NEURORECEPTOR MEDIATED VASCULAR CONTROL MECHANISMS IN THE RAT

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STATEMENT

The contents of this thesis are original and all experiments were planned and carried out by the author under the supervision and guidance of Dr. Caryl Hill, except where otherwise stated or referenced.

The reverse transcription-polymerase chain reaction (RT-PCR) methodology used for these experiments is based upon a protocol established by Dr. Maria Vidovic. Oligonucleotide primers used for these experiments were designed by Dr. Maria Vidovic, Dr. Caryl Hill, Dr. Hilton Grayson and Mr Matthew Newhouse. The studies in the arteries of the rat hepatic mesentery in Chapters 4 and 5 were initiated following discussions with Prof. Allan J. McLean. In Chapter 7, the substance P immunohistochemistry was completed by Dr. Caryl Hill and the RT-PCR study involving the use of anti nerve growth factor serum was performed by Ms Jane Ellis.

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ABSTRACT

The nervous control of blood flow is regulated by specific receptors located in the vascular smooth muscle and endothelial cells. The variability demonstrated by different vascular beds in response to nerve stimulation, both during development and in the adult, may be related to the heterogeneous expression of specific neuroreceptor subtypes. The primary aim of this thesis therefore, was to determine if such differences could be attributed to differences in receptor gene expression.

Reverse transcription polymerase chain reaction (RT-PCR) was used to screen receptor mRNA expression in the basilar, mesenteric, pulmonary, tail and hepatic arterial beds of the rat. Receptor subtypes studied included the α_1- and α_2-adrenergic, neurokinin and muscarinic receptors. Results demonstrated that the pattern of gene expression was variable, with no two arteries having the same receptor profile. Overall, some receptors showed a consistently strong pattern of expression, while others exhibited more variable profiles across the different arteries. Additional findings from this study included the previously unreported detection of mRNA for the M5 muscarinic and NK3 neurokinin receptors in all arteries studied.

The RT-PCR results examining receptor expression were consistent with the functional data of other investigators, however in order to directly correlate mRNA expression and functional protein expression in the same tissue, the hepatic arterial bed was used to further characterise neuroreceptor mediated vascular responses. A dense sympathetic innervation was demonstrated in this vascular bed, using catecholamine histochemistry and antibodies directed against the synaptic vesicle protein synaptophysin. Physiological experiments revealed that the principle receptors involved in the contractile response to nerve stimulation were the α_1A-adrenergic, P_{2X(0)}-purinergic and neuropeptide Y Y_1 receptors, all of which showed strong mRNA expression as detected using RT-PCR. Additionally, neurotransmitter release was shown to be modulated by presynaptic α_2-adrenergic and possibly P_{2X}-purinergic receptors. Sympathetic nerves were also shown to activate a dilatory response via postsynaptic β-adrenergic receptors. A dilatory response, mediated by calcitonin gene related peptide (CGRP) was also demonstrated, correlating with the detection of perivascular nerves.
immunoreactive for CGRP and the strong mRNA expression of the CGRP$_1$ receptor subtype. No role could be found for nitric oxide (NO) in nerve-mediated vasodilatory responses, however experiments did reveal an important regulatory role for NO released from the endothelium in response to vasoconstriction and increased vascular tone.

Overall, the studies in the rat hepatic arterial bed demonstrated that complex interactions occur between vasoconstrictor and vasodilatory pathways and that these responses are further modulated by presynaptic mechanisms. The major postsynaptic neuroreceptor subtypes involved were well predicted by mRNA expression levels as determined using RT-PCR, confirming that this technique is a useful adjunct in studies aimed at identifying functional vascular neuroreceptor mediated responses.

Experiments were then performed to determine if alterations in receptor mRNA expression could account for the physiological changes described in the rat mesenteric artery during development. Using RT-PCR as a screening tool, no changes were detected that could account for the maturation of the adult response, nor was there any link between the appearance of the nerves and the initial expression of mRNA for the receptors under study. This lack of positive correlation between the presence of nerves and receptor gene expression was confirmed with denervation studies, where the ingrowth of sympathetic and sensory nerve fibres was prevented over the critical first two postnatal weeks. The effectiveness of sympathectomy treatments was assessed using catecholamine histochemistry and it was interesting to note that nerve growth factor sensitive sympathetic neurones innervating the mesenteric vascular bed were resistant to permanent destruction by the compound 6-hydroxydopamine, in contrast to sympathetic neurones innervating rat iris arterioles.

In summary, this thesis reviews neuroreceptor mediated vascular control mechanisms and examines in detail the expression of neuroreceptor genes in arteries of the rat, both in the adult and during development. The contribution of the different receptor subtypes to nerve-mediated vascular responses are characterised specifically in the hepatic arterial bed. It is concluded that individual arteries possess both common and unique neural control mechanisms, which may ultimately be utilised in the development of systemic and vascular bed specific therapeutic agents.
PUBLICATIONS ARISING FROM DATA PRESENTED IN THESIS

The following papers resulting from work presented in this thesis have been published or accepted for publication:


The following papers resulting from work presented in this thesis have been submitted for publication:


A number of presentations of this work were made at scientific meetings. The following abstracts were published in conjunction with these presentations:


ABBREVIATIONS

The following is a list of abbreviations used in the text of this thesis:

\(\alpha,\beta\)-mATP: \(\alpha;\beta\)-methylene ATP
\(\beta\)-NADPH: \(\beta\)-nicotinamide adenine dinucleotide phosphate tetrasodium
5-HT: 5-hydroxytryptamine
6-OHDA: 6-hydroxydopamine
ADP: adenosine diphosphate
AMP: adenosine monophosphate
ATP: adenosine triphosphate
bp: base pairs
cAMP: cyclic 3',5'-adenosine monophosphate
cDNA: complementary DNA
CEC: chloroethylclonidine dihydrochloride
cGMP: cyclic 3',5'-guanosine monophosphate
CGRP: calcitonin gene related peptide
DMI: desipramine
DNase I: deoxyribonuclease I
EDHF: endothelium derived hyperpolarising factor
EDRF: endothelium derived relaxant factor
EDTA: ethylenediaminetetra-acetic acid
ejp(s): excitatory junctional potential(s)
GTP: guanosine triphosphate
hCGRP\textsubscript{8-37}: human CGRP\textsubscript{8-37}
IC\textsubscript{50}: half maximal inhibitory concentration
KCl: potassium chloride
L-NAME: \(\text{N}^\text{G}\)-nitro-L-arginine methyl ester
mRNA: messenger RNA
NGF: nerve growth factor
NK: neurokinin
NO: nitric oxide
NOS: nitric oxide synthase
NPY: neuropeptide Y
PACAP: pituitary adenylate cyclase activating protein
PB: phosphate buffer
PBS: phosphate buffered saline
PHI: peptide histidine isoleucine
PPADS: pyridoxal phosphate-6-azophenyl 2’-4’disulphonic acid tetrasodium
RNase: ribonuclease
RT-PCR: reverse transcription-polymerase chain reaction
UDP: uridine diphosphate
UTP: uridine triphosphate
VIP: vasoactive intestinal peptide
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CHAPTER 1
INTRODUCTION

1.1 VASCULAR CONTROL MECHANISMS

Within individual vascular beds, alterations in blood flow are regulated by vascular smooth muscle contraction and relaxation and the subsequent changes in vessel diameter (Johnson 1978b; Prys-Roberts 1984). Regulation of these vascular responses involves a variety of nervous, hormonal and local mechanisms, all of which ultimately act to sustain tissue perfusion and maintain normal blood pressure (Johnson 1978a; Katzung 1992a).

Local control mechanisms are activated by acute changes in blood flow, oxygen tension, tissue metabolic states and temperature (Prys-Roberts 1984; Redfern et al. 1995). Responses may be mediated by the endothelium, through the release of a range of endothelial derived relaxing and contracting factors (Ralevic & Burnstock 1996b) or via the vasodilatory action of sensory afferent nerves (Holzer 1992; Edvinsson et al. 1996). Hormonal control mechanisms are regulated by circulating vasoactive substances such as adrenaline and angiotensin (Guyton 1986; Reid et al. 1995) and can potentially influence vascular responses throughout the entire body. Regulation by the autonomic nervous system is superimposed over local control mechanisms and can regulate the circulation to different vascular beds with a high degree of specificity (Calaresu et al. 1975). The different nervous components involved include sympathetic vasoconstrictor neurones, parasympathetic vasodilator responses and nonadrenergic noncholinergic mechanisms (Ralevic & Burnstock 1996b; Bell 1996; Franco-Cereceda 1996). For the purpose of this thesis, further discussion of control mechanisms will be largely confined to those exerted by components of the nervous system.

Released neurotransmitter activates specific receptors, located on either smooth muscle cells or endothelial cells. Through various pathways and signaling mechanisms, contraction or relaxation of the vascular smooth muscle cell occurs in response to a
respective increase or decrease in the availability of intracellular Ca\textsuperscript{2+} (Walsh et al. 1995). This system is complicated by the fact that each nerve type contains a number of different cotransmitters, which can in turn activate a number of different neuroreceptor subtypes.

Nerve-mediated vascular responses show considerable variability, both between vascular beds and along an individual blood vessel (Hirst & Edwards 1989; Vargas & Gorman 1995; Bylund et al. 1995; Kemp et al. 1997). Changes have also been described within individual vascular beds during development and ageing (Hill & Vidovic 1992; Sandow & Hill 1998; Gurdal et al. 1995a). One hypothesis is that this heterogeneity may be due to differences in the types and subtypes of neuroreceptors expressed in individual blood vessels. This theory is particularly attractive given the recent rapid expansion of receptor subtypes being characterised by pharmacological and molecular methods (see Pharmacol. Rev, 46, 1994 for details). In this chapter, the various subtypes of neuroreceptors will be reviewed, with specific reference to the nervous system under which they function, their molecular and pharmacological classification and the vascular responses that they have been demonstrated to mediate.

1.2 VASCULAR NEURORECEPTORS

By definition, receptors recognise a specific chemical signal and convert information from that signal into a form that the cell can in turn recognise and respond to accordingly, for example, the activation of second messenger systems or changes in membrane permeability (Kenakin et al. 1992). Receptors are generally named after the endogenous ligand which activates them. Receptor subtypes are then further characterised on the basis of relative potencies and selectivities of antagonists and agonists, transduction systems and more recently, the molecular identification of specific complementary DNA (cDNA) encoding for individual receptors (Kenakin et al. 1992; Milligan et al. 1994).

Except for the P\textsubscript{2X}-purinergic receptors, all the receptors examined in this study belong to the large superfamily of G-protein coupled receptors. Strictly conserved amongst this superfamily is the basic structure of a single polypeptide with 7 putative
transmembrane domains (Majewski & Barrington 1995). Between the different receptor families and subtypes, the most conserved regions are the transmembrane domains while the greatest variability is seen in the amino terminus, carboxy tail and third intracellular loop (O'Dowd et al. 1989; Guarino et al. 1996). The third intracellular loop is believed to be important in conveying G-protein coupling specificity to the different receptor subtypes (Hein & Kobilka 1997).

G-proteins are heterotrimers of a guanosine triphosphate (GTP) binding α-subunit and regulatory β and γ subunits (Ross 1989). There are multiple α-subunits and these impart specificity to the distinct G-protein subtypes (Majewski & Barrington 1995). Once activated, G-proteins regulate the activity of particular receptor proteins or effector systems, including adenylate cyclase, guanylate cyclase and phospholipase C or they may interact directly with ion channels to alter membrane permeability (Birnbaumer et al. 1990). In general, these pathways lead to changes in intracellular Ca$^{2+}$ levels, controlled by intracellular stores or voltage-dependent/independent Ca$^{2+}$ channels (Graham et al. 1996). Classically, neuroreceptors have been broadly classified according to the type of G-protein and effector systems activated, however there is now considerable evidence that receptors can couple to multiple G-proteins and hence activate different intracellular pathways. This diversity is both tissue and species specific (Graham et al. 1996; Hein & Kobilka 1997).

1.3 ADRENERGIC NEUROTRANSMISSION

Noradrenaline, rather than adrenaline, was first identified as a sympathetic neurotransmitter in 1946 (Von Euler 1946) and many studies utilising histochemical and pharmacological techniques have confirmed its significant role in the vasculature (Wilson & Dunn 1996). The primary response to sympathetic nerve stimulation in arteries is vasoconstriction via the activation of postsynaptic α-adrenoceptors (Hirst et al. 1996). Sympathetic nerves have also been shown to mediate distinct vasodilatory responses however, and an increasing importance is being defined for cotransmitters such as adenosine triphosphate (ATP) and neuropeptide Y (NPY).
Noradrenaline acts on adrenoceptors, which were first subtyped as α and β by Ahlquist (1948), each being respectively associated with excitation (vasoconstriction) or inhibition (vasodilation). α-adrenoceptors were further classified as α₁ or α₂, based primarily on the differential sensitivities to phenoxybenzamine shown by receptors mediating pre- and postjunctional vascular responses respectively (Dubocovich & Langer 1974; Langer 1974). It has since been shown that anatomical location or physiological function is not sufficient for receptor classification (Piascik et al. 1996), however the α₁- and α₂-adrenergic subtypes are now well established in terms of agonist and antagonist sensitivities. α₁-adrenergic receptors are activated by phenylephrine and methoxamine and blocked by prazosin or WB-4101, while responses elicited by UK14304 or clonidine and antagonised by yohimbine or rauwolscine are regarded to be α₂-adrenoceptor mediated (Ruffolo et al. 1991; Piascik et al. 1996).

Functional and binding studies revealed that both the α₁-, α₂- and β-adrenergic receptor families could be further subdivided into additional subtypes and molecular biology has confirmed these subdivisions (Harrison et al. 1991a).

1.3.1 α₁-adrenergic receptors

α₁-adrenergic receptor pharmacological studies. A number of early studies postulated the existence of two types of α₁-adrenoceptors (McGrath 1982; Medgett & Langer 1984) and in the mid eighties, Morrow et al. (Morrow et al. 1985; Morrow & Creese 1986) presented evidence for α₁A and α₁B subtypes, based on the relative binding of prazosin, WB-4101 and phentolamine in rat brain preparations. The α₁A and α₁B subtypes were assigned in conjunction with respective high and low affinities for both phentolamine and WB-4101. Further studies defined the receptors as being either chloroethylclonidine (CEC) insensitive (α₁A) or sensitive (α₁B), (Minneman et al. 1987) and that they utilised different signal transduction pathways. The α₁A-adrenergic receptor was shown to control Ca²⁺ levels through the opening of dihydropyridine sensitive voltage channels, while α₁B-adrenoceptors regulated inositol phospholipid hydrolysis and increased Ca²⁺ levels from intracellular stores (Han et al. 1987). As mentioned earlier, though still broadly applicable, signaling pathways are no longer used in terms of receptor classification (Piascik et al. 1996).
**Molecular classification of α₁-adrenergic receptors.** Molecular cloning techniques were used to isolate the α₁B-adrenoceptor clone from a hamster smooth muscle cell line (Cotecchia *et al.* 1988). Screening of a bovine brain cDNA library led to the identification of a second clone which demonstrated the pharmacological profile of the α₁A-adrenergic receptor, however, lack of messenger RNA (mRNA) detection (using Northern blot analyses) in rat tissues where the α₁A subtype had been clearly defined led to the belief that a novel subtype, α₁C, had been cloned (Schwinn *et al.* 1990). A third adrenoceptor was isolated from a rat cerebral cortex cDNA library, which again demonstrated α₁A-like pharmacology and additionally showed the tissue distribution expected of the α₁A-subtype (Lomasney *et al.* 1991b). This receptor was prematurely designated the α₁A-adrenergic receptor, however at the same time, Perez *et al.* (1991), isolated a virtually identical clone from the rat brain. Subsequent analysis revealed that a sequencing error in the initial α₁A clone accounted for the minor difference and that importantly, the pharmacological profile of the new clone was not consistent with the classical α₁A-adrenergic receptor, despite its widespread tissue distribution (Perez *et al.* 1991). The subtype was subsequently classified as the α₁D-adrenoceptor (Ford *et al.* 1994). Considerable evidence has since been presented to suggest that the cloned α₁C-adrenergic receptor is in fact the α₁A-receptor, including detection of mRNA in classical α₁A-adrenoceptor tissues (Faure *et al.* 1994; Laz *et al.* 1994; Perez *et al.* 1994; Pimoule *et al.* 1995). The lack of detection of α₁AC-receptor mRNA seen in the original study (Schwinn *et al.* 1990) was attributed to rigid levels of stringency combined with weak hybridization of the bovine probe in the rat tissues (Faure *et al.* 1994; Ford *et al.* 1994).

Current nomenclature guidelines thus recognise three distinct α₁-adrenergic receptor subtypes: α₁A, α₁B and α₁D (Hieble *et al.* 1995; Alexander & Peters 1998), correlating the molecular and pharmacological properties described above.

**Low affinity α₁-adrenergic receptors.** Pharmacological studies have suggested that further subtypes of α₁-adrenergic receptors may exist in blood vessels, distinguished primarily by their low affinity for prazosin (Flavahan & Vanhoutte 1986; Muramatsu *et al.* 1990). In this system, α₁-adrenoceptors possess high (α₁H), or low (α₁L) affinity for prazosin. A third subtype α₁N, was also proposed, characterised by a relatively low sensitivity to prazosin but high affinities for HV723 and WB-4101.
(Muramatsu et al. 1990; Hancock 1996). The currently recognised receptors would be
classified as $\alpha_{1H}$-receptors under this system. Although $\alpha_{1L}$-adrenoceptors are proposed
to mediate noradrenaline induced contractions in a range of vessels, including the rabbit
aorta (Muramatsu et al. 1990; Oshita et al. 1993), mesenteric artery (Van der Graaf et
al. 1997) and cutaneous vessels (Smith et al. 1997), no corresponding molecular
equivalents have been identified and they have not officially been incorporated in the
$\alpha_1$-adrenergic receptor family (Hieble et al. 1995; Alexander & Peters 1998). Recent
studies with cloned $\alpha_1$-adrenergic receptors suggest that the human $\alpha_{1A}$-adrenoceptor
can in fact display the pharmacological properties of the both the $\alpha_{1A}^+$ and $\alpha_{1L}$-subtypes
under certain conditions (Ford et al. 1998).

$\alpha_1$-adrenergic receptor mediated vascular responses. Although noradrenergic
vasoconstrictions are predominantly mediated by $\alpha_1$-adrenergic receptors (Piascik et al.
1996), a multiplicity of $\alpha_1$-subtypes has been shown to exist in the vasculature and the
distribution of these subtypes varies with the vessel and species examined (Vargas &
Gorman 1995). Functional constriction studies have demonstrated that the majority of
responses are mediated by the $\alpha_{1A}$-subtype (Piascik et al. 1990; Vargas & Gorman 1995;
Piascik et al. 1996), for example in the renal artery (Han et al. 1990; Zhou et al. 1998),
muscular resistance vessels (Zhu et al. 1997) and in the rat mesenteric and rabbit
ovarian vascular beds (Williams & Clarke 1995; Yousif et al. 1996). $\alpha_{1B}$-adrenergic
receptors, however, have also been shown to mediate sympathetic constrictions in rat
irideal arterioles (Gould & Hill 1994) and vena cava (Sayet et al. 1993), and a
contribution by $\alpha_{1D}$-adrenergic receptors has been described in a number of different
arterial preparations including the rat aorta and iliac artery (Testa et al. 1995; Piascik et
al. 1995). Other studies have shown that vascular responses can potentially be mediated
by more than one subtype of $\alpha_1$-adrenergic receptor (Kong et al. 1994; Muramatsu et al.
1998).

Functional studies are limited to some degree by the pharmacological specificity
of the antagonists used. When looking at nerve-mediated responses, results are further
complicated by the presence of prejunctional receptors which modify neurotransmitter
release and the contribution of other postjunctival receptors to the contractile response
(Wilson & Dunn 1996). In order to overcome these difficulties, and to try and define
the physiological relevance of the different receptor subtypes, functional studies are
often correlated with ligand binding experiments or mRNA studies in an attempt to
assess receptor distribution patterns.

Messenger RNA for the $\alpha_1$-adrenergic receptors has been shown to be widely
distributed in the peripheral vasculature (Piascik et al. 1994; Guarino et al. 1996; Miller
et al. 1996). Interestingly, mRNA studies in different vessels show that message for the
$\alpha_{1A}$-adrenergic receptor dominates over that for the $\alpha_{1B}$- and $\alpha_{1D}$-receptors. This is
consistent with the apparent principal role for the pharmacological $\alpha_{1A}$-adrenoceptor in
mediating sympathetic contractile responses (Guarino et al. 1996; Piascik et al. 1996).
In some vessels, mRNA for the $\alpha_{1D}$-receptor has been demonstrated, but cannot be
correlated with a functional response (Piascik et al. 1995). The aorta has been
extensively studied, using techniques such as in situ hybridisation, ribonuclease (RNase)
protection assays, Northern blot analysis and reverse transcription-polymerase chain
reaction (RT-PCR). Results show the expression of mRNA for all three $\alpha_1$-adrenergic
receptors in this vessel (Ping & Faber 1993; Piascik et al. 1994; Rokosh et al. 1994;

1.3.2 $\alpha_2$-adrenergic receptors

$\alpha_2$-adrenergic receptor pharmacological studies. The initial classification of
$\alpha_2$-adrenergic receptors was based upon their pharmacological profile and presynaptic
location, however, it is now well established that $\alpha_2$-receptors exist on both sides of the
neuroeffector junction and that multiple subtypes exist. The pharmacological
identification of $\alpha_2$-adrenergic receptor subtypes has relied on detailed binding study
experiments, comparing the relative affinities of various agents to displace agonists and
antagonists already bound to the receptor (Ruffolo et al. 1993).

The existence of subtypes of $\alpha_2$-adrenoceptors was first suggested by Bylund in
1981 and again by Langer and Shepperson in 1982. The initial subtyping was based on
prazosin and yohimbine binding studies in human platelets and neonatal rat lung
(Bylund 1985). These tissues displayed binding sites, that in addition to showing a high
affinity for yohimbine, demonstrated low and high affinities for prazosin. The receptors
involved were designated as $\alpha_{2A}$ and $\alpha_{2B}$ respectively.
Binding studies were again used to characterise a third $\alpha_2$-adrenergic receptor from an opossum kidney derived cell line (Murphy & Bylund 1988). The receptor was initially thought to have an $\alpha_{2B}$-like pharmacological profile, however further work confirmed it was sufficiently different to classify as a new subtype: $\alpha_{2C}$ (Bylund 1988; Blaxall et al. 1991). A fourth pharmacological subtype, designated $\alpha_{2D}$, was then identified in bovine pineal gland (Simonneaux et al. 1991). Low rauwolscine and yohimbine binding affinity profiles initially suggested that this was a unique $\alpha_2$-adrenergic receptor subtype, however subsequent pharmacological, functional and molecular studies have shown the $\alpha_{2D}$-subtype to be the homologue of the human $\alpha_{2A}$-receptor in the rat, cow and mouse (MacKinnon et al. 1994; Bylund et al. 1995).

The primary signaling pathway of $\alpha_2$-adrenergic receptors is inhibition of cyclic 3',5'-adenosine monophosphate (cAMP) production via coupling to $G_i$ and inhibition of adenylate cyclase (Lomasney et al. 1991a).

**Molecular classification of $\alpha_2$-adrenergic receptors.** Molecular cloning experiments have identified three different $\alpha_2$-adrenergic receptor genes. Those derived from human tissues were initially named after their chromosome of origin. The $\alpha_2$-C10 gene was cloned from human platelets, corresponding to the $\alpha_{2A}$-adrenergic receptor (Kobilka et al. 1987) and the $\alpha_2$-C2 clone was classified as the gene encoding for the $\alpha_{2B}$-adrenergic receptor (Lomasney et al. 1990; Weinshank et al. 1990). The $\alpha_2C$ receptor ($\alpha_2$-C4), was cloned from a human kidney cDNA library (Regan et al. 1988), however it was initially incorrectly characterised as the gene for the $\alpha_{2D}$-adrenergic receptor. The rat equivalents of these receptor subtypes: RG20 (Chalberg et al. 1990), RNG$\alpha_2$ (Zeng et al. 1990) and RG10 (Lanier et al. 1991), have also been cloned and sequenced and share 82 - 90% sequence homology with the corresponding human gene (Bylund et al. 1994).

All three recombinant $\alpha_2$-adrenergic receptors have been pharmacologically characterised in cell expression systems and the profile of each corresponds closely to that of their pharmacological equivalent, taking into account the species variation seen with the $\alpha_{2A(D)}$ orthologues (Harrison et al. 1991; Bylund 1992). The current nomenclature system therefore incorporates the various molecular and pharmacological studies to define the three $\alpha_2$-adrenergic receptors as $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$ (Bylund et al. 1994; Alexander & Peters 1998).
\(\alpha_2\)-adrenergic receptor activation can influence vascular tone through a variety of mechanisms including presynaptic modulation, direct vasoconstriction via receptors located on the vascular smooth muscle and indirect vasodilation via the release of relaxant factors from the endothelium (Bylund et al. 1995; Cocks & Angus 1983).

**Presynaptic \(\alpha_2\)-adrenergic receptors.** In line with their original identification, \(\alpha_2\)-adrenergic receptors play a significant role in controlling nerve-mediated vascular responses via presynaptic inhibitory mechanisms, particularly during periods of intense sympathetic stimulation (Langer 1974; Westfall 1990; Ruffolo et al. 1993). Activation of these receptors reduces further noradrenaline release from sympathetic nerves resulting in a decrease in the postsynaptic response. In functional experiments, the presence of presynaptic \(\alpha_2\)-receptors are detected by the potentiation of vascular contractile responses after the use of selective \(\alpha_2\)-adrenoceptor antagonists (von Kugelgen & Starke 1985; Bulloch & Starke 1990). In addition to inhibiting noradrenaline release, presynaptic \(\alpha_2\)-receptors can also inhibit the release of sympathetic cotransmitters such as ATP and NPY (Brock 1995). Recent studies have investigated the subtype of presynaptic \(\alpha_2\)-adrenoceptors in a number of autonomic targets, including blood vessels, and have shown that the \(\alpha_{2A}\)-receptor is the predominant autoreceptor on sympathetic nerve terminals (Vidovic et al. 1994; Trendelenburg et al. 1997; Paiva et al. 1997). Studies in \(\alpha_2\)-adrenergic receptor deficient mice have confirmed these findings, however preliminary data suggest that the \(\alpha_{2C}\)-adrenoceptor may also contribute to the response in certain tissues (Hein et al. 1998).

**\(\alpha_2\)-adrenergic receptor mediated vasoconstrictor responses.** The contribution of \(\alpha_2\)-adrenergic receptors to noradrenaline mediated contractile responses was first suggested by Drew and Whiting (1979) with the observation that pressor responses to adrenergic agonists in anaesthetised animals could not be completely blocked by prazosin. This work was clarified further by McGrath et al. (Docherty et al. 1979; McGrath 1982), who categorised the residual component as \(\alpha_2\)-adrenoceptor mediated.

In the course of studies designed to assess the relative contribution of the \(\alpha_1\) and \(\alpha_2\) receptor subtypes to noradrenergic stimulation, it was noted that responses to \(\alpha\)-adrenergic agonists could be selectively blocked by \(\alpha_2\)-antagonists, while sympathetic nerve-mediated contractions were predominantly antagonised by \(\alpha_1\)-selective drugs.
(Ruffolo 1986). It was postulated therefore, that α₂-adrenergic receptors were located away from the neuroeffector junction, while α₁-adrenergic receptors responded primarily to released neurotransmitter (Langer et al. 1980; McGrath 1982; Medgett & Langer 1984). The extrajunctional α₂-adrenoceptors were suggested to be the target of circulating catecholamines (Wilffert et al. 1982; Langer et al. 1985). While an extrajunctional location may still be applicable for α₂-adrenoceptors in some vascular beds (Willette et al. 1991), postjunctional α₂-adrenergic receptors responsive to nerve stimulation have since been demonstrated to contribute to vasoconstriction in a number of vascular beds (Wilson & Dunn 1996). This includes the cutaneous saphenous vascular bed of the rat (Medgett & Ruffolo 1988), human digital and subcutaneous resistance arteries (Stevens & Moulds 1985; Parkinson et al. 1992) and the rat tail artery (Medgett 1985; Szabo & Hardebo 1990; Bao & Stjarne 1993; Redfern et al. 1995).

Interestingly, many of these studies note that the role of postsynaptic α₂-adrenoceptors may be underestimated because of the concurrent blockade of presynaptic α₂-receptors by relatively non selective α₂-antagonists (Stevens & Moulds 1985; Szabo & Hardebo 1990; Parkinson et al. 1992).

Unlike the α₁-adrenergic receptors, much less is known about the role and distribution of the specific α₂-receptor subtypes in vascular tissues. The α₂A-receptor is involved in agonist induced contractile responses in the pig thoracic aorta and marginal ear vein (Wright et al. 1995) and also in the contraction of rat skeletal muscle arterioles (Leech & Faber 1996), while the α₂B-receptor mediates vasoconstrictor responses to applied noradrenaline in the human saphenous vein (Smith et al. 1992). Recent studies in α₂B-receptor knockout mice have shown that this receptor may play an important role in the peripheral vasoactive response to applied α₂-agonists (Link et al. 1996; Hein et al. 1998).

Similarly, few studies have looked at the mRNA expression of the α₂-adrenergic receptor subtypes in vascular tissue. In the human aorta, mRNA was detected for all three subtypes (Eason & Liggett 1993), while in the rat, only the α₂A- and α₂B-receptors have been localised to the vasculature (Ping & Faber 1993; Blaxall et al. 1994).

α₂-adrenergic receptor mediated vasodilator responses. In opposition to their contribution to noradrenergic vasoconstrictions, α₂-adrenergic receptors can also mediate vasodilatory responses via the activation of endothelial receptors and the
subsequent release of nitric oxide (NO, see section 1.6.1). These endothelium-dependent relaxations were first described in coronary arteries (Cocks & Angus 1983) and have since been demonstrated in a variety of vessels from a number of different species, although there is considerable variation in the degree of the response between different vascular beds (Miller & Vanhoutte 1985; Angus et al. 1986; Pepke-Zaba et al. 1993; Bryan et al. 1996). In a study attempting to subtype the receptors involved in this response in pig coronary arteries, it was found that while both the α2A- and α2C-adrenergic receptors could be localised to the endothelium, only the α2A-receptor, acting via NO release, was involved in vasodilation in this tissue (Bockman et al. 1993). A study in the rat aorta has suggested that α1-adrenoceptors may also participate in endothelium-dependent vasodilations, however the relative contribution of α1- versus α2-receptors to the response was not assessed (Kaneko & Sunano 1993).

1.3.3 β-adrenergic receptors

**Pharmacological and molecular classification.** Subtypes of β-adrenergic receptors were first defined by differences in the rank order of potency of noradrenaline and adrenaline in a number of typical β-adrenoceptor mediated responses (Lands et al. 1967). The principal observation made was that while adrenaline and noradrenaline were equally effective in controlling lipolysis and cardiac stimulation (β1), adrenaline was much more effective at mediating bronchodilation or vasodepressor actions (β2). Atypical β-adrenergic responses were then described in a number of tissues, including the gastrointestinal tract and adipose tissue (Bristow et al. 1970; Arch et al. 1984). The receptor mediating these responses was characterised by its low affinity for classical β-adrenergic receptor antagonists (Emorine et al. 1989) and has since been recognised as the β3-adrenergic receptor, playing a crucial role in lipid metabolism (Strosberg 1997).

Three distinct genes encoding the respective subtypes have been cloned (Dixon et al. 1986; Frielle et al. 1987; Emorine et al. 1989). They correlate strongly with the pharmacologically defined subtypes and relatively selective agonists and antagonists have been developed (Michel et al. 1992; Summers et al. 1997; Alexander & Peters 1998). A putative β4-adrenoceptor has also been proposed on the basis of
pharmacological studies, however there is presently no definitive evidence for this subtype (Arch 1998).

Within the vascular system, β-adrenoceptors can regulate blood vessel responses via presynaptic receptors and the modulation of noradrenaline release or mediate vasodilation via postsynaptic receptors, located on either smooth muscle cells or the endothelium.

**Presynaptic β-adrenergic receptors.** In addition to possessing inhibitory α₂-adrenergic receptors, sympathetic nerve terminals also possess β-adrenoceptors which act to facilitate neurotransmitter release in a variety of tissues including blood vessels (Adler-Graschinsky & Langer 1975; Medgett *et al.* 1980; Nedergaard & Abrahamsen 1990). In the rat tail artery for example, activation of presynaptic β-adrenoceptors increases the stimulation-induced release of noradrenaline and ATP, reflected postsynaptically as an enhancement in the size of the contractile response and amplitude of the excitatory junctional potential (ejp) respectively (Encabo *et al.* 1996; Brock *et al.* 1997a).

The demonstration of the facilitatory effect of β-adrenoceptor activation is often complicated by concurrent activation of inhibitory α₂-adrenergic receptors and also that the degree of enhancement is relatively small when compared to that produced by α₂-adrenergic antagonists (Wilson & Dunn 1996; Nedergaard & Abrahamsen 1990). This has led to proposals that β-adrenoceptor positive feedback mechanisms are more important under low levels of sympathetic nerve activity or that circulating adrenaline is the primary physiological activator of these receptors (Medgett *et al.* 1980; Nedergaard & Abrahamsen 1990). Consistent with this theory, β₂-adrenoceptors are believed to be the predominant subtype on sympathetic nerve terminals, given their increased sensitivity to adrenaline (Nedergaard & Abrahamsen 1990; Reid *et al.* 1995).

**β-adrenergic receptor mediated vascular responses.** The characterisation of β-adrenergic vascular responses has been primarily studied in preconstricted vessels that are then treated with specific agonists and antagonists (Gray & Marshall 1992b; Graves & Poston 1993; Begonha *et al.* 1995). Nerve-mediated β-adrenoceptor vasodilations have, however, been demonstrated in a number of vascular beds, including the rabbit isolated facial vein (Pegram *et al.* 1976), the hepatic arterial bed of the cat (Greenway & Lawson 1969) and dog coronary arteries (Toda & Okamura 1990b). In the latter two
preparations, β-adrenoceptor vasodilations were only seen after the nerve-mediated contraction had been blocked by α₁-specific antagonists.

Although β-adrenergic receptor mediated vasodilations are generally considered to be mediated by β₂-adrenoceptors located on the vascular smooth muscle, acting via adenylate cyclase and cAMP signaling mechanisms (Lands et al. 1967; Kukovetz et al. 1981; Bea et al. 1994), various functional and localisation studies have indicated roles for both the β₁- and β₂-subtypes and additionally, β-adrenoceptors have been localised to the endothelium and been shown to activate NO vasodilatory pathways (Yamada et al. 1988; Begonha et al. 1995; Lu et al. 1995; Gray & Marshall 1992b; Graves & Poston 1993; Rebich et al. 1995). For example, in the pulmonary vascular bed of the rat, in small arteries, a significant proportion of the response is mediated by β₂-receptors and is NO independent, while in the larger arteries, β₁- and β₂-adrenoceptor responses are present and are both mediated via the endothelium and NO (Priest et al. 1997). Recent studies have also implicated a potential contribution by the β₃-adrenoceptor in some peripheral vasodilatory responses (Kuratani et al. 1994; Shen et al. 1994).

The localisation of β-adrenoceptor mRNA in the vasculature has been limited primarily to pulmonary and coronary vessels, where in situ hybridisation has demonstrated receptor transcript for both the β₁- and β₂-subtypes in the endothelium and vascular smooth muscle (Mak et al. 1996; Lu et al. 1995).

**1.3.4 γ-adrenergic receptors**

Early intracellular recordings from the smooth muscle of a number of arteries showed that sympathetic nerve stimulation produced excitatory junctional potentials (ejps) which were resistant to α-adrenergic receptor antagonists, for example arterioles of the guinea pig submucosa, rat basilar artery and the rat tail artery (Hirst & Neild 1980a; Hirst et al. 1982; Cheung 1982). It was suggested that these receptors were preferentially stimulated by neuronally released noradrenaline (junctional receptors) and were called γ receptors (Hirst & Neild 1980b). Similar α-adrenoceptor antagonist resistant ejps were described in the guinea pig vas deferens, however it was proposed that ATP, acting as a cotransmitter from sympathetic nerves, was responsible for both the ejp and first fast phase of the contraction in this tissue (Sneddon & Westfall 1984;
Sneddon & Bumstock 1984a). Excitatory junctional potentials have since been demonstrated to be mediated by ATP acting on smooth muscle purinoceptors in a number of different vessels including guinea pig submucosal arterioles and the rat tail artery where γ-adrenoceptors were first described (Evans & Surprenant 1992; Sneddon & Bumstock 1984b; McLaren et al. 1995).

There is currently no molecular equivalent for the described γ-adrenoceptor and only one antagonist, dihydroergotamine, shows any selectivity (Bramich et al. 1990; Morris 1994), however this drug also blocks contractile responses mediated by α-adrenergic and 5-hydroxytryptamine (5-HT) receptors (Muller-Schweinitzer & Weidman 1978). The role or potential contribution of γ-adrenergic receptors to sympathetic nerve-mediated vascular responses is still therefore, unproven.

1.4 PURINERGIC NEUROTRANSMISSION

In addition to being an integral part of intracellular energy metabolism, purines and related nucleotides play an important role in extracellular communication systems (Westfall et al. 1990; Burnstock 1995). The extracellular actions of purines were first described by Drury and Szent-Gyorgyi in 1929, who characterised the actions of adenosine and adenosine monophosphate (AMP) over cardiac rate, arterial pressure, vascular tone and intestinal movements. Further studies with adenosine established its potent vasodilatory actions, particularly in the coronary vasculature, where it was proposed to be the mediator of hypoxic vasodilation (Berne 1963; Berne 1980).

ATP was initially suggested to be a transmitter at sensory nerve endings, responsible for the vasodilatory response to antidromic nerve stimulation in rabbit ear vessels (Holton & Holton 1953) and it was later shown that ATP was responsible for nonadrenergic noncholinergic mechanisms in the gut (Burnstock et al. 1970). The term purinergic was initially used to describe these inhibitory visceral nerves (Burnstock 1972), however it was also shown that ATP or a related compound was released from sympathetic nerves innervating the guinea pig taenia coli (Su et al. 1971), cat nictitating membrane (Langer & Pinto 1976) and blood vessels (Su 1975). ATP has since
convincingly been demonstrated to be a cotransmitter from many different nerve types including sympathetic nerves, parasympathetic nerves, sensory nerves fibres and nonadrenergic noncholinergic visceral nerves, each innervating numerous autonomic targets (Burnstock 1993). For the purpose of this thesis however, this review will concentrate primarily on the role of ATP as a cotransmitter from perivascular sympathetic nerves.

The term purinergic receptor was used to define the receptor(s) activated by ATP or its breakdown product, adenosine, however in 1978, Burnstock (Burnstock 1978) clarified the often conflicting actions of ATP and adenosine (Burnstock 1976) by proposing that there were two different receptors, P₁ (adenosine) receptors, which were coupled to adenylate cyclase and selectively antagonised by methylxanthines and P₂ (ATP) receptors, which were not linked to cAMP responses, were unaffected by methylxanthines and were potent inducers of prostaglandin synthesis (Burnstock 1978; Burnstock 1996).

Since they were initially defined, a multitude of subtypes within both the P₁- and P₂-purinoceptor families have been described using pharmacological and more recently, molecular techniques. Within the vascular system, adenosine and ATP have both been well characterised as potent modulators of vascular tone, having presynaptic effects and postsynaptic dilatory and constrictor effects (Olsson & Pearson 1990).

1.4.1 P₁-purinoceptors

Pharmacological and molecular classification of adenosine receptors. Evidence for the existence of multiple P₁-purinoceptor subtypes was initially based on biochemical studies which demonstrated that adenosine could either inhibit (A₁/R₁) or stimulate (A₂/R₂) adenylate cyclase activity in various cell types (Van Calker et al. 1979; Londos et al. 1980). After this initial subdivision, two subtypes of the A₂ receptor: A₂A and A₂B, were also proposed, on the basis of their high and low affinities for adenosine respectively (Daly et al. 1983). Subsequent work has shown that the A₂B receptor exhibits a broadly similar agonist profile to the A₂A subtype, but can be distinguished on the basis of antagonist pharmacology (Alexander & Peters 1998). Molecular confirmation of these different subtypes has been achieved, with clones for
the $A_1$, $A_{2A}$ and $A_{2B}$ receptors being identified from a number of different species and showing similar or identical characteristics to the corresponding pharmacological receptor (Libert et al. 1989; Libert et al. 1991; Maenhaut et al. 1990; Stehle et al. 1992; Tucker & Linden 1993).

Studies on the effects of adenosine analogues in cardiac tissue and at the frog neuromuscular junction led to the proposal of a third adenosine receptor (Ribeiro & Sebastiao 1986), however its existence is still controversial, with a number of pharmacological studies presenting conflicting results (Henning 1997). Another $A_3$ receptor was identified through cloning studies (Zhou et al. 1992), however, this receptor is clearly distinct from the first suggested $A_3$ receptor (Linden 1994). The cloned receptor is able to cause inhibition of adenylate cyclase and has been linked to mast cell responses (Fredholm et al. 1996). Currently therefore, there are 4 recognised adenosine receptor subtypes: $A_1$, $A_{2A}$ and $A_{2B}$, all of which have molecular and pharmacological equivalents and the $A_3$ subtype, as defined by the $A_3$ clone (Fredholm et al. 1994; Alexander & Peters 1998).

**Sources of adenosine.** Rather than being a specific neurotransmitter, adenosine is a breakdown product, formed by the hydrolysis of ATP or AMP, derived from tissue metabolism or neurally released ATP (Zimmermann 1994; Mubagwa et al. 1996; Henning 1997). The primary role of adenosine in the vascular system is thought to be to couple tissue blood flow to metabolic state (Olsson & Pearson 1990; Rubino et al. 1995). In the heart for example, adenosine is released from the vascular endothelium and cardiac myocytes during periods of reduced myocardial oxygen supply and increased workload (Berne 1963; Mubagwa et al. 1996). In the liver, adenosine again regulates blood flow, however it maintains blood flow directly, rather than linking it to the metabolic state of the liver (Lautt 1996). In this system, adenosine is the regulator of the hepatic arterial buffer response, an intrinsic regulation mechanism that is mediated by ‘washout’ of adenosine from within the space of Mall (Lautt et al. 1985).

**Adenosine receptor mediated vascular responses.** Inhibition of noradrenaline release from perivascular nerves, mediated by adenosine, has been demonstrated in a number of vascular beds (DeMey et al. 1979; Hom & Lokhandwala 1981). The hydrolysis of ATP released as a neurotransmitter is the primary neuromodulatory source of adenosine (Olsson & Pearson 1990) and studies strongly suggest that the $A_1$ subtype
is the receptor most likely involved in the response (Jonzon & Fredholm 1984; Zimmermann 1994; Franco-Cereceda 1996).

The direct action of adenosine on vascular tissue is to cause vasodilation, although a vasoconstriction, probably mediated by $A_1$ receptors, has been described in the pulmonary vascular bed of the cat and in the renal vasculature (Lippton et al. 1992; Olsson & Pearson 1990). The vasodilatory actions of adenosine are predominantly mediated by $A_2$ receptors located on the vascular smooth muscle, with the stimulation of adenylate cyclase leading to decreased intracellular $Ca^{2+}$ levels (Olsson & Pearson 1990; Mubagwa et al. 1996; Olsson 1996). In different vascular beds, both the $A_{2A}$ and $A_{2B}$ receptors have been shown to be involved in adenosine mediated vasodilatory responses (Olsson 1996; Rubino et al. 1995). In addition, there is also functional evidence to suggest that adenosine receptors, located on the endothelium, mediate vasodilation via NO release. For example, vasodilatory responses in the guinea pig coronary artery and rat isolated aorta are partially antagonised by NO inhibitors (Vials & Burnstock 1993; Prentice & Hourani 1996) and adenosine has been shown to stimulate NO release from cultured arterial endothelial cells (Li et al. 1995a). The specific subtypes involved in these responses were not clearly identified although in the rat aorta both $A_{2A}$ and $A_{2B}$ receptors were implicated (Prentice & Hourani 1996). The signaling pathway linking adenosine receptors to the release of NO is unclear at this stage (Olsson 1996). No direct evidence has yet been presented for $A_3$-receptor mediated vasodilation (Tabrizchi & Lupichuk 1995; Olsson 1996).

The tissue distribution of adenosine receptor mRNA has recently been assessed in a number of tissues in the rat, using both in situ hybridization and the more sensitive RT-PCR technique (Dixon et al. 1996). In this study, mRNA for all 4 receptor subtypes was found to be widespread in both the CNS and periphery, including the aorta, which showed gene expression for the $A_1$, $A_{2A}$ and $A_{2B}$ receptor subtypes.

### 1.4.2 $P_2$-purinoceptors

$P_2$-purinoceptor subtypes. The subdivision of $P_2$-purinoceptors into $P_{2X}$ and $P_{2Y}$ was initially proposed by Burnstock and Kennedy (Burnstock & Kennedy 1985) based on the rank order of agonist potency of a number of ATP analogues and the activity of
selected antagonists. The P2X-purinoceptor was distinguished through its activation by α,β-methylene ATP (α,β-mATP), with the same compound selectively desensitising the receptor, while P2Y-purinoceptors were selectively activated by 2 methylthioATP and antagonised by reactive blue 2 (Burnstock 1995). Additional P2-purinergic receptors were subsequently proposed, including the P2Z receptor, which was adenosine diphosphate (ADP) selective and found on platelets, the P2Z-receptor, which was activated by ATP4- and localised to mast cells, macrophages and lymphocytes (Gordon 1986) and the P1U- and P2D-receptors, which responded preferentially to uridine triphosphate (UTP) and diadenylated nucleotides respectively (O’Connor et al. 1991; Hilderman et al. 1991).

Differences in P2-purinoceptor signaling mechanisms were also described, with the P2X-receptors operating as ligand gated ion channels (Benham & Tsien 1987; Bean 1992), whereas the P2Y-receptor appeared to be coupled to G-proteins, activating phospholipase C and intracellular Ca2+ release mechanisms (O’Connor et al. 1991).

**P2-purinoceptor nomenclature.** On the basis of cloning studies and transduction mechanisms, and in order to simplify the classification of an ever-growing number of P2-purinergic receptor subtypes, a more structured nomenclature system was introduced (Abbracchio & Burnstock 1994). Under this system, ATP activated G-protein coupled receptors were specified as P2Y, with the different cloned receptors being classified numerically in order of discovery (P2Y1, P2Y2, P2Y3 etc, Burnstock & King 1996). The family of ligand gated receptors were classified as P2X, likewise assigning numerical subscripts to each new subtype (P2X1, P2X2, P2X3 etc, Abbracchio & Burnstock 1994). Only the P2Z-receptor fell outside this structural classification scheme, mediating the formation of a large, non selective “pore” in immune cells (Dubyak 1991; Fredholm et al. 1994). Recent cloning studies have since demonstrated that the P2Z-receptor belongs to the P2X-purinoceptor family (P2X7, Surprenant et al. 1996). A recent review has suggested that P2-purinoceptors should be referred to as P2-receptors, thereby taking into consideration those receptors which are selective for uridine diphosphate (UDP) or UTP (Fredholm et al. 1997).


1.4.3 \( P_{2X} \)-purinoceptors

**Molecular and pharmacological classification of \( P_{2X} \)-purinoceptors.** To date, 7 different subtypes of \( P_{2X} \)-purinoceptors have been cloned (Fredholm *et al.* 1997; Alexander & Peters 1998). With cloning studies, it has become apparent that these receptors represent a new family of ligand gated ion channels, having a unique structural composition of two putative hydrophobic (transmembrane) domains, short intracellular amino and carboxy terminus regions and a large extracellular loop (Surprenant *et al.* 1995; Collo *et al.* 1996).

The \( P_{2X1} \) and \( P_{2X2} \)-receptors were first isolated by expression cloning in Xenopus oocytes using cDNA libraries generated from rat vas deferens and PC12 cells respectively (Valera *et al.* 1994; Brake *et al.* 1994). Multiple splice variants of \( P_{2X2} \) have since been identified (Housley *et al.* 1995; Burnstock *et al.* 1998). The \( P_{2X7} \)-receptor was isolated from dorsal root ganglion cells (Chen *et al.* 1995; Lewis *et al.* 1995). In the course of these studies, it was noted that coexpression of the \( P_{2X1} \) and \( P_{2X3} \)-channels yielded ATP activated currents similar to those seen in sensory neurons (Lewis *et al.* 1995). This observation raised the possibility, that while some native ATP-gated channels were formed by homopolymeric complexes, others could be explained by the specific heteropolymerisation of different \( P_{2X} \)-receptor subunits (Collo *et al.* 1996). The \( P_{2X4} \)-receptor was subsequently isolated from rat brain, exhibiting a unique pharmacological profile to the previously identified receptors (Bo *et al.* 1995) and the \( P_{2X5} \) and \( P_{2X6} \)-receptors were identified using PCR on coeliac and superior cervical ganglia mRNA respectively (Collo *et al.* 1996). As mentioned earlier (section 1.4.2), the \( P_{2X7} \)-receptor corresponds to the earlier defined \( P_{2Z} \)-receptor (Surprenant *et al.* 1996). Interestingly, the ADP platelet receptor (\( P_{2Y} \)) has not yet been cloned (Fredholm *et al.* 1997).

The availability of antagonists sufficiently selective to distinguish the different \( P_{2X} \)-receptor subtypes is limited (Lambrecht 1996). Current studies utilise differences in receptor sensitivities to \( \alpha,\beta\)-mATP, antagonism by pyridoxal phosphate-6-azophenyl 2'-4'disulphonic acid tetrasodium (PPADS) or suramin and the rate of receptor desensitisation, to differentiate responses mediated by the respective subtypes (Evans 1996; Collo *et al.* 1996; Fredholm *et al.* 1997).
**P$_{2X}$-purinoceptor mediated vascular responses.** While the initial response to sympathetic nerve stimulation in most blood vessels is a purinergic ejp (section 1.3.4), the contribution of ATP to nerve-mediated contractions varies greatly between vascular beds, even though both responses are mediated by postsynaptic P$_{2X}$-receptors (Kennedy 1996). In the rabbit saphenous artery, the contractile response to sympathetic nerve stimulation is due to approximately equal contributions by noradrenaline and ATP (MacDonald et al. 1992), while in the ileocolic and mesenteric arteries, the response is mainly purinergic (MacDonald et al. 1992; Ramme et al. 1987). On the other hand, in the rabbit ovarian vascular bed, noradrenaline is the major effector of nerve-mediated contractions (Yousif et al. 1996). The relative contributions of noradrenaline and ATP are also differentially affected by the frequency of nerve stimulation, with stimulation at low frequencies favouring ATP release, while higher frequencies or longer periods of stimulation increases the adrenergic component (Kennedy et al. 1986; Zimmermann 1994; Kennedy 1996).

Within the vascular system, P$_{2X1}$-receptor mRNA and protein have been localised to smooth muscle cells (Collo et al. 1996; Vulchanova et al. 1996; Chan et al. 1998; Bo et al. 1998; Nori et al. 1998) and pharmacological profiles suggest that this receptor subtype is most likely responsible for the ATP-mediated ejps and contractile responses (Burnstock 1996; Boarder & Hourani 1998). Messenger RNA for the P$_{2X2}$- and P$_{2X4}$-purinoceptors has also recently been identified in vascular tissue (Nori et al. 1998; Soto et al. 1996a).

### 1.4.4 P$_{2Y}$-purinoceptors

**Molecular and pharmacological classification of P$_{2Y}$-purinoceptors.** As for the P$_{2X}$-purinoceptor family, a large number of P$_{2Y}$-receptors have been identified through pharmacological and molecular cloning studies. Eight subtypes have been cloned from various tissues and a number of species homologues have also been identified (Burnstock & King 1996; Fredholm et al. 1997). Recently, a new subtype, provisionally called P$_{2Y11}$ was cloned from human placenta (Communi et al. 1997), however, there is currently no evidence for its presence in the vasculature (Boarder & Hourani 1998).
A paucity of subtype selective agonists and antagonists, combined with the likely expression of multiple subtypes within individual tissues, has made it difficult to associate tissue responses with specific receptor subtypes. To date, only 4 cloned subtypes: P\textsubscript{2Y1}, P\textsubscript{2Y2}, P\textsubscript{2Y4} and P\textsubscript{2Y6}, have been shown to encode functional receptors (Nicholas \textit{et al.} 1996; Alexander & Peters 1998). The best characterised P\textsubscript{2Y}-receptor antagonist is the aromatic polysulphonic acid, reactive blue 2 (basilen blue, Burnstock & Warland 1987a; Simonsen \textit{et al.} 1997) and there is evidence that PPADS may also block P\textsubscript{2Y}-receptors, showing some selectivity for P\textsubscript{2Y1}-receptors in vascular tissue (Brown \textit{et al.} 1995; Ralevic & Burnstock 1996a). Nucleotide selectivity is also used to further identify responses mediated by the different P\textsubscript{2Y}-receptor subtypes (Nicholas \textit{et al.} 1996).

**P\textsubscript{2Y}-purinoceptor mediated vascular responses.** P\textsubscript{2Y}-receptors regulate ATP-vasodilatory responses in the vascular system via both endothelial and smooth muscle cell located receptors, often co-existing in the same vascular bed. In lamb coronary arteries for example, nerve stimulation induced vasodilation via P\textsubscript{2Y}-receptors located on the smooth muscle cells, while applied ATP produced an endothelium-dependent vasodilation (Simonsen \textit{et al.} 1997). In the rabbit pulmonary artery, the receptor subtypes involved have been further characterised, with agonist studies demonstrating P\textsubscript{2Y2} (P\textsubscript{2U})-receptors on the endothelium and P\textsubscript{2Y1} (P\textsubscript{2Y})-receptors on the smooth muscle (Qasabian \textit{et al.} 1997).

Vascular endothelial cells can express both P\textsubscript{2Y1}- and P\textsubscript{2Y2}-receptors (Burnstock 1996; Pirotton \textit{et al.} 1996) and their activation induces vasodilation through a number of different pathways, including the formation and release of prostacyclin, NO and endothelium derived hyperpolarising factor (EDHF, see section 1.6.2, Boeynaems & Pearson 1990; Ralevic & Burnstock 1991; Malmsjo \textit{et al.} 1998). The physiological role of endothelial P\textsubscript{2Y}-receptors is linked to potential intraluminal sources of ATP, which includes platelets, reticulocytes and endothelial cells (Gordon 1986; Ralevic & Burnstock 1991; Pearson & Gordon 1979). Abnormal conditions such as endothelial cell damage, hypoxia or altered flow may stimulate ATP release, promoting vasodilation and inhibiting platelet aggregation, thereby limiting tissue damage (Boeynaems & Pearson 1990). Additionally, ATP may mediate vasodilatory responses.
via the breakdown of ATP to adenosine and the activation of P₁-purinoceptors (Browse et al. 1997, section 1.4.1).

There is also evidence to suggest that ATP may influence vascular responses via the prejunctional inhibition of noradrenaline release (von Kugelgen 1994). In addition to the actions of adenosine, inhibitory P₂Y-receptors have been shown to operate on perivascular sympathetic nerve terminals (Goncalves & Queiroz 1996). P₂X-receptors may also play a presynaptic role, as in the guinea pig vas deferens, pretreatment with α,β-mATP was shown to enhance the release of noradrenaline (Stjarne & Astrand 1985) and a similar effect on noradrenaline release was seen in the rat tail artery (Shinozuka et al. 1990).

1.5 NEUROPEPTIDE Y NEUROTRANSMISSION

*Neuropeptide Y.* Neuropeptide Y is a 36 amino acid peptide which was originally isolated from the porcine brain and belongs to the family of peptides which includes pancreatic polypeptide and peptide YY (Tatemoto et al. 1982). The name NPY was designated due to the peptide being tyrosine rich in its composition. Neuropeptide Y has a wide distribution and in the central nervous system, has been implicated in many physiological responses including the regulation of blood pressure, food intake, sexual activity, body temperature and the secretion of hormones (Wan & Lau 1995; Blomqvist & Herzog 1997).

A physiological role for NPY in the peripheral vascular system was initially suggested in a study on the cat submandibular gland, where a slow, long lasting, α-adrenoceptor resistant vasoconstriction was noted after sympathetic nerve stimulation (Lundberg & Tatemoto 1982). Studies in the cat spleen confirmed the potent vasoconstrictor effects of NPY and further established that NPY was released from sympathetic nerves via a guanethidine sensitive mechanism (Lundberg et al. 1984a). It has since been well established that NPY coexists with noradrenaline in most sympathetic vasoconstrictor neurons and that, in addition to exerting a direct contractile effect, NPY can also influence the response of the vasculature to other neurotransmitters (Ekblad et al. 1984; Lundberg et al. 1988; Morris et al. 1995; Wharton & Polak 1990).
Neuropeptide Y receptor subtypes. The subdivision of NPY receptors was originally based on the observation that, while the C-terminal portion of the NPY and peptide YY peptides was sufficient to activate prejunctional responses in the rat vas deferens (Y2), the whole sequence was required for postjunctional vasoconstrictor effects in vascular smooth muscle (Y1, Wahlestedt et al. 1986).

To date, the use of cloning techniques has resulted in the identification of 5 subtypes of NPY receptors (Y1, Y2, Y4, Y5, and Y6, Alexander & Peters 1998; Blomqvist & Herzog 1997). The Y4 receptor is also known as the pancreatic polypeptide receptor (Lundell et al. 1996). All belong to the 7-transmembrane G-protein coupled superfamily and mediate their responses through Gi/0, whose activation leads to inhibition of adenylate cyclase and subsequently a decrease in cAMP (Blomqvist & Herzog 1997). A Y3 receptor has also been proposed (Higuchi et al. 1988), however a corresponding clone has yet to be identified (Blomqvist & Herzog 1997).

The characterisation of NPY receptor mediated responses has been difficult due to a lack of selective and reversible antagonists, with the different subtypes being identified primarily by agonist affinity profiles for ligands such as NPY, peptide YY, pancreatic polypeptide and specific fragments of the same peptides (Blomqvist & Herzog 1997; Malmstrom 1997). Recently however, the development of non peptide Y1 selective antagonists such as BIBP 3226 (Rudolf et al. 1994), SR 120107A, SR 120819A (Serradeil-Le Gal et al. 1994; Serradeil-Le Gal et al. 1995) and 1229U91 (Lew et al. 1996), has enabled more detailed studies to be completed.

Neuropeptide Y receptor mediated vascular responses. Neuropeptide Y has three distinct regulatory effects within the vascular system. It acts as a direct vasoconstrictor, is able to potentiate the postjunctional responses of other vasoconstrictors, and it can also suppress neurotransmitter release via prejunctional receptors (Wahlestedt et al. 1990a). Of the different receptor subtypes, those with the most relevance for regulation of vascular function are the Y1 and Y2 subtypes. The cloned receptors correspond pharmacologically to the originally defined pre- and postsynaptic NPY receptors (Eva et al. 1990; Krause et al. 1992; Rose et al. 1995; Gerald et al. 1995) and in general, Y1 receptors mediate the postjunctional effects of NPY, while the Y2 receptor is considered to be predominantly presynaptic (Wahlestedt et al. 1986; Wahlestedt et al. 1990a; Westfall et al. 1990a; Grundemar et al. 1992).
agreement with the idea that the Y₁ receptor is the primary receptor involved in vascular smooth muscle responses, recent mRNA studies have demonstrated expression of the Y₁ receptor in a number of different vascular beds (Wharton et al. 1993; Nilsson et al. 1996; Bergdahl et al. 1996; Malmstrom 1997; Newhouse & Hill 1997).

Neuropeptide Y has been shown to contribute to sympathetic nerve-mediated vasoconstrictions in a number of vascular beds including human and rat mesenteric arteries (Racchi et al. 1997), pig nasal mucosa and femoral artery (Malmstrom et al. 1996), guinea pig vena cava (Malmstrom & Lundberg 1995) and rat iris arterioles (Newhouse & Hill 1997). In all these tissues, responses were mediated by Y₁ receptors, however Y₂ receptors have been shown to mediate contractile responses to applied NPY in the splenic vascular bed of the pig (Modin et al. 1991; Malmstrom 1997), the rat femoral artery (Tessel et al. 1993) and the microvasculature of the hamster cheek pouch (Boric et al. 1995). Neuropeptide Y mediated vasoconstrictions are typically slow, long lasting responses and NPY is preferentially released from sympathetic nerves by stimuli of a long duration or high frequency (Lundberg et al. 1986; Lundberg et al. 1989b; Morris et al. 1995; Newhouse & Hill 1997). The frequency dependency of NPY release has led investigators to suggest that the importance of NPY in pressor responses becomes more significant under conditions of high sympathetic nerve activity (Wahlestedt et al. 1990b).

The enhancing effects of NPY were first noted in relation to noradrenaline evoked constrictions, in response to both nerve stimulation and applied agonist (Ekblad et al. 1984; Wahlestedt et al. 1985; Hieble et al. 1989). The potentiation is not specific however, since the effects of other vasoconstrictors such as histamine (Edvinsson et al. 1984), ATP (Westfall et al. 1995; Westfall et al. 1996), 5-HT and potassium chloride (KCL) depolarisation (Andriantsitohaina & Stoclet 1988) are also enhanced in various different vascular beds. The mechanism by which NPY induces this effect is not clear, but appears to be mediated by Y₁ receptors (Tschopl et al. 1993; Westfall et al. 1995; Bergdahl et al. 1996) and is related to changes in receptor characteristics and/or second messenger systems (Westfall et al. 1995; Franco-Cereceda 1996).

At the prejunctional level, NPY can influence vascular tone by regulating the release of neurotransmitter from perivascular nerve fibres. At sympathetic nerve terminals, complex interactions between noradrenaline and NPY occur, with
prejunctional $\alpha_2$-adrenergic receptors inhibiting NPY release (Lundberg et al. 1989a) and conversely, activation of prejunctional $Y_2$ receptors inhibiting noradrenaline, and possibly also further NPY release (Westfall et al. 1990a; Grundemar & Hakanson 1993; Fredholm 1995; Westfall 1995). The presence of inhibitory $Y_1$ receptors has also been purported in some vessels (McAuley & Westfall 1992). Presynaptic inhibition of transmitter release is due to the inhibition of $Ca^{2+}$ influx via N-type $Ca^{2+}$ channels into the nerve terminal (Toth et al. 1993). This system may serve to improve the economy of the sympathetic nerve-mediated response (Grundemar & Hakanson 1993), limiting depletion of neurotransmitter during periods of high sympathetic nerve activity. In addition to its influence on sympathetic nerves, NPY has also been shown to inhibit the release of calcitonin gene related peptide (CGRP) from sensory nerve fibres innervating the rat mesenteric artery (Kawasaki et al. 1991).

In addition to the effects of NPY described above, a relatively recent study has proposed that postjunctional NPY $Y_2$ receptors may mediate vasodilatory responses in select vascular beds (Neild & Lewis 1995), potentially explaining the presence of NPY in autonomic vasodilator neurones (Morris et al. 1995).

1.6 NITRIC OXIDE AND ENDOTHELIUM DERIVED HYPERPOLARISING FACTOR

Although specific receptors do not exist for NO and EDHF, it is worth mentioning the role of these substance in relation to vasodilatory responses, with specific reference to the role of NO as a neurotransmitter in the parasympathetic nervous system and endothelium-dependent vasodilations due to both NO and EDHF.

1.6.1 Nitric oxide

Nitric oxide is synthesised from L-arginine by the enzyme NO synthase (NOS, Palmer & Moncada 1989). Three distinct isoforms of NOS have been isolated. Endothelial cells and neurones possess different isoforms, both of which are
Ca\textsuperscript{2+}/calmodulin dependent (Bredt & Snyder 1990; Busse & Mulsch 1990) and are constitutively expressed (Bredt & Snyder 1992). The third isoform, inducible NOS, is not dependent on Ca\textsuperscript{2+} for activation and its expression is induced in response to bacterial endotoxin or inflammatory cytokines (Lyons \textit{et al.} 1992; Geller & Billiar 1998). Many different cell types are capable of expressing inducible NOS, including macrophages, epithelial cells and hepatocytes (Geller & Billiar 1998).

**Endothelium-dependent NO responses.** As discussed in the preceding sections, a number of neuroreceptor subtypes can regulate NO synthesis and release. The role of the endothelium as a source of relaxant factors was first advanced by Furchgott and Zawadzki (1980), who demonstrated that the relaxation produced by acetylcholine was dependent upon the release of a diffusible substance from endothelial cells, later known as endothelium derived relaxing factor (EDRF, Moncada \textit{et al.} 1991). EDRF was subsequently identified as NO (Palmer \textit{et al.} 1987; Ignarro \textit{et al.} 1987) and was shown to mediate vasodilation in smooth muscle cells via the activation of guanylate cyclase and cyclic 3',5'-guanosine monophosphate (cGMP) signaling pathways (Rapoport & Murad 1983; McDonald & Murad 1995). Interestingly, these findings provided an explanation for the mechanism of action of vasodilators such as nitroglycerin and nitroprusside which act as NO donors (Ignarro \textit{et al.} 1981; Gruetter \textit{et al.} 1981; Moncada \textit{et al.} 1991).

In endothelial cells, NOS activity can be stimulated by receptor activation or by physical factors such as shear stress (Fleming & Busse 1995) and changes in the level of vascular tone (Vargas \textit{et al.} 1990). Endothelial NOS is also responsible for the basal or continuous release of NO in a number of vascular beds (Moncada \textit{et al.} 1991).

**Nitric oxide as a neurotransmitter in parasympathetic nerves.** Nitric oxide synthase has been colocalised with acetylcholine and vasoactive intestinal peptide (VIP) in perivascular parasympathetic nerve fibres (Lumme \textit{et al.} 1996; Goadsby \textit{et al.} 1996; Yoshida & Toda 1997) and NO nerve-mediated vasodilation has been demonstrated in a number of vascular beds including cerebral vessels of the dog and cat (Toda & Okamura 1990a; Goadsby \textit{et al.} 1996) and monkey and bovine mesenteric artery (Toda & Okamura 1992; Ahlner \textit{et al.} 1991). It is likely, therefore, that parasympathetic nerves are able to mediate vasodilatory responses via the release of NO, acetylcholine and VIP, with NO being responsible for the first fast phase of these dilations (Morris \textit{et al.} 1995;
Nitric oxide synthase has also been localised to sensory nerves (Lumme et al. 1996; Zheng et al. 1997) and nonadrenergic noncholinergic nerves in the gut (Sanders, M. & Ward 1992).

1.6.2 Endothelium derived hyperpolarising factor

In addition to NO mediated vasodilator responses, many of the neuroreceptors discussed in this chapter have also been linked to the release of EDHF and the subsequent hyperpolarisation and relaxation of vascular smooth muscle.

**Endothelium-dependent hyperpolarisations.** After the discovery of EDRF and an understanding of its actions developed, it became apparent that not all endothelium-dependent responses could be explained by the release of EDRF alone. In particular, acetylcholine was shown to induce endothelium-dependent relaxations that were associated with smooth muscle cell hyperpolarisation (Bolton et al. 1984; Taylor et al. 1988; Chen et al. 1988). Substance P also induced a similar response (Beny & Brunet 1988). These responses were attributed to a new relaxant factor called EDHF (Taylor & Weston 1988). EDHF was shown to be a labile substance (Feletou & Vanhoutte 1988), which in addition to causing hyperpolarisation, differed from EDRF in that responses could not be blocked by haemoglobin (which binds and inactivates EDRF) or methylene blue (which blocks guanylate cyclase, Chen et al. 1988; Taylor & Weston 1988). More recent studies have likewise shown the response to be resistant to specific NOS inhibitors (Cowan et al. 1993; Malmsjo et al. 1998).

**Mechanism of action of EDHF.** The hyperpolarisation of smooth muscle cells induced by EDHF is due to an increase in K⁺ conductance across the cell membrane (Komori & Suzuki 1987b; Komori & Vanhoutte 1990), which in turn decreases the activity of voltage dependent Ca²⁺ channels, reducing intracellular Ca²⁺ levels leading to relaxation (Walsh et al. 1995; Garland et al. 1995). Identity of the K⁺ channel(s) involved in this response is controversial, with different channels appearing to mediate the response in different blood vessels (Mombouli & Vanhoutte 1997). In the rabbit aorta for example, responses are inhibited by charybdotoxin, indicating large conductance Ca²⁺ sensitive K⁺ channels (Cowan et al. 1993), while in the rabbit mesenteric artery, apamin alone inhibits the response, pointing out the involvement of
small conductance Ca\textsuperscript{2+} dependent K\textsuperscript{+} channels (Murphy & Brayden 1995). In other vessels such as the rat hepatic artery, the actions of EDHF can only be abolished by a combination of charybdotoxin plus apamin (Zygmunt et al. 1997a). ATP sensitive K\textsuperscript{+} channels have also been implicated in EDHF responses (Standen et al. 1989; Cowan et al. 1993).

**Identity of EDHF.** Despite the number of studies that have implicated a role for EDHF, the chemical nature of the signal remains unknown and no firm evidence has been presented which definitely links hyperpolarisation to NO independent vasodilations (Garland et al. 1995; Cocks & Selemidis 1997). A number of candidate mechanisms have been proposed, including myoendothelial gap junctions, which could act to transfer endothelial hyperpolarisations directly to the adjacent vascular smooth muscle (Beny & von der Wied 1991; Xia et al. 1995), metabolites of arachidonic acid including epoxyeicosatrienoic acids, which are produced by the cytochrome P450 mono-oxygenase pathway (Hecker et al. 1994; Campbell et al. 1996), or endogenous cannabidiol acids, for example anandamide (Randall et al. 1996; Randall & Kendall 1998). Recent studies in the rat hepatic artery indicate that at least in that vessel, anandamide is not EDHF (Zygmunt et al. 1997b), and the identity of epoxyeicosatrienoic acids as EDHF has also been questioned (Mombouli & Vanhoutte 1997; Cocks & Selemidis 1997). The contribution of NO as a hyperpolarising agent has not been totally discounted. Nitric oxide has been shown to cause hyperpolarisation in vascular smooth muscle cells of the guinea pig uterine artery, contributing at least in part to the relaxant response to acetylcholine (Tare et al. 1990) and NO has also been reported to directly activate Ca\textsuperscript{2+} dependent K\textsuperscript{+} channels in smooth muscle cells from the rabbit aorta (Bolotina et al. 1994).

### 1.7 CHOLINERGIC NEUROTRANSMISSION

The principal transmitter of the parasympathetic nervous system is acetylcholine and two distinct receptors mediate its responses throughout the body: nicotinic receptors, which operate as ligand gated ion channels, and the metabotrophic muscarinic receptors (Ladinsky 1993). Like the sympathetic nervous system, the contribution of
cotransmitters such as NO and VIP to parasympathetic responses are achieving greater recognition in the study of vasodilatory mechanisms.

1.7.1 Muscarinic receptors

Pharmacological and molecular classification of muscarinic receptors.

Recognition of two different receptor subtypes for acetylcholine was first perceived by Dale (Dale 1914), based on the ability of acetylcholine to mimic the actions of nicotine and muscarine in different organ systems.

Atropine, found naturally in the plant Atropa Belladonna, has long been used as a muscarinic antagonist (Katzung 1992b), however it does not distinguish between any receptor subtypes. The development of more selective antimuscarinic compounds, such as the anti-ulcer drug pirenzepine, the muscarinic receptor agonist McN-A-343 and the antagonist 4-DAMP, resulted in the classification of at least three receptor subtypes (M1, M2 and M3, Hammer et al. 1980; Hammer & Giachetti 1982; Barlow et al. 1976; Doods et al. 1987; Caulfield 1993). The M1 receptor was classically demonstrated in the central nervous system and autonomic ganglia, the M2 receptor was shown to be responsible for cardiac responses, while the M3 receptor was localised to ileal smooth muscle (Caulfield & Straughan 1983; Brann et al. 1993).

The M1 and M2 genes were first cloned from porcine cerebral and cardiac tissue (Kubo et al. 1986a; Kubo et al. 1986b; Peralta et al. 1987). Using homology cloning, the human and rat M1 and M2 receptor genes, as well as three additional subtypes: M3, M4 and M5, were also soon identified (Bonner et al. 1987b; Bonner et al. 1988). Analysis of the gene sequences indicated that all belonged to the 7-transmembrane superfamily of receptor proteins and that they were derived from a highly conserved gene family, showing particularly high sequence homology over the putative transmembrane domains (Brann et al. 1993). Additionally, all the receptors were the product of intronless genes (Bonner et al. 1988). Expression of the cloned receptors in mammalian cell lines has shown that each gene encodes for their pharmacological defined native subtype, except for the M5 gene, which still lacks a clear endogenous correlate (Buckley et al. 1989; Dorje et al. 1991a; Eglen et al. 1996).
At high expression levels, muscarinic receptors can promiscuously couple to several signaling pathways (Fukuda et al. 1989), however in general, the receptors can be grouped by their coupling to either phospholipase C via $G_{q/11}$ and the mobilisation of intracellular Ca$^{2+}$ (M1, M3 and M5), or to the inhibition of adenylate cyclase via $G_{i/o}$ (M2, M4, Peralta et al. 1988; Felder 1995).

Pharmacologically, discrimination of the responses mediated by the different subtypes has been hampered by the lack of antagonists with high selectivity for one specific subtype (Caulfield 1993). Characterisation of responses has been determined instead by the rank order of affinity for a number of different antagonists, for example pirenzipine (M1), methoctramine (M2, M4), 4-DAMP (M1, M3) and p-FHHSiD (M3, Eglen et al. 1994; Eglen et al. 1996). Additionally, studies in organ systems are often further complicated by the coexpression of more than one muscarinic receptor subtype (Eglen et al. 1994; Durieux 1995).

**Muscarinic receptor mediated vascular responses.** The potent vasodilator actions of applied acetylcholine are well described in numerous vascular beds (Eglen et al. 1996). In general, the M3 muscarinic receptor is the dominant subtype, responsible for endothelium-dependent relaxations in the majority of preparations. Specific examples include feline cerebral arteries (Dauphin & Hamel 1990), the mesenteric and pulmonary circulations of the rat (Hendriks et al. 1992; McCormack et al. 1988) and both bovine and equine coronary artery (Duckles & Garcia-Villalon 1990; Brunner et al. 1991; Obi et al. 1994). In support of this, the M3 receptor was recently shown to mediate systemic vasodilatory responses to methacholine in the anaesthetised cat (Koss 1997). Vasodilatory responses mediated by M1 and M2 receptors have also been demonstrated (Chiba & Tsukada 1996; Hynes et al. 1986; Komori & Suzuki 1987a). The potential contribution of the M4 and M5 receptors to vascular responses has been difficult to ascertain due to a lack of pharmacologically selective antagonists (Dauphin & Hamel 1992; Linville & Hamel 1995).

Acetylcholine vasodilations are primarily mediated by the release of NO from the endothelium (Furchgott & Zawadzki 1980), however muscarinic receptors have also been linked to the release of EDHF (Chen et al. 1988). EDHF mediated responses have recently been demonstrated in the guinea pig coronary artery (Hammarstrom et al.)
In some vascular beds, applied acetylcholine evokes a dual response, producing vasodilation when vascular resistance is elevated and vasoconstriction under conditions of reduced tone (Altiere et al. 1994). Contractile responses have also been demonstrated when the concentration of acetylcholine is increased or when the endothelium is removed (Furchgott & Zawadzki 1980; Dauphin et al. 1994; Caulfield 1993). Contraction may be a direct effect, due to the activation of muscarinic receptors located on smooth muscle cells (Jaiswal et al. 1991; Obi et al. 1994), or it may be indirect, associated instead with the release of vasoactive factors, such as the arachidonic acid product thromboxane A₂, from the endothelium (Altiere et al. 1986; El-Kashef & Catravas 1991; Altiere et al. 1994).

The specific localisation of muscarinic receptor subtypes within the vasculature has most commonly been examined using radioligand binding studies. Expression of the M₁, M₂, M₃ and M₄ receptors has been described, however there is considerable variability, dependent on the vascular bed and species being studied (Yamanaka et al. 1986; Hynes et al. 1986; Garcia-Villalon et al. 1991; Dauphin & Hamel 1992). At the level of gene expression, freshly isolated bovine aortic endothelial cells express mRNA for the M₁, M₂ and M₃ receptors, but not M₄ or M₅ (Tracey & Peach 1992), while mRNA for the M₁ receptor has been detected in the feline cerebral vessels using Northern blots (Dauphin et al. 1991).

**Parasympathetic innervation of blood vessels.** Although muscarinic receptors appear to be present in many blood vessels, the role of these receptors is the subject of some debate, because for many vascular beds, evidence of direct parasympathetic innervation has not been demonstrated (Doods et al. 1989; Van Zwieten & Doods 1995). Parasympathetic control of coronary blood flow has been established in the dog however (Feigl 1998), and a functional cholinergic innervation has also been demonstrated in the rabbit middle cerebral artery (Van Riper & Bevan 1992), arterioles of the guinea pig submucosal plexus (Neild et al. 1990) and the rat uterus (Sato et al. 1996).

Endothelial cells have been promoted as an alternative source of acetylcholine, with studies showing the localisation of choline acetyltransferase in the vascular
endothelium (Parnavelas et al. 1985). Acetylcholine may be released from the endothelium under pathological conditions, for example hypoxia, and act locally on muscarinic receptors to stimulate the release of NO and subsequent vasodilation (Burnstock & Ralevic 1994; Ralevic & Burnstock 1996b).

1.8 VASOACTIVE INTESTINAL PEPTIDE
NEUROTRANSMISSION AND RELATED PEPTIDES

VIP belongs to the glucagon-secretin family of peptides and was first isolated from pig small intestine (Said & Mutt 1970). VIP possessed potent systemic vasodilatory actions (hence the name) and was subsequently demonstrated to coexist with acetylcholine in autonomic vasodilator neurones (Lundberg 1981). The peptides peptide histidine isoleucine (PHI) and pituitary adenylate cyclase activating polypeptide (PACAP), show considerable sequence homology to VIP (Tatemoto & Mutt 1981; Miyata et al. 1989) and have likewise been demonstrated in perivascular nerve fibres and shown to mediate vasodilatory responses, (Lundberg et al. 1984b; Edvinsson & McCulloch 1985; Cardell et al. 1991). Two forms of PACAP exist: PACAP-38 and the shorter PACAP-27, which corresponds to the N-terminal 27 amino acids of PACAP-38 (Miyata et al. 1990). It is likely that acetylcholine, VIP, PHI and PACAP are colocalised in many autonomic vasodilator neurones, although the actual combination of transmitters present may vary between different vascular beds and species (Morris et al. 1995; Morris 1995).

VIP receptors. VIP and related peptides exert their effects by binding to three different G-protein coupled receptors: VIP1 (originally named the PACAP type II receptor), VIP2 and PACAP (initially called PACAP type I). The VIP1 and VIP2 receptors recognise VIP and PACAP with comparable affinities, and are distinguished from each other by their molecular sequence (Ishihara et al. 1992; Lutz et al. 1993) and the ability of secretin to activate VIP1 but not VIP2 receptors (Usdin et al. 1994). The PACAP receptor displays a much higher affinity for PACAP than either of the VIP receptors (Arimura 1992) and again has a unique molecular sequence (Spengler et al. 1993). All three receptors are linked to Gs and activate adenylate cyclase, increasing
cAMP levels (Huang & Rorstad 1984; Miyata et al. 1989; Lutz et al. 1993; Alexander & Peters 1998). Additional PACAP receptors (1A and 1B), have been proposed on the basis of tissue differences in the potency of PACAP-27 and PACAP-38, however no molecular equivalents have been isolated (Arimura 1992; Alexander & Peters 1998).

**VIP receptor mediated vascular responses.** The vasodilatory responses mediated by VIP and related peptides show similar characteristics to the contractile effects of NPY, in that responses are slow in onset and duration (Lundberg 1981; Morris 1993; Morris 1995), and that peptide release is enhanced by high frequency nerve stimulation (Lundberg et al. 1984c; Morris 1993; Morris et al. 1995). These autonomic vasodilatory responses have been particularly well characterised in cerebral (Lee & Saito 1984; Dauphin & Mackenzie 1995), pulmonary (Hamasaki et al. 1983; Cardell et al. 1991) and pelvic (Morris 1993; Yao et al. 1996) blood vessels, where responses to both nerve stimulation and applied agonist have been assessed.

VIP receptor mediated vasodilations are endothelial independent responses, while PACAP receptor activation involves the release of NO from the endothelium (Lee & Saito 1984; Cardell et al. 1991; Franco-Cereceda 1996; Cardell et al. 1997). In agreement with these findings, mRNA for the VIP receptor has been localised to the walls of blood vessels within the rat pancreas (Usdin et al. 1994).

**1.9 SENSORY MOTOR NERVES**

Sensory motor (or primary afferent) nerves play a dual role, as they are not only able to send afferent signals to the spinal cord, but also have the ability to release transmitter from their peripheral terminals, thereby having an efferent or motor function. (Maggi et al. 1989; Holzer 1991). Within the vascular system, activation of these nerves can therefore influence vessel tone via general autonomic homeostatic reflexes, integrated within the CNS, or produce immediate local responses by the release of a number of different vasoactive peptides including the tachykinins (substance P, neurokinin A (NKA) and NKB) and CGRP (Holzer 1992; Edvinsson et al. 1996). The efferent function of sensory nerves is integral to the process of neurogenic
inflammation, which is characterised by the axon reflex or flare mechanism, vasodilation (erythema) and oedema (wheal, Brain 1996).

1.9.1 Capsaicin sensitive primary afferent neurones

Most perivascular sensory motor nerves are fine unmyelinated fibres with slow conduction velocities (C fibres) and they show a unique sensitivity to capsaicin, the pungent ingredient of the capsicum family (Holzer 1991; Morris et al. 1995). On acute application, capsaicin stimulates primary afferent nerve fibres, causing the release of transmitter from the nerve endings (Holzer 1991). It then blocks impulse conduction through the treated fibres and ultimately has a neurotoxic effect (Petsche et al. 1983; Marsh et al. 1987). Capsaicin mediates its effects by binding to specific vanilloid receptors (Holzer 1998). The vanilloid receptor subtype 1 has recently been cloned and is a non selective ion channel (Caterina et al. 1997), sensitive to the capsaicin antagonists ruthenium red and capsazepine (Maggi et al. 1988; Caterina et al. 1997). Occupation of the receptor site stimulates an inward flow of Ca^{2+} and Na^{+}, which causes the described depolarisation, desensitisation and neurotoxicity (Marsh et al. 1987; Holzer 1991). When administered systemically to newborn animals, capsaicin causes complete destruction of primary sensory neurones, while in the adult, capsaicin impairs sensory nerve responses but does not induce degeneration (Jancso et al. 1977; Holzer 1991).

Perivascular sensory nerves may contain different combinations of peptides (Morris et al. 1995) and although the tachykinins and CGRP in general induce vasodilation, there are substantial differences in the responsiveness of individual vascular beds to these neuropeptides (Edvinsson et al. 1996). Part of this variability may be related to different densities of receptors in the vascular tissue, their location on both vascular smooth muscle and endothelial cells and the presence of multiple receptor subtypes (Holzer 1992).
1.9.2 Neurokinin receptors

**Tachykinin peptides.** The tachykinins, substance P, NKA and NKB belong to a family of peptides that possess a common C-terminal sequence (Phe-X-Gly-Leu-Met-NH₂, where X = Phe or Val in mammals), which confers their unique biological activity (Lundberg 1996).

Crude extracts of substance P (initially referred to as preparation P) were first isolated from equine brain and intestine and were shown to possess atropine resistant vasodepressor and spasmogenic properties (Von Euler & Gaddum 1931). Substance P was subsequently isolated and sequenced from bovine hypothalamus (Chang et al. 1971) and was shown to be the mammalian counterpart of a family of amphibian peptides which included physalaemin (Erspamer 1981). The name tachykinin was derived from the pharmacological similarity of the peptides to bradykinin, except that they induced fast (tachy) rather than slow responses (Khawaja & Rogers 1996).

Research into the physiological significance of substance P advanced dramatically with the demonstration that substance P could be localised within the nervous system, in particular primary sensory neurons (Hokfelt et al. 1975), and with the isolation of two further mammalian tachykinins, substance K and neuromedin K, now known as NKA and NKB (Nawa et al. 1983; Kimura et al. 1983; Kangawa et al. 1983).

Studies have established that the neurokinin peptides are derived from two precursor genes: preprotachykinin A, which encodes for substance P and NKA, and preprotachykinin B which encodes for NKB (Nawa et al. 1983; Kotani et al. 1986; Bonner et al. 1987a). In regards to peptide distribution, NKA therefore coexists with substance P in most cells (though often found at a lower level), while NKB shows a more restricted pattern of distribution, being detected at lower levels in both the central nervous system and peripheral tissues (Moussaoui et al. 1992; Otsuka & Yoshioka 1993).

**Neurokinin receptors.** When the three tachykinins were tested in a variety of isolated vessels from several species, they were found to have different effects including vasodilation in vessels with intact endothelium and contraction of vessels without endothelium (D'Orleans-Juste et al. 1985; Regoli & D'Orleans-Juste 1987). This, and the finding that the relative activity of the three peptides showed different orders of potency in different systems, was taken as an indication of the presence of distinct
receptor subtypes (Lee et al. 1986; Buck & Burcher 1986; Mastrangelo et al. 1986; Regoli et al. 1987). Three receptors: NK1, NK2 and NK3, were defined by the potency of the naturally occurring agonists, with substance P being more potent at NK1 receptors, NKA acting preferentially at NK2 receptors and NKB being most potent at NK3 receptors, however it should be noted that all tachykinins are capable of acting as full agonists at all three receptor types (Regoli & D'Orleans-Juste 1987; Maggi et al. 1993). The existence of three neurokinin receptors has been confirmed through the cloning of distinct receptor genes (Yokota et al. 1989; Masu et al. 1987; Shigemoto et al. 1990) and the development of subtype selective antagonists, although marked species differences in pharmacology have been shown to exist for all subtypes (Maggi et al. 1993; Maggi 1995; Alexander & Peters 1998). All the neurokinin receptors belong to the 7-transmembrane superfamily of receptors and mediate their responses via $G_{q11}$ and stimulation of the phosphoinositol pathway (Guard & Watson 1991; Alexander & Peters 1998).

**Neurokinin receptor mediated vascular responses.** In general, the potent vasodilatory effects of substance P are mediated via endothelial located NK1 receptors and the release of NO (Zawadzki et al. 1981; Otsuka & Yoshioka 1993), however EDHF has also been shown to contribute to substance P responses (Beny & Brunet 1988; Kuroiwa et al. 1995). Endothelial NK3 receptors have recently been described in the rat, mediating NK3 agonist induced vasodilation via NO release (Mizuta et al. 1995). The neurokinins can also mediate contractile responses. For example NKA induces a contraction mediated by smooth muscle cell NK2 receptors in the rabbit pulmonary artery (D'Orleans-Juste et al. 1985) and contractile responses to substance P via NK1 receptors have been demonstrated in venous tissue (Nantel et al. 1990; Nantel et al. 1991; Patacchini & Maggi 1995).

Interestingly, many of these functional studies have been performed with the use of applied agonists, however when sensory neurogenic relaxant responses are investigated, direct neurokinin mediated vasodilations have been difficult to demonstrate (Illes & Falkenhausen 1986; Stubbs et al. 1992). Despite the finding that substance P containing perivascular nerve fibres have been detected in many blood vessels (Barja et al. 1983; Edvinsson et al. 1983), it is not clear whether endothelial neurokinin receptors are activated by neurally released transmitter (Lundberg 1996).
Substance P immunoreactivity has been shown in vascular endothelial cells (Loesch & Burnstock 1988; Milner et al. 1989) and similar to the actions of endothelial ATP and acetylcholine (see sections 1.4.4 and 1.7.1), substance P may be released in response to local conditions to activate endothelial neurokinin receptors (Couture et al. 1989; Ralevic & Burnstock 1996b).

Localisation of individual neurokinin receptor subtypes within the vascular system has been hampered to some degree by the low levels of receptor protein present and the cross reactivity shown by each receptor to the endogenous ligands (Tsuchida et al. 1990). Binding studies, however, have demonstrated NK1 receptors on the endothelium of rat iris arterioles (Hill et al. 1996), canine carotid and renal arteries (Stephenson et al. 1986; Stephenson et al. 1987) and on endothelial cells obtained from pig aorta (Saito et al. 1990). In regards to gene expression, the distribution of neurokinin receptor mRNA has been well characterised for the central nervous system and a number of peripheral tissues (Tsuchida et al. 1990; Khawaja & Rogers 1996), however no detailed studies have looked at the expression of mRNA for the different neurokinin receptors in the vascular system.

1.9.3 Calcitonin gene related peptide receptors

Calcitonin gene related peptide. CGRP is a 37 amino acid peptide that shares some sequence homology with salmon calcitonin, amylin and adrenomedullin (Bell & McDermott 1996; Brain 1996). The discovery of CGRP arose from the finding that the gene for the calcium regulating hormone calcitonin, also encoded for a novel neuropeptide, the expression of each being dependent upon alternative tissue specific RNA processing (Amara et al. 1982; Rosenfeld et al. 1983). Calcitonin is expressed in the thyroid gland, while CGRP is expressed in the nervous system (Rosenfeld et al. 1983; Bell & McDermott 1996). At least two homologous forms of CGRP exist: α- and β-CGRP, with α–CGRP being the predominant form in sensory neurones (Mulderry et al. 1988; Lundberg 1996). In the rat, the two peptides differ by only one amino acid (Amara et al. 1985).

CGRP-immunoreactive nerve fibres show a widespread distribution throughout both the peripheral and cerebral vasculature (Mulderry et al. 1985; Edvinsson et al.)
Substance P and CGRP are often colocalised (Lee et al. 1985) and in the rat, it has been noted that the content of CGRP in perivascular fibres is generally greater than that of substance P (Wharton et al. 1986).

**CGRP receptor subtypes.** The existence of multiple CGRP receptor subtypes was initially proposed on the basis of different binding affinities for CGRP analogs and on the different antagonist potencies exhibited by CGRP peptide fragments in brain and peripheral tissues (Mimeault et al. 1991; Dennis et al. 1989). The C-terminal fragment of human CGRP (hCGRP$_{8-37}$) was used as a selective antagonist to define two receptor subtypes. The CGRP$_1$ receptor which was blocked by hCGRP$_{8-37}$ and the CGRP$_2$ receptor, which was not (Dennis et al. 1990). Interestingly, neither form of the CGRP peptide ($\alpha$ or $\beta$) shows any selectivity for either of the proposed receptor subtypes (Longmore et al. 1994). Peripheral tissues in which CGRP$_2$ receptors have been identified include the rat and guinea pig vas deferens (Dennis et al. 1990; Mimeault et al. 1991) and the guinea pig urinary bladder (Giuliani et al. 1992). The CGRP$_1$ receptor predominates in the cardiovascular system (Dennis et al. 1990; Brain & Cambridge 1996).

Identification of a cDNA encoding for the human CGRP$_1$ receptor has only recently been achieved (Aiyar et al. 1996). The receptor had in fact been cloned previously from both rat and human tissues (Chang et al. 1993; Fluhmann et al. 1995), however it was not linked to CGRP in ligand studies and was therefore classified as an orphan calcitonin-like receptor. The discrepancy between these results may have been due to the different cell types used for the respective expression studies and the requirement of a specific factor to induce the appearance of the CGRP phenotype (Aiyar et al. 1996; Alexander & Peters 1998). The cloned CGRP$_1$ receptor is a G-protein coupled receptor and displays the functional and pharmacological properties of the native subtype, being linked via $G_\alpha$ to the stimulation of cAMP and antagonised by hCGRP$_{8-37}$ (Kubota et al. 1985; Aiyar et al. 1996). The cloning of the putative CGRP$_2$ receptor is still awaited.

**CGRP receptor mediated vascular responses.** CGRP is a potent vasodilator in the majority of vascular beds and amongst different species (Brain et al. 1985; Lundberg 1996). The effects of CGRP are typically long lasting (Han et al. 1990a; Gyoda et al. 1995; Morris 1995) and are due to the direct activation of receptors located on the
vascular smooth muscle and a decrease in intracellular Ca\(^{2+}\) regulated by adenylate cyclase (Lundberg 1996; Bell & McDermott 1996). However, CGRP can act via more than one mechanism to effect vasodilation. In the rat aorta for example, CGRP mediated vasodilation is dependent on the presence of an intact endothelium and NO (Brain et al. 1985; Gray & Marshall 1992a) and CGRP has also been reported to activate ATP sensitive K\(^+\) channels in arterial smooth muscle (via smooth muscle receptor activated increases in cAMP and protein kinase A, Quayle et al. 1994), leading to hyperpolarisation and hence relaxation (Nelson et al. 1990; Saito et al. 1989; Zschauer et al. 1992).

Vascular beds in which CGRP has been demonstrated as the endogenous mediator of sensory nerve vasodilations include rat and rabbit mesenteric arteries (Kawasaki et al. 1988; Han et al. 1990b; Kakuyama et al. 1998), rabbit jejunal artery (La & Rand 1993), rat iris arterioles (Hill & Gould 1997), rat hepatic artery (Bratveit & Helle 1991) and the microvasculature of the skin in a number of species (Brain 1996). In many of these studies and others where exogenous CGRP is applied directly (Hughes & Brain 1994; Kobari et al. 1995), hCGRP\(_{8-37}\) is able to block vasodilatory responses. Messenger RNA for the CGRP\(_1\) receptor has recently been localised to vascular smooth muscle cells in human cerebral arteries (Edvinsson et al. 1997) and taken together, these results confirm the important role for the CGRP\(_1\) receptor subtype in the vasculature.

One study looking at the uterine artery of the rat, has demonstrated a CGRP mediated vasodilation that could not be inhibited by hCGRP\(_{8-37}\), suggesting the presence of either CGRP\(_2\) or another, as yet unidentified CGRP receptor in this vessel (Anderson et al. 1997). CGRP\(_2\) receptors may also be present in the guinea-pig basilar artery (Jansen 1992).

1.10 DEVELOPMENTAL EXPRESSION OF NEURORECEPTORS

In the preceding pages, I have reviewed the neuronal control of vascular function and discussed the numerous receptors which mediate these responses. The functioning of this system depends upon the development and maturation of a functional neuroeffector junction, which is influenced by properties of both the ingrowing nerve
fibres and the postsynaptic tissue (Hill & Vidovic 1992). Sympathetic nerves are capable of releasing neurotransmitter when fibres are still entering their target tissue and this early period of innervation is often accompanied by significant modifications in the postsynaptic response (Hill et al. 1991; Hill & Vidovic 1992). Many of these changes involve neuroreceptors and can be due to alterations in the density of receptors, changes in the receptor subtype mediating the post-synaptic response or maturation of signaling transduction mechanisms.

1.10.1 Correlation between innervation and developmental responses

Sympathetic nervous system. The ingrowth of sympathetic nerve fibres has been correlated with changes in both the density and the coupling of adrenergic receptors. In the developing chick heart for example, the arrival of postganglionic sympathetic nerves promotes an upregulation of β-adrenergic receptor protein expression and an alteration in the functional properties of the receptors (Stewart et al. 1986). Similar changes are seen in the rat submandibular salivary gland, where the postnatal appearance of catecholamine containing nerves correlates with a dramatic increase in the density of β-adrenergic receptors and the appearance of functional stimulus-secretion coupling (Cutler et al. 1981; Bottaro & Cutler 1984).

Innervation by sympathetic nerves can also be associated with changes in the effector receptor subtype. In the rat heart, sympathetic innervation induces a switch in the chronotropic (rate) responsiveness to α₁-adrenergic receptor stimulation. The response changes from positive to negative during development (Drugge et al. 1985) and is due to the sequential functional expression of two different subtypes of α₁-adrenergic receptors (del Balzo et al. 1990). A developmental change in functional receptor type is also seen in the liver of the male rat. The metabolic response to noradrenaline is mediated predominantly by β₂-adrenergic receptors in the foetal liver and becomes an α₁-adrenergic response in the adult (Rossby & Cornett 1991). This change from a β₂- to an α₁-adrenergic response has been attributed to a number of influences including the development of the sympathetic innervation to the liver (Lautt 1980).
Parasympathetic nervous system. In the chick heart, prior to parasympathetic innervation, muscarinic receptors are present but there is no response to cholinergic drugs (Galper et al. 1977). The change in responsiveness seen with the ingrowth of parasympathetic nerves has been related to changes in signaling events beyond the receptor, specifically the expression of an inhibitory G-protein that links the muscarinic receptor to adenylate cyclase (Halvorsen & Nathanson 1984; Liang et al. 1986). The expression of non-functional muscarinic receptors prior to cholinergic innervation is also seen in the sweat glands of the rat footpad (Grant & Landis 1991b).

Neuropeptide regulation. Neuropeptides have also been implicated in developmental processes. In the mesenteric and carotid arteries of the guinea pig, nerves containing VIP, CGRP and substance P, reach a peak in density around birth and then undergo decline, consistent with a trophic role for neuropeptides in early vascular development (Dhall et al. 1986). The neuronal release of peptides has also been suggested to be involved in the regulation of prenatal cortical development (Hayashi & Oshima 1986; Pincus et al. 1992).

Studies have also suggested that neuropeptides can directly influence the developmental expression of auto- and heteroreceptors (Pincus et al. 1992). In the rat salivary gland, neonatal treatment with substance P caused a long lasting upregulation of neurokinin receptor expression (Handelmann et al. 1987) and in skeletal muscle, CGRP released from the presynaptic motor neurone has been shown to increase synthesis of the postsynaptic nicotinic acetylcholine receptor (New & Mudge 1986; Fontaine et al. 1987). Along with changes in receptor density, the nicotinic receptor in skeletal muscle also undergoes a specific receptor subunit change, with the replacement of the γ-subunit by the ε-subunit inducing a distinctive functional alteration of the receptors (Mishina et al. 1986). Innervation by the motor neurone is integral to this prenatal regulation of ε-subunit gene expression and transcription (Martinou & Merlie 1991).

1.10.2 Neuroreceptor mediated vascular responses during development

Changes in postsynaptic effector mechanisms have been described in a number of blood vessels during development. In the rat mesenteric artery for example,
intracellular recordings show a change from an adrenergic to a non adrenergic response between days 4 and 9 postnatal (Hill et al. 1983). In the rat basilar artery, electrical responses in neonatal animals are mediated primarily by excitatory β- and non α-adrenergic (γ) receptors, while in the adult, the non-α receptors predominate (Byrne et al. 1985).

More recent studies have identified changes in specific receptor subtypes. In rat iris arterioles, sympathetic nerve-mediated contractions during early development are mediated by the activation of both α₁- and α₂-adrenergic receptors, while by 21 day postnatal, only α₁-receptors are involved (Sandow & Hill 1998). In this study, although a change in receptor subtype was observed, no change was noted in the source of intracellular Ca²⁺. In rat aorta, contractile responses are mediated primarily by α₁bx-adrenergic receptors in young animals, while α₁a-receptors predominate in older animals (Gurdal et al. 1995a). Interestingly, in this tissue, functional changes are matched by parallel changes in both protein and receptor mRNA expression (Gurdal et al. 1995b). In contrast, in sheep cerebral arteries, developmental changes in α₁-adrenergic receptor responsiveness have been attributed to changes in coupling to or function of voltage gated Ca²⁺ channels, rather than alterations in receptor type or density (Longo et al. 1996).

1.11 THESIS AIMS AND OUTLINE

1.11.1 General overview

From the foregoing, it is clear that there is a great range of receptor subtypes mediating vasoconstriction and vasodilation in different vascular beds, each activating multiple signal transduction systems in either the vascular smooth muscle or endothelium. The situation is further complicated by the range of transmitters that may be released from the various types of perivascular nerves and potential interactions between pre- and postsynaptic mechanisms.
As stated in section 1.1, the variable responses to nerve stimulation that are displayed between vascular beds may be due to differences in the types and subtypes of neuroreceptors that are expressed in individual blood vessels. Such heterogeneity may also contribute to the changes that have been described in individual vascular beds during development (section 1.10.2). If such differences do exist, identifying the profile of receptor expression could potentially aid in the development of drugs targeted to specific vascular beds, thereby avoiding the systemic side effects currently seen with many cardiovascular medications (Katzung 1992b; Hoffman 1992). Such 'vascular bed specific' drugs could be targeted to individual receptor subtypes, or alternatively, the second messenger systems utilised to regulate smooth muscle cell intracellular Ca\(^{2+}\) levels. By identifying the receptors involved in functional responses to nerve stimulation, it may also be possible to manipulate interactions between vasoconstrictor and vasodilator pathways.

As highlighted in the preceding literature review, a range of techniques are available to study neuroreceptor expression in the vascular system, however a number of limitations are also evident. Radioligand binding studies for example, are limited by the selectivity of specific agonists and immunohistochemical approaches are severely impeded by a lack of available antibodies for the wide range of potential neuroreceptors. Functional experiments are again limited by drug selectivity, and also, are often complicated by interactions between both pre- and postsynaptic mechanisms and vasoconstrictor and vasodilator pathways. Given the cloning of the majority of neuroreceptor subtypes, an alternative approach is to assess receptor mRNA expression.

1.11.2 Thesis aims

The initial aim of this study therefore, was to establish if the different physiological and pharmacological responses recorded in different arteries could be attributed to differences in receptor mRNA expression. In order to gain a comprehensive overview of neuroreceptor expression in the vasculature, RT-PCR was used to directly assess the mRNA expression of a range of receptors in a number of different arterial beds of the rat. RT-PCR was chosen because it is a highly sensitive technique that allows for a relatively rapid assessment of gene expression (Pierce et al.)
1996), as compared to other less sensitive and more laborious techniques such as Northern blot analysis or RNase protection assays.

Following from this, one vascular bed: the hepatic artery, was chosen to correlate the profile of receptor mRNA expression with functional protein expression. The extrahepatic vessels, lying within the mesentery, were chosen, as the preparation allows easy access to small vessels for physiological and pharmacological experiments and additionally, is of clinical relevance in regards to the hepatic circulation in the aged and in disease states such as cirrhosis (LeCouteur & McLean 1998). For continuity, the RT-PCR results associated with this vessel are presented with the physiological findings, rather than as part of the comparative study.

The mRNA study was then further expanded to examine receptor expression in the mesenteric artery of the rat during development to determine if developmental changes could be correlated to neuroreceptor mRNA expression. Any association between the ingrowth of nerves and receptor expression was then examined by denervating the mesenteric artery over the same period and again using RT-PCR to screen receptor mRNA expression. The mesenteric artery was chosen for these experiments, as previous work from this laboratory has characterised the functional responses of this vessel in some detail, both during development and after denervation (Hill et al. 1983; Hill et al. 1985a; Hill et al. 1985b).

1.11.3 Thesis outline

The results presented in this thesis have been divided into the following sections:

- A comparative study using RT-PCR to assess neuroreceptor mRNA expression in the basilar, mesenteric, pulmonary and tail arteries of the rat.
- Characterisation of neuroreceptor mRNA expression and sympathetic nerve-mediated vasoconstriction in the arteries of the rat hepatic mesentery.
• Characterisation of neuroreceptor mRNA expression and nerve-mediated vasodilator mechanisms in the arteries of the rat hepatic mesentery.

• Assessment of neuroreceptor mRNA expression in the rat mesenteric artery during development and maturation.

• Assessment of the effect of denervation on receptor mRNA expression in the rat mesenteric artery during development.
2.1 ANIMALS

Experiments were performed on an outbred strain of Wistar-Kyoto rats of either sex (4 to 5 weeks of age), weighing 55-85g. For the developmental studies, rats were used at birth and postnatal days 7, 14, 28, 240 and 360. Animals were killed with an overdose of ether anaesthetic and cervical dislocation. In experiments where animals were perfused prior to dissection, they were anaesthetised with intraperitoneal ketamine (44mg/kg) and xylazine (8mg/kg) since a longer lasting anaesthetic was required.

2.2 DENERVATIONS

2.2.1 Sympathectomy

Chemical sympathectomy was performed with 6-hydroxydopamine (6-OHDA, Angeletti & Levi-Montalcini 1970). In preliminary experiments, animals were treated with 6-OHDA daily from days 2 to 14, however, the number of total injections was reduced due to the high mortality rates observed. In subsequent experiments, animals received subcutaneous injections on postnatal days 2, 6, 9 and 14 of 6-OHDA, 100mg/kg dissolved in physiological saline (0.9% NaCl), containing 1mg/ml ascorbic acid to prevent oxidation of the drug. Control animals received injections of the diluent only.

Immunosympathectomy was performed with antiserum directed against the protein nerve growth factor (anti-NGF, kindly supplied by Assoc. Prof. R. A. Rush, Flinders University, Australia). The antiserum was raised in sheep against mouse salivary gland NGF (Zhou & Rush 1996). Newborn animals were injected
subcutaneously with undiluted sera at 10μl/g bodyweight, with injections daily from postnatal day 0 to 6. This treatment has been shown previously to cause a complete loss of catecholamine containing fibres innervating the rat mesenteric artery (Hill et al. 1985a). Control animals were treated with physiological saline.

The efficacy of the chemical sympathectomy was determined using catecholamine histochemistry at weekly intervals up to 4 weeks of age. The efficacy of immunosympathectomy was confirmed using catecholamine histochemistry at 4 weeks of age. Iris tissue was used as a positive control for these experiments (Vidovic et al. 1987).

2.2.2 Sensory denervation

Capsaicin was used to selectively destroy substance P containing sensory-motor nerves (Jancso et al. 1977; Holzer 1991). Rats were injected subcutaneously with capsaicin (50mg/kg dissolved in 10% ethanol, 10% Tween 80 in physiological saline) on postnatal days 2 and 10. Control animals were injected with diluent at the same times.

The efficacy of capsaicin treatment was assessed with immunohistochemistry at 4 weeks of age, using antibodies directed against the sensory neuropeptide substance P.

2.3 DISSECTIONS

Arteries from 5 different vascular beds (basilar, mesenteric, pulmonary, tail and hepatic) were examined in these studies. The basilar artery was dissected from the lateral vertebral arteries to the Circle of Willis (Figure 2.1A). The mesenteric artery dissection consisted of the superior mesenteric artery intestinal branch and approximately 10 secondary branches extending as close to the jejunum and ileum as possible (Figure 2.1B). The pulmonary artery was divided into two regions: the primary branches, which consisted of the main pulmonary artery and its left and right branches before they entered the lung parenchyma and the secondary branches, which were
collected from the left lung and either the middle or inferior lobes of the right lung, starting from the first side branch within the lung tissue to the point where this vessel finally bifurcated in the lower part of the individual lobes (Figure 2.1C). The rat tail artery was collected from the base of the tail, extending two thirds along its length (Figure 2.1D). The hepatic artery was dissected from within the hepatic mesentery, from the point where it separated from the coeliac artery to the point of entry of its branches into the liver parenchyma (Figure 2.1E). To distinguish this arterial bed from the main hepatic artery and from intrahepatic vessels, the preparation is described as the arteries of the rat hepatic mesentery. For the RT-PCR and histochemical experiments the entire hepatic arterial bed was used, whilst for the physiological experiments, only second and third order branches were used (Figure 2.1F).

When iris tissue was used as a control for the denervation histochemical studies, eyes were dissected out, the lens removed and the iris was cut in half such that each piece contained both corneal and sphincter edges. When iris tissue was used as a control for RT-PCR studies the ciliary processes were included with the sphincter and dilator muscles. Other control tissues included brain, heart, superior cervical, superior mesenteric and dorsal root ganglia.

2.4 HISTOLOGY AND HISTOCHEMISTRY

2.4.1 Histology

Samples of all vascular tissue used for RNA extraction were collected after perfusion with saline, fixed in 4% paraformaldehyde overnight, washed in phosphate buffered saline (PBS) and paraffin embedded. Sections (7 μm) were cut and stained with haematoxylin and eosin to assess endothelial integrity.
Figure 2.1
Figure 2.1

Photographs illustrating the \textit{in vivo} location of the different vascular beds examined in these studies. Panel A illustrates the position of the basilar artery (arrow) located on the ventral surface of the brain stem. The mesenteric artery is shown in B. The dissection consisted of the superior mesenteric artery (arrow) and secondary branches extending to the jejunum and ileum. The pulmonary artery is shown in panel C. The heart has been deflected up to show the main pulmonary artery branches (arrows). The secondary branches were dissected separately from within the lung parenchyma. The tail artery was dissected from within the vascular groove along the ventral surface of the tail (arrow, panel D). The hepatic artery preparation is shown \textit{in situ} in panel E. The liver lobes have been deflected and the bile duct has been removed. The main hepatic artery (arrow) is shown lying over the portal vein. In panel F, the arteries of the hepatic mesentery have been dissected out and immobilised by pinning the mesentery in a tissue bath. For RT-PCR and histochemical studies the entire bed including the main artery (arrow) was used, whilst for the physiological experiments only second and third order branches were used.
2.4.2 Catecholamine histochemistry

Catecholamine histochemistry was used to assess the density and distribution of sympathetic nerves innervating whole mount preparations of the mesenteric and hepatic arteries. Irides were also used as a control tissue. The mesentery containing the mesenteric or hepatic arterial beds was dissected into cold PBS, stretched and pinned in a dish coated with Sylgard (Dow Corning, USA). Irides were cut in half and pinned with the anterior surface uppermost. Preparations were fixed in 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1M phosphate buffer (PB), pH 7, for 3h at 4°C (Furness et al. 1977), dried over phosphorus pentoxide, mounted in paraffin oil and viewed with an Olympus BH microscope (Excitation filter BP 405, Dichroic mirror DM 455, Barrier filter 475).

2.4.3 Immunohistochemistry

Whole mount preparations. Whole mount preparations of the hepatic arterial bed, prepared as described above, were used to evaluate the staining produced by antibodies directed against the synaptic vesicle protein, synaptophysin. Whole mounts were fixed in Zamboni's fixative (4% (w/v) paraformaldehyde, 0.2% (w/v) picric acid in 0.1M sodium PB, pH 7, Zamboni & De Martino 1967) for 1h at 4°C. Picric acid was removed by treatment with dimethylsulphoxide (3x10min) or 50% ethanol (3x10min) and the tissue washed and stored overnight in PBS containing 0.04% (v/v) sodium azide. Whole mounts were then treated with 0.1M glycine (3x10min), washed in PBS containing 0.01% (v/v) Triton X-100 and pre-incubated in PBS containing 0.2% (v/v) Triton X-100, 7mg ml⁻¹ λ carrageenan and 0.01% (v/v) sodium azide for 1h at room temp. Primary antibody incubation with rabbit antibodies against human synaptophysin (1:30, DAKO Denmark) was for 48h at room temperature.

Tissue sections. Tissues to be sectioned for subsequent histochemical analysis were dissected into cold PBS and then fixed in either Zamboni's fixative for 3hrs at 4°C or 4% paraformaldehyde in 0.1M PBS, pH 7.4, for 1hr at 4°C. Picric acid was removed as described above. Tissue was then washed in PBS and stored overnight in PBS containing 0.04% (v/v) sodium azide. Samples were immersed in 30% sucrose in PBS for 1hr, embedded in Tissue-Tek (Miles, USA) and frozen on dry ice. Ten μm sections
were cut and mounted on slides coated with either gelatin (0.05%, w/v) or silane (2%, w/v 3-aminopropyltrithoxysilane) and dried over phosphorus pentoxide. Slides were then stored at -20°C until further use. Prior to antibody treatment, tissue sections were treated with 0.1M glycine (2x5min) and washed with 0.1M PB (3x5min). Sections were preincubated in either PBS containing 0.2% (v/v) Triton X-100, 7mg ml⁻¹ λ carrageenan and 0.01% (v/v) sodium azide or PBS containing 2% (w/v) bovine serum albumin and 0.04% (v/v) sodium azide, for 1h at room temperature and then incubated with the primary antibody diluted in the same solution for 24-36 hrs at room temperature. Antibodies used were made in rabbits against VIP (1:2000, Furness et al. 1981), CGRP (1:2000, Gibbins et al. 1985) and substance P (1:700, Incstar, Minnesota).

**Immunoreactive labeling.** Immunoreactivity was detected after sequential incubation in biotinylated anti-rabbit secondary antibody IgG (1:650, VECTOR Labs. USA) and Texas Red conjugated to streptavidin (1:200, Amersham USA), or anti rabbit fluorescein isothiocyanate (1:40, DAKO Denmark). Preparations were then washed in PBS containing 0.1% (v/v) Triton X-100, mounted in buffered glycerol (glycerol: 0.5M sodium carbonate buffer (2:1) containing 0.6% (w/v) phenylenediamine) and viewed with an Olympus BH microscope (Excitation filter BP 545, Dichroic mirror DM 580, Barrier filter R-610 for Texas Red and Excitation filter BP 490, Dichroic mirror DM 500, Barrier filter 515 for fluorescein). Photographs were taken using an Olympus camera mounted on an Olympus BH fluorescence microscope.

2.4.4 **NADPH-diaphorase histochemistry**

NADPH-diaphorase histochemistry was used to localise NOS (Hope et al. 1991) in arterial tissue. Sections of superior mesenteric ganglia were used as positive controls (Anderson et al. 1993). Tissue sections, prepared as described previously, were washed briefly in PBS and then incubated with 50mM Tris-HCl, pH 8, 0.2% (v/v) Triton X, 0.5mM nitroblue tetrazolium (dissolved in dimethylformamide) and 1mM β-NADPH for 30min in the dark. The sections were washed briefly in 50mM Tris-HCl to terminate the reaction (Afework et al. 1992) and rinsed with PBS. Sections were counterstained with 0.01% (w/v) Neutral Red and mounted in buffered glycerol. Sections were then viewed under bright field illumination with an Olympus microscope.
2.5 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

2.5.1 Preparation of arterial tissue for RNA extraction

In general, animals were anaesthetised and perfused with a sterile saline solution containing 0.1% sodium nitrite (w/v), 0.1% bovine serum albumin (w/v) and 0.25% (v/v) heparin (1000U/ml), to remove blood cells. In some cases, where animals were not perfused, blood cells were removed manually. Each vessel was rapidly dissected into cold PBS, stripped of surrounding veins, connective tissue and fat and transferred into RNAzol B (Cinna Biotech). Six to 13 litter mates were used for each RNA preparation, based on the assumption that there is no variation in receptor expression between animals. In the developmental study, due to the different amounts of tissue collected from the various age groups, different numbers of animals of mixed sex were used for each RNA preparation: at birth - 12 to 18 rats; at 7, 14 and 28 days - 6 to 13 rats; and at 240 and 360 days - 2 to 6 rats.

2.5.2 Extraction of RNA

Tissue collected into RNAzol B solution was homogenised and total cellular RNA isolated using a single step procedure for RNA extraction, following the manufacturers recommended protocol. The RNA was precipitated from the aqueous phase with isopropanol and the pellet then washed in 75% ethanol and resuspended in diethyl pyrocarbonate-treated water. Optical density readings at 260nm were made to determine the amount of total RNA. On average, 0.5μg of total RNA was retrieved from each 1mg of arterial tissue.

As several of the receptors studied are the products of intronless genes, contaminating genomic DNA was removed by incubating total RNA with pancreatic deoxyribonuclease I (DNase I, Stratagene, USA), 10U for 45 minutes at 37°C in a 100μl total reaction volume, containing 3mM Tris base, 300μm ethylenediaminetetra-acetic acid (EDTA), 10mM MgCl₂, 1mM dithiothreitol and 40U RNase inhibitor (Stratagene...
USA). The reaction was stopped using 2μl of 0.2M EDTA (pH 8), protein extracted with phenol:chloroform isoamyl alcohol (1:1) and total RNA precipitated from the aqueous phase with isopropanol. The RNA pellet was washed in 75% ethanol and resuspended in water. Total RNA was again measured by absorbence at 260nm. This DNase step was omitted for the newborn animals as the total RNA yield was small and losses were encountered during the second RNA extraction. For the hepatic arterial preparations and the denervation experiments on the mesenteric artery, genomic DNA was removed using Message Clean DNase I (GenHunter Brookline USA) following the manufacturers recommended protocol.

2.5.3 Reverse transcription-polymerase chain reaction

To produce a cDNA pool for use in PCR reactions, RNA from each vessel was reverse transcribed in a volume of 50μl. Reactions were set up with 5μg of total RNA, 50U Stratascript RNase H reverse transcriptase enzyme (Stratagene, USA), 1mM dNTP’s (Pharmacia), 40U of RNase inhibitor and 300ng of Random Primers (GIBCO). Due to discontinuation of a product line, the Stratascript RT enzyme was replaced with 200U of Superscript II (GIBCO). Reaction temperatures were 42°C for 58min, 50°C for 58min and then 90°C for 10min to inactivate the enzyme. Experiments were also performed with oligo(dT) primers (300ng, Stratagene USA) and results were similar to those seen with random primers. Control reactions either did not contain reverse transcriptase enzyme, or did not contain RNA.

From the cDNA pool generated from each artery, 2μl was used in subsequent PCR reactions. This single cDNA pool was generated for each artery to control for variability during reverse transcription. Therefore, the amount of PCR product amplified by any particular primer pair was relative to a constant starting amount of cDNA, and between arteries was relative to a constant amount of starting RNA.

Oligonucleotide primers for use in the PCR were synthesised using an Applied Biosystems Model 380B Nucleic Acid Synthesiser and solid phase synthesis. PCR was performed with subtype specific primers for the α1(A, B, D)- and α2(A, B, C)-adrenergic, neurokinin (NK1-NK3), muscarinic (M1-M5), CGRP, NPY(Y1, Y2) and P2X(1-7)-purinergic receptors. Primer sequences, predicted fragment lengths, nucleotide position
and region of receptor amplified are shown in Table 2.1. Where possible, primers were designed to span introns, for example the neurokinin, CGRP, and purinergic receptor genes, however, some of the adrenergic, neuropeptide Y and all the muscarinic receptors are products of intronless genes. Primer design was optimised with the aid of a computer program ‘Amplify’ (Bill Engils, University of Wisconsin, USA), cross referencing primers against each other and known cDNA sequences for all the receptor types studied. The efficiency of all primer pairs was confirmed using other tissues (brain, heart, iris, superior cervical and dorsal root ganglia) in which the respective genes were known to be expressed.

PCR was performed in a capillary tube cycler (Corbett Research, Sydney, Australia) in a 20μl total reaction volume containing 2μl of the appropriate cDNA, 10mM Tris-HCL (pH 9), 50mM KCL, 1.5 mM MgCl₂ 0.01% gelatin, 0.1% (v/v) Triton X-100, dNTPs (200μM each of dATP, dTTP, dCTP and dGTP), 24pmol of each primer and 0.2U of SuperTaq DNA polymerase (P.H. Stehelin and Cie AG, Basel). Samples were heat sealed and amplifications were performed for 30-35 cycles. Annealing temperatures and extension times were determined by the melting temperatures of the individual primer pairs and predicted fragment lengths respectively (Table 2.2). Cycles involved denaturation at 94°C for 10s, annealing for 10s and then the appropriate extension time at 72°C. Cycle one involved a 2 min denaturing step and the final cycle lasted 5 min at 72°C.

2.5.4 Assessment of PCR product

Individual PCR fragments were visualised on 2% (w/v) agarose gels stained with ethidium bromide and viewed under ultraviolet light. Photographs of gels were taken on a Nova Line gel documentation system and a Mitsubishi Video copy processor. The intensity of the PCR product amplified was subjectively assessed in comparison with a standard DNA ladder of known molecular weight and a scoring system of [0] to [+++] was used, where [0] = never detected; [+] = weakly detected; [++] = strongly detected; [++++] = very strongly detected (Figure 2.2). In order to overcome differences in ethidium bromide uptake and photographic resolution between gels, each PCR product was assessed relative to the DNA ladder on the same gel as the individual product was
### Table 2.1 Oligonucleotide primer sequences for specific receptor subtypes, nucleotide positions and predicted fragment size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Nucleotide numbering</th>
<th>Region of Receptor Amplified</th>
<th>Fragment size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_{1A})</td>
<td>5'-TGGCCATCATTCTGGTATGT-3'  5'-GCAACCAACCACGATGCCCAG-3'</td>
<td>686-936</td>
<td>TM 5-6</td>
<td>251</td>
<td>(Alonso-Llamazares et al. 1993; Vidovic &amp; Hill 1995)</td>
</tr>
<tr>
<td>(\alpha_{1B})</td>
<td>5'-AGGCCCCATCTCTGTGGCCTG-3'  5'-GATGGAGATGCCTGAGGACAGAC-3'</td>
<td>370-774</td>
<td>TM 1-4</td>
<td>405</td>
<td>(Voight et al. 1990; Vidovic &amp; Hill 1995)</td>
</tr>
<tr>
<td>(\alpha_{1D})</td>
<td>5'-AGTGGGTGTCTTCCTGCGGCTT-3'  5'-GATCACTGCCCAGGGTAGGAGA-3'</td>
<td>755-1271</td>
<td>TM 1-5</td>
<td>517</td>
<td>(Lomasney et al. 1991b; Perez et al. 1991; Vidovic &amp; Hill 1995)</td>
</tr>
<tr>
<td>(\alpha_{2A})</td>
<td>5'-GCCGCCAGAACCCTCTTGCCTG-3'  5'-CCAGCGCCCTTCTCTATGAG-3'</td>
<td>196-533</td>
<td>TM 2-4</td>
<td>338</td>
<td>(Chalberg et al. 1990; Lanier et al. 1991; Vidovic et al. 1994)</td>
</tr>
<tr>
<td>(\alpha_{2B})</td>
<td>5'-AAACGCAGCCACTGGAAGGTCTC-3'  5'-ACTGGCAACTCTCATTGCTCC-3'</td>
<td>978-1433</td>
<td>IL 3</td>
<td>456</td>
<td>(Zeng et al. 1990; Vidovic et al. 1994)</td>
</tr>
<tr>
<td>(\alpha_{2C})</td>
<td>5'-CTGGCAGCCGTGGGTTTCCCTC-3'  5'-GTCGGGCAGGCGGTTAGAAAGAGAC-3'</td>
<td>160-585</td>
<td>TM 1-4</td>
<td>425</td>
<td>(Lanier et al. 1991; Vidovic et al. 1994)</td>
</tr>
<tr>
<td>NK1</td>
<td>5'-CACGCTATCGTGAGCTTCCTC-3'  5'-ACAGTACGCTGCTGAGCCGATACC-3'</td>
<td>672-1208</td>
<td>TM 1-5</td>
<td>537</td>
<td>(Yokota et al. 1989; Hill et al. 1996)</td>
</tr>
<tr>
<td>NK2</td>
<td>5'-ATCAACCTGGCCTTGGCGGACCTC-3'  5'-ATGACACTGTAAGCCCGAACCAC-3'</td>
<td>665-1107</td>
<td>TM 2-5</td>
<td>443</td>
<td>(Sasai &amp; Nakanishi 1989; Hill et al. 1996)</td>
</tr>
</tbody>
</table>
Table 2.1 continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Nucleotide numbering</th>
<th>Region of Receptor Amplified</th>
<th>Fragment size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK3</td>
<td>5'-GCAGTCTTCGGAACCTCCTA-3' 5'-CAGTACACCAGAGATGATCACAATG-3'</td>
<td>482-957</td>
<td>TM 1-5</td>
<td>476</td>
<td>(Shigemoto et al. 1990; Hill et al. 1996)</td>
</tr>
<tr>
<td>M1</td>
<td>5'-GATCACACACAGGCCCCTGCTT-3' 5'-ACACCTTTGCGTCTCA-3'</td>
<td>229-847</td>
<td>TM 1-5</td>
<td>619</td>
<td>(Bonner et al. 1987b; Shapiro et al. 1988; Phillips et al. 1996)</td>
</tr>
<tr>
<td>M2</td>
<td>5'-GCCAACAGGAAGTACGAGGTTAGCAATATT-3' 5'-GCCAACAGGAAGTACGAGGTTAGCAATT-3'</td>
<td>633-1184</td>
<td>IL 3</td>
<td>552</td>
<td>(Lai et al. 1990; Drescher et al. 1992)</td>
</tr>
<tr>
<td>M3</td>
<td>5'-GTGGGTGATGATTTGGCTCTG-3' 5'-TCTGCCGAGGAGTTGGTGTC-3'</td>
<td>591-1380</td>
<td>TM 3-5</td>
<td>790</td>
<td>(Bonner et al. 1987b; Braun et al. 1987; Drescher et al. 1992)</td>
</tr>
<tr>
<td>M4</td>
<td>5'-AGTGGCTCATCACAGTTCTGATGCCAGA-3' 5'-CACATTCCCTTGGCTTGCTGTGCTTGG-3'</td>
<td>543-1052</td>
<td>TM 5</td>
<td>510</td>
<td>(Bonner et al. 1987b; Drescher et al. 1992)</td>
</tr>
<tr>
<td>M5</td>
<td>5'-GCAGTTGACTGCTGGTTGTCA-3' 5'-TTGAGGTACCCAGGTCTTTG-3' 5'-TTGGAGGTACCCAGGTCTTTG-3'</td>
<td>1070-1663</td>
<td>TM 1-5</td>
<td>594</td>
<td>(Bonner et al. 1988; Liao et al. 1989; Phillips et al. 1996)</td>
</tr>
<tr>
<td>CGRP1</td>
<td>5'-CTCTGTGAAAGCAGTTTACACTCACAC-3' 5'-GAGACCAAAAGCCTGGAAGTG-3'</td>
<td>1244-1695</td>
<td>TM 3-7</td>
<td>452</td>
<td>(Aiyar et al. 1996; Phillips &amp; Hill 1998)</td>
</tr>
<tr>
<td>NPY1</td>
<td>5'-ATTCGCCGTCACTCTACAGGC-3' 5'-TCCACAGATGAGCTGGAGCCAC-3'</td>
<td>667-1255</td>
<td>IL 3</td>
<td>589</td>
<td>(Krause et al. 1992; Newhouse &amp; Hill 1997)</td>
</tr>
<tr>
<td>NPY2</td>
<td>5'-GGTACAGTCTCACAGCTTTTCAACC-3' 5'-CAACCTCTGCTCAGCGAAGGC-3'</td>
<td>646-1038</td>
<td>IL 3</td>
<td>393</td>
<td>(Newhouse &amp; Hill 1997)</td>
</tr>
<tr>
<td>Gene</td>
<td>Primer sequence</td>
<td>Nucleotide numbering</td>
<td>Fragment size (bp)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------------</td>
<td>----------------------</td>
<td>--------------------</td>
<td>----------------------------------------------</td>
<td></td>
</tr>
</tbody>
</table>
| P\(_{2X1}\) | 5'-GTGTGCCCTTCAATGGCAGCTGTA-3'  
5'-CCCATACAGTCCGTGGAACCTGTA-3' | 655-1052             | 397                | (Valera et al. 1994; Phillips et al. 1998)   |
| P\(_{2X2}\) | 5'-CTGCCTCCTCAGCTAACAACCTCA-3'  
5'-GAGTACGCACCTTGTGCACTTCT-3' | 881-1153             | 273                | (Brake et al. 1994; Phillips et al. 1998)   |
| P\(_{2X3}\) | 5'-ATCAAGAACAGCATCCGTTCCTCCT-3'  
5'-AGTGTGTCACATCTCACTCTC-3' | 704-1278             | 575                | (Chen et al. 1995; Phillips et al. 1998)   |
| P\(_{2X4}\) | 5'-GATGCACCTAGCAGACAGACACAC-3'  
5'-TCCACGATTTGTGCCAGACGAAT-3' | 392-787              | 396                | (Bo et al. 1995; Seguela et al. 1996; Phillips et al. 1998) |
| P\(_{2X5}\) | 5'-TTTTCTCGTTGTACACCATCGAT-3'  
5'-ATTTGTGGAGCTGAGATGAGCT-3' | 484-862              | 379                | (Collo et al. 1996, Garcia-Guzman et al. 1996; Phillips et al. 1998) |
| P\(_{2X6}\) | 5'-CAACTCTTCTTGTGACACCGCTC-3'  
5'-GGAGAGTGAATCGTACACAGTA-3' | 331-691              | 361                | (Collo et al. 1996, Soto et al. 1996b; Phillips et al. 1998) |
| P\(_{2X7}\) | 5'-GTGTGACATCATCCAGTGGTGAGCT-3'  
5'-ATCTTACTGAGAGCTCAGAGTA-3' | 1102-1668            | 567                | (Surprent et al. 1996; Phillips et al. 1998) |

TM = transmembrane region, IL = intracellular loop, bps = base pairs. Nucleotide number is based on original numbering of published sequence. Region of receptor amplified determined for the proposed 7 transmembrane receptors only, not the ligand gated P\(_{2X}\)-purinoceptor channels.
Table 2.2 PCR conditions for specific primer pairs

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Annealing temperature</th>
<th>Extension time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α_1B, α_{1D}, α_{2(A, B, C)}</td>
<td>68 °C</td>
<td>50s</td>
<td>(Vidovic &amp; Hill 1995; Vidovic et al. 1994; Phillips et al. 1996)</td>
</tr>
<tr>
<td>M1, M4, M5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α_{1A}</td>
<td>60 °C</td>
<td>30s</td>
<td>(Vidovic &amp; Hill 1995)</td>
</tr>
<tr>
<td>M2, M3</td>
<td>55 °C</td>
<td>110s</td>
<td>(Phillips et al. 1996)</td>
</tr>
<tr>
<td>NK1, NK2, NK3</td>
<td>63 °C</td>
<td>53s</td>
<td>(Hill et al. 1996)</td>
</tr>
<tr>
<td>NPY (Y_1, Y_2)</td>
<td>67 °C</td>
<td>40s</td>
<td>(Newhouse &amp; Hill 1997; Phillips et al. 1998)</td>
</tr>
<tr>
<td>P_{2X1}, P_{2X2}, P_{2X4}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGRP_1</td>
<td>50 °C</td>
<td>50s</td>
<td>(Phillips &amp; Hill 1998)</td>
</tr>
<tr>
<td>P_{2X3}</td>
<td>65 °C</td>
<td>60s</td>
<td>(Phillips et al. 1998)</td>
</tr>
<tr>
<td>P_{2X5}, P_{2X6}</td>
<td>65 °C</td>
<td>40s</td>
<td>(Phillips et al. 1998)</td>
</tr>
<tr>
<td>P_{2X7}</td>
<td>63 °C</td>
<td>60s</td>
<td>(Phillips et al. 1998)</td>
</tr>
</tbody>
</table>
Figure 2.2
Representative agarose gels illustrating the method of assessing levels of PCR product. Figures illustrate agarose gel electrophoresis of 8μl of PCR products amplified from cDNA that was reverse transcribed from DNase I treated total RNA, using multiple samples and primers to provide characteristic examples. Lane 1 in panels A and B contains 8μl of DNA molecular weight ladder (Lambda Hind III/ φX174 HaeIII). The 600 base pair (bp) band was used as a standard, where [+]= weakly detected, was described as equal to or less than the 600bp band on the ladder; [++] = strongly detected, was defined as product intensity seen to be greater than the 600bp band and [+++]= very strongly detected, was product being obviously greater than any bands on the ladder in terms of intensity. Assessment of PCR product was classified relative to the ladder present on the same gel. Although not a quantitative technique, this system allows for relative comparisons between primers, based on the constant starting amount of cDNA, and between arteries, based on the constant starting amount of RNA used for each experiment. Examples of different bands, as defined by the subjective scoring system, are illustrated in panels A and B. In panel A, bands classified as [+], [++] and [+++] are shown in lanes 2, 4 and 6 respectively. Lanes 3, 5 and 7 are primer specific controls that did not contain reverse transcriptase enzyme. Panel B demonstrates a [+] band in lane 2, while a band of [+++] intensity is shown in lane 4 and lane 6 contains a band classified as [++] . Lanes 3 and 5 represent controls that did not contain reverse transcriptase enzyme.
run, with specific bands defining the range of scoring systems as described above (see Figure 2.2 for further details). Assessment was always performed by the same person and results were averaged from the assessment of at least three experiments (except where specified).

### 2.5.5 Identity of PCR fragments

The identity of individual fragments was confirmed by sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. PCR product was purified from a 3% (w/v) low melt agarose gel using the Wizard PCR Preps purification system (Promega, USA). After assessing concentration relative to a molecular weight ladder, 60ng of DNA was combined with 8.0μl of terminator premix, 3.2pmol of primer (either reverse or forward) in a total reaction volume of 20μl. Samples were heat sealed and placed in the capillary cycler for 25 cycles at 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Sequencing products were purified using phenol:H₂O:chloroform (68:18:14) and then precipitated with 100% ethanol and 2M sodium acetate (pH 4.5). Each sample was analysed on a sequencing gel in an Applied Biosystems 370 DNA sequencer. Comparison of sequenced result and published receptor sequences was performed using the AssemblyLIGN programme (International Biotechnology’s Inc.). Fragments produced by the purinergic (P₂X₃,₇) primer pairs are still to be confirmed as such by sequencing.

### 2.6 PHYSIOLOGY

The arteries of the rat hepatic mesentery (section 2.3) were used to further characterise neuroreceptor mediated control mechanisms, using standard physiological and pharmacological techniques.
2.6.1 *In vitro preparation*

After dissection, the hepatic arterial tree was immobilised by pinning of the adjacent mesentery in a 1ml tissue bath coated with silicon resin (Sylgard, Dow Corning USA, Figure 2.1F). The vessel was continuously superfused at 2.5ml/min with Krebs’ solution (mM): NaCl 120, KCl 5.0, CaCl₂,6H₂O 2.5, MgCl₂,6H₂O 2.0, NaH₂PO₄.H₂O 1.0, NaHCO₃ 25 and D-glucose 27.7, gassed with 5% CO₂/ 95% O₂ and maintained at 34°C in the tissue bath.

Preparations were allowed to equilibrate for 45min prior to transmural stimulation (10Hz, 10s, 60V, 0.1msec pulse duration) every 20min via platinum electrodes placed a minimum of 5mm apart, on opposite sides of the preparation. Preliminary experiments were performed to determine the minimal stimulation parameters, with regard to frequency and duration, that would produce a response of reasonable size that could be blocked by tetrodotoxin. Decreasing or increasing the stimulus frequency or duration caused a respective decrease or increase in the size of the response. All experiments were performed on second or third order branches of the hepatic artery within the mesentery (mean resting vessel diameter 82.81 ± 1.91 μm, n=176). These diameters would be larger than the actual vessel diameter *in vivo* due to the pinning of the surrounding mesentery, however vessels were not occluded and blood movement was always observed during nerve stimulation.

The arteries were viewed with an inverted compound microscope (Olympus IX50, Japan) and the image visualised using a video camera mounted on the microscope and a computer monitor. Vessel diameter was continually monitored using the DIAMTRAK computer programme (T.O. Neild, Flinders University, Australia). The programme averages pixel brightness within a selected area of the video image and finds the minimum (dark line) or maximum (light line) values. This corresponds to the edges of the vessel and the difference between the edges (vessel diameter) is then calculated and sent to a digital/analgoue converter (Neild 1989). Figure 2.3 shows an artery as seen on the computer monitor, in both relaxed (A) and contracted states (B). Data was recorded and measured on a Maclab Chart Recorder (ADInstruments USA).
Figure 2.3
Figure 2.3

A small artery of the rat hepatic mesentery as seen using the DIAMTRAK programme (T.O. Neild, Flinders University, Australia). Panel A shows a vessel in a resting (unstimulated) state. Bars running parallel to the vessel represent the area within which the computer programme tracks either a dark or white line. In this example, a dark line represents the internal edge of the artery wall. Panel B shows the same vessel during transmural nerve stimulation and a contraction equivalent to 21% of resting vessel diameter is illustrated. Red blood cells can be seen to have moved within the lumen of the vessel.
2.6.2 Experimental protocol

Control experiments were performed to determine the time period over which consistent responses could be achieved and appropriate precautions were taken for light sensitive drugs, including illuminating the preparations with only long wavelength light (>610nm). The gender of animals was noted for each experiment, however sex differences did not appear to influence vascular responses to nerve stimulation.

Drugs were perfused for at least 20 min (the time between sequential nerve stimuli) and all results were obtained with the appropriate drug present in solution, except for the irreversible α-adrenergic receptor antagonist benextramine (Benfey 1982), whose effect was determined after a washout period of 20min to avoid any non-specific actions. In experiments where the effect of a second drug was examined, the first drug remained present in solution for the duration of the experiment. When α,β-mATP was used to desensitise the P2x-purinergic receptors (Kasakov & Burnstock 1983), nerve-mediated responses were not measured until the vessel had recovered from the induced contraction.

Vasoconstriction experiments. In pharmacological experiments performed to characterise vasoconstriction following nerve stimulation, Krebs’ solution contained (-)-scopolamine (10^-6M) to prevent the effects of acetylcholine on muscarinic receptors (Katzung 1992b) and capsaicin (10^-6M) to prevent the activation of sensory nerves (Holzer 1991). Only those preparations that showed a control contractile response of greater than 10% of resting vessel diameter were used for further experiments. When the effects of high K⁺ were assessed, Krebs’ solution was modified to contain 20mM KCl and 105mM NaCl, in place of the normal 5mM KCl and 120mM NaCl.

Vasodilation experiments. In experiments designed to assess vasodilator mechanisms in the arteries of the rat hepatic mesentery, vasoconstrictor responses to nerve stimulation and hence the viability of the preparation was tested prior to the addition of any drugs. Only preparations that showed a sympathetic nerve-mediated vasoconstriction were used for further experiments.

Different drug combinations were used to isolate specific nerve-mediated responses: guanethidine (5x10^-6M, Misu et al. 1976) was used to block sympathetic nerve-mediated responses; capsaicin (10^-6M) was used to prevent the release of peptides
from sensory motor nerves and scopolamine \((10^{-6}\text{M})\) was used to prevent the activation of muscarinic receptors. In those preparations where sympathetic nerve-mediated dilatory responses were studied, \(\alpha,\beta\text{-mATP} (6\times10^{-6}\text{M})\) and benextramine \((10^{-5}\text{M})\) were used to block vasoconstrictions due to the release of ATP and noradrenaline respectively. Methoxamine \((3\times10^{-5}\text{M})\) was used to preconstrict vessels in order to uncover sensory and parasympathetic nerve-mediated dilations. The potentiating effects of the NOS inhibitor \(\text{N}^\text{G}\text{-nitro-L-arginine methyl ester (L-NAME)}\) were studied on sympathetic nerve-mediated vasoconstrictions. These experiments were performed in the presence of capsaicin and scopolamine. L-NAME was used at \(10^{-5}\text{M}\), as preliminary results suggested non specific effects at higher concentrations. This drug has been shown previously to be effective at \(10^{-5}\text{M}\) in rat iris arterioles (Hill & Gould 1995; Hill & Gould 1997). 

### 2.6.3 Data analysis

The magnitude of the response to nerve stimulation was expressed as a percentage of the unstimulated (resting) vessel diameter. This was done in order to standardise nerve-mediated responses in vessels of different diameter.

At least 2 responses to nerve stimulation under control conditions were averaged to obtain a control value. For each drug or specific drug concentration, at least 2 responses were averaged once a consistent response appeared. The response in the drug solution was expressed as a percentage of the control response. Experimental values are given as the mean ± standard error (s.e.) mean of results from at least 4 preparations, where each preparation was obtained from a different animal.

Statistical significance was tested using a paired two tailed Students \(t\)-test and a \(P\) value of \(< 0.05\) was taken as significant. Contractile response curves to nerve stimulation in the presence of increasing concentrations of antagonist, were constructed using Axograph (Axon Instruments) and a Hills \(\chi^2\) equation to fit the curves. The half maximal inhibitory concentration \((\text{IC}_{50})\) was calculated directly from the curves.
2.7 DRUGS AND SOLUTIONS

The drugs used and their manufacturers are as listed. 6-hydroxydopamine (6-OHDA), (-)-scopolamine hydrochloride, benextramine tetrachloride, β-nicotinamide adenine dinucleotide phosphate tetrasodium (β-NADPH), clonidine hydrochloride, diltiazem hydrochloride, guanethidine sulphate, methoxamine hydrochloride, nifedipine, nitroblue tetrazolium, prazosin hydrochloride, DL-propranolol hydrochloride, tetrodotoxin, verapamil hydrochloride and yohimbine hydrochloride, were all manufactured by SIGMA USA. α,β-methylene ATP (α,β-mATP), 5-methyl-urapidil, BMY 7378 dihydrochloride, chloroethylclonidine dihydrochloride (CEC), GR8334, pyridoxal phosphate-6-azophenyl 2’-4’ disulphonic acid tetrasodium (PPADS) and WB-4101 hydrochloride were all from Research Biochemicals Incorporated USA. N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) was purchased from Sapphire Bioscience (NSW, Australia). SR48968 and SR142801 were kindly supplied by Sanofi Research (France). Capsaicin was purchased from Fluka Chemica (Switzerland) and the peptide antagonist, CGRP\textsubscript{8-37}, was obtained from Auspep (Australia). Desipramine (DMI) was purchased from ICN Pharmaceuticals (Australia) and felodipine was obtained from Astra Pharmaceuticals (Australia). 1229U91, an NPY, receptor antagonist (Lew et al. 1996), was kindly supplied by Prof. J. Angus and Dr. R. Murphy, (University of Melbourne, Australia) and anti sera to NGF was generously supplied by Assoc. Prof. R.A. Rush (Flinders University, Australia). All other reagents and chemicals were of standard analytical grade.

For physiological experiments, all drugs were made up daily as at least 100x stocks in deionized distilled water except for capsaicin (100% ethanol), clonidine (2% w/v ascorbic acid), prazosin (20% w/v methanol) and 5-methyl-urapidil and WB-4101 (0.1M hydrochloric acid). Diluents were tested at appropriate concentrations on the in vitro preparations to demonstrate that they had no effect on nerve-mediated responses. CGRP\textsubscript{8-37}, 1229U91 and α,β-mATP were dissolved in deionized distilled water, aliquoted and stored at -20°C until required. Dilutions of all stocks were made in Krebs’ solution for final concentrations.
CHAPTER 3

COMPARATIVE STUDY OF NEURORECEPTOR mRNA EXPRESSION IN DIFFERENT ARTERIES OF THE RAT

3.1 INTRODUCTION

Vascular constriction and dilation are primarily mediated by the activation of specific receptors located on the smooth muscle and endothelium. Individual vascular beds demonstrate variable responses to a given neurotransmitter, which may be mediated in part by the presence of different receptor populations (Hirst & Edwards 1989). This heterogeneity of vascular responses is seen in the same vessel between species, between particular vascular beds and even within different regions of the same vessel (Vargas & Gorman 1995; Bylund et al. 1995).

The postsynaptic receptors involved in the vasoconstrictor response to noradrenaline have been shown to vary. In the feline pulmonary artery, constriction is mediated by $\alpha_1$- and $\alpha_2$-adrenergic receptors (Hyman 1986), while in the rabbit ovarian vascular bed, only functional $\alpha_1$-adrenoceptors can be demonstrated (Yousif et al. 1996) and in the rat basilar artery, sympathetic nerves stimulate non $\alpha$-adrenergic receptors resulting in vasoconstriction (Hirst et al. 1982). The response to noradrenaline in the bovine cerebral vascular bed shows regional variations, with results indicating that $\alpha$-adrenoceptors populations vary from rostral to caudal locations (Ayajiki & Toda 1990).

Responses to acetylcholine show similar regional and species specific variations, however, the identification of specific receptors in individual vascular beds has been difficult due to the poor subtype selectivity exhibited by available antagonists (Caulfield 1993). Pharmacological and binding studies have suggested the presence of 4 subtypes in peripheral and cerebral blood vessels (M1- M4, Dauphin et al. 1991; Dauphin & Hamel 1992; Eglen et al. 1996; Van Charldorp & Van Zwieten 1989), while the M5 subtype has only been described in cerebral blood vessels (Linville & Hamel 1995). The M3 muscarinic receptor, located on the endothelium, is most frequently described...
as mediating dilatory responses to acetylcholine (Caulfield 1993; Eglen et al. 1996), however the M1 and M2 muscarinic receptors have also been shown to mediate endothelium-dependent vasodilations (Komori & Suzuki 1987a; Chiba & Tsukada 1996). In contrast, the same receptors (M1, M2 and M3), when located on smooth muscle cells, can mediate vasoconstrictor responses to acetylcholine (Dauphin et al. 1991; Jaiswal et al. 1991; Obi et al. 1994; Caulfield 1993).

The effects of sensory nerve neurotransmitters again indicate differences between vascular beds. In the rat mesenteric artery for example, neurokinin mediated vasodilations (either to applied agonist or sensory nerve-stimulation) could not be evoked (D'Orleans-Juste et al. 1991; Li & Duckles 1992) while in the rabbit pulmonary artery, applied SP produced an endothelial-dependent relaxation, acting through NK1 receptors, and NKA produced a contraction, acting directly via NK2 receptors located on smooth muscle cells (D'Orleans-Juste et al. 1985).

From the foregoing, it is clear that there is a great diversity of receptor subtypes involved in vasoconstriction and vasodilation in different arterial beds, influenced by both the action of several neurotransmitters and through the same transmitter activating different receptor subtypes. In this chapter, we have examined the hypothesis that specific patterns of receptor gene expression may underlie these variable physiological responses in different vascular beds. The vessels chosen for this study were the basilar, mesenteric, pulmonary and tail arteries of the rat, as they exemplify vessels in which different physiological and pharmacological responses to the same neurotransmitters have been recorded. The receptors studied were the α1(A, B, D)- and α2(A, B, C)-adrenergic, muscarinic (M1- M5) and neurokinin (NK1- NK3) receptor families. Reverse transcription-polymerase chain reaction was used to examine each of the 4 vessels for the presence of mRNA transcripts specific for each of these receptors.
as mediating dilatory responses to acetylcholine (Caulfield 1993; Eglen et al. 1996), however the M1 and M2 muscarinic receptors have also been shown to mediate endothelium-dependent vasodilations (Komori & Suzuki 1987a; Chiba & Tsukada 1996). In contrast, the same receptors (M1, M2 and M3), when located on smooth muscle cells, can mediate vasoconstrictor responses to acetylcholine (Dauphin et al. 1991; Jaiswal et al. 1991; Obi et al. 1994; Caulfield 1993).

The effects of sensory nerve neurotransmitters again indicate differences between vascular beds. In the rat mesenteric artery for example, neurokinin mediated vasodilations (either to applied agonist or sensory nerve-stimulation) could not be evoked (D'Orleans-Juste et al. 1991; Li & Duckles 1992) while in the rabbit pulmonary artery, applied SP produced an endothelial-dependent relaxation, acting through NK1 receptors, and NKA produced a contraction, acting directly via NK2 receptors located on smooth muscle cells (D'Orleans-Juste et al. 1985).

From the foregoing, it is clear that there is a great diversity of receptor subtypes involved in vasoconstriction and vasodilation in different arterial beds, influenced by both the action of several neurotransmitters and through the same transmitter activating different receptor subtypes. In this chapter, we have examined the hypothesis that specific patterns of receptor gene expression may underlie these variable physiological responses in different vascular beds. The vessels chosen for this study were the basilar, mesenteric, pulmonary and tail arteries of the rat, as they exemplify vessels in which different physiological and pharmacological responses to the same neurotransmitters have been recorded. The receptors studied were the \( \alpha_{1(A,B,D)} \) and \( \alpha_{2(A,B,C)} \)-adrenergic, muscarinic (M1- M5) and neurokinin (NK1- NK3) receptor families. Reverse transcription-polymerase chain reaction was used to examine each of the 4 vessels for the presence of mRNA transcripts specific for each of these receptors.
3.2 RESULTS

3.2.1 General observations

Messenger RNA for 13 of the 14 neurotransmitter receptors tested was expressed in at least one of the 4 rat arteries examined. The overall pattern of gene expression varied amongst the arteries with no two arteries showing exactly the same receptor profile (Table 3.1). Some receptors showed very limited expression, for example NK2 and M1, while the M4 gene was not detected in any of the vessels. The \( \alpha_{1A} \), \( \alpha_{1B} \), NK3, and M3 transcripts were strongly expressed in all vessels studied while the remaining receptor subtypes showed variable expression amongst the vessels (\( \alpha_{1D} \), \( \alpha_{2A} \), \( \alpha_{2B} \), \( \alpha_{2C} \), NK1, M2 and M5). The efficiency of all primer pairs was confirmed using other tissues (brain, heart, iris and superior cervical ganglion) in which an appropriately sized PCR product was seen at the level equivalent to \([+++]\).

All PCR fragments detected corresponded to the predicted fragment size for each receptor subtype. Fragment identity was subsequently confirmed by sequencing, with fragments corresponding to the published cDNA sequences over the region amplified with an observed similarity of 97% or greater. In each repeat of the preparations, the relative expression of receptor cDNA was consistent with previous results. When the number of PCR cycles was either reduced or increased from 35, a respective decrease or increase in the amount of product amplified was seen. For all the receptors studied, reverse transcription was done in duplicate reactions, one reaction using random primers and the other using oligo(dT) primers. The results obtained were similar. Only results obtained using random primers are illustrated. No PCR product was seen when reverse transcriptase was omitted from reactions or when RNA was omitted (Figures 3.1 and 3.2).

3.2.2 Messenger RNA expression of \( \alpha_1 \)-adrenergic receptors

The \( \alpha_{1A} \) and \( \alpha_{1B} \)-adrenergic receptor subtypes were detected consistently in each artery (Table 3.1). In the rat tail artery, the \( \alpha_{1A} \) and \( \alpha_{1B} \) subtypes were both very
Table 3.1 Profiles of receptor gene expression in the four different arteries

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basilar</td>
</tr>
<tr>
<td>( \alpha_1 )-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>( \alpha_{1A} )</td>
<td>++</td>
</tr>
<tr>
<td>( \alpha_{1B} )</td>
<td>++</td>
</tr>
<tr>
<td>( \alpha_{1D} )</td>
<td>0</td>
</tr>
<tr>
<td>( \alpha_2 )-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>( \alpha_{2A} )</td>
<td>++</td>
</tr>
<tr>
<td>( \alpha_{2B} )</td>
<td>+++</td>
</tr>
<tr>
<td>( \alpha_{2C} )</td>
<td>+</td>
</tr>
<tr>
<td>Neurokinin</td>
<td></td>
</tr>
<tr>
<td>NK1</td>
<td>+</td>
</tr>
<tr>
<td>NK2</td>
<td>0</td>
</tr>
<tr>
<td>NK3</td>
<td>+++</td>
</tr>
<tr>
<td>Muscarinic</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>++</td>
</tr>
<tr>
<td>M3</td>
<td>++</td>
</tr>
<tr>
<td>M4</td>
<td>0</td>
</tr>
<tr>
<td>M5</td>
<td>++</td>
</tr>
</tbody>
</table>

Crosses represent PCR product that corresponds to the size of the desired cDNA band. The number of crosses reflects the amount of amplified product generated after 35 cycles. [0] = never detected; [+]= weakly detected; [++] = strongly detected; [ +++ ] = very strongly detected.
strongly expressed, while the $\alpha_{1D}$ receptor mRNA was weakly detected (Figure 3.1). The primer pair for the $\alpha_{1D}$ receptor was tested on brain tissue and produced a 517bp band of strong intensity. In the other vessels the pattern of expression for the $\alpha_1$-adrenergic receptors was similar to that in the tail, except for the basilar artery in which $\alpha_{1D}$ receptor mRNA transcript was not detected (Table 3.1).

### 3.2.3 Messenger RNA expression of $\alpha_2$-adrenergic receptors

Messenger RNA encoding the three $\alpha_2$-adrenergic receptors was variably expressed amongst the basilar, mesenteric, pulmonary and tail arteries (Table 3.1). The results obtained from the rat tail artery are illustrated in Figure 3.1B. The $\alpha_{2C}$ receptor was very strongly expressed, the $\alpha_{2B}$ transcript was strongly detected and the $\alpha_{2A}$ was only weakly detected. While mRNA for all three subtypes was detected in the basilar and mesenteric arteries, in the pulmonary artery neither the $\alpha_{2A}$ nor the $\alpha_{2C}$ receptor subtypes were detected and the $\alpha_{2B}$ receptor was only weakly detected. The $\alpha_{2A^-}$-adrenergic primers have been shown to produce a strong band of 338 bp in the rat iris and superior cervical ganglion (Vidovic & Hill 1995; Vidovic et al. 1994).

### 3.2.4 Messenger RNA expression of neurokinin receptors

Expression of mRNA for NK1 and NK3 receptors was detected in all vessels while NK2 was only detected in one vessel, the pulmonary artery (Table 3.1). In the rat tail artery the mRNA for the NK1 receptor was strongly detected while the NK3 receptor was very strongly expressed (Figure 3.2A). In the basilar and mesenteric arteries, the NK1 receptor was only weakly expressed and the NK3 receptor very strongly expressed. The pulmonary artery varied from the other vessels, with the NK1 receptor being very strongly expressed, the NK3 receptor strongly expressed and the NK2 receptor weakly expressed (Table 3.1). As this was the only tissue in which the NK2 fragment was detected, the NK2 primers were tested on brain cDNA to confirm their efficiency. The appropriate 443 bp fragment was very strongly detected.
Figure 3.1

Expression of α₁- and α₂-adrenergic receptor mRNA in the rat tail artery. Figure illustrates agarose gel electrophoresis of 8μl of PCR products amplified from cDNA that was reverse transcribed from DNase I treated total RNA from the rat tail artery. In panel A, a 405 base pair (bp) product in lane 2 corresponds to the size of the desired fragment as defined by the α₁B specific oligonucleotide primers. Likewise mRNA expression for the α₁A and α₁D receptors is confirmed by the presence of bands of 251 and 517 bp in lanes 4 and 6 respectively. In panel B, a 338 bp product in lane 2 corresponds to the size of the desired fragment as defined by the α₂A specific oligonucleotide primers. Messenger RNA expression for α₂B and α₂C receptors is confirmed by the presence of bands of 456 and 425 bp in lanes 4 and 6 respectively. No PCR products were seen in the receptor specific control experiments which did not contain reverse transcriptase enzyme in the RT reactions (A, B, lanes 3, 5 and 7), and no product is seen in the control which did not contain RNA (A, B, lane 8). DNA markers (Lambda Hind III/ φX174 HaeIII) and (Pzt/Alu) appear in lanes 1 and 9 respectively (A, B).
3.2.5 Messenger RNA expression of muscarinic receptors

Muscarinic receptor mRNA expression in the 4 different arteries is illustrated in Table 3.1. In the rat tail artery, the M3 and M5 subtypes were detected, however there was no expression of the other muscarinic receptor genes in this tissue (Figure 3.2B). The PCR fragment representing M3 receptor mRNA was readily detected in all vessels, as was the M5 receptor mRNA transcript, which was very strongly expressed in the mesenteric and pulmonary vessels. Messenger RNA for the M1 receptor was detected only in the basilar artery at a low level and the M4 gene product was not detected in any of the tissues. The primers for the M1 and M4 receptors were tested on brain cDNA and the predicted 619 and 510 bp fragments respectively were very strongly detected. The M2 receptor was detected to varying extents in all vessels but the tail artery. The M2 muscarinic primer pair, when tested on heart cDNA, produced a 552 bp band of very strong intensity.

3.2.6 Comparison of the primary and secondary branches of the pulmonary artery

Previous studies have suggested that distinct regions of the pulmonary vascular bed exhibit different vasomotor responses (Liu et al. 1992; Kemp et al. 1997) and so, in the pulmonary artery, receptor expression was studied in the extrapulmonary (primary) branches and in the intrapulmonary branches (secondary). Expression of mRNA was identical for all subtypes except for $\alpha_{2c}$-adrenergic receptor, which was not detected in the primary branches but did show weak expression in the secondary branches. Only results from the primary branches are discussed.

3.2.7 Histology

Histological examination of each of the vessels after perfusion with saline confirmed the presence of the endothelial cell layer.
Figure 3.2

Expression of neurokinin and muscarinic receptor mRNA in the rat tail artery. Figure illustrates agarose gel electrophoresis of 8μl of PCR products amplified from cDNA that was reverse transcribed from DNase I treated total RNA from the rat tail artery. Panel A illustrates a 537 base pair (bp) product in lane 2 that corresponds to the size of the desired fragment as defined by the NK1 specific oligonucleotide primers. No NK2 product was amplified after 35 cycles (lane 4). Messenger RNA expression for the NK3 receptor is confirmed by a band of 476 bp in lane 6. In panel B, a 790 bp product in lane 6 corresponds to the size of the predicted fragment as defined by the M3 specific oligonucleotide primers. Likewise mRNA expression for the M5 receptor is confirmed by a band of 594 bp in lane 9. The M1, M2 and M4 products were not detected (lanes 2, 4 and 8 respectively). No PCR products were seen in the receptor specific control experiments which did not contain reverse transcriptase enzyme in the RT reactions (-RT, panel A, lanes 3, 5 and 7, B, lanes 3, 5 and 10). No -RT control is shown for the M4 primer pair. No product is seen in the control which did not contain RNA (A, lane 8 and B, lane 11). DNA markers (Lambda Hind III/ φX174 HaeIII) appear in lane 1 (A, B) and (Pzt/Alu) markers are in lane 9 (A).
3.3 DISCUSSION

Using RT-PCR to amplify mRNA, the results presented in this chapter characterise the profile of \(\alpha_1\)- and \(\alpha_2\)-adrenergic, neurokinin and muscarinic receptor subtype gene expression in the rat basilar, mesenteric, pulmonary and tail arteries, in an attempt to explain variation in the physiological responses of the described vessels. The overall pattern of receptor gene expression was variable with no two arteries having exactly the same profile. The \(\alpha_{1A}\), \(\alpha_{1B}\), NK3 and M3 receptor subtypes were strongly expressed in all vessels studied while the remaining subtypes showed a variable expression amongst the arteries. Two receptors: NK2 and M1, exhibited a very restricted distribution of mRNA, while the M4 muscarinic receptor subtype was not detected at all. For those receptors which showed a consistently strong pattern of expression, it may be postulated that they play an important role in the regulation of blood flow throughout the vasculature of the rat. On the other hand, those receptors which exhibited variable or limited expression of mRNA amongst the 4 arteries examined may underlie some of the variable responses to neural and hormonal stimulation recorded in the different vascular beds.

All of the blood vessels in this study showed dominant expression of mRNA for the \(\alpha_1\)-adrenergic receptors, in particular the \(\alpha_{1A}\) and \(\alpha_{1B}\). This finding agrees with other mRNA studies that have used both RT-PCR and RNase protection assays to assess \(\alpha_1\)-adrenoceptor gene expression (Ping & Faber 1993; Xu et al. 1997; Miller et al. 1996). In further agreement with the results presented here, low levels of mRNA detection for the \(\alpha_{1D}\)-adrenergic receptor has been reported previously in other blood vessels (Guarino et al. 1996).

Although the presence of mRNA does not necessarily imply protein expression, our findings are consistent with many functional studies which have found that the contractile response to noradrenaline in arteries is primarily due to the activation of \(\alpha_1\)-adrenergic receptors (Piascik et al. 1996). Predominantly \(\alpha_1\)-adrenoceptor mediated contractile responses have been described in the rat pulmonary (Chen & Han 1992), tail (Medgett & Langer 1984; Abel & Minneman 1986; Brock et al. 1997b) and mesenteric arteries (Agrawal et al. 1984; Pipili 1986). In the mesenteric artery, the \(\alpha_{1A}\) and
α1B-receptor subtypes have been specifically identified (Williams & Clarke 1995; Han et al. 1990). One study further classified the α1B-adrenoceptor as being predominant in the superior mesenteric artery while α1A-receptors were shown to play a greater role in the smaller resistance vessels (Kong et al. 1994). Our finding of strong α1A- and α1B-adrenoceptor gene expression agrees with these results, since the preparation contained both segments of the vascular bed. The role of the α1D-adrenergic receptor in the mesenteric, pulmonary and tail arteries has been queried, for while mRNA expression was detected in this and other studies (Piascik et al. 1995), there have been conflicting results as to whether or not the α1D-adrenoceptor contributes to contractile responses in these tissues (Piascik et al. 1995; Hussain & Marshall 1997; Yousif et al. 1998). The situation in the rat basilar artery appears to be more complex, with an atypical response to noradrenaline being described. In this vessel, nerve stimulated arterial constriction is resistant to α-adrenoceptor blockade (Hirst et al. 1982), while applied noradrenaline induces relaxation, mediated by β-adrenergic receptors (Winquist & Bohr 1982; Hempelmann & Ziegler 1993; Kitazono et al. 1993). The variable responses to noradrenaline in this and other vascular beds may be due to contributions from non-α adrenoceptors, and these need to be studied further.

Relative to the expression of the α1-adrenergic receptors, the α2-adrenoceptors demonstrated a variable and in general, weaker, expression of mRNA amongst the 4 arteries. In accord with this finding, a functional role for α2-adrenergic receptors in the rat vasculature has been controversial. This has been particularly so for the mesenteric artery (Pipili 1986; Nielsen et al. 1991) and the tail artery (Marwood et al. 1986). In both of these vessels, we have demonstrated the presence of mRNA for all three types of α2-adrenergic receptor subtypes, albeit weak for the α2A receptor. This finding supports those studies that propose a role for the α2-adrenergic receptors in these vessels. For example, in the tail artery, where postjunctional α2-adrenergic receptors are thought to contribute to applied noradrenaline and nerve-mediated contractile responses (Medgett & Langer 1984; Szabo & Hardebo 1990), and more recently have been shown to play an important role in controlling thermoregulatory responses (Redfern et al. 1995). Neuronally mediated smooth muscle cell depolarisation has also been shown to be dependent on α2-adrenoceptor activation in this vessel (Brock et al. 1997b), providing further evidence for their postsynaptic location.
In the present study, $\alpha_2$-adrenergic receptor expression in the pulmonary artery was very limited with only weak expression of the $\alpha_{2B}$ subtype. By contrast, all three subtypes of $\alpha_2$-receptors were found in the basilar artery. An atypical vasodilatory response, mediated by $\alpha_2$-adrenergic receptors, has been described in the rat basilar artery (Hempelmann & Ziegler 1993). Our results suggest that it may be due to the activation of multiple $\alpha_2$-receptor subtypes.

In all 4 arteries studied here, there was strong expression of mRNA for NK3 receptors, while the pulmonary artery was the only vessel with very strong expression of NK1 receptors. Since NK3 receptors can mediate vasodepressor responses (Couture et al. 1989) and are coupled to endothelium-dependent mechanisms similar to those of the NK1 receptors (Jansen et al. 1991; Zawadzki et al. 1981), our results suggest that NKB, or other as yet unidentified ligands for NK3 receptors, may contribute significantly to vasodilator responses in these vessels. A recent study has in fact shown that in the rat mesenteric artery, endothelial NK3, but not NK1 receptors, mediate vasodilation through the release of NO (Mizuta et al. 1995). This correlates well with the current findings and those of others, which show the rat mesenteric artery to be non-responsive to applied substance P (Li & Duckles 1992). In the pulmonary artery, results would predict that dilator responses may arise from activation of both NK1 and NK3 receptors. Expression of the NK2 receptor gene was only detected in the pulmonary artery, although only at low levels. Floch et al. (1996) have proposed that NK2 receptors may be absent from or functionally uncoupled in resistance blood vessels of the rat, based on the lack of effect of NK2 agonists in hypotensive studies. The results presented here would support the hypothesis that NK2 receptors do not play a significant role in the vasculature of the rat.

The pulmonary artery differs from all other arterial vessels in that it carries blood with a lowered oxygen concentration. It also behaves differently to other systemic vessels, constricting rather than dilating in response to hypoxia (Staub 1985). Our results indicate further that the pulmonary artery expresses an mRNA profile of receptors quite different from that of the other vessels studied. As mentioned above, $\alpha_2$-adrenergic receptor expression was very low while both NK1 and NK3 receptor expression was high. This receptor profile should favor vasodilation and increased blood flow through the lungs to maximise gaseous exchange.
The M3 muscarinic receptor gene was consistently expressed in all the 4 arteries. This is in agreement with previous functional studies which have demonstrated that the M3 muscarinic receptor mediates vasodilatory responses in the rat pulmonary (McCormack et al. 1988) and mesenteric arteries (Hendriks et al. 1992). Acetylcholine has also been shown to induce endothelium-dependent relaxations in both the rat tail and basilar arteries (Tonta et al. 1994; Mackert et al. 1997), however the specific muscarinic receptor subtypes involved have not yet been characterised. It may be postulated from the results of this study, that either the M3, or perhaps the M5, muscarinic receptor mediates this response.

One of the novel findings in this study was the consistent expression of the mRNA transcript for the muscarinic M5 receptor in all 4 vessels studied. This is the first description of M5 muscarinic receptor mRNA in peripheral tissue of the rat. Prior to this study, transcript was believed to be present only in the rat brain (Wei et al. 1994; Reever et al. 1997). The M5 receptor subtype was first cloned and expressed in 1988 (Bonner et al. 1988), however a functional role for this receptor has not yet been elucidated (Van Zwieten & Doods 1995; Reever et al. 1997). A site with a pharmacological profile similar to that of the M5 receptor has been suggested to exist in feline and human cerebral vessels (Dauphin et al. 1991; Dauphin & Hamel 1992), and recently the M5 receptor subtype was pharmacologically characterised in human and bovine cerebral microvessels (Linville & Hamel 1995). The M5 receptor, along with the M1 and M3 receptors, is coupled via G-proteins of the Gq/11 family to the inositolphosphate pathway (Felder 1995) and has recently been implicated in the generation and release of NO (Wang et al. 1994). Taken together with the demonstration here of the expression of the M5 receptor gene in all 4 arteries, these results suggest that the M5 receptor and the M3 receptor both reside on endothelial cells and are together responsible for vasodilatory responses in vascular beds of the rat.

In contrast to the M5 receptor, the M4 receptor was not detected in any of the vessels examined, which agrees with previous pharmacological studies in feline, bovine and human cerebral vasculature (Dauphin & Hamel 1992; Linville & Hamel 1995). The M4 receptor has been described in bovine intracerebral microvessels (Garcia-Villalon et al. 1991) and in porcine coronary artery, where it is responsible for contraction (Van Charldorp & Van Zwieten 1989). It is possible that we did not detect the expression of
the M4 receptor gene due to poor reverse transcription of the mRNA or inefficiency of the primer pair. Strong detection of mRNA for the M4 receptor gene in the brain, however, suggests that this is unlikely.

Expression of the M2 muscarinic receptor gene was low or absent in all vessels except the basilar artery, where strong detection levels were recorded. This correlates well with studies which describe an M2-mediated contractile response in the porcine basilar artery (Van Charlordorp et al. 1988). Similarly, the M1 receptor gene was only expressed in the basilar artery. This receptor has also been identified as mediating contractile responses in the cerebral vessels of other species (Dauphin et al. 1991; Armstead et al. 1988). As mRNA for both the M1 and M2 receptors is expressed in the basilar artery, it may be suggested that acetylcholine plays a dual role in this vessel: contraction, via M1 and M2 receptors and dilation, via M3 and M5.

In conclusion, using RT-PCR, we have characterised the profile of α-adrenergic, muscarinic and neurokinin receptor subtypes in the rat basilar, mesenteric, pulmonary and tail arteries. Amongst the 4 arteries examined a specific receptor array exists, suggesting that each artery has some unique neural and hormonal controls. The α1-adrenergic receptors were strongly expressed, agreeing with other mRNA studies and functional studies implicating a dominant α1-adrenergic role in many rat vascular beds. The α2-adrenergic receptors were detected at variable levels, corresponding with studies that have found a variable contribution from α2-adrenergic receptors to noradrenaline-induced contractions. The NK3 receptor was the dominantly expressed neurokinin receptor in all vessels except the pulmonary artery, in which the NK1 receptor was the most strongly expressed. This result is suggestive of an important functional role for the NK3 receptor in the vasculature of the rat. The discovery of mRNA for the M5 muscarinic receptor in all vessels, in addition to mRNA for the M3 receptor, is a novel finding. The pulmonary artery differed from the basilar, mesenteric and tail arteries in terms of receptor expression and this may relate to its specialised role in gaseous exchange.

Although the presence of mRNA transcript does not necessarily signify protein expression, the findings presented here correlate well with many previous functional studies. RT-PCR may therefore be a very useful tool when characterising
neuroreceptor-mediated vascular responses, particularly when the pharmacological selectivity of agonists and antagonists is limited.
4.1 INTRODUCTION

Sympathetic nerves induce vasoconstriction in most vascular beds, however the mechanism by which they mediate this response is quite variable, as they can release multiple transmitters, for example noradrenaline, ATP or NPY and these substances can in turn act on a number of different pre- and postsynaptic receptor subtypes (Sneddon 1995).

In general, sympathetic nerve-mediated contractions result from the activation of postsynaptic $\alpha$-adrenoceptors and purinoceptors (Hirst et al. 1996). The specific $\alpha$-adrenoceptors involved depend upon the vascular bed being studied. As discussed in Chapter 3, contractile responses to noradrenaline are in general mediated by $\alpha_1$-adrenergic receptors (Piascik et al. 1996), however $\alpha_2$-adrenoceptors can also participate in the response (Wilson & Dunn 1996). The purinergic component of sympathetic vasoconstrictions is mediated by $P_2X$-purinoceptors located on the vascular smooth muscle (Burnstock 1995). The proportion of the response attributable to noradrenaline versus ATP depends upon the vascular bed, the species and the experimental stimulation parameters used (Ralevic & Burnstock 1991).

Sympathetic nerve terminals possess presynaptic receptors that may either inhibit or enhance the release of the various transmitters (Langer & Arbilla 1990). The type of receptors involved again vary between different vascular beds and their nature will affect the response seen in the postsynaptic tissue. The $\alpha_2$-adrenergic receptor is most commonly characterised as inhibiting noradrenaline release, via an autoinhibitory
feedback pathway, (Wilson & Dunn 1996), however ATP and NPY release can also be inhibited by $\alpha_2$-adrenoceptors (Brock 1995).

In Chapter 3, the hypothesis that specific patterns of postsynaptic receptor gene expression might underlie some of the physiological differences seen between vascular beds was examined. RT-PCR results demonstrated that the different arteries did indeed exhibit unique patterns of receptor mRNA expression. While results correlated well with data available from the functional studies of other investigators, only limited studies have assessed receptor mRNA expression and functional protein expression in the same tissue. Such a direct correlation would strengthen the suggestion that RT-PCR studies of receptor mRNA might be a useful adjunct to studies aimed at characterising the specific postsynaptic receptors involved in neurogenic responses, particularly in situations of poor agonist and antagonist specificity and when complications arise from the activation of presynaptic receptors.

In this chapter, RT-PCR was used in conjunction with catecholamine histochemistry, immunohistochemistry and physiological techniques to investigate the specific identity of receptors involved in the constrictor response to nerve stimulation in the rat hepatic artery. Unlike the mesenteric, tail and basilar arteries in particular, the specific receptor subtypes involved in vascular responses have not been identified in this tissue, nor has there been any detailed studies of presynaptic mechanisms. Previous studies in the hepatic arterial bed of several species have, however, suggested the presence of $\alpha_1$-adrenergic and $P_{2X}$-purinergic receptors (Ballet 1990; Varga et al. 1984; Ralevic et al. 1991) and some degree of presynaptic modulation is evident (Lautt 1996a). For the physiological experiments, the smaller branches of the artery that lie within the mesentery have been studied (section 2.3, Figure 2.1F), as compared to the hepatic artery proper, as these smaller vessels are more likely to contribute to changes in vascular resistance and hence blood flow to the liver (Rossitti et al. 1995).
4.2 RESULTS

4.2.1 Catecholamine histochemistry and immunohistochemistry

Within the hepatic mesentery of the rat, the primary hepatic artery branches from the coeliac artery and in turn gives off multiple branches that supply the different lobes of the liver. A large nerve fibre bundle was consistently seen to run parallel to the artery and its branches (Figure 4.1A, 4.1B). Catecholamine histochemistry revealed a dense perivascular plexus of catecholaminergic nerve fibres. The density of this plexus was consistent over the hepatic artery proper extending up to where the smaller order vessels entered the liver parenchyma (Figure 4.1A, 4.1C). Arterioles within the mesentery were also seen to have perivascular catecholaminergic nerve fibres associated with them although there were far fewer of them than over the larger vessels (Figure 4.1B). The measured vessel diameter obtained from photographic images was greater than that calculated from the in vitro experiments due to the greater stretch required to produce flat preparations and images of high resolution.

Antibodies directed against the synaptic vesicle protein synaptophysin revealed a similar distribution of varicosities over the surface of the arteries, however the intervaricose regions did not show the same intensity of staining as was seen for the catecholamine fluorescence (Figure 4.1D).

4.2.2 Receptor mRNA expression

Messenger RNA expression of the $\alpha_1$- and $\alpha_2$-adrenergic, NPY and P$_{2X^-}$ purinergic receptor subtypes in the arteries of the rat hepatic mesentery is presented in Table 4.1. All subtypes except the NPY$_2$ and the purinergic P$_{2X0^-}$-receptors were detected (Figure 4.2, lane 9, Figure 4.3 lane 7). The $\alpha_{1A}$-adrenergic, NPY, and P$_{2X1^-}$, P$_{2X4^-}$ purinergic receptors showed very strong levels of detection (Figure 4.2, lanes 2 and 8, Figure 4.3 lanes 2 and 5). The $\alpha_{2B}$-adrenergic receptor was strongly detected (Figure 4.2, lane 6) as were the purinergic P$_{2X5^-}$ and P$_{2X7^-}$-receptors (Figure 4.3 lanes 6 and 8), while the remaining receptors showed weak levels of expression (Figure 4.2, lanes 3, 4, 72
Figure 4.1
Figure 4.1

Catecholamine histochemistry and immunohistochemistry performed on the rat hepatic artery. For the catecholamine histochemistry (panels A, B and C) whole mount preparations were fixed in 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1M phosphate buffer. Immunohistochemistry (panel D) was performed with antibodies against synaptophysin to visualise synaptic vesicles. Panel A shows the perivascular plexus of catecholaminergic fibres surrounding a second order hepatic vessel within the mesentery. Panel B shows an arteriole within the hepatic mesentery. In both panels A and B a nerve fibre bundle (fb) can be seen running parallel to the vessel. Panel C shows the catecholaminergic perivascular plexus of a secondary order vessel under higher magnification. Panel D shows the distribution of vesicles stained with antibodies against synaptophysin, detected with biotinylated anti-rabbit secondary antibodies and Texas Red conjugated to streptavidin. Scale bar represents 50\( \mu \)m in panel A and while scale bar in panel D represents 25\( \mu \)m for panels B, C and D.
Table 4.1 Expression of mRNA for $\alpha_1$-adrenergic, $\alpha_2$-adrenergic, neuropeptide Y and purinergic receptors in arteries of the rat hepatic mesentery

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>mRNA Expression Level</th>
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<tbody>
<tr>
<td>$\alpha_1$-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{1A}$</td>
<td>$+++,$</td>
</tr>
<tr>
<td>$\alpha_{1B}$</td>
<td>$+$</td>
</tr>
<tr>
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<td>$+$</td>
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<tr>
<td>$\alpha_2$-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{2A}$</td>
<td>$+$</td>
</tr>
<tr>
<td>$\alpha_{2B}$</td>
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<td>$P_{2X_7}$</td>
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Crosses represent detection of PCR product which corresponded to the size of the desired cDNA band for each set of subtype specific primers. The number of crosses reflects the amount of amplified product generated after 35 cycles. $[0]$ = never detected; $[+]$ = weakly detected; $[++]$ = strongly detected; $[+++]$ = very strongly detected.
Figure 4.2
Figure 4.2

RT-PCR products generated using primers for rat $\alpha_{1(A, B, D)}$- and $\alpha_{2(A, B, C)}$-adrenergic and neuropeptide Y ($Y_1$, $Y_2$) receptors, from cDNA from the arteries of the rat hepatic mesentery. Figure illustrates agarose gel electrophoresis of 8µl of PCR product, created after 35 cycles of amplification using subtype specific primers and cDNA that was reverse transcribed from DNase I treated total RNA. A 251 base pair (bp) product in lane 2 corresponds to the $\alpha_{1A}$-adrenergic receptor. Lanes 3 and 4 illustrate products of 405 and 517 bp corresponding to the predicted $\alpha_{1B}$- and $\alpha_{1D}$-adrenergic receptor fragments. Messenger RNA expression of the $\alpha_{2A}$-, $\alpha_{2B}$- and $\alpha_{2C}$-adrenergic receptors is confirmed by the presence of bands of 338, 456 and 425 bp respectively in lanes 5, 6 and 7. Expression of mRNA for the neuropeptide Y $Y_1$ receptor was verified by the presence of a 589 bp product (lane 8) while no product was detected with the neuropeptide Y $Y_2$ primer pair (lane 9). No PCR product was seen in receptor specific control experiments that did not contain reverse transcriptase enzyme (not shown) or did not contain RNA (lane 10). DNA molecular weight markers (Lambda HindIII/φX174 HaeIII) and (Pzt/Alu) appear in lanes 1 and 11 respectively.
Figure 4.3
Figure 4.3

RT-PCR products generated using primers for the purinergic P2X1-7-receptors, from cDNA from the arteries of the rat hepatic mesentery. Figure illustrates agarose gel electrophoresis of 8µl of PCR product, created after 35 cycles of amplification using subtype specific primers and cDNA that was reverse transcribed from DNase I treated total RNA. A 397 bp product in lane 2 corresponds to the P2X1-purinergic receptor. An extra band at 320bp is also present. Lane 3 contains a band at 273bp corresponding to the predicted P2X2-purinergic receptor. The band at 620bp is a genomic DNA fragment created with the same primers. Lanes 4, 5 and 6 illustrate products of 575, 396 and 379 bp corresponding to the predicted P2X3-, P2X4- and P2X5-purinergic receptor fragments. Messenger RNA expression for the P2X7- purinergic receptor is confirmed by the presence of a band of 567 bp in lane 8. No product was detected with the P2X5-purinergic receptor primer pair (lane 7). No PCR product was seen in receptor specific control experiments did not contain RNA (lane 9). DNA molecular weight markers (Lambda HindIII/ϕX174 HaeIII) and (P×7-Alu) appear in lanes 1 and 10 respectively.
5 and 7, Figure 4.3 lanes 3 and 4). The relative levels of mRNA expression for the different receptors were consistent amongst repeat mRNA preparations (n=3).

No predicted PCR product was seen when the reverse transcriptase enzyme was omitted from reactions or when RNA was omitted (Figure 4.2, lane 10, Figure 4.3, lane 9). The efficiency of the α-adrenergic primers has been confirmed in other arterial tissue (see Chapter 3) and neuropeptide primer pairs have been tested in brain and iris (Newhouse & Hill 1997), where an appropriately sized PCR product was detected at a level equivalent to [++++]. For the purinergic receptors, dorsal root ganglia was used as a positive control (Collo et al. 1996). All PCR fragments detected corresponded to the predicted fragment size for each receptor subtype. For the purinergic P2x1-receptor an extra band at approximately 320bp was detected. This band was not seen in sensory ganglia or brain tissue, however it was also detected in RNA extracted from mesenteric artery (see Chapter 6). On occasion, a band was also seen above the predicted fragment (Chapter 6.2.1). The band seen at 620bp in Figure 4.3, lane 3 corresponds to amplification of genomic DNA by the P2x2-purinergic primers and was seen in minus reverse transcriptase controls for this primer pair, despite the DNase treatment of the original RNA sample. Fragments corresponding to genomic DNA were not seen with other primer pairs.

4.2.3 In-vitro experiments

**General observations.** Preliminary studies indicated that a consistent contractile response could be achieved with stimulation of 10Hz, 10s (60V, 0.1ms pulse width) every 20min. Whilst decreasing or increasing the stimulus frequency or duration caused a respective decrease or increase in the size of the contraction, shorter pulses and lower frequencies of stimulation failed to elicit consistent contractions of a size sufficient to analyse. Consequently all experiments were performed using 10Hz for 10s every 20min. The average response to nerve stimulation was a contraction of 19.22 ± 0.64% (n=113) of the resting vessel diameter. The time to peak contraction from the onset of the nerve stimulus was 10.49 ± 0.15s (n=113) and the time to 90% recovery of original resting vessel diameter was 35.19 ± 1.96s (n=113, Figure 4.4A). Preparations that showed a contractile response of less than 10% of resting vessel diameter were not used.
Figure 4.4 Illustration of control contraction and the effects of applied $\alpha,\beta$-mATP on vessel diameter. Panel A shows a control response to nerve stimulation (10Hz, 10s, 0.1msec, 60V) starting 15s after time=0. Panel B shows a contraction induced by $\alpha,\beta$-mATP ($3\times10^{-6}$M, bar represents addition of drug to bath).
for further experiments. The contractile response was consistently blocked with tetrodotoxin (10^{-6}M) and guanethidine (5 \times 10^{-6}M). Early experiments indicated that preparations could be stimulated in control Krebs' containing scopolamine (10^{-6}M) and capsaicin (10^{-6}M) with the above parameters for up to 4h without a significant change in the magnitude of the nerve-mediated response (n=4). When diluent controls were tested at the appropriate concentrations, they had no effect on the nerve-mediated contraction or resting vessel diameter. When \(\alpha,\beta\text{-mATP} (3 \times 10^{-6}M)\) was applied to preparations, either before or after treatment with any other drugs, an agonist induced contraction and subsequent desensitisation occurred (Figure 4.4B). This contraction, which was equal to 35.52 \pm 1.55\% (n=72) of the resting vessel diameter, was a prolonged contraction and the vessel did not always fully recover. The average recovery was to 90.00 \pm 1.24\% (n=72) of the resting vessel diameter within 20min of the onset of the contraction.

**Components of contractile response.** To investigate what principal transmitters and receptors were involved in the contractile response to nerve stimulation, the effects of benextramine, \(\alpha,\beta\text{-mATP}\) and PPADS were tested.

Benextramine (10^{-5}M) reduced the size of the nerve-mediated contraction to 49.23 \pm 4.19\% of control (n=15, data from Figures 4.5A and 4.5B combined). When \(\alpha,\beta\text{-mATP} (3 \times 10^{-6}M)\) was used after benextramine, the contractile response was further reduced to 5.77\% \pm 3.48\% of control (n=4, Figure 4.5A). In those experiments where a residual component was present, it was removed after treatment with guanethidine (5 \times 10^{-6}M) and/or tetrodotoxin (10^{-6}M, Figure 4.5A, 4.5B). When PPADS (10^{-5}M) was used after benextramine the response was similarly reduced (Figure 4.5B).

When the experiments described above were performed in the reverse order, with \(\alpha,\beta\text{-mATP}\) or PPADS being used prior to benextramine, both drugs significantly enhanced the size of the nerve-mediated contractile response (Figures 4.6, 4.7). The addition of benextramine (10^{-5}M) after either \(\alpha,\beta\text{-mATP}\) or PPADS essentially abolished the enhanced response to nerve stimulation (Figure 4.6).

In addition to enhancing the amplitude of the contractile response to nerve stimulation, both \(\alpha,\beta\text{-mATP}\) and PPADS altered the characteristics of the contraction (Figure 4.7). In the presence of \(\alpha,\beta\text{-mATP}\), the time to peak was significantly shorter than that in control Krebs' (5.88 \pm 0.31s, n=36, P < 0.05) as was the recovery to 90\% resting vessel diameter (25.22 \pm 2.02s, n=36, P < 0.05, Figure 4.7A). A similar
Figure 4.5 Effects of the consecutive application of (A) benextramine (BNX, 10^{-5}M) and α,β-mATP (mATP, 3 \times 10^{-6}M) or (B) BNX and PPADS (10^{-5}M) on the nerve-mediated contractile response. Tetrodotoxin (TTX, 10^{-6}M) and/or guanethidine (GE, 5 \times 10^{-6}M) abolished the small residual contraction. Columns represent the means ± s.e. mean of at least 4 preparations. Results are expressed as % of the contractile response in control Krebs’ solution (Cont). Control response in (A) was 20.78 ± 2.64% of resting vessel diameter, n=7 and in (b) was 17.92 ± 1.68%, n=8. * Differs significantly from control (P < 0.05).
Figure 4.6 Effects of the consecutive application of (A) α,β-mATP (mATP, 3x10⁻⁶M) and benextramine (BNX, 10⁻⁵M) or (B) PPADS (10⁻⁵M) and BNX on the nerve-mediated contractile response. Columns represent the means ± s.e. mean of at least 6 preparations. Results are expressed as % of the contractile response in control Krebs' solution (Cont). Control response in (A) was 22.10 ± 3.62% of resting vessel diameter, n=10 and in (B) was 15.54 ± 1.32%, n=9. * Differs significantly from control (P < 0.05).
phenomenon was seen in the presence of PPADS (time to peak: 4.54 ± 0.83s, \( n=9 \), \( P < 0.05 \), recovery: 30.97± 3.37, \( n=9 \), Figure 4.7B). In the majority of experiments, \( \alpha,\beta \)-mATP (22/36) and PPADS (7/9) caused the nerve-mediated response to take on a biphasic appearance (Figure 4.7, bottom panels). A biphasic response was also seen in 6 control experiments. When this response was considered as two separate components, the first phase had a time to peak of 4.25 ± 0.17s \( (n=51) \), while the second phase had a time to peak of 10.87 ± 0.24s \( (n=51) \).

**Identification of \( \alpha \)-adrenoceptor subtypes.** In order to determine the specific postsynaptic receptor subtype responsible for the adrenergic component of the nerve-mediated constriction, a number of \( \alpha \)-adrenoceptor antagonists were used.

The effects of different concentrations of the \( \alpha_2 \)-adrenoceptor antagonist yohimbine were tested (Figure 4.8). At no concentration did yohimbine cause a reduction in the amplitude of the contractile response although concentrations of \( 10^{-8} \)M and \( 10^{-7} \)M caused a significant enhancement of the size of the nerve-mediated contraction. This potentiation was reversed at \( 10^{-6} \)M (Figure 4.8). Unlike \( \alpha,\beta \)-mATP and PPADS, yohimbine did not produce a change in the time to peak contraction and a biphasic response was never seen (Figure 4.9).

The responses to nerve stimulation in the presence of increasing concentrations of the \( \alpha_1 \)-adrenergic receptor antagonists BMY 7378 (\( \alpha_{1D} \)), 5-methyl-urapidil (\( \alpha_{1A} \)), WB-4101 (\( \alpha_{1A/1D} \)) and prazosin (\( \alpha_1 \)) were determined. The drugs showed the following potency order: prazosin > WB-4101 = 5-methyl-urapidil > BMY 7378 (Figure 4.10A), with responses being maximally inhibited by 82% for prazosin (\( 10^{-8} \)M, 82.22 ± 4.55%, \( n=4 \)), by 63% for WB-4101 (\( 10^{-7} \)M, 63.09 ± 8.86%, \( n=4 \)) and by 60% for 5-methyl-urapidil (\( 10^{-7} \)M, 59.94 ± 7.31%, \( n=4 \)). BMY 7378 was virtually ineffective at concentrations specific for the \( \alpha_{1D} \)-adrenoceptor (\( 10^{-9}, 10^{-8} \)M) and only inhibited the response at high concentrations.

Experiments were also performed with the \( \alpha_1 \)-adrenergic receptor antagonists in the presence of \( \alpha,\beta \)-mATP to eliminate the purinergic component of the contractile response and allow the curves to reach maximal inhibition. The contractile response in the presence of the specific \( \alpha_1 \)-adrenergic antagonists was expressed as a percentage of the \( \alpha,\beta \)-mATP response in order for the control response to be equivalent to 100%. In these experiments, contractile responses to nerve stimulation were extremely sensitive
Figure 4.8 Effect of increasing concentrations of yohimbine on the nerve-mediated contractile response. Columns represent the mean ± s.e. mean of at least 4 preparations. Results are expressed as % of the contractile response in control Krebs' solution (Cont), which was 19.51 ± 2.31% of resting vessel diameter, n=11. * Differs significantly from control (P < 0.05).
Figure 4.9 Representative traces showing the effect of yohimbine (10^{-8} M) on the characteristics of the nerve-mediated contraction. Top panel shows the nerve stimuli (10Hz, 10s) starting 15s after time =0. Middle panel shows a control response in Krebs' solution. Bottom panel shows the nerve-mediated response in the presence of yohimbine.
Figure 4.10 Contractile responses to nerve-stimulation in the presence of increasing concentrations of α₁-adrenergic receptor antagonists. Panel A (full symbols) illustrates the effects of BMY 7378, WB-4101, 5-methyl-urapidil and prazosin (n=4). Panel B (open symbols) illustrates the effects of the same drugs after preincubation in the presence of α₂β-mATP (3x10⁻⁶M, n=4). Results are expressed in (A) as a % of the contractile response in control Krebs' solution (20.10 ± 1.15% resting vessel diameter, n=29) and in (B) as a % of the contractile response after treatment with α₂β-mATP (22.41 ± 1.21% of resting vessel diameter vs untreated control of 15.90 ± 1.11%, n=29, P < 0.05).
to prazosin (IC\textsubscript{50}=8x10^{-10}M), WB-4101 (IC\textsubscript{50}=1x10^{-9}M) and 5-methyl-urapidil (IC\textsubscript{50}=2x10^{-9}M), and nerve-mediated contractions were essentially abolished by the higher concentrations of these drugs (Figure 4.10B). BMY 7378 again only partly reduced the response at non-specific concentrations (10^{-7}M, 10^{-6}M, Figure 4.10B).

The putative $\alpha_{1B}$-adrenoceptor antagonist, CEC, was also used both before and after treatment of the vessel with $\alpha$,\beta-mATP, however results were spurious and therefore not taken into consideration. Results are addressed in Appendix A.

**Characterisation of the biphasic response.** Given the appearance of a fast biphasic contraction after treatment with the P$_{2\text{x}}$-antagonists, but not yohimbine, a number of further experiments were performed.

In order to assess the potential involvement of postsynaptic $\alpha_{2}$-adrenoceptors, yohimbine was added after the biphasic response had been induced by either $\alpha$,\beta-mATP or PPADS. At 10^{-8}M, yohimbine did not alter the time to peak or the biphasic appearance (n=5). It did cause a trend towards an increase in the size of the fast component of the contraction however this was not significant. The noradrenaline uptake inhibitor, desipramine (3x10^{-7}M, DMI, Brock & Barrett 1997) was used to increase the availability of transmitter. When applied to control contractions, DMI caused a significant increase in the size of the contraction (136.68 ± 9.65% of control, \( P < 0.05, n=4 \)) however it did not alter the time to peak contraction or bring about the appearance of a biphasic response. With further increases in the concentration of DMI, the size of the contraction was reduced back to control values. When yohimbine (10^{-8}M) was added after DMI, there was a further increase in the size of contraction (121 ± 12.29% of the DMI treated nerve-mediated response, \( n=3 \)).

In experiments initially designed to increase vascular tone, it was noted that during exposure to increased KCl (20mM), the size of the nerve-mediated contraction was increased (142.13 ± 7.77% of control, \( P < 0.05, n=4 \)) and the time to peak contraction was shortened (5.27 ± 0.79s, \( P < 0.05, n=4 \)). KCl enhanced nerve-mediated contractions were not, however, biphasic and recovery to 90% of resting vessel diameter was not significantly different to control. The addition of 20mM KCl by itself caused a sustained contraction equal to 7.76 ± 0.84% of resting vessel diameter.
To investigate the involvement of Ca\(^{2+}\) channels in the fast response, the effect of calcium channel antagonists were tested on contractions that had been enhanced by \(\alpha,\beta\)-mATP. Nifedipine (10^{-6}M) had no effect on the size or time to peak of the fast component of the contraction \((n=3)\). Preliminary results indicate that felodipine (10^{-9}M), verapamil (10^{-6}, 10^{-5}M) and diltiazem (10^{-6}M), were also without effect.

**Nerve-mediated neuropeptide Y effects.** Treatment of the hepatic artery to remove the adrenergic and purinergic components of the contractile response uncovered a slow nerve-mediated contraction. The contraction had a time to peak amplitude of 41.57 ± 2.46s \((n=37)\) and a time to recovery to 90% of resting vessel diameter of 110.34 ± 8.75s (Figure 4.11). The amplitude of the response was 10.73 ± 1.03% \((n=37)\) of the resting vessel diameter. This slow nerve-mediated contraction was blocked by tetrodotoxin (10^{-6}M, \(n=2\)), guanethidine (5x10^{-6}M, \(n=10\)) and the NPY\(_1\) receptor antagonist 1229U91 (6x10^{-7}M, \(n=7\), Lew *et al.* 1996). While the response was only seen once under control conditions, it appeared after treatment with the following drugs: benextramine \((n=20)\), the P\(_{2X}\)-antagonists \(\alpha,\beta\)-mATP and PPADS \((n=10)\) either on their own or in combination with benextramine, and yohimbine \((n=6)\). It should be noted however that the slow contraction was not always seen after the use of any of these drugs. 1229U91 was used to block the response in those experiments where a combination of benextramine and one of the purinergic receptor antagonists had uncovered the slow contraction.
Figure 4.11 Representative tracings of contractions due to neurally released noradrenaline and ATP (middle) and NPY (bottom). Top panel shows nerve stimulus (10Hz, 10s) starting 15s after time=0. The response in control Krebs' is shown in the middle panel. The NPY-mediated response is shown in the same vessel after treatment with α,β-mATP (3×10^{-6}M) and benextramine (10^{-5}M, bottom panel).
4.3 DISCUSSION

Transmural stimulation of the arteries of the rat hepatic mesentery at 10Hz for 10s, produced a response that was consistently blocked with tetrodotoxin and guanethidine, indicating that contractions were due to the release of neurotransmitter from sympathetic nerves and did not result from direct stimulation of the vascular smooth muscle (Hausler & Haefely 1979). The principal transmitters involved in the contractile response under these stimulation parameters were noradrenaline and ATP, acting at $\alpha_1$-adrenergic and $P_{2X}$-purinergic receptors respectively, as the response was effectively blocked by the combination of benextramine, an $\alpha$-adrenoceptor antagonist (Benfey 1982) and $\alpha,\beta$-mATP, a stable analogue of ATP. The latter first activates and then desensitises $P_{2X}$-purinergic receptors, and has therefore been used extensively as an $P_{2X}$-receptor antagonist (Kasakov & Burnstock 1983; Burnstock & Kennedy 1985). The result with $\alpha,\beta$-mATP was confirmed using PPADS, a selective $P_{2X}$-receptor antagonist (Ziganshin et al. 1994).

Catecholamine histochemistry revealed a dense plexus of catecholaminergic nerve fibres surrounding the walls of the arteries in the rat hepatic mesentery. The plexus continued over the major artery and its branches, extending to the points of entry of the vessels into the liver parenchyma. This is in agreement with an earlier fluorescence study by Ungvary & Donath (1969) who showed there was an abundant adrenergic nerve supply to the hepatic arteries of a number of different species, including the rat. Antibody staining with synaptophysin revealed a very similar pattern of staining, however the intervaricose regions were not stained to the same extent. This is to be expected as synaptophysin is an integral membrane component of synaptic vesicles (Wiedenmann & Franke 1985) and has been implicated in the process of neurotransmitter secretion (Alder et al. 1995; Elferink & Scheller 1995). The similar punctate staining produced by catecholamine histochemistry and synaptophysin-like immunoreactivity confirms that these regions are the sites of neurotransmitter storage and release.

It is now well established that noradrenaline and ATP act as co-transmitters in sympathetic nerves supplying blood vessels (Kennedy 1996), however the proportion of
the nerve-mediated contractile response attributable to each depends upon both the vascular bed and the experimental conditions (Ralevic & Burnstock 1991). Stimulation at low frequencies favours purinergic responses, while higher frequencies or longer periods of stimulation increase the relative component of the adrenergic response (Kennedy et al. 1986; Kennedy 1996). In the arteries of the rat hepatic mesentery, stimulation parameters of 10Hz for 10s were required to produce a response of reasonable size that could be blocked by tetrodotoxin. These results are in agreement with a study in the rabbit hepatic artery where contractions could not be induced at frequencies of less than 8Hz (Brizzolara & Burnstock 1990). Under these stimulation conditions of 10Hz for 10s, the response appeared to be primarily adrenergic with a smaller contribution from ATP acting on P$_{2X}$-purinoceptors, since the $\alpha_1$-adrenergic antagonist, prazosin (Cambridge et al. 1977), blocked the majority of the response. On the other hand, the $\alpha$-adrenoceptor antagonist, benextramine, only reduced the response by 50%, presumably due to increased release of ATP following blockade of presynaptic receptors as discussed below.

In the arteries of the rat hepatic mesentery, the subtype of receptor responsible for the $\alpha_1$-adrenoceptor mediated component of the contraction was characterised using a number of specific $\alpha_1$-adrenoceptor antagonists. BMY 7378, initially developed as a 5-HT receptor antagonist (Yocca et al. 1987), has since been shown to be a selective antagonist of $\alpha_{1D}$-adrenergic receptors (Goetz et al. 1995; Piascik et al. 1995). In this preparation it did not affect the nerve-mediated contraction until high, non-specific concentrations were reached (Piascik et al. 1995). The hepatic artery preparation was however very sensitive to the selective $\alpha_{1A}$-adrenergic receptor antagonists WB-4101 and 5-methyl-urapidil (Hancock 1996), both in the presence and absence of $\alpha,\beta$-mATP. These sensitivities, combined with the apparent lack of effect of yohimbine, an $\alpha_2$-adrenoceptor antagonist (Langer & Shepperson 1982), strongly suggest that the contractile response to neurally released noradrenaline is mediated by $\alpha_{1A}$-adrenergic receptors. These results correlated well with the strong mRNA expression for $\alpha_{1A}$-adrenergic receptors as determined using RT-PCR. A similar experimental approach has been used to characterise $\alpha_1$-adrenergic receptor subtypes in the porcine renal artery (Zhou et al. 1998). In this study, a good correlation between mRNA expression and the pharmacological receptor profile was again described.
RT-PCR studies demonstrated strong expression of mRNA for the P2x1- and P2x4-purinoceptors and moderate expression of the P2x5- and P2x7-receptors. No gene expression was detected for the P2x6-receptor which agrees with findings in the rat mesenteric artery (Chapter 6) and aorta (Soto et al. 1996b). The P2x1-purinoceptor has previously been localised to vascular smooth muscle using both immunohistochemical and molecular techniques (Collo et al. 1996; Chan et al. 1998; Nori et al. 1998). Transcript for the P2x2- and P2x4-purinoceptors has recently been detected in a number of vessels, including the aorta and coronary vessels (Soto et al. 1996a; Nori et al. 1998), however, to our knowledge this is the first demonstration of P2x5- and P2x7-purinergic receptor mRNA in a peripheral artery. Given the insensitivity of the of P2x4-, P2x5- and P2x7-receptors to α,β-mATP (Evans 1996; Surprenant et al. 1996), it seems unlikely that these subtypes mediate the responses seen here. Based on α,β-mATP responses, a recent study in the canine coronary artery has suggested that either P2x1- or P2x3- purinergic receptors mediate contractile responses to ATP (Matsumoto et al. 1997). Our finding of low mRNA expression for the P2x3-receptor suggests that, in the arteries of the rat hepatic mesentery, the receptor subtype mediating the purinergic component of the contraction is most likely the P2x1-receptor.

It was interesting to find that yohimbine, α,β-mATP and PPADS all enhanced the size of the nerve-mediated contraction. The effect of yohimbine can be explained by the presence of presynaptic α2-adrenergic receptors, on which noradrenaline acts to inhibit further transmitter release (Langer 1974). By blocking these receptors, the negative feedback is removed and transmitter release is increased. Higher doses of yohimbine (10^-6 M) reversed the potentiation, possibly due to non-specific postsynaptic effects on α1-adrenoceptors (Bao et al. 1993). The apparently greater purinergic component of the contractile response after benextramine, compared with prazosin, suggests that these prejunctional α1-adrenoceptors also act to inhibit ATP release, as has been described in a number of different blood vessels (von Kugelgen & Starke 1985; Bulloch & Starke 1990; MacDonald et al. 1992). Experimental stimulation parameters are important considerations when assessing presynaptic modulation of transmitter release (Brock 1995), as modulation is reported to be more significant at frequencies of stimulation > 8Hz (Stjarne & Astrand 1985; Shinozuka et al. 1990).
In addition to enhancing the amplitude of the nerve-mediated contraction, the P2X-purinergic receptor antagonists also caused a reduction in the time to peak amplitude of the response and in some experiments the contraction became biphasic. Treatment with yohimbine, while also enhancing the size of the nerve-mediated contraction, did not produce any changes to the time course. A biphasic response was also seen in a small number of control experiments in the arteries of the hepatic mesentery, corresponding to the biphasic noradrenaline and ATP mediated contractions that have been reported in other vessels. (Burnstock & Warland 1987b; Chau et al. 1990; Kennedy et al. 1986; Bulloch & Starke 1990). It is possible that in our preparation, the majority of control responses contained the two phases however they were superimposed and therefore not distinguishable. The situation changed quite dramatically however with the addition of the purinergic antagonists, with the first phase being significantly enhanced.

The fast component is not due to activation of extrasynaptic α2-adrenoceptors (Langer & Shepperson 1982), as yohimbine did not block the response when added after the P2X-purinoceptor antagonist. The potential role of extrajunctional receptors was investigated further using the noradrenaline uptake inhibitor DMI, in an attempt to mimic increased noradrenaline availability. At 3x10^-7M, DMI potentiated the amplitude of the contraction but did not alter the time to peak contraction. Interestingly, higher concentrations removed this enhancement. This is most likely due to α2-adrenoceptor mediated inhibition of transmitter release (Lew & Angus 1983; Msghina et al. 1992), as the addition of yohimbine to DMI potentiated responses further increased the size of the contraction. These findings confirm the presence of inhibitory presynaptic α2-adrenoceptors in this preparation.

The ability of α,β-mATP and PPADS to enhance the size of the nerve-mediated contraction could be attributed to their action in blocking presynaptic receptors, as treatment with α,β-mATP has been shown previously to increase the release of noradrenaline from sympathetic nerves (Stjarne & Astrand 1985; Shinozuka et al. 1990). In other studies however, where an enhancement of vascular noradrenergic contractile responses by α,β-mATP has been described (Neild & Kotecha 1986; Ralevic & Burnstock 1990; MacDonald et al. 1992; Bao & Stjarne 1993), postsynaptic mechanisms are believed to be involved. Similar effects have also been reported for
suramin (Bao & Stjarne 1993; McLaren et al. 1995) and PPADS (J.Brock, pers. comm.). Interestingly, in contrast to our results, Bao & Stjarne (1993) describe α,β-mATP as delaying the onset, while increasing the amplitude and duration of nerve-mediated contractions.

In the arteries of the rat hepatic mesentery, a decrease in the time to peak was seen for nerve-mediated contractions when the vessels had been depolarized with high KCl Krebs’. The ability of KCl to mimic the enhancing actions of α,β-mATP has been described in other studies (Neild & Kotecha 1986; Ralevic & Burnstock 1990) and as α,β-mATP is able to depolarize vascular smooth muscle cells (Ishikawa 1985; Neild & Kotecha 1986; Nagao & Suzuki 1988), the change in time course may therefore be related directly to depolarisation.

Depolarisation can alter the permeability of the smooth muscle cell membrane to Ca²⁺ influx through voltage-dependent Ca²⁺ channels (Kuriyama et al. 1982; Noguera et al. 1997). In order to assess this possibility, the dihydropyridine L-type Ca²⁺ channel antagonist, nifedipine (Hille 1992) was used. Nifedipine did not affect the timing or the size of the fast response, indicating that it did not result from an increase of Ca²⁺ entry through voltage dependent Ca²⁺ channels. A number of other Ca²⁺ channel antagonists were trialled, to rule out lack of response to nifedipine associated with voltage dependent binding (Hille 1992; Sun & Triggle 1995), however these drugs also appeared to have no effect on the fast nerve-mediated contraction. The effect of depolarisation in altering the timing of nerve-mediated responses may therefore be related to other changes in Ca²⁺ availability. Thus, α,β-mATP may activate non L-type calcium channels (Kitajima et al. 1993) or the fast phase of the response may be due to the release of Ca²⁺ from intracellular stores (Kobayashi et al. 1985; Sulpizio & Hieble 1991; Zhang et al. 1997). The exact mechanism by which P₂X-antagonists potentiate noradrenaline-mediated responses is therefore still open to investigation.

It was surprising that, in the present study, nifedipine did not affect the nerve-mediated contractile response, since α₁a-adrenoceptor responses are classically mediated by Ca²⁺ influx through voltage dependent Ca²⁺ channels (Han et al. 1987; Gould & Hill 1994). A similar discrepancy has been described in other vascular tissues (Sulpizio & Hieble 1991; Lepretre et al. 1994; Kong et al. 1994) and suggests that specific α-adrenoceptors are not necessarily coupled to specific Ca²⁺ sources (Esbenshade &
Minneman 1995; Graham et al. 1996). The source of Ca\textsuperscript{2+} in this vessel remains to be determined.

In this study, a slow nerve-mediated contractile response was recorded in some experiments. This response possessed characteristics quite distinct from those of the contraction in control experiments, being much slower in the time to peak amplitude with a prolonged recovery time. Guanethidine and tetrodotoxin abolished the response, indicating that it was due to transmitter release from sympathetic nerves. Based on RT-PCR results, which indicated strong expression of mRNA for the NPY\textsubscript{1} receptor, we applied the selective NPY\textsubscript{1} receptor antagonist 1229U91 (Lew et al. 1996) and successfully blocked the response. An association between NPY\textsubscript{1} receptor mRNA expression and the presence of functional protein has also been confirmed in human cerebral arteries (Nilsson et al. 1996) and porcine renal artery (Malmstrom 1997). In blood vessels, neurally released NPY usually mediates contractions via NPY\textsubscript{1} receptors (Grundemar et al. 1992; Racchi et al. 1997) and its effects are slower to develop and more long lasting than those of its co-transmitters, noradrenaline and ATP (Morris et al. 1995).

In the present study NPY release was seen most frequently following the application of benextramine. Benextramine has been reported to be an NPY receptor antagonist (Doughty et al. 1990; Li et al. 1991; Melchiorre et al. 1994) however more recent studies have found this not to be the case (Westfall et al. 1995; Newhouse & Hill 1997). It is more likely that by blocking presynaptic α\textsubscript{2}-adrenergic receptors, benextramine removed a noradrenaline mediated inhibition of NPY release. Inhibition of NPY release by presynaptic α\textsubscript{2}-adrenergic receptors has previously been described (Dahlof et al. 1986; Haass et al. 1989). Yohimbine was also seen to induce the release of NPY, further supporting this hypothesis. The ability of α,β-mATP and PPADS to uncover the NPY response indicates a role for presynaptic inhibition of NPY release by ATP.

In summary, this chapter has shown that the arteries of the rat hepatic mesentery are subjected to complex control mechanisms involving a number of different presynaptic and postsynaptic receptor subtypes. Vasoconstriction results from the activation of postsynaptic α\textsubscript{1A}-adrenergic and P\textsubscript{2X1}-purinergic receptors and under some conditions NPY\textsubscript{1} receptors. Neurotransmitter release is modulated by presynaptic
α₂-adrenergic receptors and possibly also P₂X-purinoceptors. The major postsynaptic receptor subtypes involved were well predicted by mRNA expression as measured by RT-PCR, confirming that this technique is a useful adjunct in studies aimed at identifying functional vascular receptor subtypes.
CHAPTER 5
MULTIPLE VASODILATOR PATHWAYS EXIST IN ARTERIES OF THE RAT HEPATIC MESENTERY

5.1 INTRODUCTION

Relaxation of vascular tissue is ultimately due to a decrease in available smooth muscle cell intracellular Ca\(^{2+}\) and the subsequent dephosphorylation of myosin (Walsh et al. 1995). Intracellular Ca\(^{2+}\) levels, however, can be influenced by multiple receptor systems. Sympathetic nerves release noradrenaline and ATP to mediate vasodilation via the activation of β-adrenoceptors and P\(_{2Y}\)-purinoceptors respectively (Osswald & Guimaraes 1983; Burnstock 1996). The actions of ATP may also be mediated by the breakdown of ATP to adenosine and the activation of P\(_1\)-purinoceptors (Browse et al. 1997). Blood vessels also possess receptors for the parasympathetic neurotransmitters (Morris et al. 1995) and for the sensory neuropeptides (Maggi 1995; Aiyar et al. 1996).

Similar to vasoconstrictor mechanisms (see Chapter 4.1), the contribution of the different nervous elements and the vasodilatory mechanisms involved may vary between vascular beds and multiple receptor pathways may be present in the one vessel. In addition, the neuroreceptors mediating vasodilation may be located on either vascular smooth muscle cells or on endothelial cells. In the rabbit hepatic artery for example, applied ATP and CGRP produce vasodilation due to the activation of receptors located on the vascular smooth muscle, whereas acetylcholine and substance P mediate vasodilation via an endothelium-dependent mechanism (Brizzolara & Burnstock 1991).

Endothelium-dependent vasodilations are mainly mediated by an increase in the release of NO and in some vascular beds prostacyclin (Hecker et al. 1993) or the postulated EDHF (Garland et al. 1995). The endothelium plays an additional role in local control of blood flow, by responding to changes in haemodynamic forces, for example shear stress and vascular tone, and to locally released or circulating vasoactive...
substances (Burnstock & Ralevic 1994). Continuous or basal release of NO may also be involved in the local regulation of blood flow (Moncada et al. 1991).

Another important vasodilator pathway involves the release of NO directly from nerves (Toda & Okamura 1990a; Bredt et al. 1990). Both autonomic and sensory nerves have been demonstrated to mediate vasodilation through this pathway (Morris et al. 1995; Goadsby et al. 1996; Zheng et al. 1997).

In Chapter 4, the receptors mediating vasoconstriction in small arteries of the rat hepatic mesentery were characterised and a correlation between receptor mRNA expression and functional protein was made. An understanding of vasodilatory responses in this vascular bed is equally important, as selective use of vasodilators may aid hepatic function in chronic liver conditions such as cirrhosis (Morgan & McLean 1995) or with ageing (LeCouteur & McLean 1998). Earlier studies in the main hepatic artery of the rat have described endothelium-dependent vasodilations to applied acetylcholine via the release of EDHF (Zygmunt et al. 1994a), endothelium-dependent vasodilations to applied substance P and endothelium-independent responses to applied and neurally released CGRP (Bratveit & Helle 1991). In order to further characterise the receptors involved in nerve-mediated vasodilations in this vascular bed, a multidisciplinary approach was again employed, using histochemistry to study patterns of nerve distribution, RT-PCR to assess receptor mRNA expression and in vitro physiology to pharmacologically characterise the receptors involved in functional responses.
5.2 RESULTS

5.2.1 Histochemistry

**Immunohistochemistry.** The hepatic arteries lying within the mesentery receive a significant sensory innervation as demonstrated by the presence of perivascular fibres containing substance P and CGRP (Figure 5.1). Substance P-like perivascular immunoreactivity was detected in both varicose fibres on the surface of the vessel and in nerve bundles lying beside the artery (Figure. 5.1A, 5.1B). Fibres showing CGRP-like immunoreactivity were also detected. The fibres were in larger bundles than were seen for substance P containing fibres and the paravascular nerve bundles also appeared to be comprised of more fibres (Figure 5.1C, 5.1D). No VIP-like immunoreactivity was detected (data not shown).

**NADPH-diaphorase histochemistry.** Results using NADPH-diaphorase to localise NOS did not reveal any perivascular nerve fibres containing the enzyme, whilst there was definite punctate endothelial cell staining (Figure 5.2A, 5.2B). Occasionally, a few fibres with NADPH staining were seen in the surrounding tissue, but none were seen in association with the vessel. Efficacy of the technique was confirmed in sections of superior mesenteric ganglion, where the presence of numerous perivascular nerve baskets was demonstrated (Figure 5.2C).

5.2.2 Receptor mRNA expression

Expression of mRNA for the neurokinin, CGRP and muscarinic receptors is shown in Table 5.1. Of the sensory neuropeptide receptors, only the CGRP₁ receptor showed very high levels of mRNA expression (Figure 5.3A, lane 5), while the NK₁ receptor showed strong mRNA expression, mRNA for the NK₂ receptor was never seen and mRNA for the NK₃ receptor was only weakly detected (Figure 5.3A, lanes 2, 3 and 4 respectively). For the muscarinic receptors, the M₃ receptor was very strongly detected (Figure 5.3B, lane 4), the M₂ and M₅ receptors were strongly detected (Figure
Figure 5.1
Figure 5.1

Immunohistochemistry performed on arteries from the rat hepatic mesentery demonstrating the presence of sensory nerve fibres. In panels A and B, a fine perivascular plexus of fibres containing substance P-like immunoreactivity is detected after incubation in anti-rabbit fluorescein isothiocyanate. CGRP-like immunoreactivity is also seen (panels C and D), although fibres appear to be present in larger nerve bundles, both on the surface of the vessel and in the paravascular fibre bundles. CGRP-like immunoreactivity was detected after sequential incubation in biotinylated anti-rabbit secondary antibodies and Texas Red conjugated to streptavidin. Scale bar in panel D represents 100µm for all panels.
Figure 5.2
Figure 5.2

Sections of rat hepatic artery (panels A, B) and superior mesenteric ganglion (panel C) stained for NADPH-diaphorase reactivity to localise nitric oxide synthase. Arterial sections, shown in transverse (A) and longitudinal views (B) show no perivascular nerve fibres containing the enzyme, whilst there was punctate endothelial cell staining (arrows). Superior mesenteric ganglion was used as a control tissue (C) and staining can be seen surrounding a distinct population of neurons (arrows). Scale bar in panel C represents 100μm for all panels.
Table 5.1  Expression of mRNA for neurokinin, CGRP, and muscarinic receptors in the arteries of the rat hepatic mesentery

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>mRNA Expression Level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensory Receptors</strong></td>
<td></td>
</tr>
<tr>
<td>NK1</td>
<td>++</td>
</tr>
<tr>
<td>NK2</td>
<td>0</td>
</tr>
<tr>
<td>NK3</td>
<td>+</td>
</tr>
<tr>
<td>CGRP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Muscarinic Receptors</strong></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>0</td>
</tr>
<tr>
<td>M2</td>
<td>++</td>
</tr>
<tr>
<td>M3</td>
<td>+++</td>
</tr>
<tr>
<td>M4</td>
<td>0</td>
</tr>
<tr>
<td>M5</td>
<td>++</td>
</tr>
</tbody>
</table>

Crosses represent detection of PCR products which corresponded to the size of the desired cDNA band for each set of subtype specific primers. The number of crosses reflects the amount of amplified product generated after 35 cycles. [0] = never detected; [+ ] = weakly detected; [++] = strongly detected; [+++ ] = very strongly detected.
Figure 5.3
Figure 5.3

RT-PCR products generated from cDNA from arteries of the rat hepatic mesentery, using primers for the (A) neurokinin (NK1-NK3), calcitonin gene related peptide (CGRP1) and (B) muscarinic (M1-M5) receptors. Figures illustrate agarose gel electrophoresis of 8μl of PCR product, created after 35 cycles of amplification using subtype specific primers and cDNA that was reverse transcribed from DNase I treated total RNA. Panel A shows a 537 base pair (bp) product in lane 2 which corresponds to the NK1 receptor. No product was detected for the NK2 receptor (lane 3) while lanes 4 and 5 illustrate products of 476 and 452 bp corresponding to the predicted NK3 and CGRP1 receptor fragments. Panel B illustrates expression of cholinergic muscarinic receptors. Messenger RNA expression of the M2, M3 and M5 receptors is confirmed by the presence of bands of 552, 790 and 594 bp respectively in lanes 3, 4 and 6. No PCR product was detected for the M1 or M4 muscarinic receptors (lanes 2 and 5). No product was seen in control experiments that did not contain reverse transcriptase enzyme (not shown) or did not contain RNA (panel A, lane 6, panel B lane 7). DNA molecular weight markers (Lambda HindIII/ϕX174 HaeIII) and (Ptz/Alu) appear in A (lanes 1 and 7) and B (lanes 1 and 8) respectively.
5.3B, lanes 3 and 6) and there was no mRNA expression detected for the M1 or M4 receptors (Figure 5.3B, lanes 2 and 5 respectively).

The relative levels of expression for the different receptor subtypes were consistent amongst the different mRNA preparations. No PCR product was seen when reverse transcriptase enzyme or RNA was omitted from reactions (Figure 5.3A lane 6, 5.3B lane 7). The efficiency of all primer pairs was confirmed in other tissues where an appropriate sized PCR product was detected at a level equivalent to [+++](see Chapter 3.2.1). The CGRP₁ receptor fragment showed an 86.5% and 96.2% sequence homology to the human and rat CGRP₁ receptor sequences respectively over the region amplified (Aiyar et al. 1996; Chang et al. 1993).

5.2.3 In vitro physiology

General observations. Vasoconstrictor responses to nerve stimulation and hence the viability of the preparation was tested prior to the addition of any drugs. Only preparations that showed a sympathetic nerve-mediated vasoconstriction were used for further experiments (Figure 5.4A). The same stimulation parameters (10Hz, 10s, 0.1msec, 60V) were used for all experiments.

In those experiments where methoxamine (3x10⁻⁵M) was used to induce tone, it caused a sustained vasoconstriction that was equal to 27.28 ± 2.67% \((n=16)\) of the resting vessel diameter. In all preparations to which methoxamine was applied, the vessels demonstrated spontaneous contractile activity (described here as vasomotion, see Figure 5.7A). The vasomotion decreased in size over time however it did not cease.

The application of the NOS inhibitor L-NAME (10⁻⁴M), caused a significant increase in the size of the sympathetic nerve-mediated vasoconstriction \((267.86 ± 29.74\% \text{ of control, } n=5, P < 0.05)\) however it did not alter the contraction characteristics (time to peak contraction 9.85 ± 0.82s compared to control 9.31 ± 0.43s, time to recovery to 90% of resting vessel diameter was 31.56 ± 6.54s versus 23.20 ± 2.55s in control, \(n=5\)). The addition of L-NAME to vessels preconstricted with methoxamine caused a significant decrease in vessel diameter of 21.15 ± 3.54% of
Figure 5.4 Representative traces illustrating sympathetic nerve-mediated vasodilation. Top panels show nerve stimuli (10Hz, 10s) starting 15s after time = 0. Panel A shows a control contraction in the presence of capsaicin (10^{-6}M) and scopolamine (10^{-6}M). Panel B shows a dilation seen after treatment of the vessel with α,β-mATP (6x10^{-6}M) and benextramine (BNX, 10^{-5}M). In panel C, the dilation has been abolished by the further addition of the β-adrenoceptor antagonist propranolol (2x10^{-6}M).
control vessel diameter ($P < 0.05$, $n=6$, Figure 5.6B). This vasoconstrictor response to L-NAME was not seen under any other conditions.

**Sympathetic nerve-mediated responses.** In order to study sympathetic nerve-mediated vasodilations, vessels were pretreated with capsaicin ($10^{-6}$M) to block the activation of sensory nerves, scopolamine ($10^{-6}$M) to block muscarinic receptors and benextramine ($10^{-5}$M) and $\alpha,\beta$-mATP ($6\times10^{-6}$M) to prevent sympathetic vasoconstrictions. As described in section 4.2.3, the application $\alpha,\beta$-mATP caused an agonist induced contraction and subsequent desensitisation. The vessel did not recover fully from the contraction, with a maintained increase in tone of $12.94 \pm 1.16\%$ ($n=17$) of the resting vessel diameter. Subsequent nerve stimulation consistently produced vasodilations of $8.10 \pm 0.83\%$ ($n=24$) of the resting vessel diameter (Figure 5.4B). Time to peak dilation from the onset of the nerve stimulus was $20.94 \pm 1.39$s ($n=24$) and the time to 90% recovery of resting vessel diameter was $41.14 \pm 1.83$s ($n=24$). Treatment with L-NAME ($10^{-4}$M) did not reduce the size of the dilation (Figure 5.5A), however the $\beta$-adrenoceptor antagonist propranolol ($2\times10^{-6}$M), essentially eliminated the response (Figures 5.4C, 5.5A) and any residual component was abolished by guanethidine ($5\times10^{-6}$M, Figure 5.5A). When applied first to sympathetic control dilations, guanethidine completely abolished the response ($P < 0.05$, $n=6$).

**Sensory nerve-mediated responses.** Sensory nerve-mediated vasodilations were initially studied in vessels treated with scopolamine ($10^{-6}$M), and either $\alpha,\beta$-mATP ($6\times10^{-6}$M), benextramine ($10^{-5}$M) and propranolol ($2\times10^{-6}$M) or guanethidine ($5\times10^{-6}$M) to block sympathetic nerve-mediated responses. Under these conditions, nerve stimulation failed to elicit a vasodilation. However, when the $\alpha_{1A}$-adrenergic agonist methoxamine ($3\times10^{-5}$M), was used to increase tone in the presence of scopolamine and guanethidine, nerve stimulation evoked large dilations equal to $17.25 \pm 1.94\%$ ($n=5$) of control vessel diameter (Figure 5.6A). These dilations showed prolonged time to peak of $113.81 \pm 18.53$s from the onset of the nerve stimulus while recovery to 90% of vessel diameter was $284.07 \pm 61.99$s ($n=5$). The addition of L-NAME ($10^{-5}$M) caused a vasoconstriction (Figure 5.6B) and an apparent increase in the size of the vasodilation (Figure 5.5B), however this was not statistically significant, and was also not significant when absolute measures of vasodilation were compared ($16.05 \pm 1.51\mu$m vasodilation in control compared to $18.07 \pm 1.11\mu$m vasodilation after treatment with L-NAME).
Figure 5.5 Characterisation of sympathetic (A) and sensory (B) nerve-mediated vasodilations. Control (Cont) experiments were performed in the presence of (A) capsaicin (10^{-6}M), scopolamine (10^{-6}M), α,β-mATP (6x10^{-6}M) and benextramine (10^{-5}M) and (B) guanethidine (5x10^{-6}M), scopolamine and methoxamine (3x10^{-5}M). Histograms shows the effects of consecutive application of (A) L-NAME (10^{-5}M), propranolol (Prop, 2x10^{-6}M) and guanethidine (GE) to sympathetic dilations and in (B) L-NAME, CGRP{$_{8-37}$} (10^{-5}M) and capsaicin to sensory dilations. Columns represent the means ± s.e. means of at least 5 preparations. Results are expressed as a % of the dilatory response under control conditions. Control response in (A) was 8.14 ± 0.92% of resting vessel diameter, n=15 and (B) 21.93 ± 2.10%, n=5.

* Differs significantly from control (P < 0.05).
Figure 5.6 Representative traces illustrating sensory nerve-mediated vasodilation. Top panels show nerve stimuli (10Hz, 10s) which commenced 15s after \( \text{time} = 0 \). Panel A shows a control dilation after incubation of the vessel in scopolamine \((10^{-6}\text{M})\), guanethidine \((5 \times 10^{-6}\text{M})\) and methoxamine \((3 \times 10^{-5}\text{M})\). Panel B shows the response of the vessel after treatment with L-NAME \((10^{-5}\text{M})\) while panel C shows a significant reduction in the size of the dilation after treatment with \(\text{CGRP}_{8-37}\) \((10^{-6}\text{M})\). In D, the response has been abolished by the addition of capsaicin \((10^{-6}\text{M})\).
subsequent addition of the peptide antagonist CGRP$_{8-37}$ (10$^{-6}$M) significantly reduced the size of the dilation (Figure 5.5B, 5.6C). When expressed as a % of the L-NAME treated response, the reduction was equal to 70.54 ± 4.71% ($n=5$, $P<0.05$). The further addition of capsaicin (10$^{-6}$M) abolished the response (Figures 5.5B, 5.6D). The residual component left after treatment with CGRP$_{8-37}$ was not affected by a combination of the neurokinin receptor antagonists GR8334 (10$^{-6}$M, NK1), SR48968 (10$^{-7}$M, NK2) and SR142801 (5×10$^{-7}$M, NK3, $n=3$, data not shown). These drugs also did not affect the size of sensory nerve-mediated vasodilations when applied prior to L-NAME and CGRP$_{8-37}$ treatment.

Para sympathetic nerve-mediated responses. Parasympathetic nerve-mediated responses were initially studied after the vessel had been treated with guanethidine (5×10$^{-6}$M) and capsaicin (10$^{-6}$M). Under these conditions, nerve stimulation failed to evoke a dilation and it was necessary to increase tone with methoxamine (3×10$^{-5}$M). Even under these conditions, however, only 4 of 11 preparations showed a vasodilatory response. When seen, vasodilations had an average size of 8.12 ± 0.64% of vessel diameter ($n=4$), with a time to peak vasodilation of 15.07 ± 1.65 s from the onset of the nerve stimulus and a recovery to 90% of original vessel diameter of 27.38 ± 3.32 s ($n=4$, Figure 5.7B). The dilation was blocked by scopolamine (10$^{-6}$M, $n=3$, Figure 5.7C). Increasing the time period of the nerve stimulus from 10 to 20 s did not assist in uncovering a parasympathetic nerve-mediated dilation, nor did it alter the characteristics of dilations on the few occasions they were seen. Responses were not considered consistent enough to warrant further investigation.
5.3 DISCUSSION

This chapter has demonstrated the presence of multiple vasodilator pathways in small arteries of the rat hepatic mesentery. Abolition of sympathetic nerve-mediated vasoconstrictions uncovered a β-adrenoceptor mediated vasodilation which operates independently of NO. Sensory nerves were shown to mediate vasodilation via the activation of postsynaptic CGRP receptors. This response was again independent of NO and presumably the endothelium. While the in vitro physiology experiments were unable to demonstrate any significant parasympathetic innervation, they did reveal an important role for NO, which acts to oppose nerve-mediated vasoconstriction and increased vascular tone.

In Chapter 4, it was shown that the arteries of the rat hepatic mesentery possess a dense sympathetic innervation and that these nerves mediate a vasoconstrictor response via post synaptic α1A-adrenoceptors and P2X-purinoceptors. Once the constrictor response was abolished, a sympathetic vasodilatory component was uncovered without the need for further increases in tone. This response was due to noradrenaline acting on β-adrenoceptors, independently of NO and presumably the endothelium. These results agree with other studies in hepatic, mesenteric and coronary vascular beds, where β-adrenoceptor mediated responses were shown to be endothelium-independent (Shiraishi et al. 1997; Begonha et al. 1995; Bea et al. 1994). Participation of either β1- or β2-adrenergic receptors was found in these other vessels and further experiments are therefore planned to subtype the β-receptors involved in the rat hepatic artery, using RT-PCR to assess receptor mRNA expression and specific β-adrenergic receptor antagonists, such as atenolol (β1, Barrett et al. 1973) and ICI 118,551 (β2, Bilski et al. 1983) in physiological experiments. No role could be found for sympathetic nerves releasing ATP and mediating vasodilation via postsynaptic P2X-purinoceptors in the current study.

It is interesting to note that the time course of the sympathetic nerve-mediated vasodilations lagged behind that of sympathetic constrictions. A similar relationship between constrictor and dilator responses has been described in the dog hepatic artery (Richardson & Withrington 1977). The function of this dilatory pathway may be to attenuate or prevent excessive sympathetic noradrenaline mediated pressor responses.
Sensory innervation of the small arteries of the rat hepatic mesentery was confirmed immunohistochemically by the demonstration of fibres containing CGRP-like immunoreactivity and substance P-like immunoreactivity, agreeing with other studies that have looked at the innervation of the rat hepatic artery (Sasaki et al. 1986; Barja et al. 1983). Fibres containing CGRP-like immunoreactivity were more numerous than those for substance P. In general, the rat cardiovascular system shows this dominance of CGRP versus substance P innervation (Wharton et al. 1986). The substance P containing fibres and the majority of CGRP fibres surrounding the hepatic artery of the rat are susceptible to destruction by capsaicin (Barja & Mathison 1984; Wharton et al. 1986), indicating they are primary afferent sensory neurons.

To assess the involvement of the sensory nervous system, experiments were initially performed after the sympathetic response had been blocked with α,β-mATP, benextramine and propranolol. Unfortunately, it was not possible to see dilations under these conditions or when sympathetic nerves were blocked with guanethidine, and so vessels were preconstricted with the α1A-adrenergic receptor agonist methoxamine (Holck et al. 1983; Tsujimoto et al. 1989; Hwa et al. 1995). Methoxamine was chosen in order to mimic the natural mechanism of vasoconstriction as closely as possible (Chapter 4). In addition to inducing a sustained contraction, methoxamine also induced rhythmic contractions or vasomotion. Vasomotion may occur spontaneously or be stimulated in vessels with agonist induced tone (Gustafsson 1993). Studies in the rat carotid artery found that in 3-4 week old animals, the force of agonist induced contractions decreased with time (Eddinger & Ratz 1997), similar to the findings of this study in the arteries of the rat hepatic mesentery. Vasomotion is believed to be myogenic in origin (Morita et al. 1995) and is closely linked to Ca2+ signaling, through both voltage dependent Ca2+ channels and intracellular Ca2+ stores (Gustafsson 1993; Eddinger & Ratz 1997; Griffith & Edwards 1997).

After preconstriction with methoxamine, nerve stimulation in the presence of guanethidine and scopolamine produced a large dilation that could be blocked by capsaicin, indicating it was due to the release of transmitter from sensory nerves (Holzer 1991). At high concentrations, capsaicin has been shown to have non-selective effects, including an alteration of smooth muscle cell contractile sensitivities (Holzer 1998). These non-specific actions are reversible and are believed to be due to an action on
membrane ionic currents (Lo et al. 1995). Such non specific effects were unlikely in the current series of experiments, since control experiments demonstrated that capsaicin (10⁻⁶M) did not influence the size of nerve-mediated contractions over time periods equivalent to those of the current experiments (section 4.2.3). A similar finding has been demonstrated in rat iris arterioles (S. Sandow pers. comm.).

Treatment of the sensory nerve-mediated response with L-NAME showed an apparent increase in the size of the dilation, however this can be attributed to the further increase in vascular tone produced by the NOS inhibitor. The dilation was however, significantly reduced by the CGRP peptide antagonist CGRP₁₈₋₃₇ (Han et al. 1990b). These results, in conjunction with the immunohistochemistry and RT-PCR results indicate that sensory nerves mediate vasodilation in this tissue via the activation of postsynaptic CGRP₁ receptors, acting independently of NO. A recent study in human cerebral vessels found that CGRP₁ receptor mRNA expression was constant both in the presence and absence of the endothelium (Edvinsson et al. 1997). It is likely that in the arteries of the rat hepatic mesentery, the receptors are also located on the smooth muscle.

Treatment of the sensory nerve-mediated response with CGRP₁₈₋₃₇ did not completely abolish the dilation, however the residual component was not affected by any of the neurokinin receptor antagonists, despite the presence of substance P containing nerve fibres. ATP has previously been suggested as a co-transmitter from sensory nerves (Burnstock & Ralevic 1994), and it has also recently been described as mediating a component of the non-adrenergic non-cholinergic vasodilation in the small mesenteric artery of the rabbit, via endothelial P₂Y-purinoceptors and NO release (Kakuyama et al. 1998). Since the dilation in the present study was not affected by L-NAME, it seems likely that the residual component resulted from incomplete antagonism by the CGRP peptide antagonist when used at 10⁻⁶M. It is also possible that CGRP₂ receptors, resistant to hCGRP₁₈₋₃₇ (Dennis et al. 1990), mediate the residual component, however at this time such a possibility cannot be substantiated.

The absence of a neurokinin responses raises the question as to the role of substance P containing nerve fibres. Substance P containing fibres innervate the majority of peripheral blood vessels, however a lack of correlation between innervation and functional vasomotor response has been described previously (Barja et al. 1983;
Kawasaki et al. 1990a; Hill et al. 1996; Hill & Gould 1997). This may be because the fibres play a purely afferent role, independent of any motor or efferent function and are not involved in the regulation of tissue perfusion (Kawasaki et al. 1990a).

In contrast to the relatively fast sympathetic nerve-mediated vasodilatory response, the sensory vasodilations were slow in onset and had a protracted recovery period of up to 5 minutes in duration. These slow responses are typical of the CGRP-mediated responses seen in the mesenteric artery (Han et al. 1990a; Gyoda et al. 1995) and of neuropeptide responses in general (Morris et al. 1995). In Chapter 4, NPY was shown to mediate a slow vasoconstriction and it is therefore possible, that the sensory nerve-mediated response functions to counteract prolonged sympathetic nerve activation and NPY mediated constrictions. The demonstration of CGRP sensory vasodilations in the small arteries of the rat hepatic mesentery correlates well with other studies in the rat main hepatic artery. In these earlier studies, applied CGRP and electrical field stimulation produced endothelium-independent vasodilations (Bratveit & Helle 1991). Sensory neurogenic responses are also mediated by smooth muscle cell CGRP\textsubscript{1} receptors in the dog hepatic artery (Shiraishi et al. 1998). It is of note that the response appears to be consistent throughout the vascular bed, as compared to the rat mesenteric arterial bed for example, where different \(\alpha_1\)-adrenergic receptor subtypes dominate the physiological response in the larger conduit vessels versus the smaller resistance vessels (Kong et al. 1994).

Parasympathetic nerve-mediated responses were investigated after preconstriction with methoxamine, as preliminary experiments in the presence of guanethidine and capsaicin, showed no evidence of a dilatory response. Even with preconstriction, however, dilatory responses were infrequent and small when compared to sensory nerve-mediated responses. Investigation of these responses indicated that they did not appear to be due to differences in animal weight, sex or tissue preparation procedures. It appears therefore, that the parasympathetic nervous system makes a minimal contribution to the regulation of blood flow in this vascular bed, which is in agreement with previous studies (Greenway & Stark 1971). The lack of VIP-like immunoreactivity or NADPH-diaphorase staining further confirmed the absence of a functional parasympathetic innervation, as acetylcholine, VIP and NO have been shown
to be co-localised in other parasympathetic nerves supplying blood vessels (Lundberg 1981; Morris et al. 1995; Yoshida & Toda 1997).

These results agree with others that suggest there is little evidence for a parasympathetic innervation to the majority of vascular beds (Doods et al. 1989; Van Zwieten & Doods 1995). Vessels that have been shown to possess a functional cholinergic innervation include the arterioles of the guinea pig submucosal plexus (Neild et al. 1990), rabbit middle cerebral arteries (Van Riper & Bevan 1992), rat uterine arteries (Sato et al. 1996) and the lingual artery of a number of different species (Yoshida & Toda 1997). RT-PCR results from the current study, however, do suggest the presence of muscarinic receptors in the arteries of the rat hepatic mesentery. These results are supported by immunohistochemistry of hepatic vessels using antibodies directed against the M3 muscarinic receptor, which show endothelial cell staining (Hill, unpublished results) and a series of studies by Zygmunt et al (Zygmunt et al. 1994a; Zygmunt et al. 1994b; Zygmunt & Hogestatt 1996), that describe applied acetylcholine as mediating an endothelium-dependent relaxation in this vascular bed. The specific receptor subtype has not been characterised, although the dilation is believed to be mediated by both NO and EDHF.

Although NO has been described as a neurotransmitter in a number of different vascular beds (Goadsby et al. 1996; Zheng et al. 1997), no evidence was found in this study for the presence of NOS in perivascular nerves. There was however, good evidence that NO is released from endothelial cells in this tissue. Previous studies have confirmed that NADPH-diaphorase reactivity is a reliable indicator of endothelial NOS and the results presented here demonstrated endothelial cells with a characteristic punctate staining (O'Brien et al. 1995).

NO release from endothelial cells can be due to a number of different mechanisms including receptor activation, basal release, shear stress or changes in the level of vascular tone (Moncada et al. 1991; Fleming & Busse 1995; Vargas et al. 1990). No evidence was seen for basal release of NO in the present study, as the application of the NOS inhibitor L-NAME (Rees et al. 1990) did not cause a vasoconstriction under conditions of resting tone. In other vessels, for example, rat iris arterioles and feline superior mesenteric artery (Hill & Gould 1995; Macedo & Lautt 1997) application of L-NAME produces a pronounced vasoconstriction. Lack of basal
release of NO has been described in the hepatic artery previously (Greenblatt et al. 1993; Macedo & Lautt 1997) and may imply that the hepatic arterial vascular bed exhibits less constitutive synthesis of NO than other vascular beds (Mathie et al. 1991). Interestingly though, in our study, L-NAME did cause a vasoconstriction in vessels that had been preconstricted with methoxamine. The ability of NOS inhibitors to cause vasoconstriction in vessels with increased tone has been described in other vascular beds (Crawley et al. 1990; Vargas et al. 1990) and may be due to an increase in shear stress associated with decreased vessel diameter or increased perfusion pressure (Fleming & Busse 1995; Macedo & Lautt 1998). It may also be due to an increase in NO release directly associated with an increase in tension or vascular tone (Crawley et al. 1990; Vargas et al. 1990).

Further evidence for the stimulated release of NO from endothelial cells was seen by the ability of L-NAME to potentiate sympathetic nerve-mediated vasoconstrictions. This potentiating effect is not specific to this vascular bed (Vo et al. 1991; Macedo & Lautt 1998), nor is it specific to sympathetic nerve-mediated contractile responses (Vo et al. 1992; Abdullah et al. 1997). These effects of L-NAME are most likely due to an inhibition of increased NO release following increased vascular tone and/or shear stress produced during the sympathetic vasoconstriction. It has been proposed that this is a local vascular reflex mechanism, designed to oppose vasoconstriction, compared to basal release which acts to control vascular tone (Vargas et al. 1990; Macedo & Lautt 1998). This pathway therefore has a significant role in the regulation of local haemodynamics (Greenblatt et al. 1993). How changes in vascular tone can affect the activity of endothelial NOS is uncertain, although changes in intracellular Ca\(^{2+}\) have been demonstrated (Adeagbo et al. 1994; Fleming & Busse 1995).

In the present experiments, the NOS inhibitor L-NAME, was used to eliminate the involvement of NO in nerve-mediated vasodilatory responses in the arteries of the rat hepatic mesentery. It is likely that the concentration of drug used in these experiments was sufficient to inhibit NO release, as this concentration has inhibited NO effects in rat iris arteriole preparations investigated in the same laboratory (Hill & Gould 1997, section 2.6.2). The effectiveness of L-NAME at 10\(^{-5}\)M in preventing release of NO from the endothelium, as described above, further confirms the efficacy of the drug...
and suggests that the endothelium is intact in these preparations. Further confirmation of these two points would require histological examination of the structure of the hepatic arteries after incubation in vitro, along with the abolition by L-NAME of acetylcholine induced vasodilations.

In summary, this chapter has shown that there are multiple vasodilatory pathways present in the small arteries of the rat hepatic mesentery. Sympathetic and sensory nerve-mediated responses are due to the activation of β-adrenergic and CGRP₁ receptors respectively. Interestingly, while none of the neuroreceptor-mediated responses appear to be linked to release of NO, endothelial NO was shown to be released in response to increased vascular tone and vasoconstriction.

An understanding of the vasodilatory mechanisms in the hepatic arterial bed is of clinical significance when considering patients with altered hepatic drug metabolism. One mechanism that may explain reduced liver function in these patients is the oxygen limitation theory, which describes the presence of a functional barrier causing reduced oxygen diffusion to the hepatocytes (Morgan & McLean 1995; LeCouteur & McLean 1998). By increasing oxygenated blood flow to the liver it may be possible to improve hepatocyte function, as vasodilators have been able to improve liver function in cirrhotic rats (Reichen & Le 1986). More selective use of vasodilators may help to avoid systemic hypotension. Cirrhosis is also characterised by a generalised vascular hyporesponsiveness (Safka et al. 1997). Nitric oxide mediated vasodilation appears to be integrally related to these abnormalities and reduction of NO production has been shown to correct arterial vasodilation in experimental cirrhosis (Niederberger et al. 1995). The fact that NO plays such an important modulatory role in hepatic arterial flow would need to be taken into consideration if systemic inhibition of NO production is to be used as a therapeutic measure. In line with these clinical considerations, future studies applying a similar experimental approach to a cirrhotic rat model, are currently being planned.
The innervation of autonomic targets is accompanied by significant modification of neuroreceptor function in the postsynaptic tissue. These changes (as reviewed in section 1.10.1) can be summarised as increases in the density of receptors, maturation of the coupling of the receptor to the appropriate intracellular second messenger systems or changes in the receptor subtype or subunit mediating the post-synaptic response. Specific regulation of receptor expression may be achieved through transcription of DNA or translation of mRNA into protein.

In the mesenteric artery of the rat, the sympathetic and sensory nerve fibre plexuses develop over the first three postnatal weeks (Hill et al. 1983; Rayner & van Helden 1994). Functionally mature nerve-mediated contractile responses cannot be elicited before 14 days postnatal (Serio et al. 1996), correlating with the appearance of adult-like ejps (Hill et al. 1983). Prior to this period, intracellular recordings from animals aged 4 to 9 days old, showed slow depolarizing potentials which were mediated by α-adrenoceptors. From day 9 onwards, ejps, which were resistant to α-adrenergic antagonists were recorded (Hill et al. 1983). Following denervation studies in this vascular bed, electrical responses similar to those seen during the early stages of development were recorded (Hill et al. 1985a; Hill et al. 1985b), suggesting that a similar sequence of events occurs during regeneration as occurs during development.

A comparable developmental pattern has been described in the mouse vas deferens. Sympathetic nerve fibre networks develop rapidly, with the nerves extending along the length of the vas deferens by day 9 postnatal and adult-like densities being
present at 25 days. Excitatory junctional potentials, however could not be detected until
day 18 and mature responses were not recorded until after 30 days (Furness et al. 1970). In a more recent study, development of the adult nerve-mediated response in the mouse vas deferens has been associated with the maturation of fast purinergic transmission. Furthermore, an increase in P$_{2X1}$-purinoceptor mRNA expression has been demonstrated between postnatal days 10 and 42, correlating temporally with these physiological changes during development (Liang et al. 1998).

The innervation and responses of the mesenteric artery also change with ageing and hypertension. A decrease in the number of sensory nerves around the mesenteric artery was observed during the development of hypertension in rats (Kawasaki et al. 1990b) and an impairment of endothelium-dependent vasodilation due to acetylcholine has been described in both aged and hypertensive rats (Fujii et al. 1993; Atkinson et al. 1994). In the rat aorta, changes seen in $\alpha_1$-adrenergic receptor responsiveness with maturation and ageing (Gurdal et al. 1995a) have been attributed to alterations in the expression of specific $\alpha_1$-adrenergic receptor subtypes (Gurdal et al. 1995b). Recent studies have also suggested phenotypic changes in the functional $\alpha$-adrenoceptor subtypes mediating vasoconstriction in the mesenteric artery of rats aged 16 to 52 weeks (Smith & McGrath 1996).

In earlier chapters, it was shown that RT-PCR is a useful tool when characterising vascular receptor subtypes. To determine whether the loss of $\alpha$-adrenergic responsiveness and the development of ejps is associated with the transcriptional regulation of specific receptor subtypes, RT-PCR was used to study the developmental mRNA expression of neurotransmitter receptors in the rat mesenteric arterial bed. The receptor subtypes studied were those of the $\alpha$-adrenergic, neurokinin, muscarinic and P$_{2X}$-purinergic receptor families. In order to assess alterations in receptor mRNA levels with maturation, experiments were extended to also include rats aged between 8 and 12 months.
6.2 RESULTS

6.2.1 General observations

Results showed that all receptor genes expressed at 28 days postnatal were already expressed by day 7 (Tables 6.1, 6.2 and 6.3). Overall, two developmental patterns of receptor mRNA expression were apparent, with some receptors showing no change in their relative detection levels, being either strongly (α1A, α2B, NK3, P2X1, P2X2, P2X4 and P2X7) or weakly (α2A, α2C, NK1, P2X3, P2X5) expressed at all ages. Relative to these receptors and taking into account the constant starting amount of RNA in each age group, some receptor subtypes showed a developmental increase in their expression up to 14 days postnatal (α1B, α1D, M2, M3 and M5).

All PCR fragments detected corresponded to the predicted fragment size for each receptor subtype and the relative expression of cDNA was consistent between repeats of experiments. For the purinergic P2X1-receptor, extra bands were seen above and below the predicted fragment (Figure 6.3, lane 2). These bands were not seen in control DRG tissue, however they were also detected in RNA extracted from the hepatic artery (Chapter 4.2.2). Splice variants exist for the P2X2-receptor and it may be that these bands represent splice variants of the P2X1-receptor which have not yet been identified. Sequencing of the fragments is planned in order to identify these cDNA products. Occasionally, use of the P2X2-purinergic primers produced a band at 620bp (Figure 6.3, lane 3). This band corresponded to amplification of genomic DNA and was seen in minus reverse transcriptase controls for this primer pair, in spite of the DNase treatment of the original RNA sample. Fragments corresponding to genomic DNA were not seen with other primer pairs (Figures 6.1 and 6.2). No PCR product was detected when RNA was omitted from reactions (Figures 6.1, 6.2 and 6.3).

The efficiency of the primer pairs was confirmed using other tissues (Chapters 3 and 4). For all primer pairs except the purinergic receptors, results were obtained using random primers and oligo(dT) primers. Results were similar and only results using random primers are illustrated. Only random primers were used for the purinergic receptor experiments. The results from the mature rat preparations at 240 and 360 days of age did not differ, and therefore have been combined.
6.2.2 *Messenger RNA expression of receptor subtypes throughout development*

**α-adrenoceptors.** The profile of receptor mRNA expression for the α-adrenergic receptors at the different postnatal ages is shown in Table 6.1. The expression of some receptors showed no changes with development, being either strongly (α1A, α2B) or weakly expressed (α2A, α2C) at all ages studied. Relative to the expression of these receptors, others showed a developmental increase in expression up to 14 days postnatal (α1B, α1D).

PCR products representing the expression of α1-adrenergic receptors at 4 weeks of age are shown in Figure 6.1A. Oligonucleotide primers specific for the α1A subtype amplified a very strong cDNA band of 251 bp (Figure 6.1A, lane 4) and strong bands were also detected for the α1B- and α1D-adrenergic receptors (Fig. 6.1A, lanes 2 and 6 respectively).

The mRNA transcripts encoding the α2-adrenergic receptor subtypes are shown in Figure 6.1B. PCR products of 338 and 425 bp corresponded to the desired fragments for the α2A and the α2C primer pairs respectively (Figure 6.1B, lanes 2 and 6), while a strong band was generated with the α2B specific primers (Figure 6.1B, lane 4).

**Neurokinin and muscarinic receptors.** Messenger RNA expression of the neurokinin and muscarinic receptor subtypes at different postnatal ages is presented in Table 6.2. The NK1 and NK3 receptors showed no change in expression levels, being either weakly or strongly detected at all ages studied respectively. In contrast, the M2, M3 and M5 muscarinic receptors showed a relative increase in receptor mRNA detection up to 14 days postnatal. The NK2 and M4 receptor subtypes were not detected in any age group.

Expression of neurokinin receptor mRNA levels at 4 weeks of age is illustrated in Figure 6.2A. A faint band of 537 bp (lane 2) represents product amplified by the NK1 receptor primer pair, while no PCR product was generated with the NK2 primer pair (lane 4). A very strong band of 476 bp corresponds to the desired fragment as defined by the NK3 primers (lane 6).

Figure 6.2B shows the expression of gene transcripts for muscarinic receptors at 4 weeks of age. A weak band of 552 bp (lane 4) represents the M2 muscarinic receptor.
Table 6.1 Developmental profile of α-adrenergic receptor mRNA expression in the rat mesenteric artery

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Age Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Birth days</td>
</tr>
<tr>
<td>α₁-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>α₁A</td>
<td>++</td>
</tr>
<tr>
<td>α₁B</td>
<td>+</td>
</tr>
<tr>
<td>α₁D</td>
<td>0</td>
</tr>
<tr>
<td>α₂-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>α₂A</td>
<td>+</td>
</tr>
<tr>
<td>α₂B</td>
<td>++</td>
</tr>
<tr>
<td>α₂C</td>
<td>+</td>
</tr>
</tbody>
</table>

Crosses represent PCR product that corresponds to the size of the desired cDNA band. The number of crosses reflects the amount of amplified product generated after 35 cycles: [0] = never detected; [+] = weak detection; [++] = strong detection and [++++] = very strong detection.
Figure 6.1
Figure 6.1

Expression of α-adrenergic receptor mRNA in the mesenteric artery of 4 week old rats. Figure illustrates agarose gel electrophoresis of 8μl of PCR product amplified from cDNA that was reverse transcribed from DNase I treated total RNA. In A, the 405 base pair (bp) product in lane 2 corresponds to the size of the desired fragment as defined by the α1B specific oligonucleotide primers. Likewise, mRNA expression for the α1A and α1D receptors is confirmed by the presence of bands of 251 and 517 bp in lanes 4 and 6 respectively. In B, a 338 bp product in lane 2 corresponds to the size of the desired fragment as defined by the α2A specific oligonucleotide primers. Messenger RNA expression for the α2B and α2C receptors is confirmed by the presence of bands of 456 and 425 bp in lanes 4 and 6 respectively. No PCR products were seen in the receptor specific control experiments which contained no reverse transcriptase enzyme in the RT reactions (A, B: lanes 3, 5 and 7), and no product is seen in the control which did not contain RNA (A, B: lane 8). DNA markers (Lambda Hind III/ φX174 HaeIII) and (Pzt/Alu) appear in lanes 1 and 9 respectively (A, B).
Table 6.2 Developmental profile of neurokinin and muscarinic receptor mRNA expression in the rat mesenteric artery

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Birth 7 days</th>
<th>14 days</th>
<th>28 days</th>
<th>240 - 360 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurokinin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NK2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NK3</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Muscarinic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>M4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M5</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Crosses represent PCR product that corresponds to the size of the desired cDNA band. The number of crosses reflects the amount of amplified product generated after 35 cycles: [0] = never detected; [+] = weak detection; [++] = strong detection and [++++] = very strong detection.
Figure 6.2
Figure 6.2

Expression of neurokinin and muscarinic receptor mRNA in the mesenteric artery of 4 week old rats. Figure illustrates agarose gel electrophoresis of 8μl of PCR product amplified from cDNA that was reverse transcribed from DNase I treated total RNA. In panel A, the 537 base pair (bp) product in lane 2 corresponds to the size of the desired fragment as defined by the NK1 specific oligonucleotide primers. No NK2 product was amplified after 35 cycles (lane 4). Messenger RNA expression for the NK3 receptor is confirmed by a band of 476 bp in lane 6. In panel B, the band of 552 bp in lane 4 represents the M2 muscarinic receptor. A 790 base pair product in lane 6 corresponds to the size of the predicted fragment as defined by the M3 specific oligonucleotide primers. Messenger RNA expression for the M5 receptor is confirmed by a band of 594 bp in lane 9. The M1 and M4 products were not detected (lanes 2 and 8 respectively). No PCR products were seen in the receptor specific control experiments which did not contain reverse transcriptase enzyme (-RT) in reactions (A: lanes 3, 5 and 7, B: lanes 3, 5, 7 and 10). The -RT control is not shown for the M4 primers. There is no product seen in the control which did not contain RNA (A: lane 8, B: lane 11). DNA markers (Lambda Hind III/ φX174 HaeIII and Ptz/Alu) appear in (A) lanes 1 and 9 respectively and in (B) (Lambda Hind III/ φX174 HaeIII) lane 1.
Stronger bands of 790 and 594 bp (lanes 6 and 9) depict the levels of M3 and M5 muscarinic receptor mRNA respectively. No PCR product was seen for the M1 or M4 oligonucleotide primer pairs (lanes 2 and 8).

**P₂ₓ-purinoceptor expression.** Expression of the purinergic P₂ₓ(1-7)-receptors at the different ages is shown in Table 6.3. All subtypes were detected at all ages except for the P₂ₓ₆-purinoceptor, which was not seen at any age. When comparing the different age groups, there was no difference in the relative levels of expression amongst the receptors.

Expression of mRNA for the P₂ₓ-purinoceptors in the mesenteric artery of 7 day old rats is shown in Figure 6.3. The P₂ₓ₁- and P₂ₓ₄-purinoceptors showed very strong levels of mRNA expression (lanes 2 and 5 respectively). Strong bands were seen with oligonucleotide primers specific for the P₂ₓ₂- and P₂ₓ₇-purinoceptors (lanes 3 and 8), while only weak bands were detected for the P₂ₓ₃- and P₂ₓ₅-purinoceptors (lanes 4 and 6). No product was detected for the P₂ₓ₆-purinoceptor (lane 7).

6.2.3 **Messenger RNA expression of receptor subtypes in maturity**

An upregulation of receptor mRNA expression was detected for the P₂ₓ₃-, P₂ₓ₅- and P₂ₓ₇-purinoceptors in rats aged 240 to 360 days old. An upregulation was also seen for the α₂B, M2, and M3 receptors while a down regulation was noted for the α₁D and NK3 receptor subtypes. For these receptors, however, fluctuations were within the ranges previously detected during development. The M1 muscarinic receptor was only detected in the mature rats, albeit at a low level of expression.
**Table 6.3.** Developmental profile of P$_{2\times}$-purinergic receptor mRNA expression in the rat mesenteric artery

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Age Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days</td>
</tr>
<tr>
<td>P$_{2\times}$-Purinergic</td>
<td>++</td>
</tr>
<tr>
<td>P$_{2\times}$1</td>
<td>+++</td>
</tr>
<tr>
<td>P$_{2\times}$2</td>
<td>++</td>
</tr>
<tr>
<td>P$_{2\times}$3</td>
<td>+</td>
</tr>
<tr>
<td>P$_{2\times}$4</td>
<td>+++</td>
</tr>
<tr>
<td>P$_{2\times}$5</td>
<td>+</td>
</tr>
<tr>
<td>P$_{2\times}$6</td>
<td>0</td>
</tr>
<tr>
<td>P$_{2\times}$7</td>
<td>++</td>
</tr>
</tbody>
</table>

Crosses represent PCR product that corresponds to the size of the desired cDNA band. The number of crosses reflects the amount of amplified product generated after 35 cycles: [0] = never detected; [+ ] = weak detection; [++] = strong detection and [+++ ] = very strong detection.
Figure 6.3
Expression of $P_{2X}$-purinergic receptor mRNA in the mesenteric artery of 7 day old rats. Figure illustrates agarose gel electrophoresis of 8μl of PCR product amplified from cDNA that was reverse transcribed from DNase I treated total RNA. Lane 2 shows a very strong PCR product at 397 base pairs (bp), which is the predicted band for the $P_{2X1}$-purinoceptor primer pair. Extra bands are present above and below the desired fragment. The $P_{2X2}$- and $P_{2X3}$-receptors are represented by bands at 273 and 575 bp respectively in lanes 3 and 4. A very strong 396 bp product in lane 5 corresponds to the size of the predicted fragment as defined by the $P_{2X4}$ specific oligonucleotide primers. Messenger RNA expression for the $P_{2X5}$- and $P_{2X7}$-receptors is confirmed by bands of 379 and 567 bp in lanes 6 and 8 respectively, while no product was detected for the $P_{2X6}$-receptor (lane 7). There is no product seen in the control which did not contain RNA (lane 9). DNA markers (Lambda Hind III/ $\Phi X174$ HaeIII) and (Pitz/Alu) appear in lanes 1 and 10 respectively.
6.3 DISCUSSION

During postnatal development, intracellular recordings from smooth muscle cells of the rat mesenteric artery, showed a change from an \( \alpha \)-adrenergic response seen up to 9 days postnatal, to a nonadrenergic response mediated by ejps and presumably ATP after this period (Hill et al. 1983; Ishikawa 1985). In this chapter, it has been shown that there were no changes in \( \alpha \)-adrenergic receptor gene expression to explain the loss of the \( \alpha \)-adrenergic response to sympathetic nerve stimulation and similarly, there was no increase in receptor mRNA expression for any of the 7 subtypes of P2X-purinoceptors to account for the development of ejps in this tissue. This is in contrast to the rat superior cervical ganglion, where the \( \alpha_{1B} \)-adrenergic receptor gene was transiently expressed during development (Vidovic & Hill 1997) and to studies in the mouse vas deferens, where an increase in P2X1-purinoceptor mRNA levels corresponded to the appearance of ejps and a fast purinergic contractile response (Liang et al. 1998).

While the absence of changes in receptor mRNA expression may suggest that regulation occurs at the level of protein expression, receptor mRNA and protein expression have been positively correlated in a number of tissues (Grant & Landis 1991b; Gurdal et al. 1995b; Li et al. 1995; Rokosh et al. 1996). Other nerve induced changes may therefore account for the change in the physiological response. For example, alterations in receptor affinity profiles, changes in the rate of receptor turnover or alterations in receptor-coupling to second messenger pathways (Miller et al. 1996). In several tissues, receptors are expressed prior to innervation but are not effectively coupled to their signal transduction pathways during early development. The appearance of agonist induced functional responses has been correlated with the presence of nerves in these systems (Galper et al. 1977; Ludford & Talamo 1980; Bylund et al. 1982; Malfatto et al. 1990; Seagrave et al. 1996b). As a specific example, in the sweat glands of the rat footpad, non-functional muscarinic receptors are present in the early developmental period and, though a more complex innervation pattern exists in this tissue, with the sympathetic nerves undergoing a phenotypic conversion in response to sweat gland factor (Habecker & Landis 1994), the coupling of muscarinic receptors to sweat gland secretion does not occur until functional sympathetic cholinergic innervation is established (Grant & Landis 1991b). Sensory nerves may also play a role
in the development of this response, as the number of fibres showing substance P-like immunoreactivity in the sweat glands reach a peak during the second week of postnatal development (Hill et al. 1988), being the same time as the sympathetic nerves are beginning to undergo their phenotypic conversion (LeBlanc & Landis 1986).

Alternatively, the changes in physiological responses seen in the rat mesenteric artery could be due to the development of specialised neuromuscular junctions (Llewellyn-Smith 1984; Hill & Vidovic 1992; Luff 1994), or changes in neuronal phenotype (Nagao et al. 1994). In guinea pig seminal vesicles, for example, electrical field stimulation in newborns evokes a tonic contraction mediated by α-adrenoceptors, however at 2 weeks of age a fast phasic component appears, mediated by ATP at P_2X^7-purinoceptors (Pinna et al. 1997). Variations in the ratio of noradrenaline and ATP released from the sympathetic nerves are thought to contribute to this change.

As described earlier, the sympathetic and sensory innervation of the mesenteric artery develops during the first three weeks postnatal (Hill et al. 1983; Rayner & van Helden 1994). Since the overall profile of receptor mRNA expression did not change over this critical developmental time period, it would appear that the initial transcription of receptor genes studied in this tissue is established independently of innervation. A similar result was obtained in the rat iris. In the iris, sympathetic innervation also occurs over the early postnatal period however the pattern of α-adrenoceptor expression was again consistent from birth through to 13 months of age (Vidovic et al. 1987; Vidovic & Hill 1995).

Although RT-PCR, as used in this study, is not a quantitative technique, it was of interest to note that, while all the receptors expressed by day 7 postnatal were also expressed at 4 weeks, when the relative amounts of product amplified were considered, two general patterns became apparent. There were those receptors that showed constant mRNA expression levels from birth through to maturity, these included the α_{1A^-}, α_{2B^-} adrenergic, NK3, P_{2X^1^-}, P_{2X^2^-}, P_{2X^4^-} and P_{2X^7^-}purinergic receptors, which were strongly detected in all age groups, and the α_{2A^-}, α_{2C^-}adrenergic, NK1, P_{2X^3^-} and P_{2X^5^-}purinergic receptors which were weakly expressed in all age groups. On the other hand, the α_{1B^-}, α_{1D^-}adrenergic, M2, M3 and M5 muscarinic receptors showed an increase in mRNA expression during the first two postnatal weeks. This period corresponds to the period of ramification of sympathetic and sensory nerve fibres over the arterial surface.
(Hill et al. 1983; Rayner & van Helden 1994) and is therefore suggestive of an inductive influence. A similar phenomenon has been described in the rat heart, where developmental expression of $\alpha_1$-, $\beta$-adrenergic and muscarinic receptors peaks around the first or second week after birth, a time when innervation is likewise maturing rapidly (Robinson 1996). The time course of cholinergic innervation of the mesenteric artery is unknown, but presumably mirrors that of the other two nerve populations.

The influence of adrenergic control over blood flow during ageing has been questioned, some studies finding no decline in function (Duckles et al. 1985), others showing decreased postsynaptic $\alpha_2$- but not $\alpha_1$-adrenergic responsiveness (McAdams & Waterfall 1986), while yet other studies show altered expression of specific $\alpha_1$-adrenergic receptor subtypes (Gurdal et al. 1995b; Smith & McGrath 1996). This study has shown no significant changes in mRNA expression for any of the adrenergic receptors in the mesenteric artery associated with maturation. These results are in agreement with another recent RT-PCR study, which likewise found no changes in $\alpha_1$-adrenergic receptor mRNA levels in the mesenteric artery of rats aged 3 to 24 months old (Xu et al. 1997). Interestingly, they did note decreased expression of specific receptor subtypes in the aorta and renal artery. In the rat tail artery, age related decreases in contractile responses have been attributed to postreceptor mechanisms and impaired endothelial NO production, rather than changes in $\alpha$-adrenoceptor density or function (Vila et al. 1997). In the rat aorta, altered $\beta$-adrenoceptor mediated relaxation has been linked to blunted activation of adenylyl cyclase (Chin et al. 1996).

Alterations in receptor signaling pathways may also be responsible for the described decrease in response of blood vessels to acetylcholine with ageing (Hynes & Duckles 1987; Atkinson et al. 1994; Fujii et al. 1993), as we saw no down-regulation of muscarinic receptor mRNA expression in mature animals. Altered coupling of muscarinic receptors to signal transduction mechanisms has been reported in the rat submandibular gland and cerebral cortical membranes, where a loss or reduced coupling to phospholipase C is seen with ageing (Seagrave et al. 1996a; Narang et al. 1996).

Changes in purinergic receptor mediated responses have been associated with ageing in the rat tail artery, where the relative contribution of ATP to neurogenic contractions was seen to decrease (Bao et al. 1989). Given the results presented here, in which the only change noted was a relative increase in some $P_{2X}$-purinoceptor mRNA
levels, it would suggest that any functional alterations are at either the prejunctional or postreceptor levels. In contrast to our results, a recent study using *in situ* hybridization detected no mRNA transcript for the $P_{2X1}$, $P_{2X2}$, or $P_{2X4}$ receptors in the superior mesenteric artery of adult rats (Nori et al. 1998). This discrepancy may however, be explained by the differences in sensitivity between the RT-PCR and *in situ* hybridization techniques. Alternatively, it may be that there is a differential distribution of mRNA for the $P_{2X}$-receptors in the superior mesenteric artery versus mesenteric resistance vessels, as has been described for $\alpha_1$-adrenergic receptors in this vascular bed (Kong et al. 1994). Further experiments assessing receptor mRNA expression from each region would help clarify these results.

Results also demonstrated that the level of neurokinin receptor mRNA expression remained constant from birth through to maturity, despite reports of decreased vasodilatory responses to sensory nerve stimulation and decreases in the density of sensory fibres, during ageing and hypertension in the rat mesenteric arterial bed (Kawasaki et al. 1990b; Li & Duckies 1993).

In conclusion, this chapter has examined the mRNA expression of $\alpha$-adrenergic, neurokinin, muscarinic and $P_{2X}$-purinergic receptor subtypes during the development and maturation of the rat mesenteric artery, in an attempt to explain changes seen in responsiveness to nerve stimulation during these periods. In general, there was no correlation between the appearance of the nerves and the initial mRNA expression of the receptors studied. The overall profile of receptor expression did not change over the developmental period. There was also no evidence for the loss of the $\alpha$-adrenergic response to nerve stimulation being due to the cessation of mRNA expression for any of the $\alpha$-adrenergic receptors, nor was there any increase in $P_{2X}$-purinergic receptor mRNA expression to correlate with the appearance of ejps. The changes seen in responsiveness to sympathetic nerves during development in this vessel may therefore result from nervous regulation of receptor function at the level of translation of mRNA into protein, alterations in the coupling of receptors to their effector systems, the development of specialised neuromuscular junctions or neuronal modifications.

While the mRNA levels for some receptors remained constant throughout development, others showed an apparent increase in mRNA detection which mirrored postnatal increases in nerve density. Thus, while there was little evidence for the
influence of nerves on the absolute mRNA expression of neuroreceptors in the mesenteric artery of the rat, results do suggest that the adult levels of mRNA for the \( \alpha_{1B} \), \( \alpha_{1D} \)-adrenergic receptors and muscarinic receptors could require the presence of nerves.
7.1 INTRODUCTION

The ramification of autonomic nerve fibres in target tissues occurs extensively in the early postnatal period in the rat (De Champlain et al. 1970; Owman et al. 1971). In the vascular system, although innervation patterns may vary between different vascular beds, adult-like innervation densities are established within the first 4 weeks of life (Todd 1980; Cowen et al. 1982).

In Chapter 6, the hypothesis that alterations in receptor mRNA expression may explain the physiological changes that are seen in the rat mesenteric artery during development was examined. RT-PCR results showed that there were no changes in \( \alpha \)-adrenoceptor mRNA expression to explain the loss of \( \alpha \)-adrenergic receptor responsiveness during development, nor was there an increase in receptor mRNA expression for any of the 7 subtypes of P2X-purinoceptors that correlated with the development of ejps in this tissue. All receptors present at 28 days were expressed by day 7, however, a relative upregulation of receptor mRNA was described for some receptors during the first two postnatal weeks. Although some fibres are present at birth, this time period is temporally associated with the ramification of the sympathetic and sensory nerve fibres over this vascular bed (Hill et al. 1983; Rayner & van Helden 1994). The results therefore suggested that the nerves may be required for the induction of the adult patterns of mRNA expression.

The role of innervation in the establishment or maintenance of receptor populations appears to be variable. In rat sweat glands, removal of sympathetic nerves, either neonatally or in the adult, did not affect the expression of muscarinic receptor
protein (Grant & Landis 1991b; Grant et al. 1991), while in the rat kidney, radioligand binding studies indicated that denervation produces a relative increase in $\alpha_1$, $\alpha_2$, and $\beta$-adrenergic receptor density (Yamada et al. 1986). In contrast, a recent study of the rat mesenteric artery noted a decrease in the density of adult $\alpha_{1A}$-adrenergic receptor protein following sympathetic denervation (Stassen et al. 1998). Very few studies in the vascular system have looked at the influence of denervation on receptor mRNA expression, however transcriptional regulation of $\alpha_1$-adrenergic receptor genes (and parallel changes in receptor density) have been described in cardiac myocytes in response to chronic hypoxia and noradrenaline exposure (Li et al. 1995; Rokosh et al. 1996).

Given the relative changes noted for some receptor subtypes during development in the rat mesenteric artery, the purpose of this chapter was to assess the influence of the sympathetic and sensory nerves in the development and maintenance of gene expression for the studied neuropeptide receptor families. To do this, the innervation of the mesenteric vascular bed was prevented over the first 4 postnatal weeks, using 6-OHDA and antisera directed against NGF to destroy sympathetic nerves and capsaicin to destroy substance P containing sensory nerve fibres. These treatments have been shown in numerous studies to destroy the respective fibre types (Angeletti & Levi-Montalcini 1970; Cohen 1960; Wakade 1979; Jancso et al. 1977; Holzer 1991). RT-PCR was used to screen the effect on receptor mRNA expression for the $\alpha_{1(A,B,D)}$ and $\alpha_{2(A,B,C)}$-adrenergic, muscarinic (M1-M5), neurokinin (NK1 - NK3) and P$_{2X(1-7)}$-purinoceptor families. Catecholamine histochemistry and immunohistochemistry, using antibodies directed against substance P, were used to assess the effectiveness of the respective denervation treatments.
7.2 RESULTS

7.2.1 Efficacy of denervation treatments

Chemical sympathectomy. The effectiveness of treatment with 6-OHDA, injected on days 2, 6, 9 and 14 postnatal, was assessed in the mesenteric artery of rats aged 7, 14, 21 and 28 days old. Iris tissue was used as a control. At 7 days postnatal (Figure 7.1) control animals showed the early establishment of a sympathetic nerve plexus around the mesenteric artery and in the iris, demonstrated by the presence of smooth weakly fluorescent nerve fibres (Figure 7.1A, 7.1C). Animals that had been treated with 6-OHDA showed a complete lack of fibres in both tissues (Figure 7.1B, 7.1D). Large paravascular fibre bundles could be seen running parallel to the mesenteric artery in control and treated animals.

At 14 days postnatal (Figure 7.2) the mesenteric arteries and irides of control animals showed an increase in the density of the nerve fibre plexuses compared to that at 7 days postnatal (Figure 7.2A, 7.2C). In treated animals, both tissues showed a complete lack of fibres (Figure 7.2B, 7.2D). Paravascular fibre bundles running beside the mesenteric artery appeared to contain degenerating fibres (Figure 7.2B).

At 21 days postnatal (Figure 7.3), the irides of treated animals completely lacked the dense catecholaminergic fibre plexus seen in control animals (Figure 7.3C, 7.3D). In the mesenteric artery however, in the treated animals, some weakly fluorescent fibres could be seen (Figure 7.3B, arrows).

At 28 days postnatal (Figure 7.4), evidence of nerve regeneration along the mesenteric artery was more apparent. While the density of the plexus was significantly less than that in control animals, weakly fluorescent fibres could be seen forming a plexus over the surface of the blood vessel (Figure 7.4B). In contrast, iris tissue from 6-OHDA treated animals at 28 days showed no evidence of regeneration of sympathetic nerve fibres, either over the iris muscle or on the surface of the irideal arterioles (Figure 7.4D).

Immunosympathectomy. Figure 7.5 illustrates the effectiveness of postnatal anti-NGF treatment on the mesenteric artery and iris arterioles of 28 day old rats. Anti-
Figure 7.1
Figure 7.1

Catecholamine histochemistry of stretch preparations of mesenteric artery (A, B) and irides (C, D) of 7 day old rats treated postnatally with diluent (A, C) or 6-hydroxydopamine (6-OHDA, panels B and D). In the irideal preparations the pupillary sphincter is located at the top of the photomicrograph. In the mesenteric artery, paravascular fibre bundles can be seen running parallel to the vessel. In control animals (A, C) smooth weakly fluorescent fibres can be seen over the surface of both tissues. In 6-OHDA treated animals (B, D) there is a complete lack of fibres. Scale bar in D represents 100μm for panels A, B, C and D.
Figure 7.2
Figure 7.2

Catecholamine histochemistry of stretch preparations of mesenteric artery (A, B) and irides (C, D) of 14 day old rats treated postnatally with diluent (A, C) or 6-hydroxydopamine (6-OHDA, panels B and D). In control animals, fluorescent varicose fibres formed a plexus over both the mesenteric artery (A) and dilator muscle of the iris (C). In 6-OHDA treated animals (B, D) there was a complete lack of fibres. Paravascular bundles beside the artery appeared to contain degenerating fibres (B). Scale bar in D represents 100μm for panels A, B, C and D.
Figure 7.3
Figure 7.3

Catecholamine histochemistry of stretch preparations of mesenteric artery (A, B) and irides (C, D) of 21 day old rats treated postnatally with diluent (A, C) or 6-hydroxydopamine (6-OHDA, panels B and D). In control animals, fluorescent varicose fibres formed a dense catecholaminergic plexus over both the mesenteric artery (A) and dilator muscle of the iris (C). In 6-OHDA treated animals, a few weakly fluorescent fibres can be seen running across the surface of the mesenteric artery (B, arrows) however there are no fibres over the surface of the iris dilator muscle (D). Scale bar in D represents 100μm for panels A, B, C and D.
Figure 7.4
Figure 7.4

Catecholamine histochemistry of stretch preparations of mesenteric artery (A, B) and irides (C, D) of 28 day old rats treated postnatally with diluent (A, C) or 6-hydroxydopamine (6-OHDA, panels B and D). In control animals, a dense plexus of catecholamine containing fibres can be seen over both the mesenteric artery (A) and over arterioles and the dilator muscle of the iris (C). In 6-OHDA treated animals, varicose fluorescent fibres can be seen forming a fine plexus over the surface of the mesenteric artery (B, arrows). In contrast, there was no indication of any catecholamine containing fibres being present over the iris arterioles or dilator muscle (D). Scale bar in D represents 100μm for panels A, B, C and D.
NGF treatment totally prevented the establishment of the sympathetic nerve plexus, demonstrated by the lack of a catecholamine containing fibre plexus over either the mesenteric artery or the irideal arterioles and dilator muscle (Figure 7.5B, 7.5D). In mesenteric arteries and irides of control animals, a dense plexus of sympathetic nerve fibres was seen (Figure 7.5A, 7.5C).

Sensory denervation. Capsaicin treatment was shown to effectively destroy the primary afferent sensory nerve fibres supplying the mesenteric arteries in 28 day old rats. In arteries from control animals, a fine perivascular plexus of fibres containing substance P-like immunoreactivity was seen (Figure 7.6A, 7.6B). Animals treated with capsaicin failed to show any fibres containing substance P-like immunoreactivity in association with the mesenteric arteries (Figure 7.6C).

7.2.2 Receptor mRNA expression

General observations. Assessment of PCR product was performed using the subjective assessment method as described in section 2.5.4. For denervation experiments, PCR was performed simultaneously on treated and control samples and PCR product was run on the same gel for each set of samples to further reduce variability in assessment (Figure 7.7).

All PCR fragments detected corresponded to the predicted fragment size for each receptor subtype. The relative expression of cDNA was consistent between repeats of experiments. For each mRNA preparation, results represent data obtained from at least 2 separate RNA preparations except for the anti-NGF treated animals. Due to the limited availability of anti-sera, data was obtained from only one set of animals. For the purinergic P2X1-receptor, extra bands were seen above and below the predicted fragment as described previously (section 4.2.2 and section 6.2.1). No PCR product was detected when RNA was omitted from reactions (Figure 7.7).

For those receptors which demonstrated very strong levels of cDNA amplification at 35 cycles, PCR experiments were repeated at reduced numbers of cycles until the level of expression was equal to [+] or [++] as defined previously. This was in order to better assess the relative levels of mRNA expression between control and treated groups.
Figure 7.5
Catecholamine histochemistry of stretch preparations of mesenteric artery (A, B) and irides (C, D) of 28 day old rats treated postnatally with saline (A, C) or antiserum to nerve growth factor (anti-NGF, panels B and D). In control animals, a dense sympathetic nerve fibre plexus is seen lying over the mesenteric artery (A) and arterioles and dilator muscle of the iris (C). In anti-NGF treated animals, the mesenteric artery (B) and iris (D) are completely devoid of any fluorescent fibres. Scale bar in B represents 100\(\mu\)m for panels A and B, while scale bar in D represents 100\(\mu\)m for panels C and D.
Figure 7.6

Substance P-like immunoreactivity in sections of mesenteric artery from 28 day old rats treated postnatally with diluent (A, B) or capsaicin (C) to selectively destroy primary afferent sensory nerve fibres. In control animals, a fine perivascular plexus of fibres containing substance P-like immunoreactivity was present, however in animals treated with capsaicin no fibres could be seen in association with the vessels (C). Scale bar in C represents 100μm for panels A, B and C.
Expression of mRNA following sympathectomy. Expression of receptor mRNA was assessed after chemical sympathectomy with 6-OHDA and immunosympathectomy using antisera directed against NGF. Levels of mRNA expression for the α-adrenergic, neurokinin and muscarinic receptors after 35 cycles of amplification are shown in Table 7.1. For the majority of receptors, no difference was seen between mRNA levels of control and treated samples. Receptors that did demonstrate a difference in mRNA expression after denervation were the α2A-adrenergic and the NK2 and M1 muscarinic receptors. After 6-OHDA treatment, the α2A-adrenergic receptor showed a relative increase in mRNA expression from [+] to [++] (Figure 7.7B). In contrast, the α2A-receptor was detected at the level of [++] in both control and anti-NGF treated samples. For the NK2 and M1 receptors, while absent from control groups, both receptors were detected after treatment with anti-NGF. The α1B-adrenergic and M5 muscarinic receptors showed different levels of expression in 6-OHDA control versus anti-NGF control tissues, however, within each experimental group, there was no difference between control and treated tissues (Table 7.1). For the P2X-purinoceptors, no differences were seen in mRNA expression between control and anti-NGF treated tissues after 30 cycles of amplification (Table 7.2).

Some small changes in receptor mRNA expression were seen for some receptor subtypes after sympathectomy treatment however these changes were not significant enough to change the classification levels used in the present paper. For example, after anti-NGF treatment, the α1D-adrenergic and NK1 receptors (Figure 1.1 A) showed a slight increase in the amount of product detected when compared to their respective controls. When the expression of the very strongly expressed receptors, like the α1A-, α1B-, α1D+, α2A-, α2B-adrenergic, NK3, P2X1- and P2X4-purinergic receptors, was assessed at reduced numbers of cycles, the P2X4-purinoceptor was the only subtype to show any changes with denervation, with an increase in expression relative to its control seen after anti-NGF treatment.

Expression of mRNA following sensory denervation. The profile of α-adrenergic, neurokinin and muscarinic receptor mRNA expression in the rat mesenteric artery after postnatal treatment with capsaicin is shown in Table 7.3. Results were obtained after 35 cycles of amplification. Neither the NK2 nor M1 muscarinic receptors were expressed in mesenteric arteries of control rats, while both were detected
Table 7.1. Profile of mRNA expression for neuroreceptors in the rat mesenteric artery following chemical- and immuno-sympathectomy

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 6-OHDA Control Anti-NGF</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
</tr>
<tr>
<td>α₁-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>α₁A</td>
<td>+++</td>
</tr>
<tr>
<td>α₁B</td>
<td>++</td>
</tr>
<tr>
<td>α₁D</td>
<td>++</td>
</tr>
<tr>
<td>α₂-Adrenergic</td>
<td></td>
</tr>
<tr>
<td>α₂A</td>
<td>+</td>
</tr>
<tr>
<td>α₂B</td>
<td>++</td>
</tr>
<tr>
<td>α₂C</td>
<td>+</td>
</tr>
<tr>
<td>Neurokinin</td>
<td></td>
</tr>
<tr>
<td>NK1</td>
<td>+</td>
</tr>
<tr>
<td>NK2</td>
<td>0</td>
</tr>
<tr>
<td>NK3</td>
<td>+++</td>
</tr>
<tr>
<td>Muscarinic</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>0</td>
</tr>
<tr>
<td>M2</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>++</td>
</tr>
<tr>
<td>M4</td>
<td>0</td>
</tr>
<tr>
<td>M5</td>
<td>++</td>
</tr>
</tbody>
</table>

Crosses represent detection of PCR product which corresponds to the size of the predicted cDNA band for each set of α-adrenergic, neurokinin and muscarinic subtype specific primers. Treated animals received postnatal injections of either 6-hydroxydopamine (6-OHDA) or antisera to nerve growth factor (anti-NGF) while control animals were treated with the respective diluent only. The number of crosses reflects the amount of amplified product generated after 35 cycles. [0] = never detected; [+ ] = weakly detected; [++] = strongly detected; [+++] = very strongly detected.
Table 7.2. Expression of mRNA for the P2X-purinoceptors in the rat mesenteric artery after immunosympathectomy

<table>
<thead>
<tr>
<th>P2X-Purinoceptor Subtype</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X1</td>
<td>+++</td>
</tr>
<tr>
<td>P2X2</td>
<td>+</td>
</tr>
<tr>
<td>P2X3</td>
<td>+</td>
</tr>
<tr>
<td>P2X4</td>
<td>+++</td>
</tr>
<tr>
<td>P2X5</td>
<td>+</td>
</tr>
<tr>
<td>P2X6</td>
<td>0</td>
</tr>
<tr>
<td>P2X7</td>
<td>++</td>
</tr>
</tbody>
</table>

Crosses represent detection of PCR product which corresponds to the size of the predicted cDNA band for each set of P2X-purinergic subtype specific primers. Treated animals received postnatal injections of antisera to nerve growth factor (anti-NGF) while control animals were treated with diluent only. The number of crosses reflects the amount of amplified product generated after 30 cycles. [0] = never detected; [+]= weakly detected; [++] = strongly detected; [+++]= very strongly detected.
Table 7.3. Profile of mRNA expression for neuroreceptors in the rat mesenteric artery after sensory denervation

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Control</th>
<th>Capsaicin Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Adrenergic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1A</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>α1B</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>α1D</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α2-Adrenergic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α2B</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α2C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurokinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NK2</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>NK3</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Muscarinic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M5</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Crosses represent detection of PCR product which corresponds to the size of the predicted cDNA band for each set of α-adrenergic, neurokinin and muscarinic subtype specific primers. Treated animals received postnatal injections of capsaicin while control animals were treated with diluent only. The number of crosses reflects the amount of amplified product generated after 35 cycles. [0] = never detected; [+] = weakly detected; [++] = strongly detected; [+++] = very strongly detected.
Figure 7.7
Expression of receptor mRNA from the mesenteric artery of 28 day old rats treated with capsaicin (A) or 6-hydroxydopamine (6-OHDA, panel B), using primers specific for the NK1 (A) and α2A-adrenergic (B) receptors. Figures illustrate agarose gel electrophoresis of 8μl of PCR product created after 35 cycles of amplification. In panel A, a weak band of 539 bp in lane 2 represents product amplified from control animals while lane 4 shows NK1 product amplified from animals treated postnatally with capsaicin. A slight increase in the intensity of the band amplified from capsaicin treated animals is evident, however it was not significant under the described classification system (section 2.5.4). Lane 7 shows a positive control amplified from brain tissue under the same conditions. Panel B illustrates a band in lane 2 of 338bp, corresponding to the α2A-adrenergic receptor. While the control sample produced a band classified as [+] (lane 2), product amplified from animals treated postnatally with 6-OHDA showed a relative upregulation to [++] (lane 4). A very strong band was also amplified from brain tissue (lane 7). No PCR product was seen in receptor specific control experiments which did not contain reverse transcriptase enzyme (A, B: lanes 3 and 5) or did not contain RNA (A, B: lane 6). DNA molecular weight markers (Lambda HindIII/ φX174 HaeIII) and (Psz/Alu) appear in lanes 1 and 8 respectively (A, B).
at a low level after treatment with capsaicin. While none of the other receptors showed a change in their relative levels of mRNA expression, the NK1 receptor did show a slight upregulation after capsaicin treatment however this was not significant enough to warrant a change in classification (Figure 7.7A). When the expression of the adrenergic, neurokinin and muscarinic receptors was tested at 30 cycles, the $\alpha_{1D}$-adrenergic receptor showed a relative increase in expression compared to its corresponding control. None of the other receptors showed any change in expression relative to the control tissue at reduced numbers of cycles.
In the previous chapter, it was shown that mRNA expression for the $\alpha_{1B}$- and $\alpha_{1D}$-adrenergic, M2, M3 and M5 muscarinic receptors increased up to 14 days postnatal. Since this time period also corresponded to the development of the sympathetic and sensory nerve plexuses in this vascular bed (Hill et al. 1983; Rayner & van Helden 1994), an inductive influence of the nerves on receptor expression was suggested. The present study has shown, however, that neither sympathetic nor sensory denervation produced any decreases in the mRNA expression of these receptors nor any other members of the $\alpha_1$- and $\alpha_2$-adrenergic, neurokinin, muscarinic and $P_{2X}$-purinergic receptor families. These results are in agreement with other developmental and denervation studies which show that innervation is not mandatory for receptor protein expression in other autonomic targets; for example muscarinic receptors in rat sweat glands (Grant & Landis 1991b; Grant et al. 1991), $\beta$-adrenoeceptors in the rat parotid gland and heart (Ludford & Talamo 1980; Yamada et al. 1980) or $\alpha_1$-, $\alpha_2$- and $\beta$-adrenergic receptors in the rat kidney (Yamada et al. 1986).

Small increases in mRNA expression of the NK1, NK2, M1 and $\alpha_{1D}$-adrenergic receptors were found after anti-NGF and capsaicin treatment and for the $\alpha_{2A}$-adrenergic receptor after 6-OHDA treatment. The similarity of the anti-NGF and capsaicin treatments in producing these effects suggests that, in addition to preventing the ingrowth of sympathetic nerve fibres, anti-NGF may have had an influence on the development of the sensory nerves. Postnatal anti-NGF treatment has been shown previously to reduce the number of substance P and CGRP containing fibres supplying the rat mesenteric artery (Aberdeen et al. 1991). When considered together, these results are consistent with the continued responsiveness that substance P containing sensory neurones show to NGF for normal phenotypic development and maintenance during the postnatal period (Kessler & Black 1980; Yip et al. 1984; Johnson et al. 1986; Lewin & Mendell 1993).

In apparent contrast with the results of our study, a recent paper has described a parallel relationship between the density of sympathetic innervation and the density of $\alpha_{1A}$-adrenoceptors in rat blood vessels (Stassen et al. 1998). The presence of the $\alpha_{1A}$-
receptor was confirmed by the detection of mRNA. Denervation of the mesenteric artery was associated with a decrease in ligand binding of approximately 50% (Stassen et al. 1998). Unfortunately however, a reassessment of $\alpha_{1A}$-adrenoceptor mRNA levels was not made after chemical sympathectomy in this study, and so it is not clear whether the decrease in protein expression was accompanied by a corresponding change in mRNA expression.

RT-PCR was used in this study as it allowed us to screen the relative expression of a large number of receptor subtypes, an approach not easily made with other methods (Pierce et al. 1996). Queries have, however, been raised regarding the estimation of template using PCR due to sample to sample variation (Hellstrom-Lindahl & Nordberg 1996). In this thesis, an attempt has been made to correct for such variation by using common RNA and cDNA pools, thereby allowing for relative comparisons between receptor subtypes within each mRNA preparation. Furthermore, control and treated samples underwent RT-PCR simultaneously and PCR product was assessed on the same gel to further decrease substrate variability. With these considerations, results nevertheless support the conclusion that the expression of receptor mRNA is established independently of innervation in the rat mesenteric artery, confirming the findings of Chapter 6. While slight changes in the expression of some receptors was detected in both the developmental and denervation studies, more sensitive quantitative techniques, for example RNase protection assays, Northern blot analysis or quantitative RT-PCR (Ping & Faber 1993; Vidovic & Hill 1997; Kelleher et al. 1998) would be required to follow up these interesting findings.

6-hydroxydopamine is taken up selectively by sympathetic nerves and its accumulation in the adult leads to the destruction of the nerve terminals without affecting the cell body (Wakade 1979). In neonates, it causes a permanent destruction of sympathetic ganglia (Angeletti & Levi-Montalcini 1970), due to an increased ability of the nerves to take up exogenous amines during development (Jaim-Etcheverry & Zieher 1971). When 6-OHDA is injected into adult animals, the nerves innervating the vascular system have been shown to recover very rapidly from 6-OHDA induced damage, with studies in the renal and mesenteric vascular beds exhibiting near normal nerve fibre plexuses within 4 weeks of treatment (Finch et al. 1972; Lorez et al. 1975), as compared to the heart and iris for example, which take up to 30 weeks to recover.
from the same treatment (Lorez et al. 1975). It appears that resistance of vascular neurones to 6-OHDA is also present in the neonate, as our study showed a very rapid regrowth of fibres along the mesenteric artery once treatment had ceased. Similar results have been described in the renal artery of the rat, where normal responses to electrical field stimulation were seen 8 weeks after early postnatal treatment with 6-OHDA (Finch et al. 1973). Resistance to 6-OHDA does not, however, appear to be a feature of all sympathetic neurones innervating the vasculature, since the arterioles of the iris remained denervated throughout the study period.

One of the mechanisms of 6-OHDA mediated denervation in the neonate may involve the deprivation of essential trophic factors via destruction of the nerve terminals (Schmidt et al. 1988). Despite being separated from their targets for the first two postnatal weeks during the 6-OHDA treatment, the sympathetic neurones innervating the mesenteric artery were able to reestablish a nerve plexus over the arterial wall. This interesting observation supports the idea that there is variation amongst neuronal populations in their degree of sensitivity to neurotoxic agents or requirement for neurotrophic factors (Hill et al. 1985a). For example, differential sensitivities between prevertebral and paravertebral ganglia have been demonstrated for NGF, 6-OHDA and guanethidine (Hill et al. 1985a; Schmidt et al. 1988). The absence of any sympathetic fibres in the mesenteric artery following anti-NGF treatment however, confirms that these sympathetic neurones do require NGF for their survival, as do the neurones innervating the arterioles of the iris.

In conclusion, this chapter has shown that the expression of mRNA for neuroreceptors in the rat mesenteric artery occurs independently of innervation during the early postnatal period. While some receptors showed what appeared to be a relative upregulation of mRNA expression after denervation, more sensitive, quantitative analysis is required to confirm this finding. It was also noted that the effects of anti-NGF treatment and capsaicin treatment were similar and that some autonomic neurones innervating the vasculature were more resistant to neonatal destruction by 6-OHDA than others, in spite of both being dependent on NGF for survival.
Can differences in receptor mRNA expression account for the variable functional responses that are demonstrated amongst different vascular beds? The results, presented in Chapters 3, 4 and 5, do indicate that the overall pattern of gene expression was variable amongst the basilar, mesenteric, pulmonary, tail and hepatic arteries of the rat, with no two vessels showing exactly the same profile. Some receptors showed a consistently strong pattern of expression in all vessels examined, while others exhibited heterogeneous or limited profiles. It may be postulated that those receptors which demonstrated a uniform expression play a significant role in the general control of vessel tone, while those which showed a more variable pattern may contribute to the diverse responses to nerve stimulation that are seen in different vascular beds. Thus, mRNA for the $\alpha_1$-adrenergic receptors demonstrated strong expression levels in all vessels studied, correlating with studies that show these receptors dominate sympathetic nerve-mediated responses in most vascular beds (Chapter 4, Vargas & Gorman 1995; Guarino et al. 1996), as compared to the $\alpha_2$-adrenergic receptors for example, which exhibited a more limited and in general, weaker expression of mRNA amongst the different arteries. On the other hand, the pulmonary and hepatic arteries exhibited unique receptor profiles, consistent with their specialised roles in the vasculature.

Although the expression of mRNA does not directly imply the presence of functional protein, a close correlation between neuroreceptor mRNA expression and functional protein expression was demonstrated in these studies of the arteries of the rat hepatic mesentery (Chapters 4 and 5). Thus, the principal receptors involved in the contractile response to sympathetic nerve stimulation were the $\alpha_{1A}$-adrenergic, P$_{2X(0)}$- and NPY $Y_1$-receptors, all of which demonstrated strong mRNA expression. A role for the CGRP$_1$ receptor in sensory nerve-mediated vasodilations was also predicted using RT-PCR. These results suggest that RT-PCR is a useful screening tool when attempting to characterise neuroreceptor mediated vascular responses. The
demonstration of strong mRNA expression can thus be used to predict the involvement of particular receptor subtypes. Once confirmed in functional studies, such information may be used for the development of drugs targeted to either general systemic vascular responses or to specific vascular beds and hence organ systems as required.

Particularly novel findings of the present studies included the detection of mRNA for the M5 muscarinic and NK3 neurokinin receptors in all vessels studied. No physiological role has yet been defined for the M5 muscarinic receptor (Reever et al. 1997), although studies have suggested that it may participate in vasodilatory responses in the cerebral vasculature (Dauphin et al. 1991; Dauphin & Hamel 1992; Linville & Hamel 1995). Until specific antagonists become available, it will be difficult to demonstrate a functional role for this receptor, however given its widespread distribution, other investigators may now consider its involvement in more detail. Similarly, the NK3 receptor has previously been largely discounted in studies in the rat vascular system. The results presented in this thesis suggest that NKB, or other as yet unidentified ligands for NK3 receptors, may contribute significantly to vasodilator responses in this species.

In the vasodilator studies, it became apparent that a number of receptor subtypes showed relatively strong mRNA expression levels, for example the NK1 and M3 muscarinic receptors, however no nerve-mediated functional role could be demonstrated for these receptors. Both of these receptors are most commonly expressed on the endothelium (Otsuka & Yoshioka 1993; Eglen et al. 1996). In blood vessels that have multiple smooth muscle cell layers, such as the arteries of the rat hepatic mesentery, it is unlikely that endothelial receptors are exposed to neurally released transmitter, given the intervening physical barriers and degradation by enzymes such as angiotensin converting enzyme, neutral endopeptidases and acetylcholinesterase (Khawaja & Rogers 1996; Ralevic & Burnstock 1996b; Katzung 1992a). The circulation represents a source of vasoactive agents, for example adrenaline, however for substance P and acetylcholine, blood levels are generally low due to their rapid breakdown (Burnstock & Ralevic 1994). What then is the endogenous source of vasodilator substances and the physiological role of endothelial located receptors? It has been demonstrated that endothelial cells can themselves release acetylcholine and substance P in response to abnormal situations - for example hypoxia or altered flow (Parnavelas et al. 1985;
Milner et al. 1989; Ralevic et al. 1990; Ralevic & Burnstock 1996b). It is possible then, that endothelial receptors are activated by locally derived products and assume importance in pathological situations, rather than being involved in the maintenance of vascular tone under normal conditions.

The studies in the arteries of the rat hepatic mesentery also indicated that complex interactions occur between pre- and postsynaptic mechanisms and between vasoconstrictor and vasodilator pathways. For example, a high degree of presynaptic inhibition of neurotransmitter release was noted and sympathetic nerves were shown to mediate both vasoconstrictor and vasodilator responses. Classical slow, peptide mediated responses were demonstrated and additionally, it was demonstrated that NO was released from the endothelium in response to sympathetic vasoconstrictions and increased vascular tone. The timing of these different responses strongly suggest that control mechanisms are in place to attenuate or prevent excessive sympathetic nerve-mediated pressor responses. Such interactions offer a potential target for the manipulation of blood flow in this and other vascular beds. For example, stimulation of presynaptic inhibitory pathways to limit sympathetic neurotransmitter release could be used as a method to increase blood flow, instead of postsynaptic inhibition of α-adrenoceptor mediated contractions or activation of postsynaptic dilatory pathways. Interactions between second messenger systems may also be targeted in the design of vascular bed specific drugs. In conjunction with this possibility, future experiments are planned to identify the source of Ca²⁺ mediating α₁A-adrenergic sympathetic contractions in the arteries of the rat hepatic mesentery.

The present study has only investigated nerve-mediated responses in the arteries of the rat hepatic mesentery under one set of stimulus parameters (10Hz, 10s, 0.1msec, 60V). These parameters were chosen because they produced a consistent response of reasonable size that could be blocked by tetrodotoxin. Previous studies have suggested that sympathetic fibres may fire at frequencies greater than 10Hz (Folkow 1952; Kennedy et al. 1986) and in the perfused rat liver preparation, stimulations of 10Hz have been described as falling within the physiological range of frequencies (Gardemann et al. 1987). Experimental conditions however, can influence neurotransmitter release (Brock 1995; Kennedy 1996) and it is yet to be determined
whether contractile and vasodilator mechanisms vary with different stimulus parameters in this vascular bed.

This thesis sets the background for an examination of the control of blood flow in chronic liver conditions such as cirrhosis. By selectively increasing hepatic blood flow it may be possible to improve liver function and therefore alleviate patients clinical symptoms, yet avoid systemic effects such as postural hypotension (Morgan & McLean 1995; Katzung 1992b). In line with these clinical considerations, future studies in a cirrhotic rat model are currently planned. For these experiments, cirrhosis will be induced in adult rats by oral dosing with carbon tetrachloride and pentobarbitone (Proctor & Chatamra 1982). Once the disease is established, the extra hepatic arterial bed will be examined in a similar fashion to that described in this thesis, with RT-PCR being used to screen for any alterations in receptor mRNA expression and a combination of immunohistochemistry and physiology applied to assess innervation patterns and functional responses respectively. If any significant differences in nerve-mediated function are noted, results can be applied to an in situ perfused liver preparation (Gardemann et al. 1987), which could then be utilised as a model for therapeutic drug trials.

Reverse transcription-polymerase chain reaction was used to determine if alterations in receptor mRNA expression could account for the physiological changes described in the rat mesenteric artery during development. No changes were detected that could account for the maturation of the physiological response, nor was there any correlation between the appearance of the nerves and the initial expression of the receptors studied. A relative increase in the level of mRNA expression was noted for some receptor subtypes up to day 14 postnatal, however denervation studies indicated that the absolute expression of receptor mRNA expression was not affected by the absence of sympathetic or sensory nerve fibres. Taken together, the results suggest that the initial expression of receptor genes is established independently of innervation during the prenatal period. Likewise, the establishment of adult levels of mRNA expression do not depend on the development of a neural plexus. Mismatches between receptor localisations and neurotransmitter types have been described in the brain (Herkenham 1987), rat iris arterioles (Hill et al. 1996) and in the sweat glands of the rat footpad (Grant & Landis 1991a; Grant et al. 1991). The described physiological
changes in the mesenteric artery may therefore result from changes in the expression levels of receptor protein, maturation of coupling pathways or prejunctional modifications such as changes in neuronal phenotype. As an intact innervation is essential for the normal structural and functional development of vascular tissue (Bevan & Tsuru 1981; Bevan 1989), interactions between the developing neurones and the target tissue may still be responsible for regulating the changes in vascular responsiveness described, possibly via a specific trophic factor, and further work assessing such interactions is warranted.

Reverse transcription-polymerase chain reaction has been used extensively in this thesis. It has the advantage of being a highly sensitive procedure which allows for rapid analysis of a large number of receptors in different vessels. Additionally, for the functional experiments, the technique eliminated problems associated with the presence of presynaptic receptors, as protein synthesis occurs exclusively in the neuronal cell body and dendrites, well removed from the effector site (Edstrom et al. 1969). On the other hand, a limitation of the method is that results did not indicate if the receptors were expressed on the endothelium or vascular smooth muscle. Experiments were initially considered in which the endothelium would be denuded physically (for example perfusion with distilled water), however preliminary results indicated that although the endothelium could be damaged, it was not possible to ensure that all cells had been removed. To ascertain the specific location of receptors therefore, techniques such as immunohistochemistry, radioligand binding studies, in situ hybridisation or in situ RT-PCR could be utilised (Dorje et al. 1991b; Hill et al. 1996; Bates et al. 1997).

Another caveat that must be considered when using RT-PCR are the limitations of the technique as a quantitative method. Due to its exponential nature, concerns regarding comparisons of RT-PCR product have been raised, as slight differences arising from experimental variations have the potential to be significantly magnified (Hellstrom-Lindahl & Nordberg 1996; Kelleher et al. 1998). Care was taken therefore, not to over interpret the quantitation of RT-PCR results presented in this thesis. While any variability arising from RNA extraction applies to all techniques which assess mRNA expression, the relative quantitation of gene transcripts amplified using RT-PCR relies upon the consistency of the RT reaction, PCR amplification and product measurement steps (Hall et al. 1998). As described previously, an attempt has been
made in these studies to standardise the procedure by using a constant amount of RNA and cDNA to control for variability in the different steps. Interestingly, a recent study has shown that standard RT-PCR is in fact more reproducible than previously thought (Hall et al. 1998), suggesting that the relative comparison of results between samples may not be unjustified.

For the absolute quantification of gene transcript, techniques such as competitive RT-PCR or Northern blot analysis are required. In both the comparative and developmental studies presented in this thesis, RT-PCR was used to screen the expression of a large number of receptor subtypes. In such circumstances, competitive RT-PCR is not a suitable approach, given that a mimic would need to be constructed for each of the receptor subtypes examined. A multigene competitor RNA (Wang et al. 1989) may have been developed, however this approach would have also been limited by the small amounts of total RNA retrieved from the arterial preparations and the desire to investigate a large number of receptor subtypes. The effectiveness of Northern blots may have also been hampered by the relatively low levels of message present in vascular tissue (Graham et al. 1996) and the requirement to load more of the limited RNA sample. The results presented in Chapter 7 have, however, highlighted some receptors which may undergo significant alterations in mRNA expression levels after denervation, for example the NK2, M1 muscarinic and α2A-adrenergic receptors and future experiments utilising competitive RT-PCR may be warranted for these specific receptor subtypes.

No previous study has examined the expression of neuroreceptor mRNA for such a broad range of receptors or in such a variety of vascular beds as described in this thesis. Each vessel expressed mRNA for a wide range of receptor subtypes and it is of interest to speculate as to why multiple receptor subtypes exist within the vasculature, given that for many, the endogenous ligands do not distinguish between them. For example, all three subtypes of α1-adrenergic receptors recognise adrenaline and noradrenaline with similar affinities (Graham et al. 1996) and the NK1 receptor can mediate responses to all three tachykinins (Maggi 1997). Additionally, multiple receptor subtypes can be co-expressed in the one vessel but regulate vascular tone by nearly identical pathways (Piascik et al. 1996). It has been suggested that the various subtypes may regulate functional responses in ways that are not yet understood, or that
the presence of such homologous subtypes is of no significance and purely represents
the presence of redundant proteins (Hein & Kobilka 1997). Receptor specific and
multiple receptor knockout mice may play a part in answering some of these questions,
as has recently occurred for the \( \alpha_2 \)-adrenergic receptors (Link et al. 1996; MacMillan et
al. 1996; Hein et al. 1998). Alternatively, animals designed to allow temporally specific
down-regulation of gene expression may be useful, since such animals may overcome
complications of potential compensation by remaining receptor subtypes. Elucidating
the regulation and physiological significance of so many receptor subtypes therefore
remains a challenge, both within the vascular system and throughout the body as a
whole.
A.1 INTRODUCTION

In the process of characterising the α₁-adrenoceptor responsible for mediating sympathetic vasoconstriction, we used the putative α₁B-adrenoceptor antagonist CEC (Han et al. 1987). Results were consistent with the subtype being an α₁A-adrenoceptor however some unusual responses were noted, particularly when the drug was used in combination with the P₂X-purinoceptor antagonist α,β-mATP. A study by Bultmann and Starke (1993) has shown that CEC is able to act as a partial α₂-agonist, with properties similar to its parent compound clonidine (Leclerc et al. 1980), and so experiments were repeated with the α₂-agonist clonidine.

A.2 RESULTS

When the α₁B-adrenoceptor antagonist CEC (10⁻⁵M) was used, it had no effect on the nerve-mediated contractile response (Figure A1, panel A). The further addition of α,β-mATP (3x10⁻⁶M) however, reduced the response to 26.17 ± 5.96% of control (Figure A1, panel A). When applied in the reverse order, α,β-mATP produced an enhanced response as previously described (170.83 ± 35.26% of resting vessel diameter), while the subsequent addition of CEC reduced the response to 21.06 ± 4.25% of control (n=4). Clonidine (10⁻⁶M) also did not significantly affect the size of the nerve-mediated contraction, however the further addition of α,β-mATP reduced the
Figure A1  Effects of the consecutive application of (A) chloroethylclonidine (CEC, \(10^{-5}\)M) and \(\alpha,\beta\)-mATP (mATP, \(3 \times 10^{-6}\)M) and (B) clonidine (Clon, \(10^{-6}\)M) and mATP on the nerve-mediated contractile response. Columns represent the mean±s.e. mean of at least 4 preparations. Results are expressed as % of the contractile response in control Krebs’ solution (Cont). Control response in (A) was 20.64±1.80% of resting vessel diameter, \(n=6\) and in (B) was 20.40±1.78%, \(n=4\). * Differs significantly from control (\(P < 0.05\)).
contraction to 16.35 ± 1.73% of control (Figure A1, panel B). Neither CEC nor clonidine affected the resting vessel diameter when applied to the preparation at these concentrations.

The use of CEC in combination with α,β-mATP uncovered slow nerve-mediated contractions on 4 occasions, as did the primary use of clonidine, however this was only seen twice. When analysed, these responses were typical of the NPY-mediated sympathetic vasoconstriction (contractions size equal to 9.28 ± 1.42% of resting vessel diameter, time to peak of 43.60 ± 4.08s and a recovery to 90% of resting vessel diameter of 127.10 ± 18.78s, n=6).

A.3 DISCUSSION

On its own, CEC had no effect on the nerve-mediated response, however when it was used in conjunction with α,β-mATP, the response to nerve stimulation was significantly reduced. In the rat vas deferens, CEC has been shown to have α2-agonist properties, acting on presynaptic α2-adrenergic receptors and inhibiting the release of noradrenaline and ATP (Bultmann & Starke 1993). We therefore applied clonidine to the rat hepatic artery preparation and were able to reproduce the CEC results. In our preparation it seems therefore, that the application of CEC or clonidine alone does not produce a significant inhibition of neurotransmitter release (possibly due to the high levels of inhibition already present). When combined with α,β-mATP, however, their agonistic effects are potentiated and neurotransmitter release is significantly reduced, thereby causing a reduction in the size of the contraction. The ability of α,β-mATP to potentiate the effect of clonidine has also been described in the rat tail artery, where both noradrenaline release and subsequent vasoconstriction were reduced (Shinozuka et al. 1990). The authors had no ready explanation for this finding.

CEC and clonidine occasionally uncovered contractions that were characteristic of the sympathetic NPY-mediated contraction. Other experiments suggested that this response was uncovered by the use of α2-adrenergic receptor antagonists (see Chapter 4). The appearance of an NPY-contraction while using potential α2-agonists therefore
adds more doubt to the mechanism of action of these drugs. An explanation however, may be found in the results of a recent study, which suggest that in addition to acting as a prejunctional α₂-agonist, inhibiting transmitter release, CEC may also act as an antagonist on a different population of prejunctional receptors, concurrently increasing transmitter release (Guimaraes et al. 1997). Following from this hypothesis, one may speculate that different prejunctional α₂-adrenergic receptors modulate noradrenaline and NPY release in the arteries of the rat hepatic mesentery.

Clonidine and CEC have both been shown to have non-specific sites of action. Clonidine for example, has been shown to have dual effects in a number of vascular beds, acting as a classical α₂-agonist and an α₁-antagonist (Kong et al. 1991; Nomura et al. 1995; Silva et al. 1996). CEC likewise has multiple effects. It binds to all α₁-adrenergic receptors to some degree (Michel et al. 1993; O'Rourke et al. 1997) and in addition to acting as an α₂-agonist at both pre- and postjunctional locations (Bultmann & Starke 1993; Nunes & Guimaraes 1993), can also antagonise α₂-adrenoceptor-mediated responses (Michel et al. 1993; Guimaraes et al. 1997). The suitability of these drugs in experiments designed to characterise receptor subtypes, at either pre- or postjunctional locations, is therefore of questionable or limited value.
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