberdeen et al., 1990; 1992) it seemed feasible that
they may represent the source of enhanced NA-evoked ATP release seen in vasa deferentia removed from sympathectomised animals. Thus, in order to investigate the role of sensory nerves in NA-evoked release from sympathectomised tissues, some rats were pretreated with capsaicin prior to chemical sympathectomy. This regime has been shown to reverse the proliferation of CGRP-containing sensory fibres seen in rat cerebral arteries after sympathectomy (Mione et al., 1992). Sympathectomy was achieved by pretreatment with guanethidine which has been shown to be highly selective in both adult (Burnstock et al., 1971) and neonatal (Eränko & Eränko, 1971) rats and it is particularly effective in tissues receiving a dense sympathetic innervation such as the rat vas deferens (Sjöstrand, 1962; see Johnson & Manning, 1984 for review).

Thus the main aim of the current study was to investigate, using selective neurotoxins, whether or not NA can evoke the release of ATP from sensory nerves innervating the rat vas deferens, and to establish whether or not the proliferation of these sensory fibres can explain the rise in NA-evoked ATP release seen after sympathectomy.
8.3 Materials and Methods

The vasa deferentia from control and pretreated rats (see below) were prepared and mounted onto platinum electrodes (which also served as tissue holders) as described in Chapter 2. Age-matched rats were pretreated with guanethidine, capsaicin, guanethidine plus capsaicin or the appropriate vehicles as described in section 2.8. The preparations were continuously perfused using a Watson-Marlow pump with Krebs' at a rate of 2.5 ml/min and allowed to equilibrate for 90 min prior to experimentation.

The vasa deferentia were superfused with Krebs' containing NA (50 μM) for periods of 1 min at 30 min intervals. The preparations were exposed to NA 3 times during the course of each experiment. The first period of superfusion with NA (S1) acted as a control; the second exposure to NA (S2) was carried out in the presence or absence of prazosin (1 μM). The last period of NA superfusion (S3) was used either as a control or recovery. Any changes in the NA-induced release of ATP were quantified as the S2/S1 ratio.

All superfusate samples were collected in ice-cold polystyrene vials and directly placed on ice; they were assayed for ATP as described in Chapter 2. ATP release is expressed in pmol/min/g of vas deferens.
8.4 Results

8.4.1 ATP release

'Control' tissues

Superfusion of the vasa deferentia removed from saline-injected ('guanethidine-control') rats with NA (50 μM) evoked a statistically significant increase in the efflux of ATP (n=9, P < 0.001). The levels of ATP released rose from 7.15 ± 1.15 to 46.88 ± 8.67 pmol/min/g (Figure 8.1B). This substantial increase in the release of ATP was accompanied by a large monophasic contraction but no spontaneous activity was seen (Figure 8.1A). The release of ATP from these tissues did not significantly differ from the 'no treatment' control (see Table 8.1). In fact, the release of ATP was not significantly affected by any of the vehicle ('control') pretreatments (see Table 8.1) and the NA-evoked responses were very similar to that shown in Figure 8.1A. No spontaneous contractions were seen in any of the 'control' tissues.

Perfusion of saline pretreated vasa deferentia with prazosin (1μM) for 10 min prior to superfusion with NA (50 μM) and prazosin (1 μM) together significantly reduced the NA-evoked release of ATP (n=8, P < 0.001. Figure 8.2B): the S2/S1 ratio was reduced from 0.582 ± 0.111 to 0.389 ± 0.091. Prazosin also significantly reduced the NA-evoked release of ATP from tissues pretreated with either capsaicin vehicle or capsaicin vehicle plus saline. In
the latter, the $S_2/S_1$ ratios were reduced from 0.722 ± 0.089 to 0.407 ± 0.122. Prazosin had no significant effect on the basal release of ATP in any of the 'control' preparations but the NA-evoked contraction was always substantially reduced (e.g. Figure 8.2A) and frequently abolished, despite the fact that the release of ATP was not totally inhibited.

**Guanethidine pretreatment**

In the vasa deferentia removed from guanethidine-sympathectomised rats, superfusion with NA evoked a statistically significant ($n=7, P < 0.001$) increase in the efflux of ATP. The levels of ATP released increased from 10.92 ± 1.78 to 195.19 ± 40.1 pmol/min/g (Figure 8.3B). In these tissues, the amount of NA-evoked ATP release was significantly higher than in vasa deferentia removed from control rats ($n=7, P < 0.005$). Subsequently, the NA-evoked mechanical response of sympathectomised tissues was considerably increased and the vasa deferentia were spontaneously active (Figure 8.3A). The basal release of ATP was not significantly increased (Figure 8.3B).

In preparations removed from sympathectomised rats, prazosin (1 µM) significantly decreased the NA-evoked efflux of ATP ($n=7, P < 0.001$) and reduced the $S_2/S_1$ ratio from 0.734 ± 0.112 to 0.169 ± 0.06. The spontaneous release of ATP was unaffected by prazosin (Figure 8.4A). In contrast to 'control' preparations, the vasa deferentia of guanethidine-pretreated rats frequently elicited a residual contraction to NA in the presence of prazosin.
(although the response was not always as marked as shown in Figure 8.4A). The residual contractile response was abolished by superfusion with prazosin and α,β-methylene ATP (1 μM, not shown). The spontaneous mechanical responses were inhibited by prazosin.

Prazosin appeared to have a much greater effect in guanethidine pretreated rats. In vasa deferentia taken from sympathectomised animals it reduced the NA-evoked release of ATP by approximately 80% compared to a decrease of about 40% in 'saline-injected' rats.

Capsaicin pretreatment

Superfusion of vasa deferentia from capsaicin only pretreated rats with NA (50 μM) evoked a statistically significant increase in the efflux of ATP. The release rose from basal level of 6.41 ± 1.25 to 31.52 ± 6.8 pmol/min/g. The release of ATP from these tissues did not significantly differ from 'control' vasa deferentia (see Table 8.1). Similarly, the subsequent mechanical response closely resembled that of a control.

Once again, prazosin significantly reduced the NA-evoked release of ATP (n=6, P < 0.05). The S2/S1 ratio was reduced from 0.838 ± 0.107 to 0.311 ± 0.061.

Capsaicin and guanethidine pretreatment

In vasa deferentia removed from capsaicin plus guanethidine pretreated rats NA still evoked a statistically significant increase in the efflux of ATP. The levels of ATP released rose from 7.06 ± 1.93 to 64.8 ±
9.79 pmol/min/g (Figure 8.5B). The concomitant contraction is shown in Figure 8.5A. As seen in guanethidine-only pretreated rats, the vasa deferentia removed from these animals were also spontaneously active although once again the basal release of ATP was not significantly increased by this pretreatment regime (Figure 8.5B). Superfusion with prazosin (1 µM) substantially reduced the NA-evoked release of ATP from these tissues (Figure 8.6B). The S2/S1 ratio was decreased from 0.731 ± 0.055 to 0.377 ± 0.062. The resting levels of ATP release were unaffected by prazosin and frequently, in the presence of prazosin, NA was able to evoke a small residual response (Figure 8.6A).

The NA-evoked release of ATP was significantly greater in tissues removed from capsaicin plus guanethidine pretreated rats than in vasa deferentia taken from the appropriate 'control' animals (n=6, P < 0.01) but the increase was not nearly so marked as with guanethidine alone. Prazosin reduced the release of ATP by a similar amount from both capsaicin plus guanethidine and 'control' pretreated rats.
8.4.2 Catecholamine content

The effects of the various pretreatment regimes on NA content in the vas deferens is shown in Table 8.2. Pretreatment with guanethidine alone (n=1, P < 0.001) or together with capsaicin (n=5, P < 0.005) caused a statistically significant decrease in the NA content of the rat vas deferens.
8.5 Discussion

In this study, the NA content of vasa deferentia removed from sympathectomised rats was significantly reduced after chronic guanethidine-pretreatment, such that less than 1% of the NA remained. In these tissues the NA-evoked release of ATP was significantly enhanced compared to appropriate controls. Similar findings have recently been reported in the guinea-pig (Vizi et al., 1992). The most likely source of this enhanced efflux of ATP is the sensory nerves since they store and release ATP and are known to proliferate after chronic sympathectomy (Yodlowski et al., 1984; Carvalho et al., 1986; Aberdeen et al., 1990; 1992) due to increased availability of nerve growth factor (Kessler et al., 1983). We therefore reasoned that if sensory neurons were a major source of the enhanced NA-evoked ATP release seen after chronic sympathectomy, then destruction of these nerves in conjunction with sympathectomy should prevent this substantial rise in the NA-evoked release of ATP. Indeed, after combined sensory and sympathetic denervation the NA-evoked release of ATP was substantially less than after sympathectomy alone. Furthermore, since capsaicin selectively destroys only primary afferent sensory fibres (Jancso et al., 1987) and since we have already shown that capsaicin plus guanethidine pretreatment completely abolished CGRP-containing fibres in the rat cerebral arteries, when guanethidine alone increased the number of sensory nerves present (Mione et al., 1992), we must
conclude that these nerves are involved in the release of ATP. This is consistent with previous reports of storage and release of ATP from sensory nerve fibres (Holton & Holton, 1954; Jahr & Jessel, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985). The demonstration that there is a substantial release of ATP after sympathectomy may explain the excruciating (post-sympathectomy) pain experienced after peripheral nerve lesion (Schon et al., 1985); the nociceptive actions of ATP have previously been reported (Coutts et al., 1981).

The vasa deferentia removed from sympathectomised rats were clearly 'supersensitive', inasmuch as they were spontaneously active and the mechanical responses to exogenous NA were substantially greater both in magnitude and duration (Fleming et al., 1973; Lee et al., 1975; Fleming, 1976). It is unlikely that the observed increase in the NA-evoked secretion of ATP was due to prejunctional supersensitivity per se because if the enhanced ATP release was purely due to a rise in [NA] available at α1-adrenoceptors (since there is no longer the possibility of any uptake into sympathetic nerves) then capsaicin would have been without effect. Thus, it appears that after chronic sympathectomy compensatory changes occur which are responsible for the rise in the NA-evoked release of ATP.

In many tissues, including the vas deferens, the proliferation of sensory nerves is accompanied by a substantial increase in substance P and calcitonin gene-
related peptide (CGRP) content (Kessler et al., 1983; Carvahlo et al., 1986; Nielsch & Keen, 1987; Aberdeen et al., 1990; 1992). The peptide CGRP is a potent neuromodulator, it has for example been shown to increase the rate of synthesis and insertion of acetylcholine receptors into cultured chick myotubes (Fontaine et al., 1986; New & Mudge, 1986). The effects of this peptide on adrenoceptor synthesis are not known but merit further investigation since changes are known to occur within the α-adrenoceptor population (but not α1) after sympathectomy (Abel et al., 1985; Rohde & Huidobro-Toro, 1988). The finding that prazosin decreased the NA-evoked release of ATP still further in guanethidine-only pretreated rats is consistent with the view that, after denervation, there is an increase in the number of α1-adrenoceptors. However, it is unlikely that any CGRP-mediated effects on adrenoceptor synthesis represent the main mechanism whereby, in sympathectomised tissues, NA could evoke an increase in ATP release. Since in cultured myotubes the maximum increase in acetylcholine receptor synthesis was 50% (New & Mudge, 1986); in vasa deferentia removed from sympathectomised rats the NA-evoked release of ATP is increased almost 600%. Sympathectomy has been shown to enhance the NA-dependent release of ATP from the guinea-pig vas deferens by a similar magnitude (Vizi et al., 1992).

It is also notable that, in vasa deferentia removed from sympathectomised rats, NA, even in the presence of
prazosin is still able to evoke a sizeable contraction. While this may provide further evidence for the denervation induced supersensitivity to ATP (and α,β-methylene ATP) previously reported by Rohde and Huidobro-Toro (1988) it may be due to, even in the presence of prazosin, the larger levels of ATP released in these pretreated tissues. This is of particular interest since Katsuragi et al. (1991) have proposed the existence of an ATP-evoked ATP releasing system in the guinea-pig vas deferens.

The results presented in this study also show that in control rats, part of the NA-evoked release of ATP from the rat vas deferens, at least 50%, is dependent on α₁-adrenoceptor activation. This substantiates our earlier claim that the indirect sympathomimetic tyramine, acts in this tissue to release NA which in turn evokes the release of ATP (Chapter 7, this thesis). Similar findings have recently been obtained in the guinea-pig vas deferens where the release of ATP was in part dependent on the actions of NA on α₁-adrenoceptors (Katsuragi et al., 1990; Vizi et al., 1992) although in neither study was the efflux of ATP due to smooth muscle contraction per se. It is generally presumed that the α₁-dependent portion of the NA-evoked ATP release arises from a non-neuronal source, probably the smooth muscle (Vizi & Burnstock, 1988; Katsuragi et al., 1990; Vizi et al., 1992). The demonstration that capsaicin pretreatment alone did not reduce the NA-evoked release of ATP from the rat vas
deferens gives further credence to this view since NA obviously does not exert its effects on the α1-adrenoceptors present on sensory nerve endings (Starke, 1977; Vizi, 1979; Wiesenfeld-Hallin & Hallin, 1984) in control tissues, thus making it more likely that under normal circumstances, the α1-dependent portion of the NA-evoked release of ATP arises from a non-neuronal source.

The mechanism whereby NA can evoke the release of ATP from sensory nerves in sympathectomised rat vasa deferentia is unclear but since a major portion of this release is inhibited by prazosin and the enhanced NA-evoked ATP release is greatly reduced by pretreatment with capsaicin, it seems likely that the release of ATP occurs in the main via the actions of NA on α1-adrenoceptors on sensory nerves (Starke, 1977; Vizi, 1979; Wiesenfeld-Hallin & Hallin, 1984).

In conclusion, the findings of this study indicate that sensory nerves appear to be a major source for the enhanced NA-evoked ATP release only in vasa deferentia removed from sympathectomised rats. These neurons are not a source for the NA-dependent release of ATP in control tissues. A large part of this release appears to arise from a non-neuronal store, probably the smooth muscle. These results provide an interesting insight into the neuronal interactions occurring between sympathetic and sensory nerves in the rat vas deferens.
Table 8.1 The release of endogenous ATP by NA from the vasa deferentia of 'control' and pretreated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal efflux of ATP (pmol/min/g ± S.E.M)</th>
<th>NA-evoked efflux of ATP (pmol/min/g ± S.E.M)</th>
<th>'n'</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>1.45 ± 1.41</td>
<td>30.18 ± 1.39</td>
<td>6</td>
</tr>
<tr>
<td>Guanethidine control</td>
<td>7.15 ± 1.15</td>
<td>46.88 ± 8.67</td>
<td>9</td>
</tr>
<tr>
<td>Capsaicin control</td>
<td>4.63 ± 0.85</td>
<td>30.04 ± 5.32</td>
<td>5</td>
</tr>
<tr>
<td>Capsaicin + guanethidine control</td>
<td>4.80 ± 0.58</td>
<td>28.38 ± 6.15</td>
<td>5</td>
</tr>
<tr>
<td>Capsaicin pretreated</td>
<td>5.37 ± 1.14</td>
<td>31.52 ± 6.83</td>
<td>6</td>
</tr>
<tr>
<td>Guanethidine pretreated</td>
<td>10.2 ± 1.78</td>
<td>195.5 ± 40.5 **</td>
<td>7</td>
</tr>
<tr>
<td>Capsaicin + guanethidine pretreated</td>
<td>7.06 ± 1.93</td>
<td>64.68 ± 9.79 *</td>
<td>6</td>
</tr>
</tbody>
</table>

Where 'n' represents the number of observations. Asterisks represent significant difference from appropriate control values (* P < 0.02, ** P < 0.005)
Table 8.2 The noradrenaline content of samples taken from the prostatic region of control and pretreated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NA content of vas deferens (µM/g tissue ± S.E.M)</th>
<th>'n'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>12.90 ± 0.50</td>
<td>5</td>
</tr>
<tr>
<td>Capsaicin vehicle</td>
<td>16.4 ± 0.90</td>
<td>5</td>
</tr>
<tr>
<td>Capsaicin vehicle + saline</td>
<td>13.7 ± 0.40</td>
<td>5</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>12.7 ± 0.52</td>
<td>3</td>
</tr>
<tr>
<td>Guanethidine</td>
<td>0.11 ± 0.03 **</td>
<td>4</td>
</tr>
<tr>
<td>Guanethidine + capsaicin</td>
<td>0.11 ± 0.04 **</td>
<td>5</td>
</tr>
</tbody>
</table>

Where 'n' denotes the number of samples. ** were shown to be statistically different from the appropriate 'control' (** P < 0.001).
Figure 8.1
Shows the effects of perfusion of the rat vas deferens from saline-injected animals with NA (50 μM) for periods of 1 min at 30 min intervals on the release of ATP.
A. Perfusion with NA (50 μM) for 1 min at 30 min intervals elicited a large phasic contraction which was maintained. B. Shows the effects of three successive NA (50 μM) perfusions on the endogenous release of ATP (n=9). The release of ATP mean ± S.E.M. is expressed as pmol ATP release/min/g of rat vas deferens.
pmoles ATP released/min/g of vas deferens

[Graph showing pmoles ATP released/min/g of vas deferens over time with bars for different conditions.]
Figure 8.2

The effects of prazosin (1 µM) on the NA-evoked responses and release of ATP in the vas deferens from 'control' rats. A. 10 min perfusion with prazosin (1 µM) before perfusion with NA (50 µM) and prazosin (1 µM) together, abolished the NA-induced contraction. B. The NA-evoked release of ATP was significantly reduced while the basal ATP efflux was unaffected. (** P < 0.01, n = 8).

pmoles ATP released/min/g of vas deferens

[Diagram showing bar graph with bars labeled NA and B, compared to a control (indicated by a cross) and a treatment (Prazosin 1 μM)]
Figure 8.3
Shows the effects of perfusion with NA (50 µM) on ATP release in the vasa deferentia from rats pretreated with guanethidine (60 mg/kg/day (s.c) for 6 weeks). A. Note that the NA-evoked contractions are much larger and that the vasa deferentia are now spontaneously active. B. Guanethidine-pretreatment substantially increased the NA-induced release of ATP (n=7).
pmoles ATP released/min/g of vas deferens
Figure 8.4

The effects of prazosin (1 μM) on the NA-evoked responses and ATP release in the vasa deferentia of guanethidine-pretreated rats (60 mg/kg/day (s.c) for 6 weeks). A. Prazosin (1 μM) significantly reduced the response to NA although it failed to completely abolish it, a fast twitch-like contraction remaining. B. Prazosin significantly decreased the NA-evoked release of ATP in these tissues (*** P < 0.001, n=7).
pmoles ATP released/min/g of vas deferens

Graph showing ATP release with various x-axis values ranging from 0 to 350.
The effects of perfusion with NA (50 μM) for 1 min at 30 min intervals on the contractile response and endogenous release of ATP in vasa deferentia removed from rats pretreated with capsaicin (50 mg/kg (s.c) on first and second day of life) followed by guanethidine pretreatment (60 mg/kg (s.c) daily for 6 weeks). A. Shows the NA-evoked contraction which is greater in both magnitude and duration. Note the vasa deferentia were again spontaneously active. B. The enhanced endogenous release of ATP in response to perfusion with NA (50 μM). Note that the release of ATP is not nearly so markedly increased in these rats (n=6).
Figure 8.6

Shows the effects of perfusion with prazosin (1 μM) on the NA-evoked contraction and ATP release from vasa deferentia previously pretreated with both capsaicin (50 mg/kg on day 1 and 2 of life) followed by guanethidine pretreatment (60 mg/kg/day (s.c.) for 6 weeks). A. Prazosin substantially lowered the NA-evoked contraction but did not abolish it. B. The NA-evoked release of ATP was significantly reduced in the presence of prazosin (***P < 0.001, n=6).
pmoles ATP released/min/g of vas deferens
CHAPTER 9.

General Discussion
9.1 General Discussion

Since each of the previous experimental chapters has its own detailed discussion, this chapter is intended to provide a general overview of the thesis and to suggest, where applicable, future experiments which may further our knowledge in this field.

Cotransmission is said to occur when two (or more) neurotransmitters are concomitantly released from the same nerve and then act on the same target cell so that the final response is the resultant effect of both (or all) the transmitters (Burnstock, 1976; Campbell, 1987). During the last 15 years, compelling evidence has accumulated to indicate that ATP is released from sympathetic nerves as a cotransmitter along with NA (Stjärne, 1989; Burnstock, 1990b; Von Kügelgen & Starke, 1991). Much of the evidence supporting this view arises from work on the vas deferens, where ATP is believed to be the transmitter mediating the fast initial component of contraction, the 'twitch' and the electrical events preceding it, the EJPs: both of these responses are resistant to α-adrenoceptor blockade (Sneddon & Burnstock, 1984a; Sneddon & Westfall, 1984; Allcorn et al., 1986). Further evidence to support the view that, in the guinea-pig vas deferens the EJPs and the twitch are mediated by ATP is presented in Chapter 5 of this thesis. In this study, EJPs and the contractile response produced by stimulation of the sympathetic nerves and by exogenous
ATP were inhibited by both $\alpha,\beta$-meATP and the dinucleotide. APsA. APsA and $\alpha,\beta$-meATP were approximately 100 times more potent than ATP in producing phasic contractions of the guinea-pig vas deferens and approximately equipotent in their production of desensitization of this tissue. In all but one aspect, the effects of APsA and $\alpha,\beta$-meATP were remarkably similar: the two agents differed only in that the antagonistic effects of APsA on mechanical responses elicited by field stimulation and EJPs were more readily reversed on washout. This finding may be due to the existence, in sympathetic neurones, of a novel enzyme capable of asymmetrically hydrolysing diadenosine polyphosphates. Such an enzyme, an ecto-5'-nucleotidase polyphosphate hydrolase has recently been characterized in chromaffin (Rodriguez-Pascal et al., 1992) and endothelial cells (Ogilvie et al., 1999). More recently, diadenosine compounds have been shown to be released from chromaffin cells and synaptosomes prepared from rat brain and torpedo electric organ (Pintor & Miras-Portugal, 1993). In rat brain, the effects of diadenosine polyphosphates are believed to be mediated via a new purinoceptor, the $P\alpha$ receptor (Pintor & Miras-Portugal, 1993) and in the rat vas deferens APsA and AP$\alpha$A act at a receptor that is pharmacologically distinct from the $P\alpha$-purinoceptor (Stone & Paton, 1999).

This thesis also presents clear evidence to corroborate the view that ATP is released as a cotransmitter from the sympathetic nerves innervating the
rat and guinea-pig vas deferens. In Chapter 3, electrical stimulation of the guinea-pig vas deferens elicited the characteristic biphasic contraction (Swedin, 1971; McGrath, 1978) and evoked a substantial increase in the release of endogenous ATP; both of these responses were severely reduced by treatments known to prevent neurotransmission and in particular by agents known to impair sympathetic transmission. For example, the release of ATP and the subsequent contraction were greatly reduced by TTX, 6-OHDA and the sympathetic neuron blocker, guanethidine. On the other hand, neither the twitch nor the release of ATP were reduced by reserpine although almost 99% of the tissue NA content was lost. This is in agreement with previous studies which have shown that reserpine has little effect on fully facilitated EJPs in the guinea-pig vas deferens (Sneddon et al., 1982; Sneddon & Westfall, 1984; Cunnane & Manchanda, 1989). Thus, whilst intact sympathetic nerves are a prerequisite for stimulus-evoked release of endogenous ATP and the initial phase of contraction in the guinea-pig vas deferens, the classical sympathetic transmitter NA, is not. This finding ends previous speculation that, in some tissues, the α1-adrenoceptor resistant, NANC responses are mediated by NA acting on a unique type of adrenoceptor, the ɣ-receptor (Hirst & Neild, 1980; 1981). This is in agreement with the recent findings of Evans & Suprenant (1992) who have conclusively demonstrated that neither the EJPs nor the nerve stimulation-evoked contraction of
the guinea-pig submucosal arteries are mediated by NA acting on the \( \gamma \)-receptor. Indeed, both responses were antagonised by the \( P_2 \)-receptor antagonist, suramin. Furthermore, since the release of ATP and the initial component of the contraction were not decreased by reserpine, it seems unlikely, in the guinea-pig vas deferens at least, that these responses arise secondary due to the action of NA on \( \alpha_1 \)-adrenoceptors (Fredholm & Hedqvist, 1980; Fredholm et al., 1982; Vizi & Burnstock, 1988). It is also unlikely that the \( \alpha_1 \)-adrenoceptor antagonist resistant responses may be due to newly synthesized NA acting on \( \alpha_1 \)-adrenoceptors (Duval et al., 1985). Since, in Chapter 3 we have shown that prazosin did not significantly antagonise the twitch response nor the release of ATP in reserpinized tissues.

The demonstration in this thesis, that neither ATP release nor the subsequent twitch response were reduced by reserpine was in disagreement with Vizi and his colleagues (Sperlagh & Vizi, 1992; Vizi et al., (1992) who demonstrated that after reserpinization, the basal release of ATP was significantly increased whilst evoked release significantly decreased throughout. It is unclear why such a disparity should occur since pretreatent methods are the same. it is possible however, that with significantly increased basal levels of ATP there was an increased concentration of adenosine at the prejunctional nerve terminal which in turn, via \( P_1 \)-purinoceptors decreased the evoked release of ATP. Evidence for the
existence of Pi-purinoceptors capable of modulating the
evoked release of ATP is given in Chapter 5 (see below).

Further evidence to suggest that ATP was indeed released as a cotransmitter from sympathetic nerves was
presented in Chapter 4. In this study, the evoked release of ATP was not significantly reduced when the mechanical
response was almost completely abolished by the application of prazosin and α,β-meATP. Similar results
were reported by Lew & White (1987). These findings suggest that release of ATP is not due to contraction of
the smooth muscle per se and this is borne out by the demonstration by Katsuragi et al. (1990) that K⁺ induced
contractions do not evoke the release of ATP from the guinea-pig vas deferens. However, in two recent reports
it has been shown that, even in the guinea-pig vas deferens, the release of ATP could arise secondarily to the
efflux of NA and that this release was in part due to the effects of NA acting on α-adrenoceptors (Katsuragi et
al., 1990; Vizi et al., 1992). In the rat vas deferens, similar studies have shown that almost 80% of the ATP
released by sympathetic stimulation occurs as a direct result of the effects of NA acting on α-adrenoceptors
(Vizi & Burnstock, 1988). Similar findings are presented in Chapter 7 of this thesis, where part of the
tyramine-evoked release of ATP was also dependent on α1-adrenoceptor stimulation. However, since only a portion of
the tyramine-evoked release of ATP was inhibited by prazosin (approximately 40%) this cannot be the only
mechanism whereby tyramine can increase the secretion of ATP. Exactly how tyramine can evoke the release of ATP in a manner independent of α₁-adrenoceptor activation is not yet clear but several possibilities exist. For example, tyramine has been shown to enhance the release of NPY from the rat vas deferens and from synaptosomes prepared from this tissue (Cheng & Shen, 1987). The release of NPY was inhibited by 6-OHDA and desipramine and therefore it was concluded that secretion arose prejunctionally from sympathetic nerves via a mechanism involving the neuronal carrier (Cheng & Shen, 1987). Furthermore, since NPY has been shown to be released by tyramine and since NPY and ATP are known to be costored with NA only in the LDV's (Fried et al., 1985), it seems feasible that tyramine may act there to evoke the release of ATP, NA and NPY.

It is also feasible that the α₁-independent portion of ATP release evoked by tyramine arises due to the ATP enhancement of ATP release recently reported by (Katsuragi et al., 1991). In their study, interaction of α,β-meATP with P₃-purinoceptors increased the release of ATP but not that of ACh or NA and the authors therefore concluded that ATP was probably released postjunctionally from the smooth muscle. However, the existence of prejunctional P₂ₓ-purinoceptors has already been proposed (Stjärne & Astrand, 1985) and so it is also possible that α,β-meATP enhances the release of ATP by interacting with P₂ₓ-purinoceptors located on the sympathetic nerves. P₂ₓ-purinoceptors may also be present prejunctionally in the
the guinea-pig ileum (Moody & Burnstock, 1985). α,β-
meATP, at much lower concentrations, significantly
enhanced the basal and the tyramine-evoked release of ATP.

Alternatively, tyramine may act to release NA which
in turn may release ATP from sympathetic nerve terminals
by mimicking the actions of the neurotoxin 6-OHDA, which
has been shown to enhance the release of both ATP and
[^3H]-NA from rabbit pulmonary artery (Katsuragi et al.,
1987). This possibility is supported by the finding that
in vivo, NA or a metabolite of NA has been shown to mimic
the damaging effects of 6-OHDA (Texiera et al., 1989). It
is interesting to note that the neurotoxic effects of NA
in this study by Texiera et al. (1989) appeared to be
focussed on the LDV, a well established intraneuronal
source of ATP (Lagercrantz, 1976) and the only neuronal
store for NPY (Fried et al., 1985). Like tyramine, only
part of the exogenous NA-evoked release of ATP from the
rat vas deferens is dependent on stimulation of α-
adrenoceptors.

The release of ATP occurring secondary to the α-
adrenoceptor mediated actions of NA has hitherto been
accepted as evidence that much ATP is released from a
postjunctional pool (Fredholm & Hedqvist, 1980; Hedqvist
et al., 1982; Vizi & Burnstock, 1988; Katsuragi et al.,
1991; Vizi et al., 1992). While NA has been shown to
behave in a tyramine-like manner to enhance its own
release from the mouse vas deferens (Vizi et al., 1985;
1986) and to release [^3H]-purines and NA by mimicking the
neurotoxin 6-OHDA, the demonstration that sympathectomy does not reduce but rather substantially increases the NA-evoked release of ATP has been presented as evidence for the lack of involvement of sympathetic neurons in NA-dependent ATP secretion (Vizi et al., 1992).

In agreement with Vizi et al. (1992) the results presented in Chapter 8 also show that sympathectomy, produced in this study by chronic guanethidine pretreatment, significantly enhances the NA-evoked release of ATP from the vasa deferentia of the rat. The sensory neurons are the most likely source of the enhanced release of ATP since after combined sensory and sympathetic denervation, the NA-evoked release of ATP is substantially less than after sympathectomy alone. The mechanism whereby NA can evoke the release of ATP from sensory nerves in sympathectomised tissues is unclear but since a major portion of this release is inhibited by prazosin and the enhanced NA-evoked release is greatly reduced by capsaicin, it seems likely that the release of ATP occurs in the main via the actions of NA on \( \alpha_1 \)-adrenoceptors on sensory nerves (Starke, 1977; Vizi, 1978; Wiesendfeld-Hallin & Hallin, 1984). The fact that NA-evoked ATP release from the rat vas deferens is not decreased after sympathectomy nor reduced by capsaicin alone provides further evidence to support the view that under normal circumstances, the \( \alpha_1 \)-adrenoceptor dependent release of ATP arises from an non-neuronal source, probably the smooth muscle. This view is strengthened by the
demonstration that methoxamine, an \( \alpha \)-agonist which is not taken up into sympathetic nerves, can also stimulate the release of ATP (Vizi et al., 1992).

However, the smooth muscle cannot be the only site of ATP release since prazosin failed to completely inhibit the efflux of ATP. The source of the remaining portion of the NA-evoked release of ATP is as yet unclear but it may in part due to stimulation of ATP release by ATP as discussed above (Katsuragi et al., 1991).

The fact that sympathectomy increased rather than decreased the NA-evoked release of ATP tends to suggest that sympathetic nerves are not involved in the \( \alpha \)-independent release of ATP but it is important to appreciate that the significant rise in ATP release seen after sympathectomy may mask a much smaller decrease in the release of ATP due to the inability of NA to act on these nerves. Thus it is difficult in this study to predict what role, if any, sympathetic nerves play in the \( \alpha \)-adrenoceptor mediated release of ATP. Further research is required to elucidate the role of ATP released from sources other than sympathetic nerves. In particular, the role of the cholinergic neurons innervating the rat vas deferens regarding NA-evoked release of ATP should be investigated.

The fact that ATP can be released from source(s) other than sympathetic nerves has important implications. For example, ATP released by NA or indeed by ATP (Katsuragi et al., 1991) may contribute to the contractile
response and may thus constitute an important physiological amplification method (Von Kügelgen & Starke, 1991). For example, the fact that NA can evoke the release of ATP may explain why NA applied iontophoretically to guinea-pig submucosal arterioles can produce a depolarisation which resembles the EJP normally produced by stimulation of the sympathetic nerves innervating this tissue (Hirst & Neild, 1980; 1981), especially when NA is applied close (< 10 nM) to nerves (Hirst & Neild, 1981). The fact that NA may mediate the EJPs in guinea-pig submucosal arterioles by releasing another substance from nearby nerves has been suggested (Neild, 1981) and in fact, in a recent study, Evans and Suprenant (1992) showed that EJPs in the guinea-pig submucosal arterioles are antagonized by the P2-purinoceptor antagonist, suramin.

The results in Chapter 4 also show for the first time, the concomitant release of NA, ATP and NPY. However, with the exception of a few blood vessels, the spleen and the mouse vas deferens (where it produces only a weak contraction: Lundberg & Tatemoto, 1982; Edvinsson, 1985; Fried et al., 1985; Stjärne & Lundberg, 1986; Kascube & Zetler, 1989) NPY is considered to be a neuromodulator rather than a neurotransmitter. The demonstration that electrical stimulation could evoke the release of NA, ATP and NPY strongly suggests that both small and large dense core vesicles undergo exocytosis since only the large dense core vesicles contain NPY (Freid et al., 1985; De
although it is still not clear whether or not NA and ATP are released together from the same or different vesicle subtypes. Recent studies have shown that the release of ATP and NA from the vasa deferentia of several species can be differentially controlled (Trachte, 1985; Trachte, 1988; Trachte et al., 1989; Ellis & Burnstock, 1989a, b; Hata et al., 1991; Donoso et al., 1992; Sperlágh & Vizi, 1992; this thesis (Chapter 3). The existence of multiple storage sites involved in transmitter release, whether they be different vesicle subtypes or subpopulations of sympathetic nerves (Burnstock, 1990b) is fundamental to the belief that the release of ATP occurs independently to that of NA.

Regardless of which vesicle subtypes are involved in the release of NA, ATP and NPY in the vas deferens, the very existence of multiple transmitter/modulator substances, able to interact with their own and each others pre- and postjunctional receptors make any discussion of factors controlling release and any postjunctional effects witnessed, exceptionally complex. For example, ATP is rapidly and sequentially dephosphorylated to adenosine by ectonucleotidases (Pearson, 1985; Gordon, 1986; Meghji, 1993) and the adenosine generated by this process is a potent neuromodulator of transmitter release in both the peripheral and the central nervous system (Fredholm & Dunwiddie, 1988; Williams, 1989).

In the vas deferens, adenosine has been shown to
inhibit the release of NA (Fredholm & Hedqvist, 1976; Clanachan et al., 1977; Wakade & Wakade, 1978). Its effects on the evoked release of ATP from the guinea-pig vas deferens are presented in Chapter 5, where adenosine was shown to inhibit the evoked release of ATP via prejunctional P1-purinoceptors. Studies in the mouse and rat vas deferens have suggested that the P1-purinoceptor subtype inhibiting the twitch response and EJPs most closely resembles the A1-receptor (Blakeley et al., 1988; Hourani et al., 1993). Future studies with more selective A1-receptor antagonists could investigate which adenosine receptor subtype controls transmitter secretion in the guinea-pig vas deferens.

In Chapter 5, we were also able to demonstrate that adenosine plays an important physiological role in neurotransmission in the guinea-pig vas deferens since, in this study, the effects of endogenous adenosine were significantly enhanced by the adenosine uptake inhibitor, dipyridamole. These findings were supported by the fact that the P1-receptor antagonist, 8-PT, was able to significantly enhance the basal release of ATP. This suggests that transmitter secretion is, under normal circumstances, maintained by endogenous adenosine.

NA is also a potent inhibitor of neurotransmitter release, inhibiting both its own release and that of other transmitters via interactions with prejunctional α1-adrenoceptors (Starke, 1987). In the vas deferens, α1-adrenoceptor agonists reduce the amplitude of EJPs.
(Blakeley et al., 1981; Illes & Starke, 1983; Brock et al., 1990) and have recently been shown to decrease the release of ATP from the guinea-pig (Sperlagh & Vizi, 1992; Breisner et al., 1993) and mouse vas deferens (von Kügelgen et al., 1989; 1991). Thus, in this thesis we have shown that ATP is released as a cotransmitter from the sympathetic nerves of the guinea-pig and rat vas deferens. The multiplicity of transmitters and receptors involved and the interactions between the receptor subtypes makes interpretation of postjunctonnal and release data complex and leaves several questions unanswered. In particular, much must be done to ascertain the exact role of ATP released by either NA or ATP from sources which are not sympathetic and the contribution of this release to sympathetic neurotransmission. Furthermore, future studies must investigate the morphological apparatus that enables the release of ATP and NA to be differentially controlled.
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245


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255


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