The Autonomic Nervous System And The Heart

Jane Yvonne Barnes, Bsc. (Hons)

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Declaration

I declare that the work for this thesis was undertaken during my PhD studentship at the Cardiovascular Research Unit, Departments of Medicine and Cardiology, Royal Infirmary Edinburgh, and written up thereafter. I was the principle contributor to all sections except where indicated in the text.

Jane Y Barnes

February 1997

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Abbreviations

ACh	acetylcholine
AcCoA	acetyl coenzyme A
AChE	acetylcholinesterase
ANOVA	analysis of variance
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CK	creatine kinase
CoA	coenzyme A
CPP	coronary perfusion pressure
DENS	data evaluation of non-linear standard curves
dP/dt	left ventricular contractility
EDTA	ethylene diamine tetra acetic acid
G6P-DH	glucose 6 phosphate dehydrogenase
G protein	guanosine triphosphate binding protein
GPT	glutamate pyruvate transaminase
GS	ganglion stimulation
HCl	hydrochloric acid
HK	hexokinase
HPLC	high pressure liquid chromatography
HR	heart rate
LDH	lactate dehydrogenase
LSG	left stellate ganglion
LVP	left ventricular pressure
MI	myocardial infarction
NA	noradrenaline
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
PI	phosphoinositide
SCD	sudden cardiac death
SD	standard deviation
SIDS	sudden infant death syndrome
SUNDS	sudden unexpected nocturnal death syndrome
VF	ventricular fibrillation
VT	ventricular tachycardia

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Abstract

The mechanisms by which the release of acetylcholine from cholinergic neurones in the heart is regulated are not well understood. A reliable and sensitive assay for acetylcholine is lacking. Most studies of cardiac parasympathetic neurotransmission have used indirect methods such as changes in heart rate and contractility.

A novel high pressure liquid chromatography (HPLC) method (electrochemical detection) was adopted to assay acetylcholine overflow from the *in situ*, perfused, innervated rat heart. Sample concentrations of 20 nM and above could be accurately measured (inter-assay coefficient of variation, 6 %). Bilateral vagal nerve stimulations (15 Hz, 60 s, 0.8 mA) in the presence of neostigmine, produced the expected negative inotropic and chronotropic effects which were reversibly abolished by 1 μM atropine. Acetylcholine was not detected in coronary effluent. Neostigmine, 50 μM, inhibited over 91 % of the *in vitro* myocardial acetylcholinesterase activity. Infused acetylcholine, 200 nM, was quantitatively recovered. These results suggested that the concentration of acetylcholine released from the parasympathetic neuroeffector junction was below the detection limit for the assay. Increasing the frequency and voltage of the vagal nerve stimulation to cause asystole did not produce a detectable amount of acetylcholine. Neither did the presence of atropine, which has been reported to quadruple the acetylcholine overflow from the perfused chicken heart. Bioassay of acetylcholine, using the leech dorsal muscle preparation, was twice as sensitive as the HPLC method, but still failed to detect acetylcholine.

An HPLC method was also set up for the measurement of noradrenaline in coronary effluent samples from perfused, innervated rat hearts. The sensitivity was 0.5 nM noradrenaline (inter-assay coefficient of variation, 4.9 %). Four repeated left stellate ganglion stimulations (5 Hz, 60 s, 0.8 mA), S1-S4, separated by a 15 min recovery period, demonstrated a progressive decline in noradrenaline overflow. The influence of duration of ganglion stimulation (30 or 60 s) and coronary flow rate (5 or 10 ml g^{-1} min^{-1}) on the noradrenaline overflow and functional response to four consecutive sympathetic ganglion stimulations, was examined. Baseline (pre-stimulation) mechanical performance of the preparation was stable. Lactate production was low and did not vary significantly between the groups. Stimulation induced changes in heart rate and left ventricular pressure were stable throughout the four stimulations (coronary flow rate 5 ml g^{-1} min^{-1}). Noradrenaline overflow and contractility, however, showed a progressive decline from S1 to S4 ($p < 0.05$). Reducing the stimulus duration from 60 to 30 s delayed the onset of the decline in noradrenaline

overflow and +dP/dt, while preventing the decline in the magnitude of the -dP/dt response. Increasing the coronary flow rate, completely prevented the decline in the response to nerve stimulation.

Perfusing the heart with very large amounts of catecholamines may protect against prolonged ischaemia (so-called ischaemic preconditioning). The ability of sympathetic nerve stimulation (5 Hz, 5 min) to precondition the rat heart against dysfunction and tissue enzyme depletion after a 30 min period of stop-flow ischaemia was investigated. Noradrenaline and lactate release, left ventricular pressure, heart rate and contractility during the first 30 min of reperfusion were not altered by preconditioning. Tissue creatine kinase levels after 4 hours reperfusion also did not differ. Recovery of coronary perfusion, however, was slightly improved in the preconditioned groups ($p < 0.05$).

In conclusion, vagal stimulation of the perfused, innervated rat heart results in marked negative inotropic and chronotropic changes, without detectable acetylcholine overflow. Repeated left stellate ganglion stimulation (S1-S4) results in reduced noradrenaline overflow and haemodynamic effects, that can be prevented using a coronary perfusion flow rate of 10 ml g^{-1} min^{-1}. Left stellate ganglion stimulation does not confer a protective effect against ischaemia. Future studies of the role of the parasympathetic nervous system require more sensitive methods to detect acetylcholine.

CHAPTER 1

Introduction and literature review

1.1 The cardiac autonomic nervous system

The autonomic nervous system and its control of cardiac function has long been a subject of interest by investigators. In 1921, Loewi performed his classic, and much quoted experiment on frog heart which was the first demonstration of chemical neurotransmission. A profusion of experiments followed, during which the principles of chemical neurotransmission were elucidated. More recently, the importance of autonomic nerve function in determining the outcome of acute cardiac ischaemia or infarction has been investigated.

Sudden cardiac death following acute myocardial ischaemia remains one of the highest causes of mortality in the western world (Bennett, 1996; Immonen-Räihä *et al.*, 1996). Death commonly occurs within minutes of the onset of severe chest pain or heart attack, in many cases, before the patient has reached hospital. In a study by Armstrong, in which patients were followed up for 4 weeks after suffering a myocardial infarction, 45 % of the deaths over this period, occurred within an hour of the onset of symptoms (Armstrong *et al.*, 1972). Holter monitoring has shown that the majority of such deaths result from the onset of ventricular fibrillation, often preceded by episodes of ventricular tachycardia (Nikolic *et al.*, 1982), generated through a re-entrant mechanism (Janse *et al.*, 1980; Janse and Wit, 1989).

Evidence of autonomic activation is common during the first hour of an episode of acute myocardial ischaemia. In an important study by Webb and colleagues (Webb *et al.*, 1972), over 80 % of patients admitted to hospital suffering from the advent of their first myocardial infarction showed symptoms of autonomic overactivity during the first hour of the ischaemic event. Experimental studies have demonstrated an important role for the autonomic nervous system in the genesis of arrhythmias. In general, sympathetic neural influences are thought to facilitate the formation of arrhythmias in the ischaemic and non-ischaemic myocardium (Sheridan *et al.*, 1980; Verrier and Lown, 1984; Penny, 1984; Schwartz *et al.*, 1976a and b; Schwartz, 1985; Schwartz *et al.*, 1985), while parasympathetic influences are thought to oppose this (Ferrari *et al.*, 1992; Billman, 1989; De Ferrari, 1987; Das and Bhattacharya, 1972; Hohnloser *et al.*, 1986).

Evidence that the cardiac autonomic nervous system may contribute to arrhythmogenesis during myocardial ischaemia in the *in vivo* situation, comes from studies of

parasympathetic tone using the methods of heart rate variability and baroreflex sensitivity testing (see section 1.1.6 for detailed description). These techniques have shown that where parasympathetic responses are impaired, as indicated by a reduced heart rate variability or baroreflex sensitivity, the myocardium is more vulnerable to the onset of ventricular arrhythmias (Schwartz *et al.*, 1988a). The degree of impairment of parasympathetic neural function is a good predictor of outcome following myocardial infarction, both in animals (Schwartz *et al.*, 1988a; Hull *et al.*, 1990) and humans (La Rovere *et al.*, 1988; Kleiger *et al.*, 1987; Odemuyiwa *et al.*, 1994). Furthermore, the strength of pre-infarction parasympathetic reflexes may be able to identify those individuals at greater risk from arrhythmias in the event of an episode of acute ischaemia (Schwartz *et al.*, 1988a; Adamson *et al.*, 1994).

Understanding the behaviour of the cardiac autonomic nervous system during acute ischaemia may therefore, lead to the development of interventions to both identify and treat those at greatest risk from arrhythmias. In order to understand and interpret the effect of changes in autonomic nerve function during ischaemia, however, it is important to first understand the behaviour of the autonomic nervous system under normal conditions.

1.1.1 Anatomy of cardiac autonomic innervation

There are two limbs of the autonomic nervous system - sympathetic and parasympathetic. In control of cardiac function, these two limbs generally (although not always) have opposing effects. Sympathetic nerve activation causes increases in heart rate, force of contraction and automaticity. Parasympathetic nerve activity leads to decreases in all of the above. In some respects, however, the two systems do have synergistic effects. For example, both noradrenaline and acetylcholine shorten action potential duration and thus reduce the refractory period.

Although both obey the same principles of stimulus secretion coupling, the anatomy of the two systems is different (for review see Corr *et al.*, 1986; Löffelholz and Pappano, 1985). Sympathetic efferent preganglionic neurones have their cell bodies in the lateral horn of the grey matter in the cervical-thoracic vertebrae. Fibres leave the spinal cord in the spinal nerves. Once outside the spinal cord, they exit the spinal nerves and run to the paravertebral sympathetic chain - an interconnected chain of sympathetic ganglia which lie on either side of the spinal column. Cardiac sympathetic nerves synapse in the left and right stellate ganglia (the product of a fusion between the inferior cervical and first thoracic ganglia).

Efferent sympathetic impulses are transmitted to the heart via ansae subclaviae - branches of the right and left stellate ganglia. Postganglionic axons primarily enter the myocardium following the coronary arteries. Although overlap does exist, left sided cardiac

nerves mainly innervate the anterior aspect of the ventricles and the AV node, whereas the right-sided cardiac nerves innervate the sinus node and the posterior portion of the ventricles.

The parasympathetic cardiac innervation originates from the dorsal vagal nuclei in the medulla and is conducted to the heart in the left and right branches of the vagus nerve. Ganglia are located in the cardiac vagal plexus or the atrial walls. Postganglionic neurones are much shorter than those of the sympathetic system and infiltrate the atria and nodal tissues along blood vessels. It was once thought that there was no vagal innervation to the ventricular myocardium. Acetylcholine (Brown, 1976), choline acetyltransferase (Roskoski *et al.*, 1975) and muscarinic receptors (Fields *et al.*, 1978) have all, however, been shown to exist in ventricular tissue. It is now known that some postganglionic axons enter the ventricular myocardium via the AV groove. Although the density of parasympathetic innervation in the ventricles is substantially less than that in the atria, vagal nerve stimulation is able to exert important effects on ventricular automaticity and repolarization, both through an interaction with the sympathetic nervous system and also through direct electrophysiological actions (Rardon and Bailey, 1983).

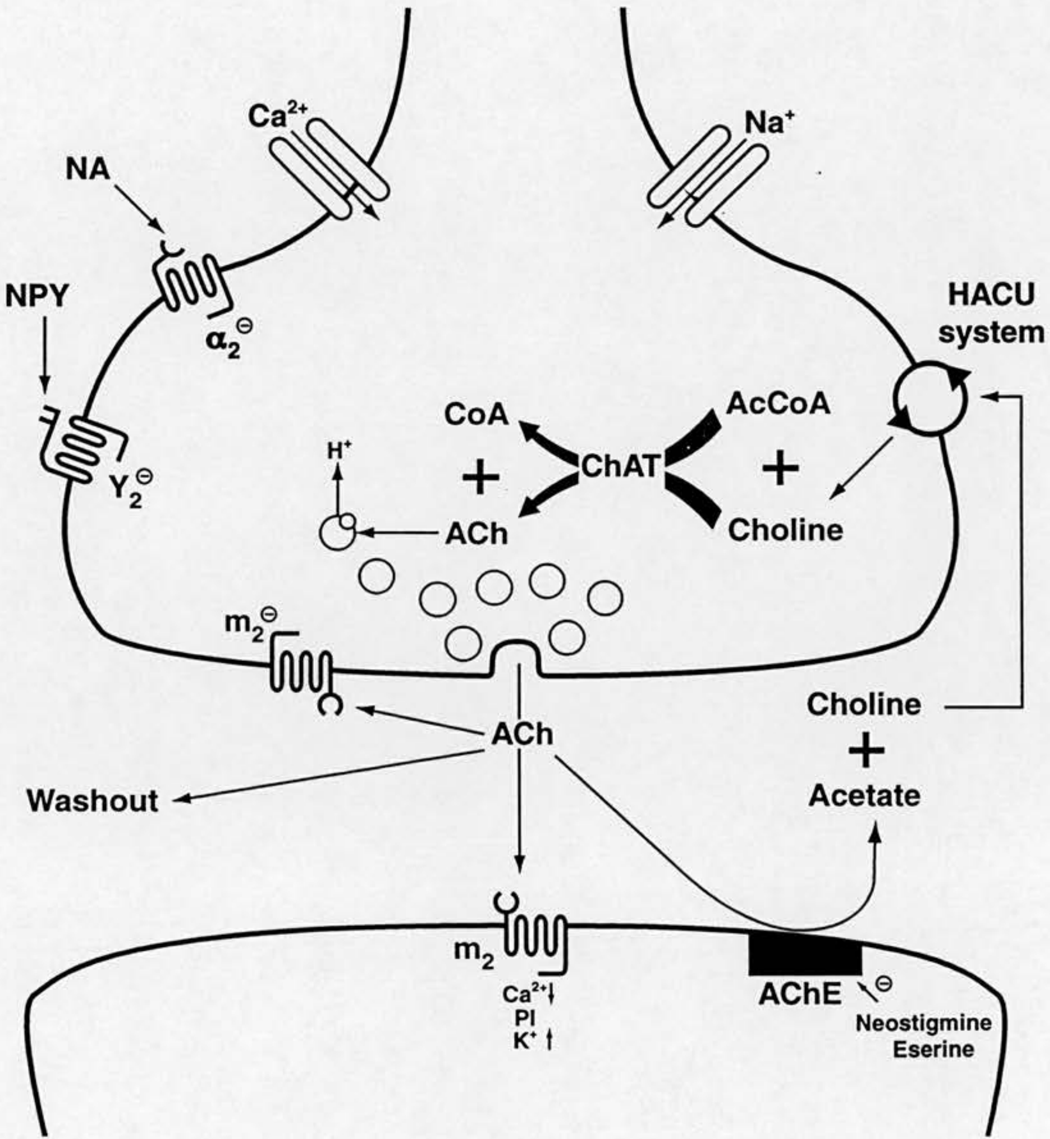
1.1.2 Physiology of the parasympathetic nervous system

a) Synthesis and storage of acetylcholine

Acetylcholine is synthesised within the axoplasm of cholinergic nerve terminals (for review see Löffelholz *et al.*, 1984). The acetyl moiety from acetyl coenzyme A is transferred to choline in a reaction catalysed by the enzyme, choline acetyltransferase. The original source of the acetyl groups has not been determined in the heart, although in brain tissue, they are derived from pyruvate. In comparison, much more is known about the second precursor, choline.

Choline is a charged polar molecule which cannot easily cross the cell membrane. Its transfer into the nerve cell is the rate limiting step in acetylcholine synthesis. Choline for the reaction is taken up from the interstitial space by a high affinity choline uptake (HACU) carrier (Wetzel and Brown, 1983). The carrier molecule is sodium dependent and activated by stimulation of the vagus nerves. Plasma levels of choline in mammalian species are of the order of 5-10 μM (Bligh, 1952). Under normal physiological conditions, this is sufficient to maintain adequate acetylcholine synthesis (Wetzel and Brown, 1983; Dieterich *et al.*, 1978).

Figure 1.1 *Schematic illustration of acetylcholine synthesis, storage, release and clearance in cholinergic nerves*



Acetylcholine is synthesised locally by choline acetyltransferase (ChAT) and stored in small, clear vesicles. Acetylcholine release is triggered by depolarisation. At the end-plate, acetylcholine acts on both presynaptic and postsynaptic muscarinic-m_2 receptors. The presynaptic m_2 receptors inhibit acetylcholine release - an effect which is shared by both Y_2 purinergic and α_2 adrenergic receptors. Released acetylcholine is degraded by the membrane-bound enzyme, acetylcholinesterase into choline and acetate. Choline may be taken up into the presynaptic terminal by a high affinity choline uptake carrier (HACU) where it is reincorporated into acetylcholine.

Hydrolysis of released acetylcholine by acetylcholinesterase inactivates acetylcholine and also liberates choline into the extracellular space. A certain amount of this choline is reuptaken into the nerve terminal although this is likely to be a relatively unimportant source of choline for acetylcholine synthesis (Löffelholz and Pappano, 1985). A further supply of extracellular free choline is obtained from the breakdown of phospholipids - glycerophosphocholine and phosphorylated choline. This accounts for the choline efflux evident in unstimulated isolated hearts, perfused with choline free medium (Corradetti *et al.*, 1982).

The formation of acetylcholine is catalysed by choline acetyltransferase - a cytosolic enzyme. The distribution of choline acetyltransferase within the heart has been mapped, and supports observations by histologists that postganglionic cholinergic innervation is low in mammalian ventricles (Slaviková and Tucek, 1981) compared to the atria.

Following synthesis, the acetylcholine is packaged into synaptic storage vesicles inside which, the concentration of the transmitter can be as high as 800 mM (Zimmermann, 1988).

b) Acetylcholine release

Release of acetylcholine from the vesicles is triggered either by the arrival of an action potential or by high extracellular potassium levels (for review see Kilbinger, 1988). The exact mechanism of the release has not been elucidated, but it is dependent on an influx of extracellular calcium. Agents which affect interneuronal calcium influx can modify acetylcholine release. Magnesium, cobalt and manganese ions all inhibit acetylcholine release by interfering with the slow inward calcium current in the neurone (Ginsborg and Jenkinson, 1976; Glitsch and Pott, 1978). Likewise, barbiturates have been reported to reduce the release of cardiac acetylcholine. Pentobarbital infused into the isolated, perfused chicken heart caused a substantial inhibition of stimulation induced acetylcholine release (Lindmar *et al.*, 1979).

Acetylcholine release can also be modulated by a variety of endogenous agents (Wetzel and Brown, 1985; Wetzel *et al.*, 1985). Mechanisms of presynaptic modulation of neurotransmitter release by endogenous substances are discussed in the next section.

Following its release from the presynaptic membrane, acetylcholine diffuses across the synaptic cleft to the postsynaptic membrane where it combines with muscarinic receptors. Despite the existence of several different species of the muscarinic acetylcholine receptor (Kubo *et al.*, 1986; Bonner *et al.*, 1987), only receptors of the m_2 subtype have been identified in mammalian cardiac tissue (Deighton *et al.*, 1990; Maeda *et al.*, 1988). The density of receptors is highest in areas with the greatest parasympathetic innervation.

Postsynaptic muscarinic receptors are coupled to a variety of ion channels and second messenger systems, through a range of G proteins. Different subclasses of the m_2 receptor, with different affinities for acetylcholine, have been hypothesised to be coupled to the same effector via different G proteins, allowing fine control of the response (Ikegaya *et al.*, 1990).

Among its other actions, acetylcholine or cholinergic agonists binding to cardiac postsynaptic muscarinic receptors can inhibit adenylate cyclase and the production of 3',5'-cyclic adenosine monophosphate (cAMP) (Delhaye *et al.*, 1984; Hescheler *et al.*, 1986; Breitweiser and Szabo, 1985), stimulate the production of cyclic guanosine monophosphate (cGMP) (Cramb *et al.*, 1987; George *et al.*, 1970), open potassium channels (Szabo and Otero, 1990; Pfaffinger *et al.*, 1985) and interact with the PI cycle (Cockroft, 1987).

c) Presynaptic modulation of neurotransmitter release

Mechanisms of presynaptic modulation of neurotransmitter release can be classified into four groups. Presynaptic mechanisms affecting both the parasympathetic and the sympathetic postganglionic nerve terminals are discussed below.

i) Feedback modulation

Receptors located on the presynaptic neurone, allow neurotransmitter, present in the synaptic cleft, to influence its own release. The best characterised example of feedback modulation occurs when noradrenaline released from the neuroeffector junction, acts at presynaptic α_2-adrenoceptors on sympathetic nerves to inhibit the further release of noradrenaline (Starke, 1987; Haass *et al.*, 1989). The mechanism of this effect is an inhibition of voltage-gated calcium channels (Rand *et al.*, 1987). It appears that acetylcholine is able to similarly influence its own release from the cholinergic neuroeffector junction. Pharmacological blockade of muscarinic receptors with atropine causes an increase in the stimulation-evoked release of acetylcholine (Löffelholz *et al.*, 1984). Although the mechanism has not been investigated in the heart, in other tissues which have been examined, such as the guinea pig ileum and the cat urinary bladder, presynaptic muscarinic receptors cause inhibition of N-type calcium channels (Wessler, 1996).

ii) Axo-axonal modulation

Neurotransmitters released from adjacent nerves of a different type, have presynaptic receptors on their neighbouring nerves. In the heart, the best example of this is the reciprocal arrangement which exists between parasympathetic and sympathetic postganglionic nerves. Cholinergic agonists or vagal stimulation inhibits stimulated noradrenaline overflow from isolated, perfused rat hearts (Löffelholz and Muscholl, 1969; Fuder *et al.*, 1985; Levy, 1984;

Lavallée *et al.*, 1978). Similarly, acetylcholine release from incubated rat atria, evoked by either field stimulation or high potassium, was significantly inhibited by α-adrenoceptor agonists (Wetzel *et al.*, 1985; McGrattan *et al.*, 1987). As yet, it has not been demonstrated that noradrenaline release evoked by sympathetic nerve stimulation is able to inhibit the overflow of vagally mediated acetylcholine overflow. Although, in the dog heart *in vivo*, sympathetic nerve stimulation reduces heart rate variability, the effect is mediated through the adrenergic co-transmitter neuropeptide Y and not through presynaptic α-adrenoceptors (Warner and Levy, 1989; Potter, 1987; Hall and Potter, 1990).

iii) Trans-synaptic (retrograde) modulation

Released neurotransmitter binding to postsynaptic receptors on the effector cell, may stimulate the release of a substance which is able to diffuse back and interact with presynaptic exocytotic mechanisms. Examples of such substances include: adenosine, bradykinin, histamine, serotonin and prostaglandins. Thus, adenosine which is released from myocardial cells following sympathetic stimulation (Fredholm and Sollevi, 1981), acts at presynaptic A_1-adenosine receptors on the sympathetic nerves and inhibits further release of noradrenaline (Haass *et al.*, 1989).

iv) Neurohormonal modulation

Autacoids or hormones produced by remote tissues are carried in the blood and influence neurotransmitter released at presynaptic autonomic nerves. Examples would be the facilitation of noradrenaline release by adrenaline acting at presynaptic β_2-adrenoceptors on cardiac sympathetic nerves (Adler-Graschinsky and Langer, 1975; Yamaguchi *et al*, 1977). Also, the inhibition of presynaptic acetylcholine release from parasympathetic nerves by circulating noradrenaline acting at α_2-adrenoceptors. Other substances in this category include angiotensin II, histamine, dopamine, enkephalines and serotonin (Corr *et al.*, 1986).

d) *Electrophysiological and metabolic effects of myocardial acetylcholine*

General parasympathetic effects on cardiac electrophysiology include negative inotropic and chronotropic changes, decreased automaticity and pacemaker activity, decreased duration of action potential and refractory period. The rate of electrical conduction through the myocardium may also be slowed. These changes are effected through alterations to the membrane ionic conductances, particularly potassium, which occur during the five phases of the cardiac action potential.

For example, acetylcholine exerts its negative chronotropic effect, primarily by opening inwardly rectifying potassium channels $I_{K(ACH)}$ during phase 4 of the action potential in

the nodal tissues (Boyett *et al.*, 1995; Spear *et al.*, 1979). This effect opposes the spontaneous depolarisation mediated by I_f (DiFrancesco and Tromba, 1988) and increases the time taken to reach the pacemaker potential. Acetylcholine is coupled to the potassium channel through m_2-muscarinic activation of a heterotrimeric, pertussis toxin sensitive G protein (Kurachi *et al.*, 1992; Pfaffinger *et al.*, 1985). Through its actions on inwardly rectifying potassium channels, acetylcholine is also able to hyperpolarise the cell membrane and depress the pacemaker potential, making the heart less excitable and less prone to the induction of re-entrant arrhythmias (Baumgarten and Fozzard, 1986; Mubagwa and Carmeliet, 1983).

In addition to its negative chronotropic effect, acetylcholine is able to antagonise the positive inotropic effect of β-adrenergic stimulation, mediated by increased inward calcium influx during the plateau phase of the action potential (Josephson and Sperelakis, 1982). As discussed later (see section 1.1.3c), the β-adrenergic effect is mediated by cyclic AMP and m_2-muscarinic receptor activation is coupled to adenylate cyclase activity by an inhibitory G protein. The muscarinic stimulation of guanylate cyclase activity and cGMP (George *et al.*, 1970) may also play a role in this inhibition (Beavo *et al.*, 1971; Endoh, 1979). Acetylcholine also increases the repolarising inward potassium current during phase 3 of the action potential, resulting in lengthening of the refractory period.

In addition to the acute effects on ionic conductance, acetylcholine acting at muscarinic receptors can also exert more long-term effects through alteration of membrane phospholipid metabolism. Stimulation of muscarinic receptors causes the 'PI effect' - an increase in the breakdown of phosphatidylinositol (Cockroft, 1987; Brown and Brown, 1983; Quist, 1982). Changes in the phospholipid metabolism may be directly linked to effector sites through mechanisms involving alterations to physical properties of the membrane (fluidity etc.). Furthermore, although the muscarinic stimulation of myoinositol-D-1,4,5-trisphosphate (IP_3) formation is thought to be physiologically unimportant in the mammalian heart (Hawthorne and Simmonds, 1989), formation of the second metabolite, diacylglycerol, and the subsequent activation of protein kinase C, may play a role in the modulation of contractility over the longer term. Protein kinase C is proposed to achieve this through desensitisation of β-adrenergic receptors (Limas and Limas, 1985), and phosphorylation of sarcoplasmic reticulum to enhance the calcium transport ATPase (Limas, 1980).

e) Inactivation of released acetylcholine

Clearance of acetylcholine from the synaptic cleft in the heart is by both enzymatic hydrolysis and diffusion / washout (for reviews, see Löffelholz *et al.*, 1984; Löffelholz and Pappano, 1985). During the former, acetylcholine is hydrolysed into its constituent parts by the enzyme

acetylcholinesterase. Acetylcholinesterase is located on the basement membrane, past which the acetylcholine must diffuse to reach the postsynaptic membrane. This process is relatively slow within the mammalian heart and allows much of the released acetylcholine to diffuse out of the extracellular space and into the vascular lumen from where it is washed out. Even so, the use of cholinesterase inhibitors markedly potentiates the actions of acetylcholine within the mammalian heart (Osterrieder *et al.*, 1981; Glitsch and Pott, 1978), leading to a 2-3 fold increase in the evoked output (Dieterich *et al.*, 1977). In the perfused chicken heart, the mean extracellular concentration of acetylcholine following a short burst of vagal stimulation was found to decline in a multiexponential fashion. The concentration was reduced by 50 % after only 2.5 seconds (Lindmar *et al.*, 1982) although 20 % of the acetylcholine remained unhydrolysed after a further 20 seconds. In contrast to the mammalian heart, acetylcholine overflow is readily detectable in the coronary effluent of perfused, vagally stimulated avian hearts, even in the absence of cholinesterase inhibition (Kilbinger and Löffelholz, 1976; Dieterich *et al.*, 1977). The avian ventricle has a much more extensive parasympathetic innervation than its mammalian counterpart (Löffelholz and Pappano, 1985) and vagal stimulation results in a much larger acetylcholine overflow.

1.1.3 Physiology of the sympathetic nervous system

a) Synthesis and storage of noradrenaline

Noradrenaline is synthesised within the cytosol of the adrenergic nerve terminals (for a detailed review, see Knight *et al.*, 1989; Philippu and Matthaei, 1988). There are several enzymatic steps in the process, the first of which is rate limiting and involves the hydroxylation of L-tyrosine by tyrosine hydroxylase. The resulting DOPA is acted on by DOPA decarboxylase to form dopamine, which is then converted to noradrenaline by dopamine β-hydroxylase.

Storage of noradrenaline is in both small and large dense cored vesicles within the nerve terminal. Very little noradrenaline is present in the cytosol, but the concentration in the vesicles is very high (0.3 - 1.0 M). A reserpine sensitive active transport system carries the noradrenaline across the vesicular membrane. Inside the vesicles, noradrenaline is complexed to a protein, chromogranin A, and ATP, which reduces the osmolarity of the vesicle contents and helps to prevent the noradrenaline from leaking out. Neuropeptide Y (NPY), a co-transmitter, is also stored within the large dense cored vesicles and released at the same time as noradrenaline, ATP and chromogranin A.

b) Noradrenaline release

Noradrenaline release from adrenergic nerve terminals is triggered in much the same way as acetylcholine from cholinergic nerve terminals. Depolarisation caused either by an action potential or high extracellular potassium concentration allows an influx of calcium into the postganglionic adrenergic nerve terminal through voltage sensitive calcium channels. This stimulates synaptic storage vesicles to fuse with the presynaptic membrane by a mechanism which has yet to be fully elucidated. Noradrenaline release is accompanied by release of the co-transmitters NPY and ATP, which themselves may modulate further exocytotic release via presynaptic Y_2 and P_{2Y} receptors respectively (Lundberg, 1996). The presynaptic modulation of exocytotic noradrenaline release is discussed in more detail in section 1.1.2c (see Figure 1.2).

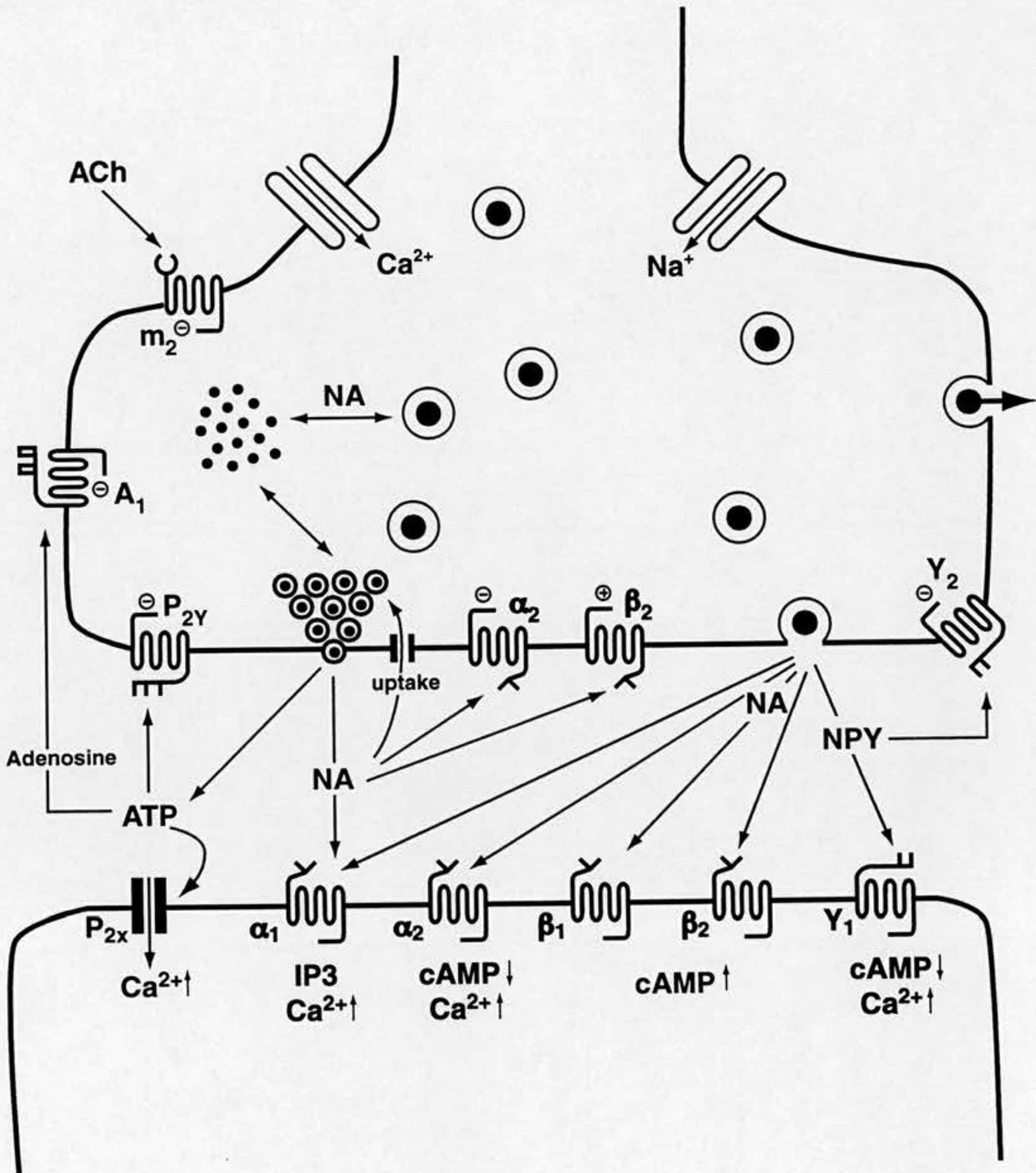
c) Effects of released noradrenaline on cardiac electrophysiology and metabolism

There are five distinct types of adrenoceptor, and at least four of them are present in the cardiac junctional tissues. Of the four, α_1, β_1 and β_2 receptors are located postsynaptically, whereas α_2 receptors are found prejunctionally. β_3-adrenoceptors are expressed in adipose tissue, where they play a role in the modulation of lipolysis (Barbe *et al.*, 1996), and the alimentary tissues, where they mediate muscle relaxation (De Boer *et al.*, 1995). Their presence in the heart has yet to be confirmed (Kaumann and Molenaar, 1996).

Stimulation of α_1-adrenoceptors causes an increase in cytosolic free calcium levels (Bylund *et al.*, 1994). This is achieved by triggering the release of calcium from intracellular calcium stores (Charest *et al.*, 1983) through the G protein-mediated activation of phospholipase C (Bylund *et al*, 1994). The calcium binds to calmodulin and the resulting complex acts as a second messenger. α_1-adrenoceptors have also been linked to a variety of other signal transduction mechanisms, including the activation of phospholipases A_2 and D, and effects on cyclic nucleotides (Minneman, 1988). In the myocardium, α_1-adrenoceceptor stimulation leads to increased inotropy, reduced automaticity of pacemaker cells, lengthening of the action potential and increased glucose uptake (Clark and Patten, 1984).

Cardiac beta-adrenoceptors are divided into two subtypes - β_1 and β_2. Both subtypes are present in human cardiac tissue, although β_1 appears to predominate (Stiles *et al.*, 1983). Myocardial β-adrenergic stimulation leads to positive inotropic and chronotropic changes, shortening of action potential duration, increased automaticity of pacemaker cells and increased conduction velocity.

Figure 1.2 *Schematic illustration of the major pre- and post-synaptic interactions at the adrenergic nerve terminal*



Noradrenaline is synthesised locally in nerve endings and stored in both small and large dense-cored vesicles. The co-transmitter, neuropeptide Y (NPY) is also stored in the large dense-cored vesicles, along with ATP which is present in both vesicle types. Depolarization causes the vesicles to migrate and fuse with the presynaptic membrane, releasing their contents. Following release, noradrenaline activates a variety of postjunctional α and β-adrenoceptors. In addition, it activates prejunctional α_2 adrenoceptors, inhibiting calcium entry and thereby reducing noradrenaline release. Active reuptake of noradrenaline into the presynaptic nerve terminal occurs via uptake$_1$. Following reuptake, noradrenaline is repackaged into storage vesicles. The co-transmitters NPY and ATP also possess both pre- and post-synaptic receptors. ATP acts postsynaptically at P_{2x} purinergic receptors. It also acts on presynaptic $P_{2}y$ purinergic receptors to inhibit noradrenaline release, and can be degraded by ecto-ATPase to form adenosine which has similar actions at presynaptic A_1 adenosine receptors. NPY also has an inhibitory effect on noradrenaline release through its actions at presynaptic Y_2 neuropeptide receptors, and acetylcholine diffusing from adjacent cholinergic nerve terminals activates presynaptic m_2 muscarinic receptors to achieve the same result.

All three β-adrenoceptor subtypes are linked to adenylate cyclase activity through a stimulatory G protein, G_s (Pelzer *et al.*, 1990). There is no evidence for subtype variability in this mechanism (Tate *et al.*, 1991). When an agonist such as noradrenaline binds to a β-adrenoceptor, adenylate cyclase activity is stimulated through the action of G_s, resulting in increased levels of intracellular cAMP. Cyclic AMP exerts a variety of intracellular effects through phosphorylation of a range of cAMP-dependent protein kinases. These have targets at calcium and potassium channels, sarcolemmal proteins such as phospholamban, contractile proteins and metabolic enzymes responsible for glycogenolysis and lipolysis.

Cyclic AMP-dependent protein kinase A, phosphorylates components of voltage-gated calcium channels, allowing increased calcium influx during the early part of the action potential (Kameyama *et al.*, 1996). This increased calcium influx is responsible for the positive inotropic changes which are characteristic of β-adrenergic stimulation.

There is evidence that G_s is able to regulate calcium, potassium and sodium ion channels directly, even when the cAMP pathways are blocked (Brown and Birnbaumer, 1988; Yatani *et al*, 1988; Pelzer *et al.*, 1990). Although still controversial, the effect is very rapid and has been proposed to allow beat to beat regulation of ion currents by sympathetic stimulation of the heart (Pelzer *et al.*, 1990).

In addition to its effects on myocardial contractility and action potential duration, β-adrenoceptor stimulation exerts a positive chronotropic effect. This is achieved by increasing the rate of spontaneous depolarisation of atrial pacemaker cells during phase 4 of the action potential through an increase in the pacemaker current (Noble, 1984).

Alpha$_2$-adrenoceptors too, are able to regulate adenylate cyclase activity. They have an inhibitory effect on adenylate cyclase activity via a pertussis toxin sensitive G protein, G_i (Limbird, 1988). Presynaptic α_2-adrenoceptors are located on both parasympathetic and sympathetic postganglionic neurones, where they inhibit the overflow of neurotransmitter (Farnebo and Hamberger, 1971; Kirkepar and Puig, 1971; Paton and Vizi, 1969). The inhibition is mediated by a reduction in the calcium influx through voltage-gated calcium channels, following depolarisation of the synaptic membrane. In the past, this was thought to be mediated through a reduction in adenylate cyclase activity. The failure of inactivation of G_i with pertussis toxin, to reliably abolish the α_2-mediated inhibition of neurotransmitter release called this into question (Nichols *et al.*, 1988). Recently, a cGMP-dependent protein kinase mechanism has been proposed (Haddad *et al.*, 1995).

d) Deactivation and clearance of released noradrenaline

Unlike acetylcholine, there is no synaptic enzyme whose specific purpose is to inactivate noradrenaline. Instead, under normal physiological conditions, the majority of the released noradrenaline is removed from the synaptic cleft by two uptake systems, $uptake_1$ and $uptake_2$.

$Uptake_1$ is relatively more important in the clearance of noradrenaline from the synaptic cleft (Iversen, 1973; Goldstein *et al.*, 1988; Dart *et al.*, 1984b) and carries noradrenaline back across the synaptic membrane. It is an active transport system that is powered by the electrochemical Na^+ gradient. Its action is blocked by cocaine, phenoxybenzamine, amphetamines and tricyclic antidepressants such as desipramine. Blockade of $uptake_1$ causes an increased overflow of noradrenaline (Dart *et al.*, 1983) and increased haemodynamic response during sympathetic nerve stimulation (Dart *et al.*, 1984a). Although $uptake_1$ has a high affinity for noradrenaline (it does not transport the β agonist isoprenaline), it has a low maximum rate. Once inside the axoplasm, the majority of noradrenaline is repackaged into storage vesicles. A small amount is metabolised by monoamine-oxidase (MAO) into the inactive metabolite 3,4-dihydroxy-phenyl-ethyl-glycol.

$Uptake_2$ is located in the postsynaptic membrane and transports noradrenaline into the extra-neuronal cells. Inside the cells, noradrenaline is degraded by the enzyme catechol-O-methyl transferase (COMT), producing the metabolite nor-metanephrine. Although $uptake_2$ has a high maximum rate in comparison to $uptake_1$, it has a much lower affinity for noradrenaline, and so plays a less important role in its clearance (Iversen, 1973; Goldstein *et al.*, 1988; Dart *et al.*, 1984b). $Uptake_2$ has a higher affinity for adrenaline and isoprenaline, both of which can also be methylated by the enzyme COMT. $Uptake_2$ is blocked by corticosteroids but is not sensitive to any of the compounds that inhibit $uptake_1$.

Washout of noradrenaline from the synaptic cleft into the circulation also occurs (Dart *et al.*, 1983) although reuptake is quantitatively more important at low flow rates. In the presence of uptake blockade, washout becomes the principal method of synaptic clearance of noradrenaline. Studies on the isolated rat heart, perfused at 5 ml g^{-1} min^{-1} in the presence of desipramine, have shown that following a 1 minute sympathetic nerve stimulation, 98 % of the noradrenaline overflow appears in the coronary effluent within the first minute (Du, Ph.D thesis, 1991). Sensitive and robust methods exist for the analysis of noradrenaline and have been successfully applied to direct measurement of the neurotransmitter in coronary effluent from isolated hearts. This has proved to be a useful indicator of sympathetic activity and has provided important insights into the behaviour of the sympathetic nervous system and the process of chemical neurotransmission during cardiac ischaemia (Dart and Du, 1993).

1.1.4 Parasympathetic / sympathetic interactions

Interaction of the two halves of the autonomic nervous system occurs at all levels, permitting the fine control of its actions. In general, the interactions can be classified as presynaptic or postsynaptic mechanisms.

a) Presynaptic interactions

Interaction of sympathetic and parasympathetic nerves occurs in the control of neurotransmitter release from presynaptic storage vesicles (see section 1.1.2c). The presynaptic sympathetic nerve terminals express muscarinic receptors, and parasympathetic terminals express α-adrenoceptors. In both cases, the presynaptic receptors have been shown to be capable of reducing the overflow of neurotransmitter from the nerve terminal. As already discussed, this type of modulation is known as axo-axonal modulation. Trans-synaptic modulation may also occur where for example, stimulation of the sympathetic nerves causes the effector cell to release a substance such as adenosine or a prostaglandin, which diffuses to a specific receptor on the presynaptic parasympathetic terminal and affects acetylcholine release.

b) Postsynaptic interactions

Postjunctional interaction of the two branches of the autonomic nervous system is indicated by the ability of acetylcholine to antagonise the effects of noradrenaline infusion (Dempsey and Cooper, 1969).

Interaction can occur at any of the post-receptor stages beyond G protein activation. For example, β-adrenergic receptors are coupled to adenylate cyclase via a G protein (Gs). Binding of noradrenaline to a β-adrenergic receptor will stimulate adenylate cyclase activity and cause an increase in the cytosolic concentration of cyclic AMP. This in turn, activates cyclic AMP-dependent protein kinase which is involved in the cleavage and regulation of key metabolic enzymes and the control of certain ion channels. The simultaneous stimulation of cholinergic, muscarinic receptors acts to oppose the adrenergic effects at more than one level. First, muscarinic receptors are coupled to an inhibitory G protein (Gi), which inhibits adenylate cyclase activity and therefore reduces the build up of cyclic AMP. In addition, muscarinic receptors are coupled to guanylate cyclase activity, stimulating the production of cyclic GMP which has been shown to antagonise the actions of cyclic AMP (Hartzell and Fischmeister, 1986).

Interaction of the autonomic nervous system at both the presynaptic and postsynaptic levels allows the two halves to be in equilibrium with each other. Direct measurement of

acetylcholine overflow from stimulated rat heart preparations would allow further investigation and understanding of the mechanisms of presynaptic modulation of acetylcholine overflow and the effect of ischaemia on these mechanisms.

1.1.5 Autonomic activation during myocardial ischaemia

Signs of autonomic activation are common during acute myocardial infarction. Acute ischaemia or infarction and reperfusion is able to trigger cardiac reflexes (Sleight, 1964; Webb *et al.*, 1972) by the stimulation of both chemosensitive and mechanosensitive receptors. In a study by Webb and colleagues, the incidence of autonomic disturbance was recorded in patients within the first hour of the onset of symptoms of their first episode of acute myocardial infarction. Subjects were excluded if they had suffered a previous myocardial infarction, had experienced ventricular arrhythmic events or received therapy with either beta blockers or digitalis. Sinus bradycardia, AV block or transient hypotension were taken as evidence of parasympathetic hyperactivity, whereas sinus tachycardia and transient hypertension denoted sympathetic hyperactivity. Within 30 minutes, most patients showed signs of either parasympathetic or sympathetic overactivity, with the former being more frequent in cases of inferior infarction. Symptoms of excessive sympathetic activation were the more usual outcome in cases of anterior infarction (Webb *et al.*, 1972; Ninomiya *et al.*, 1986; Thames *et al.*, 1978). These site-specific effects of ischaemia are thought to reflect the greater extent of vagal innervation and density of vagal afferent receptors located in the infero-posterior wall of the left ventricle (Inoue and Zipes, 1987a).

There is also evidence that both branches of the autonomic nervous system can be activated simultaneously during myocardial ischaemia. Circulating levels of plasma catecholamines are raised during the 48 hours following myocardial infarction (Nadeau and de Champlain, 1979; Forfar *et al.*, 1984; Goldstein, 1981). Catecholamines are released from the peripheral tissues in response to pain, distress and anxiety, resulting in a high sympathetic drive. A simultaneously enhanced parasympathetic tone may override this sympathetic activity (Lombardi *et al.*, 1984). Atropine treatment caused subjects who had previously displayed bradycardia and symptoms of parasympathetic overactivity to become tachyarrhythmic, indicating a coexisting sympathetic overactivity (Webb *et al.*, 1972).

In contrast to the above studies, some investigators have observed that ischaemia or infarction can inhibit normal cardiac reflex activity soon after the onset of coronary occlusion (Barber *et al.*, 1985; Inoue *et al.*, 1988). In addition, the local effects of ischaemia, such as tissue acidosis and potassium efflux may lead to the interruption of normal neural function, both within the ischaemic territory and at sites apical to it which are served by axons lying

within the ischaemic zone. In one study (Inoue and Zipes, 1988), heterogeneous loss of functional efferent sympathetic innervation in non-infarcted apical sites occurred as early as 5-20 minutes after the onset of transmural ischaemia created by coronary occlusion. The extent of this acute denervation was found to increase with time. Following chronic experimental denervation, either chemically with 6-hydroxy-dopamine, or surgically, the affected tissue becomes hyper-responsive to certain agonists. This phenomenon is known as denervation supersensitivity and has been documented for both the sympathetic and parasympathetic nervous systems (Cannon, 1939). A postsynaptic mechanism appears to be involved (Vatner *et al.*, 1985). Sympathetic supersensitivity is characterised by an exaggerated shortening of the refractory period in response to adrenergic agonist infusion. Shortening of the refractory period is pro-arrhythmic, particularly when the electrophysiological changes are heterogeneous across the myocardium (Herre *et al.*, 1988; Inoue and Zipes, 1987b). Likewise, electrophysiological inhomogeneities caused by vagal denervation, may be arrhythmogenic too.

Whether ischaemia triggers or inhibits cardiac reflexes may depend on a number of factors. The severity of the ischaemia and degree of collateral circulation, the size and position of the ischaemic zone and whether it is transmural or not may all be important in determining the outcome. Different time courses may also be involved.

1.1.6 Autonomic nervous system and sudden cardiac death

Under experimental conditions, increased sympathetic neural input during ischaemia is recognised to be pro-arrhythmic, causing ventricular tachyarrhythmias and lowering of the threshold to precipitate VF. Furthermore, sympathetic denervation or β blockade can protect the ischaemic myocardium against arrhythmias (Schwartz and Zaza, 1986; Schwartz and Stone, 1980). Traditionally, parasympathetic influences have been regarded as antiarrhythmic, an action which is thought to be due to their anti-adrenergic property rather than any direct antiarrhythmic effect.

With the introduction of two non-invasive methods to measure parasympathetic tone, the relationship between autonomic nerve function *in vivo,* and the development of lethal arrhythmias following myocardial infarction has been examined more closely in humans too.

Tonic vagal activity can be determined by the analysis of heart rate variability from 24 hour ECG recordings. Small variations in the beat to beat length of the R-R interval are measured. The variability increases with increasing parasympathetic tone. Frequency analysis of the ECG recordings can also be accomplished. When the frequencies are divided into 4 bands - from high to ultra low, each band is thought to represent different components of the

autonomic nervous system. The high frequency band (0.15 - 0.4 Hz) is thought to reflect tonic parasympathetic activity (Berger *et al.*, 1986).

Parasympathetic reflex activity can also be assessed by evaluating baroreflex sensitivity in the control of heart rate. Arterial baroreceptors are stimulated by increasing the blood pressure with graded doses of a vasoconstrictor such as phenylephrine. Reflex sympathetic tone can be gauged in the same way, using a vasodilator to lower blood pressure.

Measurement of both heart rate variability and baroreflex sensitivity have been found to be useful in predicting the outcome after myocardial infarction in experimental and clinical situations (for review see Barron and Lesh, 1996). Experimentally, the canine model of sudden cardiac death has been most extensively studied. Myocardial infarction is surgically induced and then one month later, the dogs are run on a treadmill at the same time as transient ischaemia is induced by inflation of a surgically implanted balloon. Those that develop VF are classified as 'susceptible' and the others as 'resistant'. One month after infarction, both parasympathetic tonic (Hull *et al.*, 1990; Adamson *et al.*, 1994) and reflex (Schwartz *et al.*, 1988b) activity measured by heart rate variability and baroreflex sensitivity respectively, have been shown to be significantly more impaired in the susceptible as opposed to resistant dogs.

In Adamson's study, pre-infarction heart rate variability was not different in the two groups of dogs (Adamson *et al.*, 1994). Beta blockade, however, caused a much greater increase in heart rate variability in those animals which later were shown to be resistant to the development of VF. Confounding variables, such as the extent of the collateral circulation, were also not different in the two groups.

Pre-infarction differences in baroreflex sensitivity too may be present (Schwartz *et al.*, 1988a). Baroreflex sensitivity and heart rate variability are reduced after a myocardial infarction, but steadily improve over a period of about 3 months back to control levels (Schwartz *et al.*, 1988b; Lombardi *et al.*, 1987). This time period appears to fit the window in which post-infarction patients are at most risk of sudden cardiac death (Myerburg *et al.*, 1992).

Several investigators have examined the predictive value of baroreflex sensitivity and heart rate variability testing in patients who have suffered an infarction. Both depressed heart rate variability and baroreflex sensitivity are associated with increased risk of sudden death in the period following an infarction (Kleiger *et al.*, 1987; La Rovere *et al.*, 1988). The strength of the association is greatly diminished after 6 months post-infarction (Odemuyiwa *et al.*, 1994).

Studies comparing the prognostic value of the two tests have found that baroreflex sensitivity carries the greatest predictive power (Farrell *et al.*, 1992). La Rovere and

colleagues studied a group of 78 patients suffering their first myocardial infarction (La Rovere *et al.*, 1988). The follow up period was 2 years. Baroreflex sensitivity of less than 3.0 ms / mm Hg carried a 50 % mortality rate, compared to only 3 % in patients with a baroreflex sensitivity greater than 3.0 ms / mm Hg.

The superiority of baroreflex sensitivity testing over heart rate variability in determining arrhythmic risk is probably due to the nature of the two tests. Whereas heart rate variability measures tonic parasympathetic activity, baroreflex sensitivity measures the ability of the parasympathetic system to respond to increased sympathetic activity in the cardiovascular system. Thus, parasympathetic reflex activity is more likely to be an important factor in limiting the potentially dangerous electrophysiological changes which occur when the already destabilised myocardium is put under stress.

1.1.7 Catecholamine release in the ischaemic myocardium

Despite the widely held view that myocardial ischaemia induces neuronal noradrenaline release, investigators have found difficulty in demonstrating an enhanced noradrenaline overflow from the ischaemic tissue without reperfusion (Riemersma and Forfar, 1982). In studies of stop flow ischaemia, where the duration of ischaemia is longer than 10 minutes, reperfusion causes the overflow of noradrenaline. The amount of released noradrenaline is related to the duration of the ischaemia. The kinetics of this overflow fits a single compartment model and suggests that the noradrenaline accumulates in the extracellular space during ischaemia and is not released by reperfusion itself. For shorter ischaemic episodes, reperfusion overflow of noradrenaline cannot be demonstrated, possibly due to increased neuronal reuptake which occurs during early myocardial ischaemia (Riemersma and Forfar, 1982).

Two mechanisms of noradrenaline overflow from acutely ischaemic myocardium have been proposed. During the first few minutes of ischaemia, the activation of sympathetic reflexes is postulated to result in limited local release of noradrenaline. Activation of cardiac sympathetic efferents has been demonstrated within seconds of the onset of coronary artery occlusion (Malliani *et al.*, 1969). Noradrenaline overflow, however, was not measured. Indeed, increased noradrenaline overflow from the ischaemic tissue, corresponding to this activity has been hard to demonstrate both in dogs (Forfar *et al.*, 1983; McGrath *et al.*, 1981), and in man undergoing balloon angioplasty (Schömig *et al.*, 1990). Increased neuronal reuptake during early ischaemia may have removed any noradrenaline that was released before it could wash out (Richardt *et al.*, 1990). When neuronal reuptake was taken into consideration, McCance and Forfar could document enhanced spillover of noradrenaline in patients during episodes of angina (McCance and Forfar, 1989). Evidence that reflex sympathetic activation during early

ischaemia causes neuronal catecholamine release also comes from denervation studies. In the *in vivo* conscious dog model, left stellectomy or left stellate ganglion blockade protected the acutely ischaemic heart from VF. This was presumably by preventing the deleterious release of myocardial catecholamines (Schwartz and Stone, 1980), although the results of denervation studies should be interpreted with care. Denervation may also reduce the rate of potassium efflux from the ischaemic zone (Martin and Meesmann, 1985) and chronic or chemical denervation facilitates the development of preexisting collaterals (Jones *et al.*, 1978; Römer *et al.*, 1981) both of which are antiarrhythmic. In addition to the above evidence of sympathetic activation, cAMP levels are raised within 3 minutes of the onset of ischaemia and coincide with the appearance of ventricular arrhythmias (Opie *et al.*, 1982). Reflex induced endogenous noradrenaline release occurs by calcium dependent exocytosis and is sensitive to calcium channel blockers (Göthert *et al.*, 1979).

The overflow of noradrenaline after longer periods of ischaemia (between 15 and 40 minutes duration) has been easier to demonstrate both with reperfusion (Abrahamsson *et al.*, 1984;) and histochemical studies (Holmgren *et al.*, 1981). This noradrenaline overflow has been demonstrated in isolated heart preparations, and is proposed to be due to a non-exocytotic, non-calcium dependent mechanism (Kurtz *et al.*, 1995a and b; Schömig *et al.*, 1985). The above authors have hypothesised that the compromised energy state of the tissue allows noradrenaline to leak out of the vesicles and into the cytoplasm. Provision of glucose in the perfusion medium delays the noradrenaline release, presumably by improving the energy supply (Abrahamsson *et al.*, 1983; Schömig *et al.*, 1987). The sodium ion gradient is reversed in the ischaemic cells and it is proposed that this reverses the normal direction of the $uptake_1$ carrier, causing it to pump noradrenaline out of the cell and into the extracellular space (Schömig *et al.*, 1985). Desipramine, the $uptake_1$ blocker, has been shown to prevent this release (Kurtz *et al.*, 1995a; Schömig *et al.*, 1987; Carlsson, 1987). This noradrenaline release occurs in the absence of extracellular calcium (Abrahamsson *et al.*, 1984), and has also been shown to occur without the concurrent release of the co-transmitter NPY, which would be expected, were an exocytotic mechanism responsible.

The physiological significance of this non-exocytotic noradrenaline release has yet to be established. In the isolated rat heart, suppression of non-exocytotic noradrenaline release with desipramine (1 μM) reduced the incidence of VF during ischaemia (Kurz *et al.*, 1995a). In the *in vivo* situation, however, the release occurs at a time when the plasma levels of catecholamines are already raised due to enhanced release from sympathetic nerves and adrenal medulla (Goldstein, 1981; Nadeau and de Champlain, 1979). The effect of any local release from the ischaemic tissue at this time may be overwhelmed by the effect from the high

circulating catecholamine concentration. Furthermore, differentiating between the two sources of noradrenaline in the *in vivo*, un-anaesthetized situation is likely to be impossible.

1.1.8 Contribution of the sympathetic nervous system to arrhythmogenesis

Three principal arrhythmogenic mechanisms have been identified during ischaemia; re-entry, enhanced automaticity and triggered automaticity. The autonomic nervous system is able to influence all three (for review see Janse and Wit, 1989).

Re-entrant mechanisms are proposed to underlie the majority of serious ventricular arrhythmias during ischaemia in the clinical as well as the experimental setting (Janse *et al.*, 1980). Conduction occurs through a loop of tissue in a circular motion. A unidirectional conduction block must be present in part of the loop. Under these conditions, an impulse entering from outside can only travel in one direction around the conduction loop. Normally, the impulse would die out after a single circuit, as it would not be able to reexcite the refractory tissue in the first half of the loop. During ischaemia, slow conduction velocity and shortened refractory periods may allow the impulse to continue propagating and generate a circus movement, or re-entrant rhythm. Interventions which increase the inhomogeneity of conduction velocity or repolarisation within the myocardium, facilitate the formation of re-entrant circuits. Sympathetic activation is proposed to do this, as the innervation is not homogeneous throughout the myocardium. Sympathetic nerve stimulation was observed to increase the dispersion of refractoriness in the myocardium (Schwartz and Zaza, 1986), whereas a slow infusion of a low dose of exogenous noradrenaline was found to have the opposite effect (Han and Moe, 1964).

Enhanced or abnormal automaticity of Purkinje or ventricular myocardial cells, as a result of myocardial infarction, has also been proposed to lead to the genesis of arrhythmias (for a detailed review see Gilmour and Zipes, 1986). Under normoxic conditions, automaticity only occurs in pacemaker cells as a result of a spontaneous depolarisation during phase 4 of the action potential. Electrophysiological studies have demonstrated the presence of spontaneous activity in depolarised specimens of diseased human ventricle (Singer *et al.*, 1981; Gilmour *et al.*, 1983). Such cells may provide an ectopic focus within the infarction zone and, particularly under the influence of catecholamines which increase the firing rate (Imanishi and Surawicz, 1976), contribute to the genesis of re-entrant arrhythmias. This mechanism of arrhythmogenesis has never been proved to occur *in vivo*. It is deemed to be an unlikely mechanism by some investigators, owing to the fact that the automaticity should be suppressed in ischaemic tissue by the elevated potassium concentrations which are present (Janse and Wit, 1989).

The third mechanism of arrhythmogenesis during ischaemia, is triggered activity due to delayed after-depolarisations. Delayed after-depolarisations are small oscillations in the membrane potential which occur at the end of the action potential, when the membrane has returned to its resting state. They are caused by fluctuations of calcium ions (Ducceschi *et al.*, 1996) and are generally sub-threshold in magnitude. Under certain conditions, such as ischaemia, the amplitude of the oscillations can increase above the threshold level and an action potential is triggered. A tachyarrhythmia results if the process is repeated. Catecholamines increase the influx of calcium ions, increasing the amplitude of the oscillations and facilitating the development of triggered activity (Roden, 1993).

Autonomic mechanisms are also involved in the pathology of long QT syndrome, a hereditary condition which is characterised by an abnormally long QT interval on ECG. Those affected have a high risk of sudden death due to the onset of lethal ventricular arrhythmias. Prolongation of the surface QT interval is believed to reflect imbalance of the ventricular autonomic innervation, resulting in increased dispersion of refractoriness and increased vulnerability to re-entrant arrhythmias. Left stellectomy, either alone or in conjunction with β blocker therapy, can protect against the onset of lethal ventricular arrhythmias in sufferers of the syndrome (Schwartz, 1985).

1.1.9 Contribution of the parasympathetic nervous system to arrhythmogenesis

It is well documented that the parasympathetic nervous system can protect the myocardium against the formation of serious ventricular arrhythmias during the early phase of acute myocardial ischaemia. During experimental ischaemia, vagal nerve stimulation raised the VF threshold (Kent *et al.*, 1973) and reduced the incidence of ventricular arrhythmias (Myers *et al.*, 1974; Scherlag *et al.*, 1970). Likewise, cholinesterase inhibitors protected against arrhythmias (Das and Bhattacharya, 1972; Harrison *et al.*, 1974), an effect which was abolished by atropine (Harrison *et al.*, 1974). The antiarrhythmic effect of cholinergic stimulation is more pronounced during the early stages of experimental myocardial ischaemia. Parasympathetic nerve stimulation was ineffective, or even exacerbated the genesis of arrhythmias after experimental myocardial ischaemia had progressed beyond the first few hours (Scherlag *et al.*, 1974; Kerzner *et al.*, 1973). This pro-arrhythmic effect may have resulted from vagally-mediated slowing of the atrial pacemaker rate, thus abolishing the overdrive pacing of areas of enhanced automaticity in the ventricles. Ventricular cells exhibiting enhanced automaticity may contribute to the formation of re-entrant arrhythmias (see section 1.1.8). Mortality owing to the sudden onset of malignant ventricular arrhythmias, is at its highest during the first hour of acute myocardial ischaemia (Webb *et al.*, 1972). In a

recent study, muscarinic agonists protected against ventricular arrhythmias during an episode of transient ischaemia in conscious dogs one month after a previous myocardial infarction (De Ferrari *et al.*, 1993). In the same model, superior vagal reflexes have been linked to a decreased risk of sudden death (De Ferrari *et al.*, 1991).

The parasympathetic nervous system is proposed to exert its antiarrhythmic effect, primarily through its antiadrenergic actions. This includes the presynaptic inhibition of noradrenaline release (Löffelholz and Muscholl, 1969; Levy and Blattberg, 1976) and antagonism of the postsynaptic effects of noradrenaline. In opposing the effects of the sympathetic nervous system, the parasympathetic system exhibits the phenomenon of accentuated antagonism (Levy, 1971). In other words, vagal effects are more powerful when there is a coexisting sympathetic drive. Thus vagal stimulation opposes the tendency of catecholamines to facilitate the genesis of re-entrant and triggered arrhythmias as described in the preceding section.

In addition to its antiadrenergic actions, various investigators support a role for a more direct antiarrhythmic action on myocardial electrophysiology. Blair and colleagues demonstrated that *in vivo*, atropine shortened the effective refractory period in feline ventricle, even after sympathectomy (Blair *et al.*, 1980). Similarly, Prystowsky's group showed the same effect of atropine in humans treated with the β blocker propanolol (Prystowsky *et al.*, 1981). For many years, studies on preparations of ventricular myocardium, *in vitro*, were unable to demonstrate a significant effect of exogenous acetylcholine application on action potential characteristics (Hoffman *et al.*, 1953). Recent work has demonstrated that while acetylcholine prolongs effective refractory periods in canine epicardium, it is without effect in endocardial tissues (Litovsky and Anzelvitch, 1990). Previous studies in which a direct effect of acetylcholine on ventricular refractoriness was not found, had all used endocardial preparations. Similar results have now been reported in feline ventricular tissues (Rosenshtraukh *et al.*, 1994). The mechanism of this direct action of acetylcholine on ventricular refractory period appears to be via activation $I_{K(ACh)}$ (Koumi *et al.*, 1995; Yang *et al.*, 1996). Lengthening of the effective refractory period opposes the formation of re-entrant arrhythmias.

1.2 Ischaemic preconditioning

Brief episodes of ischaemia and reperfusion which themselves do not cause myocardial damage, prior to a longer sustained period of myocardial ischaemia delay the onset and decrease the severity of lethal cell injury in the ischaemic tissue (for reviews see: Cohen and Downey, 1996; Losano *et al.*, 1996). This unexpected phenomenon was first described by Murry and co-workers in the dog heart (Murry *et al.*, 1986). Four 5 minute circumflex coronary occlusions interspersed with 5 minute periods of reperfusion immediately before a 40 minute occlusion, reduced the infarct size to 25 % of that produced in the control group by a single 40 minute occlusion. They described the phenomenon as ischaemic preconditioning and noted that it was not effective when the episode of sustained ischaemia was extended to 3 hours duration. Since these initial experiments were published, ischaemic preconditioning has been observed in both *in vivo* and *in vitro* preparations of all the species which have been studied. These include rabbit (Thornton *et al.*, 1990; Liu *et al.*, 1991), rat (Liu and Downey, 1992; Yellon *et al.*, 1992), dog (Murry *et al.*, 1986; Li *et al.*, 1990), and pig hearts (Schott *et al.*, 1990). But ischaemic preconditioning is not limited to intact preparations. The phenomenon has also been demonstrated in isolated ventricular myocytes (Armstrong *et al.*, 1994).

Various preconditioning protocols have been described. The minimum duration of ischaemia which has been reported to be effective in an animal model is a single episode of 2 minutes duration (Banerjee *et al.*, 1993). Although a duration of 90 seconds may be sufficient to cause protection in humans undergoing angioplasty (Deutsch *et al.*, 1990). More commonly, single or multiple episodes of 5 minutes duration have been used in conjunction with periods of 5 minutes of reperfusion. Only a few studies have ever been designed to investigate the relative efficacy of different preconditioning protocols and most investigators have chosen to use a protocol which has previously been successful in that model. In one such study by Miura and co-workers, the infarct size-limiting effect of single or multiple episodes of 5 minutes of ischaemia interspersed with 5 minutes of reperfusion were compared. Multiple episodes of preconditioning ischaemia were not more effective than a single episode at limiting infarct size (Miura *et al.*, 1992). In the dog, a single episode of 5 minutes of preconditioning ischaemia was as effective as either six or twelve 5 minute episodes (Li *et al.*, 1990).

In addition to its effect on infarct size, ischaemic preconditioning improves functional recovery after global ischaemia (Cave, 1995) and reduces the number of serious ventricular

arrhythmias during reperfusion (Podzuweit *et al.*, 1989; Komori *et al.*, 1990; Parratt and Vegh, 1994).

1.2.1 Proposed mechanisms of ischaemic preconditioning

At the time when the experiments for this thesis were carried out, efforts were being made to elucidate the mechanism of ischaemic preconditioning. Initial experiments explored several different potential targets for preconditioning. An effect on collateral flow was ruled out early on (Murry *et al.*, 1986), as were effects on mitochondrial function (Vander Heide *et al.*, 1991) and stimulation of endogenous antioxidants (Turrens *et al.*, 1992). Other avenues of investigation have explored the possibility of effects on: ischaemia-induced osmotic swelling (Armstrong *et al.*, 1994), ATP-dependent potassium channels (Grover *et al.*, 1990; Gross and Auchampach, 1992), neutrophil accumulation (Mizamura *et al.*, 1995), haemodynamic function (Schjott *et al.*, 1994; Goto *et al.*, 1993) or the rate of ATP utilisation in the ischaemic myocardium (Murry *et al.*, 1990). Currently, the end effector for ischaemic preconditioning remains to be elucidated. In recent years, however, progress has been made to identify the pathways involved in triggering the preconditioning response. It was postulated that substances such as adenosine, released by the ischaemic tissue, might be the first step in the process. Infusions (5 min) of adenosine or a specific adenosine A_1 receptor agonist in rabbit heart, were indeed found to mimic ischaemic preconditioning (Liu *et al.*, 1991). Furthermore, adenosine antagonists were able to abolish the infarct reducing effect of ischaemic preconditioning (Liu *et al.*, 1991). The involvement of adenosine in the preconditioning response was also reported in dog (Grover *et al.*, 1992; Hoshida *et al.*, 1994), pig (Yokota *et al.*, 1995) and subsequently in human hearts (Claeys *et al.*, 1996). These results seem to support the existence of a common pathway to trigger the preconditioning response in all these species. The mechanism of preconditioning in the rat heart, however, appears to be independent of adenosine (Asimakis *et al.*, 1993; Li and Kloner, 1993).

Myocardial adenosine receptors are coupled to the intracellular protein kinase C second messenger system (Cohen and Downey, 1996; Henry *et al.*, 1996). Early studies to examine a possible link between protein kinase C activation and ischaemic preconditioning found that activators of protein kinase C such as phorbol esters and water-soluble diacylglycerol compounds could mimic preconditioning (Mitchell *et al.*, 1993). Inhibitors of protein kinase C, such as staurosporine and polymyxin B, abolished the expected response (Ytrehus *et al.*, 1994). A variety of other myocardial receptors are coupled to protein kinase C including bradykinin (Minshall *et al.*, 1995), angiotensin II (Sadoshima and Izumo, 1993), endothelin (Irons *et al.*, 1993) and α_1-adrenergic receptors (Kaku *et al.*, 1991). It now appears

that there may be a number of different triggers for the ischaemic preconditioning response, which are all coupled to the end effector via a common second messenger system.

1.2.2 Catecholamines and Ischaemic Preconditioning

At the time when the experiments for this thesis were undertaken, very few investigations of the potential role of catecholamines in the mechanism of ischaemic preconditioning had been carried out. One of the first publications described how exogenous noradrenaline infusion in the rat heart, acting through α_1-adrenergic receptors, could mimic ischaemic preconditioning (Banerjee *et al.*, 1993). Reserpine pre-treatment or α_1-adrenergic blockade abolished ischaemic preconditioning, leading the authors to conclude that ischaemic preconditioning is mediated through α_1-adrenoceptor activation. At around the same time, Thornton's group published the results of their work in rabbits. They used tyramine infusions to release endogenous catecholamines and found that this could mimic preconditioning in the rabbit heart *in vivo* (Thornton *et al.*, 1993). The ability of tyramine to mimic ischemic preconditioning could be blocked by both an α_1-adrenergic receptor antagonist or by a nonselective adenosine receptor antagonist. The α_1-adrenergic antagonist was, however, unable to abolish the effect of ischaemic preconditioning to limit infarct size. They concluded that although catecholamines could cause a preconditioning effect in the rabbit, they were not implicated in the mechanism of ischaemic preconditioning. They postulated that the catecholamine release caused increased oxygen demand and metabolic stress, provoking adenosine release and leading to the preconditioning response. Adenosine overflow, however, was not measured.

One explanation for the discrepancy in the results of the above two studies, was that the mechanism of ischaemic preconditioning in the rat heart differs from that in the rabbit. Experiments with adenosine in the two species at the time supported this view (Liu *et al.*, 1994; Li and Kloner, 1993; Asimakis *et al.*, 1993). The results of a third study (Toombs *et al.*, 1993), however, did not. They reported that the infarct-limiting effect of ischaemic preconditioning was abolished in reserpinized rabbit preparations *in vivo*, leading to the conclusion that endogenous noradrenaline is involved in the genesis of the ischaemic preconditioning response.

All these investigations used large pharmacological interventions. Where exogenous noradrenaline infusions were carried out (Banerjee *et al.*, 1993; Bankwala *et al.*, 1994) unphysiologically high concentrations of noradrenaline were used (up to 30 nmol kg^{-1} min^{-1}). Whether *endogenous* noradrenaline release, induced by sympathetic nerve activation, could result in the preconditioning of the rat heart remains to be seen. This is an important question

since repeated episodes of chest pain, accompanied by increased cardiac sympathetic activity, could result in a preconditioning effect clinically.

1.2.3 Potential clinical applications of ischaemic preconditioning

To date, ischaemic preconditioning remains a mainly laboratory phenomenon. Various authors have discussed the potential applications of preconditioning in the clinical or surgical setting (Cohen and Downey, 1996). Evidence now exists that patients who experience episodes of anginal pain in the period before a major coronary event, have a better outcome than those whose first symptom is the pain of a myocardial infarction (Kloner *et al.*, 1995). Clinically, the ultimate aim would be to be able to render a patient in a chronically preconditioned state. This would provide an alternative treatment for those deemed to be at risk from coronary thrombosis in whom surgery or angioplasty was not appropriate, or whilst waiting for these elective procedures. In the event of severe myocardial ischaemia, it would delay the onset of irreversible cell damage and lengthen the time when effective thrombolytic therapy could be given to achieve myocardial salvage. In the clinical setting, preconditioning the heart with ischaemia is far from ideal. In addition to the dangers associated with inducing even transient ischaemia, the efficacy of ischaemic preconditioning to cause myocardial protection is short-lived. The initial window of protection was generally reported to be less than 2 hours long (Van Winkle *et al.*, 1991; Sack *et al.*, 1993). A second window of protection may develop up to 4 days later (Sack *et al.*, 1993), but this too is likely to have a finite length. Ideally, a pharmacological agent would be developed which would overcome both the necessity of inducing transient ischaemia and the temporary nature of the protection. Recently, investigators were unable to maintain rabbits in a chronically preconditioned state using either repetitive episodes of ischaemia or adenosine A_1-selective analogue administration (Tsuchida *et al.*, 1994; Cohen *et al.*, 1994). Tolerance developed to both interventions within 36 hours. Potentially, there is much larger scope for the routine use of ischaemic preconditioning in the surgical as opposed to the clinical environment. Either transient ischaemia or a pharmacological agent could be used to reduce the risk of complications during surgery and the recovery from bypass procedures. Encouragingly, experiments during heart surgery have indicated a benefit of several brief episodes of aortic cross-clamping preceding the principal ischaemic episode, on the recovery from heart bypass or transplant procedures (Yellon *et al.*, 1993).

1.3 Summary of objectives

The experiments detailed in this thesis aimed to investigate the following:

1) Can a novel, sensitive HPLC method be used to quantify acetylcholine overflow from the *in situ*, innervated perfused rat heart preparation following vagal nerve stimulation?
2) To what extent does the severity or duration of global ischaemia affect the vagally mediated overflow of acetylcholine?
3) Which of the concomitants of ischaemia such as reduced oxygen supply, reduced glucose supply and extent of tissue acidosis, have the greatest effect on vagally mediated acetylcholine overflow?
4) In the normoxically perfused rat heart, does the coronary flow rate affect the stimulation evoked overflow of autonomic transmitters: acetylcholine and noradrenaline?
5) How do different severities and durations of global ischaemia affect the presynaptic interaction of the parasympathetic and sympathetic nervous systems with respect to the overflow of acetylcholine and noradrenaline?
6) Does noradrenaline infusion or sympathetic ganglion stimulation improve functional recovery following global ischaemia in the rat heart?
7) Do pre-ischaemic noradrenaline infusion or sympathetic ganglion stimulation reduce the post-ischaemic reperfusion washout of noradrenaline from the rat heart?

CHAPTER 2

Materials and methods

2.1 Animal Experiments

2.1.1 Animals

Male, Sprague Dawley rats (200 - 300 g) were used (Bantin and Kingman, Hull, UK). They were housed in an area with a 12 hour light / dark cycle. The temperature was maintained at 20 °C, the humidity was controlled at 60 ± 5 %, and food and water were available *ad libitum*. A standard rat and mouse laboratory chow diet was fed (Bantin and Kingman), and between two and four animals were kept in each cage.

2.1.2 Langendorff Perfusion

An *in situ*, perfused, innervated rat heart model was used (Dart *et al.*, 1984b). Rats were anaesthetised with pentobarbitone sodium (60 mg kg^{-1} *ip*) and heparinised (200 units rat^{-1} *iv*) via the femoral vein. The animal was immobilised, ventral surface uppermost. The chest wall was removed and the ascending aorta was cannulated with a stainless steel cannula (internal diameter, 0.8 mm), which was tied into place for Langendorff perfusion. The time from the first incision into the chest wall to the commencement of perfusion was not more than 60 s.

Coronary effluent was drained from the right atrium via a polythene cannula (internal diameter, 0.86 mm), which was introduced via the inferior *vena cava* and tied into position such that it did not irritate the atrial wall. Recovery of the coronary effluent was maximised by tying off the right and left pulmonary vessels, and also the superior *vena cava*. After occlusion of the pulmonary vessels, the lungs were removed.

The perfusate was a Krebs-Henseleit solution (see Table 2.1 for composition), which was continuously gassed with a mixture of oxygen / carbon dioxide in the ratio 95 to 5. The temperature of the perfusate as it entered the aorta was 37 °C. Mean (± SD) pH, pO_2 and pCO_2 at the beginning of each experiment were as follows:

pH 7.39 ± 0.03

pO_2 579 ± 11 mm Hg

pCO_2 44.3 ± 0.4 mm Hg

Table 2.1.1 *Composition of the Krebs-Henseleit solution*

Ion	Conc. (mM)
Na^+	148.00
K^+	4.00
Ca^{2+}	1.85
Mg^{2+}	1.05
Cl^-	140.00
HCO_3^-	25.00
PO_4^{3-}	0.50
Glucose	11.00
EDTA	0.03

During the nerve stimulation experiments, the flow rate was controlled with a peristaltic pump (model 202U, Watson Marlow) and was set at 5 or 10 ml g^{-1} min^{-1}, depending on the experiment.

A free flow model of perfusion was, however, used for the series of preconditioning experiments - in line with what had been used by other investigators. In this model, the coronary perfusion pressure is held constant. The level of perfusate in the perfusion column was maintained at a constant level of 100 cm above the heart by the use of two peristaltic pumps an inflow - and a faster outflow pump (MHRE 200, Watson Marlow) which returned fluid to the reservoir.

Each preparation was allowed to equilibrate for 35 or 45 minutes (depending on the protocol) and, during this time, the amount of coronary effluent collected per minute was determined.

Preparations were excluded if they were found to have:

a) Percentage recovery of coronary effluent lower than 80 % (or 7.5 ml min^{-1} for free flow).

b) Persistent arrhythmias during the equilibration period,

c) An insufficient response to nerve stimulation (less than 15 % increase in heart rate and dP/dt),

d) Poor contractile function (dP/dt less than 2500 mm Hg s^{-1}).

In those experiments with a fixed flow rate, the mean percentage recovery of coronary effluent was 93.1 ± 2.5 %. Any preparations in which it was less than 80 % were discarded (5 out of 50 experiments).

In free flow experiments, the mean coronary flow rate after the 45 min equilibration period was 9.6 ± 1.0 ml g^{-1} min^{-1} (n = 27). Preparations with a coronary flow rate of less than 7.5 ml g^{-1} min^{-1} were discarded (2 out of 29 experiments).

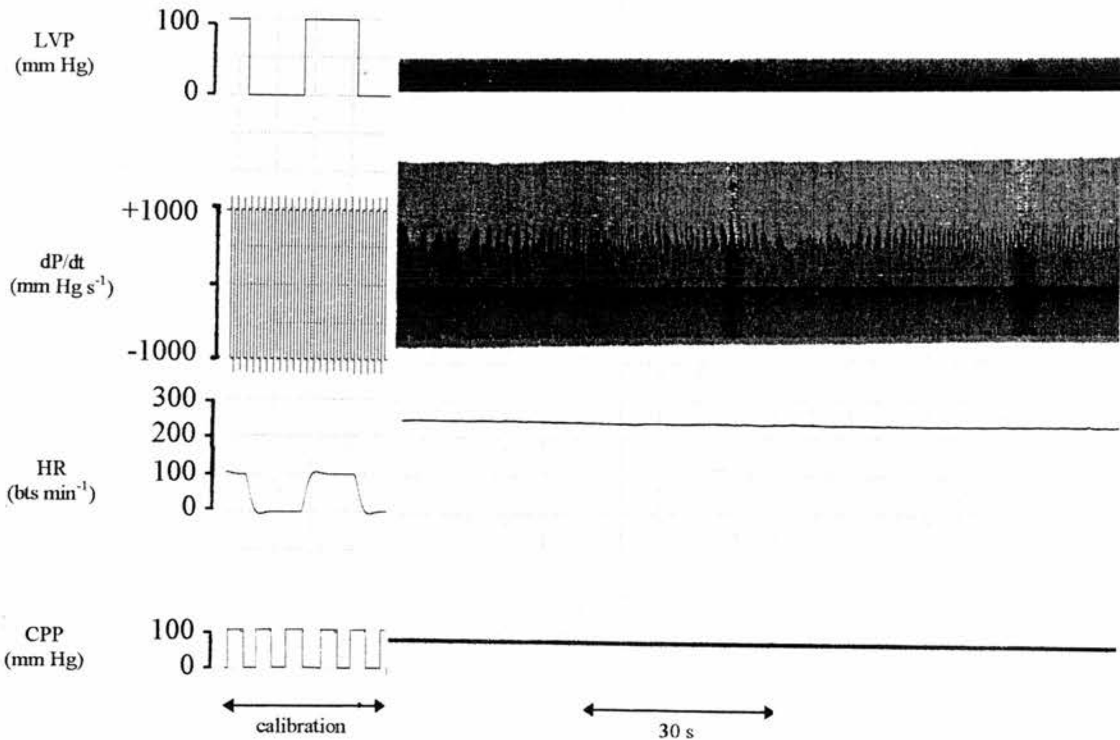
2.1.3 Measurement of Functional Parameters

Heart rate, dP/dt and left ventricular pressure were continuously measured via a pressure transducer (model E.M. 751, Elcomatic) connected to a polythene cannula (length 2.5 cm, internal diameter, 0.8 mm) which was carefully introduced into the left ventricular cavity through a small hole in the apex. The frequency-response curve for the system was flat and deviated by less than 5 % at 40 Hz.

The coronary perfusion pressure was monitored with an identical pressure transducer connected to a side arm in the aortic cannula. Under normal conditions, the coronary perfusion pressure at a flow rate of 5 ml g^{-1} min^{-1} was approximately 44 mm Hg.

Information from the transducers was relayed to a polygraph (Department of Medical Physics, Royal Infirmary Edinburgh) which was connected in series to a multichannel recorder (Gould TA 2000, Gould). The equipment was calibrated between 0 and 100 mm Hg once a week using a pneumatic transducer tester (DPM-1B, Bio Tek). An electronic calibration was carried out at the start of each preparation, using the mechanism built into the polygraph. This calibration was also from 0 - 100 mm Hg.

Figure 2.1 *Typical trace of left ventricular pressure (LVP), heart rate (HR), dP/dt and coronary perfusion pressure (CPP) at the end of the equilibration period.*



2.1.4 Nerve Stimulations

Once the heart was perfused, the cervicothoracic vagal nerves and the left stellate ganglion were exposed, and superfusion with warmed oxygenated buffer commenced to prevent the nerves from becoming either hypoxic or hypothermic. The nerves were continuously superfused throughout the experiment, except during periods of nerve stimulation when, to isolate the stimulus from the surrounding tissue, the nerves and surrounding tissues were gently blotted dry.

Nerve stimulations, of either 30 or 60 s duration, were carried out with a dual channel stimulator (S 48, Grass) connected to a signal isolating unit (SIU 7, Grass).

• *vagus nerves*

Bilateral vagus nerves were dissected free at the cervicothoracic level. A ligature was placed around them at the anterior end and the nerves were severed anterior to the ligature in order to abolish any vagal reflexes. A bipolar, platinum, stimulating electrode was placed under the nerves with the cathode closest to the heart. The stimulus was a square wave pulse of 0.8 mA amplitude and 2.0 ms duration.The stimulation frequency was 15 Hz.

• *sympathetic nerves*

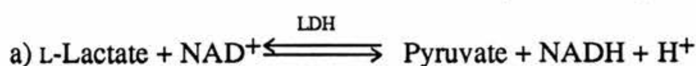
Sympathetic nerve stimulation was carried out through the left cervicothoracic stellate ganglion, which was exposed with the help of a surgical microscope (Wild M7A, Wild UK Ltd) at the level of the first rib (Dart *et al.*, 1983). Preganglionic fibres were cut and a bipolar platinum electrode was positioned under the ganglion with the help of a micromanipulator (Prior of England). The stimulus was a square wave pulse of 0.8 mA amplitude and 2.0 ms duration.The stimulation frequency was 5 Hz.

2.2 Lactate

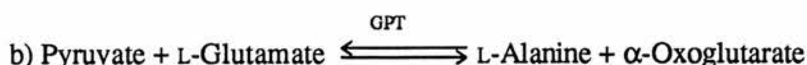
Lactate concentrations in the coronary effluent were assayed spectrophotometrically using a centrifugal analyser (Cobas Bio, Roche Diagnostics) and an enzymatic lactate assay kit (Kit number 256 773, Boehringer Mannheim).

2.2.1 Reaction Principle

Lactate reacts with NAD^+ in the presence of lactate dehydrogenase, to form pyruvate, NADH and H^+. The NADH formed is measured spectrophotometrically and is proportional to the amount of lactate which has reacted. The conversion of lactate is incomplete as the equilibrium lies far to the left. Complete conversion of lactate cannot therefore be achieved. The removal of pyruvate through a second reaction moves the equilibrium for the initial reaction to the right and so allows all the lactate to be reacted quantitatively:



a) L-Lactate + $NAD^+ \overset{LDH}{\rightleftharpoons}$ Pyruvate + NADH + H^+



b) Pyruvate + L-Glutamate $\overset{GPT}{\rightleftharpoons}$ L-Alanine + α-Oxoglutarate

2.2.2 Method

Measurements were carried out at a wavelength of 340 nm. Aliquots of sample or standard (30 µl) were analysed with 250 µl of reagent, 60 µl of diluent and 5 µl of enzyme solution. The incubation temperature was 37 °C, and Precinorm S (880 µM lactate, Boehringer Mannheim) was used as the quality control. The standard was 1 mM L-lactate (Boehringer Mannheim). Samples were analysed in duplicate.

The inter- and intra-assay coefficients of variation for the analysis of Precinorm S were 3.7 (n = 29) and 0.2 % (n = 10) respectively.

2.3 Creatine kinase

Creatine kinase (CK) activity was assayed in freshly prepared ventricular homogenates (see below) diluted 1:40 (v/v) with 2 % bovine serum albumin (A6003 Sigma) containing 10 mM Tris, pH 7.4.

The assay was carried out spectrophotometrically using a Cobas Bio centrifugal analyser (Roche) and an enzymatic CK assay kit (Unimate 3CK, Roche).

2.3.1 Tissue Homogenisation

Excised hearts were dropped into ice cold homogenisation buffer (0.25 M sucrose containing 1 mM disodium EDTA and 0.1 mM dithiothreitol, pH 7.4). The hearts were trimmed to remove the atria, major blood vessels and excess fat or connective tissue, and the wet weight of the ventricles was recorded.

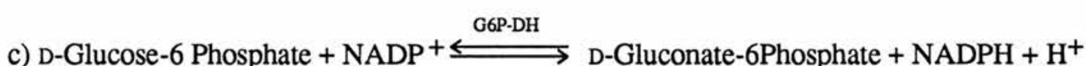
Using scissors, the tissue was finely minced on ice and placed into a Potter Elvjehem glass homogeniser (50 ml, Jencons) kept on ice. Homogenisation buffer, 25 ml per gram of tissue, was measured out and divided into two portions, one of which was added to the glass homogeniser with the tissue. Ten strokes of a well fitting teflon plunger were used to break up the tissue. The resulting homogenate was poured into a centrifuge tube and the remaining portion of homogenisation buffer was used to rinse the homogeniser.

The homogenate was spun in a centrifuge (Centra7R, IEC) at 1000g, 4 °C, for 10 min. The supernatent was poured off into an ice cold beaker, the pellet was resuspended in a second aliquot of homogenisation buffer and the spin was repeated.

The two supernatents were combined and used for the determination of creatine kinase activity (and protein concentration).

2.3.2 Reaction Principle

CK was activated by N-acetylcysteine and measured by the photometric determination of NADPH formation. The rate of NADPH formation through the following reactions, was directly related to the CK activity:



Samples were assayed in duplicate.

The quality control was Precinorm C (Boehringer Mannheim).

Results were expressed in Units mg^{-1} protein.

The inter- and intra-assay coefficients of variation for the analysis of Precinorm C were 3.4 (n = 29) and 0.75 (n = 10) % respectively.

2.4 Protein

Measurement of membrane protein concentration in myocardial homogenates was carried out on the Cobas Bio centrifugal analyser using the method of Lowry *et al.* (1951) as modified by Clifton *et al.* (1988). The assay utilises a colour change following the reduction of Folin and Ciocalteu's phenol reagent (1927), measured at 750 nm.

The main reagent, made up fresh each day, was a cocktail of the following three stock solutions and double distilled water.

A) 10 % (w/v) Na_2CO_3 in 0.5 M NaOH

B) 1 % (w/v) $CuSO_4.5H_2O$

C) 2 % (w/v) Na^+/K^+ tartrate

The volume ratio of the components (A:B:C:water) in the final mixture was 10:0.5:0.5:40. A 1:3 dilution (v/v with double distilled water) of the Folin Ciocalteu's phenol reagent (Roche) was used as the start reagent for the reaction.

The protein standard solution was bovine serum albumin, fraction V (A6003, Sigma) dissolved in homogenisation buffer. Standards of 0, 50, 100, 200, 300 and 400 mg L^{-1} were run with each estimation. Sample protein concentration was calculated from the non-linear calibration curve, using the DENS (data evaluation of non-linear standard curves) mode. Samples were run in duplicate.

The inter-assay coefficient of variation, calculated from the 300 mg L^{-1} standard, was 3.4 % (n = 5). The intra-assay coefficient of variation was 0.6 % (n = 26).

2.5 Catecholamines

Catecholamines were extracted from the coronary effluent using alumina in the presence of an internal standard - dihydroxybenzylamine (DHBA). The catechols were then eluted using HCl and analysed by HPLC with electrochemical detection.

2.5.1 Extraction Principle

The extraction principle is adapted from a published method (Howes *et al.*, 1985) and exploits the fact that catecholamines will bind tightly to alumina under alkaline conditions, but will be eluted from it at low pH.

2.5.2 Reagents

a) Acid washed alumina, activity grade 1 (Sigma).

b) Tris EDTA buffer - 1 M Tris base (Sigma), 0.05 M disodium EDTA (BDH) pH 8.8 with HCl.

c) Internal standard - 91 nM solution of DHBA (D4471, Sigma), diluted with water, daily, from a 4.5 mM stock solution in 0.2 N HCl. The stock solution was stored in brown glass at 4 °C.

d) 0.2N Hydrochloric acid (BDH).

All the reagents were of the highest grade available, to avoid interference from contaminants. Water was double distilled and then deionised.

2.5.3 Extraction Procedure

Sample or standard (2 ml) was added to 15 ml polystyrene tubes (Elkay) containing 2 ng of the internal standard solution and a small amount of alumina (approximately 20 mg).

Tris / EDTA buffer (2 ml) was added and the tubes were capped and mixed on a rotary mixer (SB1, Stuart Scientific) for 10 min. The catecholamines were now tightly bound to the alumina.

The alumina was allowed to settle and most of the water layer was aspirated to waste, following which the alumina was washed twice with 10 ml of deionised water. Catecholamines were eluted from the alumina by the addition of 150 µl of 0.2 N hydrochloric acid to the tubes, followed by vortex mixing of the contents for 1 min.

Alumina was sedimented (1 min at 1700g), and the acid phase (130 µl) was analysed by HPLC. Care was taken not to introduce any grains of alumina into the HPLC system as they could cause blockages affecting peak shape and retention time.

A standard curve for noradrenaline and adrenaline was included with every run. Stock solutions of noradrenaline and adrenaline (1 mM in 0.2 N HCl) were stored in the fridge. On

the day of the assay they were diluted, first with 0.2N HCl and then with water, to give final concentrations of 1, 5, 10, 15 and 20 nM. Each batch of samples also contained a blank and a zero.

The percentage recoveries for noradrenaline and adrenaline through the method, determined from the 10 nM standard, were 91 and 89 % respectively. The within assay coefficients of variation for 10 nM concentrations of noradrenaline and adrenaline were 2.0 and 1.9 % respectively. The between assay coefficients of variation for the same concentrations were 4.9 and 4.5 %

When stored at -70 °C, a quantity of pooled samples and diluted standards were found to be stable for up to 3 months. All the experimental samples generated were, however, analysed within 6 weeks of collection.

2.5.4 HPLC Analysis of Catecholamines

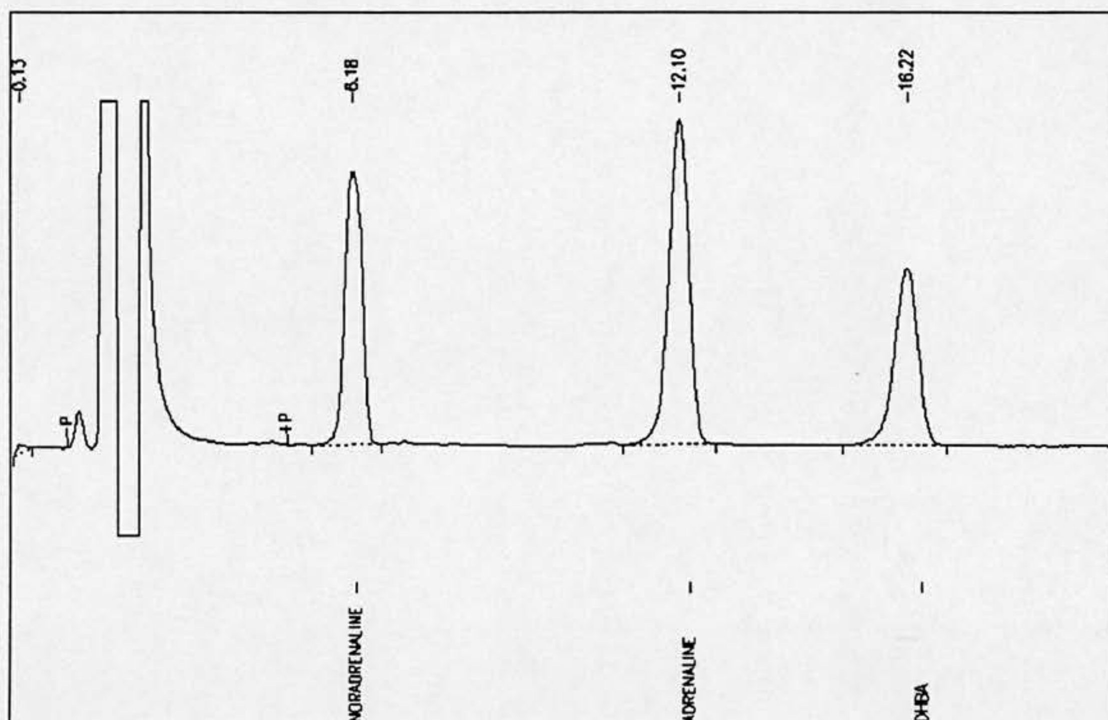
The reverse phase HPLC separation followed by electrochemical detection of catecholamines was adapted from the method of Moyer and Jiang (1978).

2.5.5 HPLC Apparatus

The HPLC system consisted of an isocratic pump (LC250, Perkin Elmer), connected to an autosampler (ISS200, Perkin Elmer) fitted with a 100 µl PTFE sample loop (Anachem). The analytical column was an ODS2, 3 µm cartridge column (10 cm × 4 mm, Phase Separations) fitted with a reverse phase guard cartridge. Compounds were detected electrochemically (LC4C, Bioanalytical Systems) using a glassy carbon electrode at a potential of + 0.7 V. Data handling was carried out with a 950 interface box (PE Nelson) and Turbochrom 3 software (PE Nelson). Precolumn tubing was narrow bore PTFE tubing (0.005" internal diameter, Anachem).

The mobile phase was 0.18 M potassium phosphate, pH 3.5, containing 0.2 mM disodium EDTA and 2 mM heptane sulphonic acid (H8901, Sigma) as an ion pairing agent. Double distilled and deionised water, mentioned already, was filtered and degassed under vacuum through a 0.22 µm hydrophilic membrane (Durapore, Millipore). The flow rate was 1 ml min^{-1}.

Figure 2.2 *Chromatogram showing 10 pmoles, on column, of each of noradrenaline, adrenaline and DHBA.*



The numbers denote the time elapsed from the injection, in minutes

2.5.6 Analysis

Batches of approximately twenty samples and standards (100 μl per injection) were run together. The system was calibrated with noradrenaline, adrenaline and DHBA (see Figure 2.2). Standards were made up in 0.2 N HCl. Calibration curves were constructed for amounts of: 1; 5; 10; 15 and 20 pmoles on column.

In the majority of samples, the only peaks to be observed were those which were due to noradrenaline and DHBA. There was, however, a small peak which could not be identified in a few samples, which ran approximately 40 s before noradrenaline. It did not interfere with the assay and attempts to identify it with known metabolites were unsuccessful.

All standards were purchased from Sigma Chemical company. The inter- and intra-assay coefficients of variation for 10 pmoles, on column, of noradrenaline were 3.5 % and 1.8 % (n = 10) respectively. Standards were run in duplicate, but due to insufficient volume, samples were run singly.

2.6 Chemicals

All chemicals were purchased from MERCK-BDH, except where stated, and were analytical grade. Water was double distilled and, for catecholamine extractions and analyses, was deionised (UHQII, Elga.).

2.7 Statistical analysis

Data was checked for normality using frequency distribution charts in microsoft excel 5.0. Other analyses were carried out using the MINITAB version 7 software (CLE.COM Ltd.,Birminham).

Normally distributed groups were compared using one- or two-way analysis of variance (ANOVA). Where a follow-up test was required, unpaired Student's T-tests were performed and Bonferoni corrections were applied. All results are expressed as mean ± one standard deviation. Statistical significance was accepted at $p < 0.05$.

Skewed data was analysed using the non-parametric Kruskal-Wallis test. Follow-up testing was done with the Mann-Whitney test and appropriate Bonferoni correction.

The intra-assay coefficient of variation for the protein results was determined using the following formula to calculate the standard deviation:

Standard deviation = $\sqrt{(\Sigma x^2 / 2n)}$ where x is the difference in the duplicate values of the samples and n is the number of duplicates.

CHAPTER 3

Acetylcholine methodology

3.1 Introduction and methods

Measurement of noradrenaline levels in the coronary effluent of isolated, perfused hearts has led to a greater understanding of the workings of the sympathetic nervous system. Comparatively little is, however, known about the functioning of the parasympathetic nervous system owing to the lack of a sufficiently sensitive acetylcholine assay with which to study it.

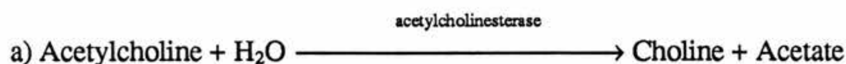
The aim of the study was to investigate the behaviour of the parasympathetic nervous system in the *in situ*, perfused, innervated rat heart during several different severities of ischaemia, by employing a recently developed HPLC assay to measure levels of acetylcholine present in coronary effluent.

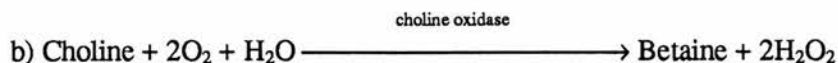
3.1.1 HPLC Assay

The assay kit was purchased from Biotech Instruments (Bioanalytical Systems).

• *assay principle*

Acetylcholine and choline in the sample were separated on a polymer analytical column (Bioanalytical Systems Ltd) and passed as discreet, resolved bands into a reactor column (Bioanalytical Systems Ltd). The reactor column contained enzymes which were covalently bonded to the support phase and catalysed the following reactions:





Hydrogen peroxide was detected electrochemically (LC4C, BAS) using an oxidative reaction at a platinum electrode held at +0.6 V vs Ag/AgCl. Within the effective concentration range, the amount of hydrogen peroxide liberated was directly proportional to the amount of acetylcholine or choline present in the sample.

The precolumn HPLC system was identical to that described in the preceding chapter for catecholamine analysis (see Section 2.5.5). The run time for each sample was 20 min.

Retention times for acetylcholine and choline were 6.2 ± 0.5 (n = 25) and 7.9 ± 0.6 (n = 25) min respectively.

• *mobile phase*

The optimum mobile phase was found to consist of 0.1 M Tris (T6791, Sigma) containing 0.1 M sodium perchlorate monohydrate (10313 4Y, BDH) made up in double distilled, deionised water. Kathon CG reagent, 0.005 % v/v (CF-2150, BAS) was present as an anti-bacterial agent. The pH was adjusted to 8.50 ± 0.05 with HCl.

• *standards*

Acetylcholine and choline standards were made up in a solution of glacial acetic acid (0.3 % v/v) adjusted to pH 5 with dilute sodium hydroxide and containing 0.005 % v/v Kathon CG reagent. Standards and samples of perfusate 'spiked' with known amounts of acetylcholine and choline, were stable at room temperature for at least 24 hours. All samples were analysed within 8 hours of collection.

Standard curves were constructed for acetylcholine and choline for on column amounts of 5, 10, 20, 30 and 40 pmole (see Figure 3.2). The injection volume was 100 µl.

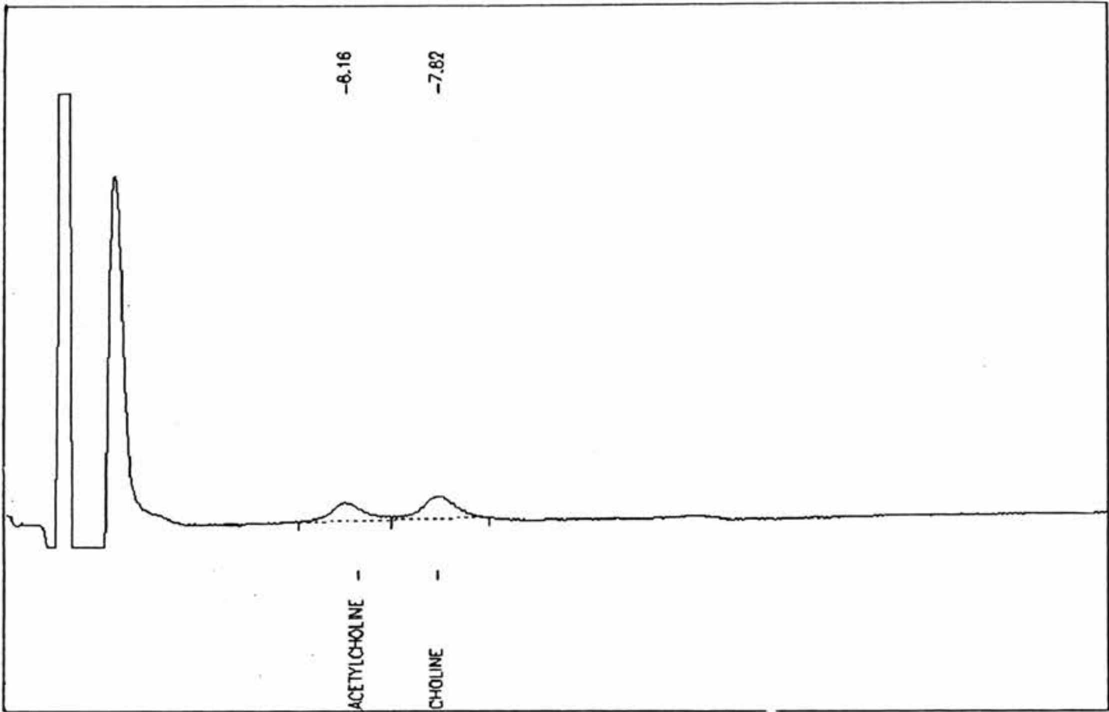
The detection limit for acetylcholine was found to be 2 pmole on column which, with an injection volume of 100 µl, equates to a sensitivity of 20 nM.

The intra-assay coefficient of variation for 20 pmole of acetylcholine in a 100 µl injection was 4.8 % (n = 10). The inter-assay coefficient of variation for the same standard was 6.0 % (n = 10).

• *peak separation*

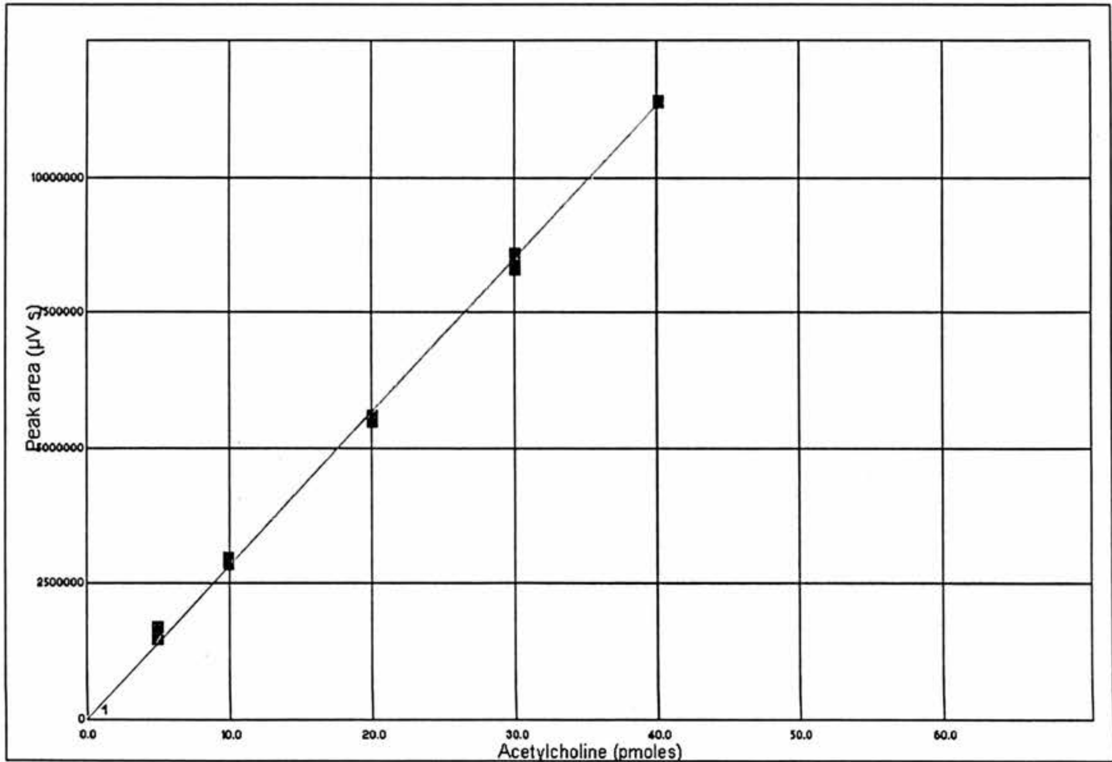
This was a commercial assay and information regarding the nature of the solid phase was not available to us. The fine-tuning of the separation was, therefore, limited by our lack of knowledge of the chemistry involved. Nevertheless, it was possible to alter the ionic strength, pH and flow rate of the mobile phase to optimise the separation. Despite this, baseline separation of acetylcholine and choline was not quite achieved and both compounds exhibited some peak-tailing, particularly at higher concentrations (see Figure 3.1 and 3.3).

Figure 3.1 *Separation of 5 pmole of acetylcholine and choline (100 μl injection)*



Numbers denote the time after injection in minutes

Figure 3.2 *Standard curve for acetylcholine (5 - 40 pmole).*



Each acetylcholine standard was injected 3 times
Injection volume was 100 μl

3.1.2 Protocol for pilot experiments

The basic model was the *in situ*, innervated, perfused rat heart as described in Section 2.1.2. The flow rate was controlled at 5 ml g^{-1} min^{-1}.

• *use of drugs*

To prevent the rapid hydrolysis of released acetylcholine, neostigmine methyl sulphate (N2126, Sigma), a reversible acetylcholinesterase inhibitor, was included in the perfusate at a concentration of 50 μM. Neostigmine also interacted with the enzyme in the reactor column, but at the concentration used in these experiments it has been demonstrated that the enzyme present on the reactor column is in sufficient excess for pharmacological experiments (data shown in manufacturer's literature). Moreover, neostigmine elutes after acetylcholine.

During initial experiments, choline chloride (C7017, Sigma) was also present in the perfusate at a concentration of 10 μM in order to provide a substrate for acetylcholine resynthesis (Dieterich *et al.*, 1978; Wetzel and Brown, 1983). Despite attempts to improve the separation of acetylcholine and choline (see Section 3.1.1), the compounds were not quite baseline resolved. When choline chloride was present in the perfusate at the above concentration, picomolar amounts of acetylcholine appeared as a shoulder on the choline peak and the assay sensitivity was reduced by a factor of ten. During initial experiments, vagus stimulation did not produce detectable amounts of acetylcholine in the presence of choline chloride. Thus, to maximise the assay sensitivity for acetylcholine, choline chloride was excluded from the perfusate for the remaining experiments.

• *vagus nerve stimulation*

Bilateral vagus nerve stimulations were carried out as described in Section 2.1.4. The duration of each stimulation was 30 s and there was a 15 min recovery period between each episode of nerve stimulation. Each preparation underwent four episodes of nerve stimulation at 45, 60, 75 and 90 min after aortic cannulation.

• *sample collection*

Total coronary effluent was collected in consecutive 30 s samples. Sampling began 30 s before the onset of each nerve stimulation and continued for 180 s. A single aliquot (100 μl) from each sample was injected directly onto the HPLC system.

• *treatment groups*

In ten preparations there was a 45 min equilibration period followed by four vagus nerve stimulations. The reproducibility of the response to vagus nerve stimulation was determined and the coronary effluent was analysed for the presence of acetylcholine.

In a further five preparations, the functional response to a single vagus nerve stimulation was determined at the end of the 45 min equilibration period. Atropine (A0132, Sigma), a muscarinic antagonist, was then infused into the aortic cannula via a syringe infusion pump (2400-001, Harvard Apparatus), to reach a final concentration of 1 μM in the perfusate. After 15 min, a second vagus nerve stimulation was carried out to confirm that the functional changes observed during vagus nerve stimulation were indeed due to the action of acetylcholine on muscarinic receptors and were therefore abolished by atropine.

3.2 Results and discussion

3.2.1 Haemodynamic response to vagus nerve stimulation

The effect of bilateral vagus nerve stimulation on the various measured haemodynamic parameters is shown in Table 3.2.1. There was no significant change in any of the pre-stimulation values during the 60 min measurement period (1 way ANOVA, p = NS). Heart rate was significantly reduced by vagus stimulation during all four stimulations (2 way ANOVA, followed by paired T-test, $p < 0.05$), although the response declined significantly through the experiment (1 way ANOVA, $p < 0.05$). Vagus nerve stimulation caused a significant reduction in both +dP/dt and -dP/dt during the first two, but not the subsequent two stimulations (2 way ANOVA, followed by paired T-test, $p < 0.05$). Left ventricular pressure was not significantly altered by vagus stimulation.

3.2.2 Use of atropine to verify vagus nerve stimulation

The results are shown in Table 3.2.2. In the absence of atropine, heart rate and dP/dt were significantly reduced by nerve stimulation (Stim 1) when compared to pre-stimulation values. The addition of 1 μM atropine to the perfusate 15 min before a second nerve stimulation (Stim 2) completely abolished this response.

Table 3.2.1 *Haemodynamic parameters during vagus stimulation (mean ± SD values, n = 10)*

HAEMODYNAMIC PARAMETER	PRE-STIMULATION VALUES				#VAGUS STIMULATION, MIN VALUES				MAX CHANGE			
	45 min	60 min	75 min	90 min	45 min	60 min	75 min	90 min	45 min	60 min	75 min	90 min
+dP/dt (mm Hg s^{-1})	1908 ± 395	1792 ± 246	1863 ± 309	1850 ± 342	*1460 ±378	*1425 ± 419	1525 ± 513	1613 ± 530	391 ± 102	390 ± 74	338 ± 312	238 ± 326
- dP/dt (mm Hg s^{-1})	908 ± 163	840 ± 89	925 ± 184	888 ± 103	* 675 ± 140	* 720 ± 115	800 ± 216	775 ± 222	233 ± 52	120 ± 76	125 ± 155	113 ± 175
HR (Beats min^{-1})	248 ± 12	247 ± 22	234 ± 16	238 ± 6	*118 ± 20	*124 ± 32	*161 ± 38	*166 ± 47	146 ± 57	123 ± 44	73 ± 27	71 ± 43
LVP (mm Hg)	51 ± 6	46 ± 6	48 ± 11	47 ± 8	43 ± 10	44 ± 11	47 ± 18	45 ± 13	8.8 ± 4.8	2.5 ± 6.5	1.6 ± 10.4	1.6 ± 7.6

HR - Heart rate
LVP - Left ventricular pressure
During vagus stimulation, the minimum values of HR, LVP and dP/dt were recorded.
* Significantly different from the pre-stimulation value (2 way ANOVA, followed by paired T-test, $p < 0.05$).

Table 3.2.2 *The effect of atropine on the cardiac response to vagus nerve stimulation (n = 5). Values are mean ± SD.*

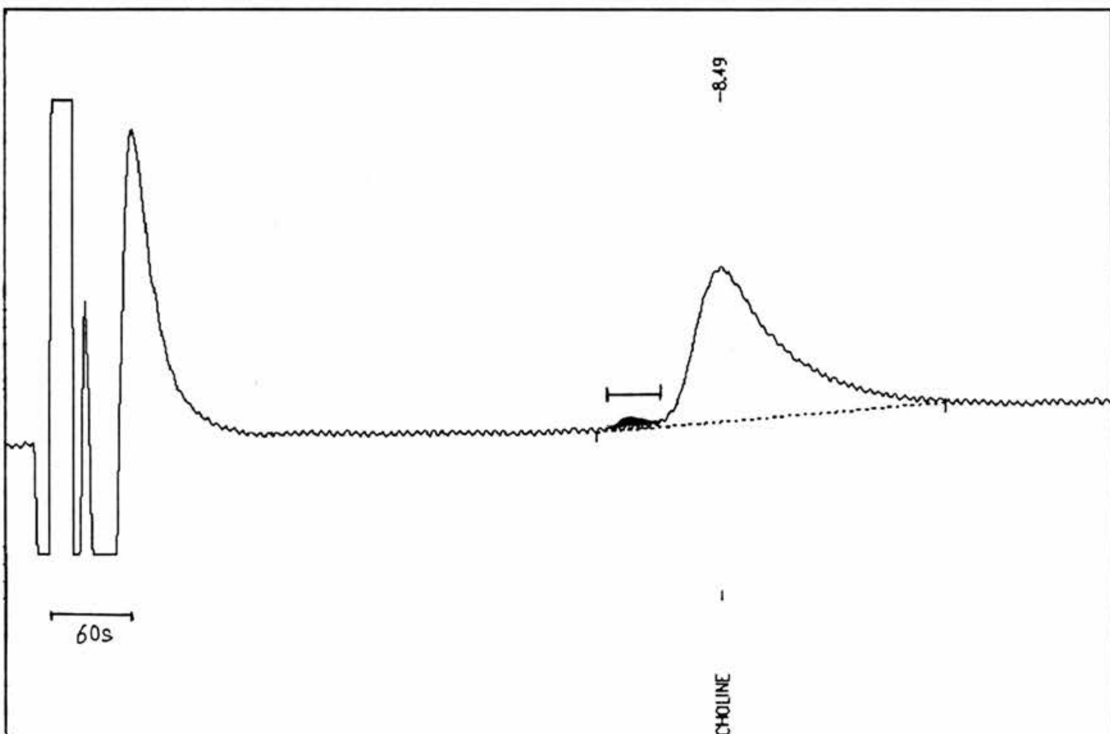
FUNCTIONAL PARAMETER	NO ATROPINE		ATROPINE (1μM)	
	PRE-STIM	STIM 1	PRE-STIM	STIM 2
HEART RATE max (beats min^{-1})	255 ± 11	120* ± 25	248 ± 8	253 ± 15
+ dP/dt max (mm Hg s^{-1})	2010 ± 265	1460* ± 308	1950 ± 288	2100 ± 320
- dP/dt max (mm Hg s^{-1})	1000 ± 148	700* ± 69	940 ± 92	940 ± 92

* $p < 0.05$ vs pre-stimulation without atropine (paired t-test)

3.2.3 Presence of acetylcholine in coronary effluent

A typical HPLC chromatogram of coronary effluent collected during vagus stimulation is shown in Figure 3.3. No traces of acetylcholine were detected in any of the samples of coronary effluent which were analysed, although choline was detected, even in unstimulated samples.

Figure 3.3 *Analysis of acetylcholine in coronary effluent collected during vagus nerve stimulation*



⊢—⊣ The inserted peak represents the peak resulting from 2 pmoles of acetylcholine (detection limit).
99.6 pmole of choline was detected, equivalent to 5.0 nmoles g^{-1} min^{-1}.
The retention time for choline was 8.49 minutes.
The injection volume was 100 μl.

3.2.4 Discussion

Vagus nerve stimulation was confirmed both by the reduction in heart rate and dP/dt and also by the abolition of this response in the presence of 1 μM atropine.

The haemodynamic response to vagus nerve stimulation was reproducible for the first two stimulations only. The deterioration in the response to the third and fourth stimulations was possibly due to the absence of choline chloride in the perfusate. This may have led to the progressive failure of ganglionic transmission as the ganglionic stores of acetylcholine were exhausted (Dieterich *et al.*, 1978). Alternatively, the relatively low flow rate (5 ml g^{-1} min^{-1}), may have caused a mild hypoxia, affecting the nerve function (see Chapter 4). Other investigators have employed similarly low flow rates in experiments to look at sympathetic nerve function (Du and Dart, 1993) and the interaction of the parasympathetic and sympathetic nervous system (Du, Ph.D. thesis, University of Edinburgh, 1991). A low flow rate was chosen for the experiments detailed in this chapter, in order to maximise the chances of observing acetylcholine in the coronary effluent. Attempts to differentiate between the above two mechanisms were not made as, at this stage, the experiments were not quantitative and the haemodynamic responses were only used as a marker of acetylcholine release.

The apparent absence of acetylcholine in the coronary effluent raised three possibilities:

a) The acetylcholinesterase on the reactor column of the HPLC system was inhibited by the neostigmine present in the samples and, consequently, only choline was detected.

b) Myocardial acetylcholinesterase was not sufficiently inhibited by the concentration of neostigmine used, to prevent the hydrolysis of released acetylcholine before it could be washed out of the synaptic cleft.

c) The concentration of released acetylcholine was too low to be detected by the HPLC method employed.

Further experiments were devised to differentiate among the above possibilities.

3.3 Further experiments

3.3.1 Inhibition of reactor column acetylcholinesterase

The manufacturer's literature, supplied with the column, suggested that 50 μM neostigmine would not affect the assay unless the column had deteriorated to such an extent that the amount of enzyme present was seriously reduced. The following experiments were carried out to see if this was the case.

• *protocol*

The following series of injections (100 μl) were carried out:

a) 200 nM acetylcholine standard made up in diluent (10 injections).

b) Coronary effluent from a stimulated heart (10 injections) but alternating with 10 injections of a 200 nM acetylcholine standard made up in diluent.

c) 200 nM acetylcholine standard made up in coronary effluent from a stimulated heart (10 injections).

The mean ± SD area for the 200 nM acetylcholine standard was calculated from the first set of standard injections. This was compared with the same statistic from the other two sets of standard injections to determine if there were any carry-over or direct effects from the presence of neostigmine in the perfusate.

• *results and discussion*

There were no significant differences in the observed acetylcholine result between any of the three groups of standards, indicating that neostigmine, or other substances in the coronary effluent samples, did not interfere with the HPLC analysis of acetylcholine (see Table 3.3.1).

Table 3.3.1 *Recovery of 20 pmoles of added acetylcholine in the absence and presence of 50 μM neostigmine*

	STANDARD GROUP		
	a (n = 10)	**b (n = 10)**	**c (n = 10)**
MEAN SAMPLE ACETYLCHOLINE CONTENT (pmoles)	19.3 ± 0.8	20.1 ± 1.3	19.7 ± 1.0
PERCENTAGE RECOVERY	96.5	100.5	98.5

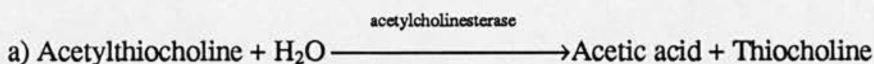
3.3.2 *In vitro* myocardial acetylcholinesterase activity

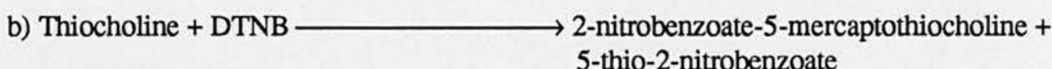
Myocardial acetylcholinesterase activity was measured in crude atrial and ventricular homogenates as well as atrial and ventricular microsomal preparations. These measurements were carried out both in the presence and the absence of an inhibitor.

Acetylcholinesterase activity was measured using a spectrophotometric assay, (Ellman *et al.*, 1961), modified for use on a centrifugal analyser (Cobas Bio, Roche).

• *spectrophotometric assay of acetylcholinesterase*

Acetylthiocholine is hydrolysed by acetylcholinesterase to acetic acid and thiocholine. The thiocholine then reacts with DTNB (Ellman's reagent) to form the yellow anion, 5-thio-2-nitrobenzoate, which is measured at 410 nm. The rate of formation of the coloured compound is directly proportional to the amount of enzyme present in the sample.





The following solutions were made up

1) Phosphate buffer, 100 mM, pH to 8.0 with NaOH.
2) Phosphate buffer, 100 mM, pH to 7.0.
3) Buffered Ellman's reagent, DTNB (D8130, Sigma) 10 mM, made up in solution 2) and containing 17.85 mM sodium bicarbonate (S6014, Sigma).
4) Acetylthiocholine perchlorate (A7407, Sigma) 75 mM.

The freshly prepared reagent was a combination of 9 ml phosphate buffer (pH 8.0), 60 µl acetylthiocholine (substrate) and 300 µl DTNB solution.

Each sample (5 µl) was analysed with 300 µl of the above reagent and the course of the reaction was followed for 6 min. The linear portion of the reaction was used to calculate the rate. The reaction took place at 37 °C. Samples were analysed in duplicate.

The intra-assay coefficient of variation for a sample with a mean activity of 254 Units L^{-1} was 2.7 % (n = 10). The inter-assay coefficient of variation for the same sample was 7.5 % (n = 10). The approximate range of sample activities measured on the Cobas was 100 to 600 Units L^{-1}.

• preparation of tissue homogenates

Hearts were perfused for 10 min to flush out blood.

In the case of crude membrane preparation, the hearts were excised into ice cold phosphate buffer (100 mM, pH 8.0). The atria and ventricles were separated and any excess fat or connective tissue was removed. The atria and ventricles were minced with scissors, before being homogenised separately over ice, using ten strokes of a close fitting teflon plunger (Dounce homogeniser). The ratio of tissue to phosphate buffer was 400 mg to 20 ml. The homogenate was filtered through gauze before analysis.

Hearts for microsome preparation were excised into ice cold Tris / HCl buffer (50 mM Tris containing 25 mM sucrose, pH 7.4). They were treated in the same way as the crude preparations but the ratio of tissue to buffer for the homogenisation was 1 g to 20 ml.

Following the gauze filtration step, the filtrate was centrifuged for 10 min at 16,000 g_{av}, 4 °C (JA20 rotor). The pellet was discarded and the supernatant was centrifuged for a further 25 min at 42,000 g_{av}, 4 °C in a Beckman rotor (45 Ti). The microsomal pellet was gently resuspended in 2 ml phosphate buffer before analysis.

• protocol

Three different fractions of atrial and ventricular homogenates were analysed. These were crude homogenate, a microsomal preparation and the post-microsomal supernatant.

Microsomal membranes were prepared from the pooled tissues of 15 hearts. Crude homogenate was prepared from the pooled tissues of 3 hearts. Solutions of neostigmine methyl sulphate, Eserine (E8375, Sigma), an irreversible acetylcholinesterase antagonist, and Quinidine sulphate (Q0875, Sigma), an inhibitor of pseudocholinesterase were made up in phosphate buffer.

Aliquots (180 µl) of the tissue fractions were pipetted into the Cobas sample cups. To these were added 20 µl of one of the inhibitors or phosphate buffer such that the final concentration of inhibitor in the sample was as follows

Neostigmine, 50 µM

Eserine, 100 µM

Quinidine, 20 µM

Duplicate samples (5 µl) were analysed for cholinesterase activity and protein content (see Section 2.4). Results were expressed as Units mg^{-1} protein. A reagent blank of phosphate buffer was also run.

• *results and discussion*

In the control group, approximately 70 % of the observed enzyme activity was located in the atria, with only 30 % of the activity attributable to the ventricles. The level of activity in the crude homogenate was approximately 75 % of that in the microsomal fractions when the results were corrected for protein concentration. Very little enzyme activity (≤ 13 Units mg^{-1} protein) was detected in the supernatant fractions (see Table 3.3.2).

The enzyme activity in samples treated with quinidine sulphate was not significantly different from samples which only contained phosphate buffer, indicating that there was no pseudocholinesterase present in the samples. If pseudocholinesterase activity had been detected, it would have indicated that there was erythrocyte contamination in the homogenate.

Both eserine (100 μM) and neostigmine (50 μM) inhibited acetylcholinesterase activity to a similar degree, causing 90 to 95 % inhibition in the crude homogenates and the microsomal fractions.

Table 3.3.2 *The effect of inhibitors on myocardial acetylcholinesterase activity (Units mg^{-1} protein).*

TISSUE FRACTION	TREATMENT			
	Control 100 mM	Quinidine 20 μM	Eserine 100 μM	Neostigmine 50 μM
HA	91	77	7	6
HA	91	89	7	6
HV	38	38	4	4
HV	38	38	4	4
MA	120	120	10	10
MA	120	120	20	10
MV	60	50	3	3
MV	50	50	3	3
SA	12	12	5	5
SA	13	13	5	3
SV	6	6	4	4
SV	6	6	4	4

KEY
HA = Homogenised atria
HV = Homogenised ventricles
MA = Atrial microsomes
MV = Ventricular microsomes
SA = Atrial supernatant
SV = Ventricular supernatant

These results show that in tissue homogenates, up to 95 % of myocardial acetylcholinesterase activity is inhibited by a 50 μM concentration of neostigmine. A two times higher concentration of eserine, a more efficacious anticholinesterase, caused a similar degree of enzyme inhibition.

Naturally, these are *in vitro* results and may not be exactly representative of the *in vivo* situation. It seems likely, however, that the relative amount of inhibitor to enzyme would be more favourable *in vivo*, where the membrane surface area would be expected to be smaller.

3.3.3 *In vivo* myocardial acetylcholinesterase activity

• *protocol*

Three hearts were perfused for 45 min from a 500 ml perfusion column containing oxygenated Krebs-Henseleit buffer with 50 μM neostigmine. The flow rate was 5 ml min^{-1} and the perfusion pressure was maintained at a constant 100 cm H_2O by the use of a recirculating system (as described in Section 2.1.2). Perfusion and superfusion were then switched to a second, identical recirculating perfusion system containing the same Krebs-Henseleit buffer and neostigmine solution spiked with 200 nM acetylcholine. A second group of three hearts (control group) underwent the same protocol, but in the absence of neostigmine.

Samples of coronary effluent and superfusate were taken 5, 7 and 9 min after the switch to acetylcholine infusion, and analysed for acetylcholine content.

The amount of acetylcholine recovered from the coronary effluent samples was compared to that recovered from the samples of superfusate which had undergone the same treatment, except that they did not pass through the myocardium.

• *results and discussion*

There was no observed difference between the amount of acetylcholine present in the superfusate samples from the two groups (see Table 3.4.3).

In the group containing neostigmine, there was no observed difference between the amount of acetylcholine present in the samples of coronary effluent or superfusate. The coronary effluent samples from the control group, however, contained less acetylcholine than the superfusate samples (paired T-test, $p < 0.05$).

Table 3.3.3 *effect of 50 μM neostigmine on mean ± SD acetylcholine result (pmoles).*

SAMPLE	TREATMENT GROUP	
	Control (n = 3)	**50 μM Neostigmine (n = 3)**
Superfusate (n = 3)	17.9 ± 1.5	18.8 ± 1.0
Coronary effluent (n = 3)	# 14.0 ± 2.5	18.3 ± 1.2

Significantly lower than superfusate (paired T-test, $p < 0.05$).

The results show that 50 μM neostigmine will prevent the significant hydrolysis of 200 nM infused acetylcholine. Assuming that the infused acetylcholine partitions within the myocardium in a similar manner to that released during nerve stimulation, a 50 μM concentration of neostigmine should also prevent the significant hydrolysis of acetylcholine released during nerve stimulation.

3.3.4 The effect of stimulation frequency and voltage

- *increase the efficacy of the nerve stimulus*

The magnitude of the haemodynamic response to sympathetic nerve stimulation depends upon the frequency and voltage of the stimulus (Du, unpublished observations). This is reflected in the amount of noradrenaline overflow.

The frequency and voltage of the vagus nerve stimulus can also be observed to influence the magnitude of the haemodynamic response and presumably, therefore, the amount of acetylcholine release.

By changing, first the frequency (15 - 50 Hz) and then the voltage (10 - 40 Volts) of the vagus nerve stimulus to elicit the maximum possible response, it was hoped to increase the amount of acetylcholine released from the nerve terminal to a level that could be detected by the HPLC assay.

Despite altering the stimulus to cause asystole, no acetylcholine was detected in any samples (not shown).

- *use of atropine*

It has been observed that atropine (1 μM), a muscarinic antagonist, caused an approximately four fold increase in acetylcholine overflow from stimulated preparations of cat superior cervical ganglia and rat cortical slices (Kato *et al.*, 1975). This was possibly as a result of the inhibition of a negative feedback effect on acetylcholine release when the acetylcholine was prevented from binding to the muscarinic receptors.

Although the use of atropine would abolish any haemodynamic changes during vagus nerve stimulation, it was decided to use it just to see if it could cause the overflow of a detectable amount of acetylcholine.

In a total of eight hearts, the viability of the vagus nerves was tested by doing a 5 s control stimulation at the end of the 45 min equilibration period. Atropine was then infused at the rate of 100 µl min^{-1}, to reach a final concentration in the perfusate of 1 µM. A second bilateral vagus nerve stimulation (15 Hz, 20 V, 30 s) was then performed, and samples collected and analysed as previously described.

In 5 out of 8 preparations, no acetylcholine was detected. In 3 preparations, however, there was a very small baseline disturbance which coincided with the position of the acetylcholine standard peak and looked as though it might be due to the presence of a tiny amount of acetylcholine.

Assuming that the 'peak' was indeed due to acetylcholine and not to a contaminant, and assuming too that a four fold increase in acetylcholine overflow was also caused by atropine in this preparation, it would indicate that at least a 20 to 30 fold increase in the concentration of acetylcholine in the samples was needed before this assay could be reliably employed to measure acetylcholine overflow from the isolated rat heart during vagus nerve stimulation.

3.3.5 Sample concentration

The basic samples consisted of a 100 µl injection of the perfusate. Previously, no attempt had been made to clean them up as the method had been successfully employed by other investigators to measure levels of acetylcholine in 100 µl samples of brain microdialysate (Kendrick *et al.*, 1992).

Assuming that acetylcholine was present in the samples, but in concentrations too low to be detected by the HPLC system, the following methods were employed to concentrate acetylcholine in the samples.

• *reduction of perfusion flow rate during nerve stimulation*

The flow rate was reduced from 5 ml g^{-1} min^{-1} to 0.5 ml g^{-1} min^{-1} during, and for 3 min after episodes of nerve stimulation, in an attempt to increase the concentration of acetylcholine in the samples. Acetylcholine was not detected.

• *evaporation*

A 100 nM standard solution of acetylcholine in perfusate was prepared. Aliquots (2 ml) of the standard solution and perfusate were freeze dried overnight and then reconstituted in 200 μl of double distilled water, 100 μl of which was injected onto the HPLC system.

Unfortunately, the resulting high salt concentration caused a massive baseline disturbance when injected, and any acetylcholine peak would have been masked. As a result it was decided to try and extract the acetylcholine from the salt matrix.

• *solvent extraction*

A solid phase extraction method for extracting acetylcholine from physiological salt solutions could not be located, and so the following solvent extraction, (Kato *et al.*, 1975) was used.

Aliquots (4 ml) of the 100 nM standard solution of acetylcholine in perfusate were shaken with an equal volume of a 10 mg ml^{-1} solution of tetraphenylboron (T4125, Sigma) in heptanone (H7883, Sigma) to extract the acetylcholine out of the aqueous layer.

After centrifugation, to separate the aqueous and organic layers, 3 ml of the organic layer was removed and shaken with 1.5 ml of 1 N HCl to back-extract the acetylcholine into the aqueous layer.

Following a further centrifugation step, the organic layer was discarded and the aqueous layer was dried down under a vacuum (Büchi Rotavapor-P, Orme Scientific). The residue was redissolved in 300 μl of double distilled, deionised water. Aliquots (100 μl) of the resulting solution were injected onto the HPLC.

Unfortunately, injection of the extracted samples caused an offscale response during the solvent peak which did not return to baseline before the acetylcholine peak eluted. The acetylcholine peak could not be detected.

3.3.6 Bioassay of acetylcholine

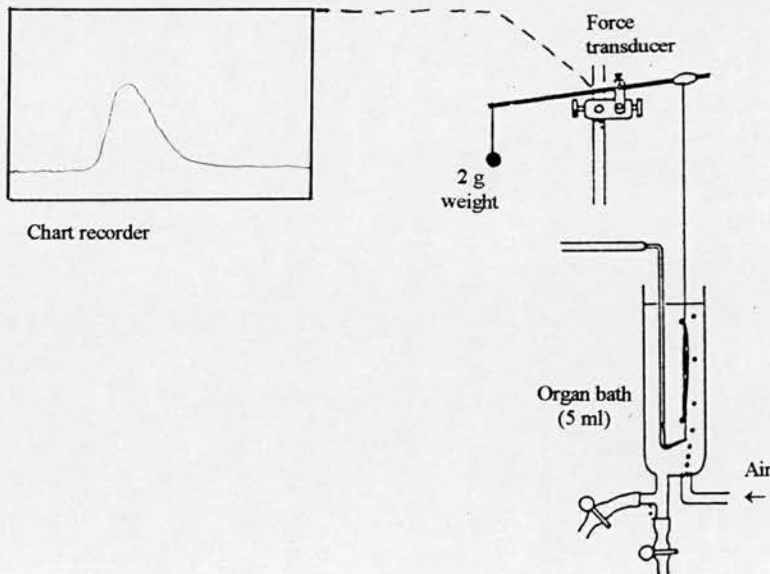
It was therefore decided that bioassay using a preparation of the leech dorsal muscle might be a suitable and more sensitive assay system for acetylcholine (Feldberg and Gaddum 1934).

• *protocol*

Ten specimens of *Hirudo medicinalis* were obtained (Biopharm UK Ltd). They were stored at 4 °C in a muslin bag containing a commercial gel (Hirudogel, Biopharm UK) to keep them moist. A longitudinal strip of dorsal muscle was dissected from each specimen and was set up in an organ bath filled with cold frog Ringer's solution (containing in mM; 111 NaCl, 1.8 KCl, 0.4 $NaH_2PO_4.2H_2O$, 11 glucose, 4.8 $NaHCO_3$, 1.08 $CaCl_2$). The Ringer's solution

also contained the acetylcholinesterase inhibitor eserine (30 μM). The organ bath was continuously bubbled with air. The top of each muscle section was tied with thread and connected to the arm of a force transducer (see Figure 3.6). A small weight (approximately 2 g) was attached to the opposite end of the transducer arm, such that the muscle underwent isotonic contractions.

Figure 3.4 *Leech dorsal muscle preparation for acetylcholine bioassay.*



All preparations underwent a 2 hour equilibration period, during which time the organ bath was regularly flushed with fresh frog Ringer's solution. At the end of this period, the chart recorder was zeroed and a dose response curve was constructed for acetylcholine, using four concentrations of acetylcholine made up in a 5 in 7 dilution of Krebs-Henseleit solution with double distilled water. The Krebs buffer was diluted to be isotonic with the Ringer's solution.

Standards and samples were 5 ml in volume. They remained in contact with the muscle preparation for 60 s, after which the organ bath was flushed repeatedly with fresh Ringer's solution. Sufficient time was left between additions to the organ bath to allow the muscle to return to it's resting length (generally, around 10 min).

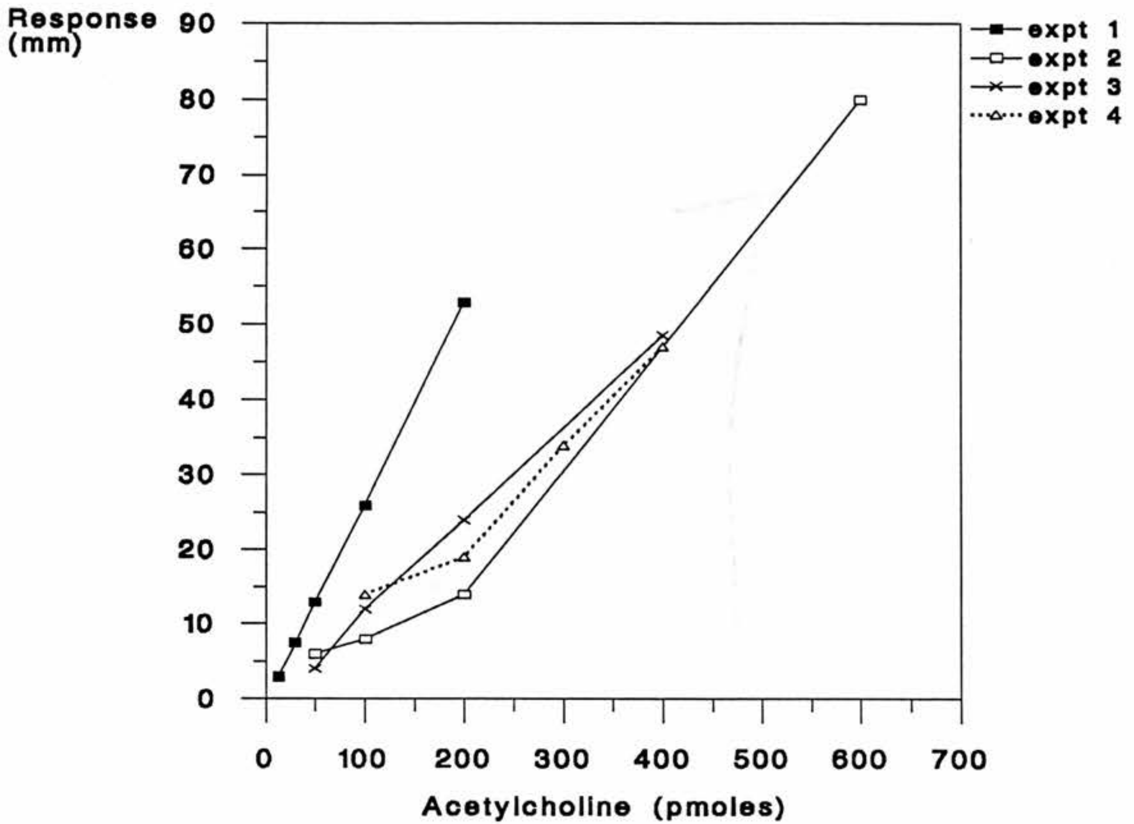
Samples of coronary effluent from perfused hearts containing neostigmine or eserine, were collected for 60 s. The sampling period began 60 s before vagus nerve stimulation, and continued for 180 s. Samples were diluted in the same way as the standards before addition to the organ bath. A total of 10 experiments were carried out.

• *results and discussion*

Blank samples consisting of diluted Krebs-Henseleit solution did not cause any significant change to the baseline.

Sensitivity to acetylcholine varied between the preparations (see Figure 3.5 for examples of dose response curves). A significant response was, however, observed in 75 % of preparations, upon addition of 50 pmoles of acetylcholine to the organ bath. This would equate to a sample sensitivity of 10 nM, twice the sensitivity achieved with the HPLC assay. The most sensitive preparation exhibited a sensitivity to acetylcholine of 5 nM.

Figure 3.5 *Dose response curves for bioassay of acetylcholine on leech dorsal muscle (experiment numbers 1-4)*



Despite the superior sensitivity of the assay, however, no acetylcholine was detected in any of the samples of coronary effluent which were analysed.

3.4 CONCLUSIONS

Taking all these experiments into account, it would seem that the most likely explanation for the absence of acetylcholine in samples of coronary effluent, is that acetylcholine is only released in very small quantities upon vagus nerve stimulation, and that as it washes out of the myocardium it becomes too dilute to be detected by the HPLC assay.

Unlike the sympathetic innervation, which is predominantly ventricular (Jacobowitz *et al.*, 1967), the majority of the parasympathetic nerve terminals are located within the atria (Kent *et al.*, 1974, Pardini *et al.*, 1987). The observed distribution of acetylcholinesterase within the myocardium would also support this (Löffelholz and Pappano 1985). Thus, acetylcholine has a much smaller target tissue than noradrenaline, and it is perhaps not surprising that coronary effluent from stimulated hearts would appear to contain substantially less acetylcholine than noradrenaline.

Avian and amphibian hearts demonstrate a larger evoked acetylcholine overflow than those of mammals. Studies with isolated perfused chicken hearts have yielded measurable (by bioassay) quantities of acetylcholine into the coronary effluent, even in the absence of vagus nerve stimulation (Dieterich *et al.*, 1977). It is possible that the perfused innervated chicken heart may have been a more suitable preparation in which to perform these experiments. The rat heart was chosen as we had considerable experience of the model and the sympathetic ganglion stimulation-evoked overflow of noradrenaline during normoxia and ischaemia. Furthermore, although avian hearts liberate substantially larger amounts of acetylcholine in response to nerve stimulation, tissue acetylcholine concentrations do not differ from those recorded in mammals. Furthermore, acetylcholinesterase activity may even be lower in avian than mammalian hearts (Dieterich *et al.*, 1977). This raises the possibility that the stimulus-secretion coupling in avian hearts is different from that in mammals. Consequently, information about the interaction of the parasympathetic and sympathetic branches of the autonomic nervous system, obtained in an avian preparation, might not be applicable to the mammalian situation, where it is of most interest. In recent years, the publication of several reports of multiple muscarinic receptor subtypes in chick atrial and ventricular tissues (Tietje and Nathanson, 1991; Gadbut and Galper, 1994), provides more concrete evidence that cholinergic neurotransmission in avian hearts may indeed differ from that in mammalian hearts. Mammalian atrial and ventricular tissues have been found to contain muscarinic receptors of the m_2 subtype alone.

In conclusion, until such time as a good sample clean up and concentration method becomes available, the HPLC analysis of acetylcholine in the coronary effluent of stimulated mammalian hearts will not be an appropriate method to monitor the overflow of this neurotransmitter.

CHAPTER 4

Ganglion stimulation experiments

4.1 Introduction

In the past, investigators have employed a variety of different coronary flow rates when studying autonomic nerve function in the buffer-perfused, isolated rat heart. Flow rates as low as 3.5 ml g^{-1} min^{-1} have been used during experiments to examine sympathetic stimulation-evoked noradrenaline overflow (Dart *et al.*, 1983), although flow rates of 5 ml g^{-1} min^{-1} have been reported more frequently (Kurz *et al.*, 1995a; Du and Dart, 1993). Similarly, studies of the haemodynamic response to vagus nerve stimulation in the *in situ*, innervated perfused rat heart, have utilised flow rates of 5 ml g^{-1} min^{-1} (Du, Ph.D. thesis, University of Edinburgh, 1991). The perfusion buffers for these studies are based on Krebs-Henseleit solution. The composition of these buffers does not vary greatly, except for the inclusion of pyruvate in some, but not all of them. The early studies (Dart *et al.*, 1983; Dart *et al.*, 1984a and b; contained pyruvate (1.8 mM), whereas pyruvate was not included in the perfusion buffer of later studies where a flow rate of 5 ml g^{-1} min^{-1} was used (Du and Dart, 1993; Du, 1991; Kurz *et al.*, 1995).

Buffer flow rates of around 5 ml g^{-1} min^{-1} were justified by past investigators on the basis that they were close to the flow rate of blood through the coronary arteries *in vivo* (Malic *et al.*, 1976). Perhaps more importantly, however, they were limited by the sensitivity of the radioenzymatic assay for noradrenaline. Higher flow rates, similar in magnitude to those achieved with free flow at a constant perfusion pressure of 100 cm H_2O in the buffer perfused heart (approximately 10 ml g^{-1} min^{-1}), may have been preferable, but the assay could not cope with the resulting sample dilution. This is not a problem with the superior sensitivity achieved with the HPLC assay.

The experiments described in the previous chapter, were carried out with a buffer flow rate of 5 ml g^{-1} min^{-1}. The haemodynamic response to vagus nerve stimulation was not maintained over the course of 4 consecutive stimulations. This effect persisted when exogenous choline was provided for acetylcholine resynthesis (Dieterich *et al.*, 1978). In addition to the above observation, while the HPLC assay for catecholamines was being set up and verified, a control group for sympathetic stimulation from a previously published experiment, using a flow rate of 5 ml g^{-1} min^{-1}, was repeated. Again, it was noted that the

magnitude of the noradrenaline overflow was not maintained over the course of the four stimulations, despite the fact that the *in situ*, isolated, innervated rat heart model has been reported to be stable under these conditions (Results not shown). The reason for the discrepancy in these results, with those of Du, was not apparent. The experimental conditions were identical to those reported by Du, and the only difference was the assay method for noradrenaline. In Du's experiments, a radioenzymatic method for determination of catecholamine concentrations in coronary effluents was used (Da Prada and Zürcher, 1976), compared to the alumina extraction and HPLC analysis detailed in Section 2.5.

Studies have demonstrated the improved contractile performance of blood-perfused compared to buffer-perfused hearts, owing to the superior myocardial oxygen supply (Masuda *et al.*, 1994). We hypothesised that at buffer flow rates of 5 ml g^{-1} min^{-1}, either the nerves may already be slightly hypoxic, or the myocytes might be releasing factors that inhibit nerve function. Despite their un-physiological nature, higher flow rates may be appropriate in the buffer perfused, innervated heart to provide sufficient oxygen for the maintenance of function. The following experiments were designed to investigate the model stability under different flow and nerve stimulation conditions.

4.2 Protocol

4.2.1 Stability of nerve stimulation model

Animals were allocated to one of three experimental groups:

Group A_1, 5 ml g^{-1} min^{-1} flow rate, 60 s duration of nerve stimulation, (n = 6).

Group A_2, 5 ml g^{-1} min^{-1} flow rate, 30 s nerve stimulation, (n = 6).

Group B_1, 10 ml g^{-1} min^{-1} flow rate, 30 s nerve stimulation, (n = 6).

Hearts were cannulated and perfused as described in Section 2.1.2. Each preparation underwent a 40 minute equilibration period followed by four consecutive left stellate ganglion stimulations (5 Hz, 20 V), at t = 0, 15, 30 and 45 minutes.

The coronary venous effluent was collected in two consecutive 120 s aliquots, starting 120 s before the beginning of the nerve stimulation. Duplicate 2 ml portions of each aliquot were assayed for noradrenaline content using the HPLC method. Lactate concentrations were analysed in duplicate in coronary effluent collected before the first ganglion stimulation (S1) and during nerve stimulation, as described in Section 2.3.1. Left ventricular pressure, heart

rate and dP/dt were continuously monitored as described in Section 2.2.1. Maximum stimulation evoked changes were recorded.

4.3 Results

4.3.1 Model stability - baseline haemodynamic function and biochemical data

Baseline haemodynamic function was defined as the values of LVP, HR and ±dP/dt in the absence of ganglion stimulation, measured immediately prior to each episode of stimulation. Baseline haemodynamic function of the isolated rat heart preparation was stable in all three experimental groups over the 45 minute observation period.

Table 4.3.1 *Baseline haemodynamic function of the isolated rat heart perfused at 5 or 10 ml g^{-1} min^{-1}. Mean ± SD values are presented.*

Exp. Group	Flow Rate *	Time (mins) #	HR (beats min^{-1})	LVP (mm Hg)	+ dP/dt (mm Hg s^{-1})	- dP/dt (mm Hg s^{-1})
		0	250 ± 32	53 ± 9	2071 ± 360	1044 ± 175
A_1	5	15	258 ± 29	52 ± 8	2159 ± 378	1021 ± 134
(n = 6)		30	249 ± 36	56 ± 10	2206 ± 388	1032 ± 151
		45	245 ± 41	54 ± 9	2176 ± 375	994 ± 153
		0	269 ± 48	48 ± 6	1763 ± 364	850 ± 265
A_2	5	15	270 ± 47	51 ± 12	1975 ± 457	988 ± 184
(n = 6)		30	258 ± 60	56 ± 12	1950 ± 332	1000 ± 163
		45	278 ± 28	55 ± 13	1900 ± 327	925 ± 150
		0	318 ± 21	83 ± 17	2588 ± 232	1625 ± 350
B_1	10	15	304 ± 29	81 ± 15	2588 ± 232	1713 ± 307
(n = 6)		30	294 ± 34	83 ± 18	2700 ± 294	1650 ± 235
		45	301 ± 28	76 ± 17	2725 ± 568	1388 ± 312
2 way ANOVA:	Flow rate:		p < 0.001	p < 0.05	p < 0.001	p < 0.001
	Time:		NS	NS	NS	NS

* units of ml g^{-1} min^{-1}
# Minutes after the end of the equilibration period.

Baseline values of LVP, HR and ± dP/dt were all significantly enhanced ($p < 0.01$; $p < 0.05$; and $p < 0.01$ respectively) at all time points in group B_1 (10 ml g^{-1} min^{-1} flow rate), compared to groups A_1 and A_2 (flow rate 5 ml g^{-1} min^{-1}). Baseline haemodynamic parameters were not

different in the two groups perfused at 5 ml g^{-1} min^{-1} (1 way ANOVA, p = NS). Unstimulated lactate overflow in hearts perfused with 5 ml g^{-1} min^{-1} was 1.7 ± 0.8 and 1.5 ± 0.8 μmol g^{-1} min^{-1} in groups A_1 and A_2 respectively. In the hearts perfused at increased flow rate (10 ml g^{-1} min^{-1}), lactate overflow was higher (2.9 ± 2.5 μ mol g^{-1} min^{-1}). Owing to the increased variation, the effect was not significant (unpaired T-test, p = NS).

Noradrenaline was not detected in any unstimulated samples (< 0.5 pmol ml^{-1}; not shown).

4.3.2 The effect of stimulus duration on the response to repetitive ganglion stimulation

Group A_1 (60 s stimulus duration, 5 ml g^{-1} min^{-1}) was compared with group A_2 (30 s stimulus duration, 5 ml g^{-1} min^{-1}). Mean ± SD values were normalised by expressing them as a percentage of the first ganglion stimulation S1. The results are summarised in Table 4.3.2. Actual values of S1 are mentioned in the text or presented in Table 4.3.3.

Table 4.3.2 *The effect of stimulus duration (60 or 30 s) on the response to repetitive ganglion stimulation (data are presented as percent of S1 (S1 = 100 %).*

Exp. Group	*Stim Dur. (s)	Stim No.	NA Overflow (% of S1)	Delta HR (% of S1)	Delta LVP (% of S1)	Delta +dP/dt (% of S1)	Delta -dP/dt (% of S1)
		S1	100	100	100	100	100
A_1	60	S2	71 ± 36	83 ± 30	108 ± 33	79 ± 29	81 ± 22
(n = 6)		S3	49 ± 33	90 ± 36	108 ± 42	76 ± 40	72 ± 26
		S4	40 ± 31	90 ± 71	83 ± 75	72 ± 45	59 ± 26
		S1	100	100	100	100	100
A_2	30	S2	94 ± 29	74 ± 37	71 ± 22	68 ± 25	80 ± 30
(n = 6)		S3	65 ± 9	75 ± 33	71 ± 35	65 ± 17	70 ± 22
		S4	46 ± 11	55 ± 19	64 ± 45	41 ± 14	58 ± 17
2 way	60 vs 30 s:		p < 0.05	NS	NS	NS	NS
ANOVA	S1 - S4:		p < 0.001	NS	NS	p < 0.01	p < 0.01

* Stimulus duration.

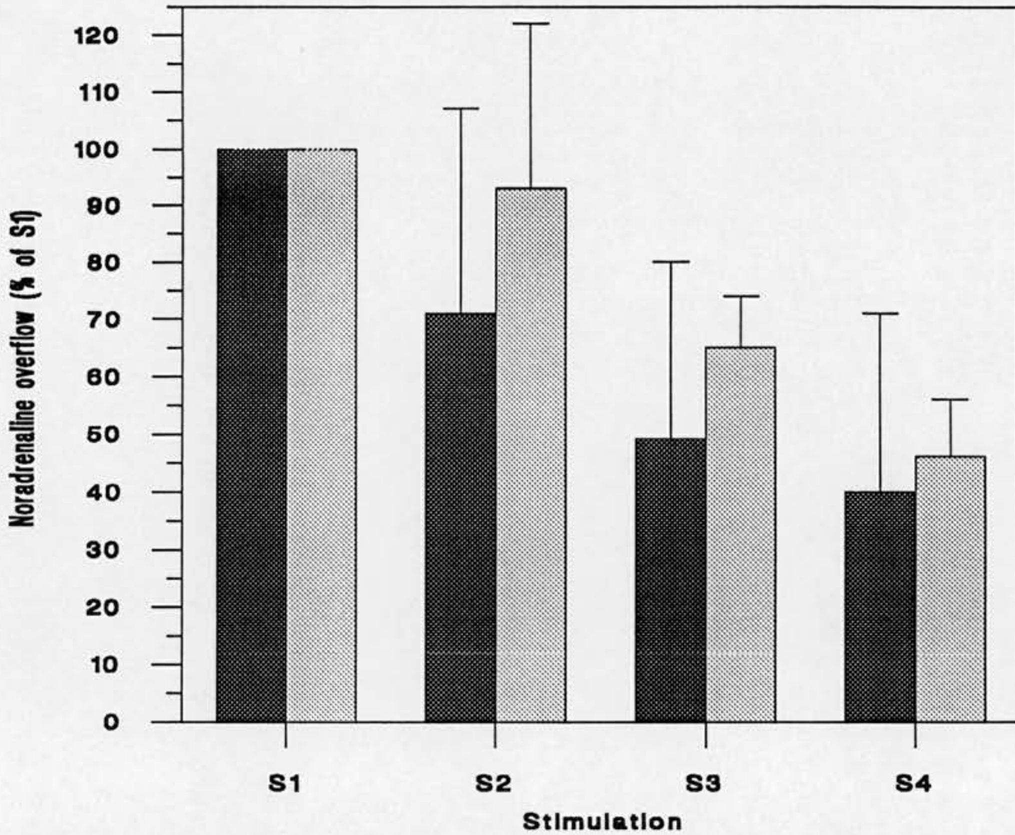
• *Noradrenaline overflow*

Left stellate ganglion stimulation evoked a significant noradrenaline overflow during the two minute collection period in all experiments. The amount of noradrenaline overflow during S1 was not significantly different in the two groups (145 ± 58 and 161 ± 40 pmol g^{-1} min^{-1} for groups A_1 and A_2 respectively).

In both groups, noradrenaline overflow declined significantly over the course of the four ganglion stimulations (ANOVA, p < 0.001). Ganglion stimulation evoked noradrenaline

overflow declined more rapidly when the duration of the stimulation was 60 s rather than 30 s (2 way ANOVA, $p < 0.05$; see Table 4.3.2 and Figure 4.1).

Figure 4.1 *The effect of stimulus duration on noradrenaline overflow during four consecutive left stellate ganglion stimulations.*



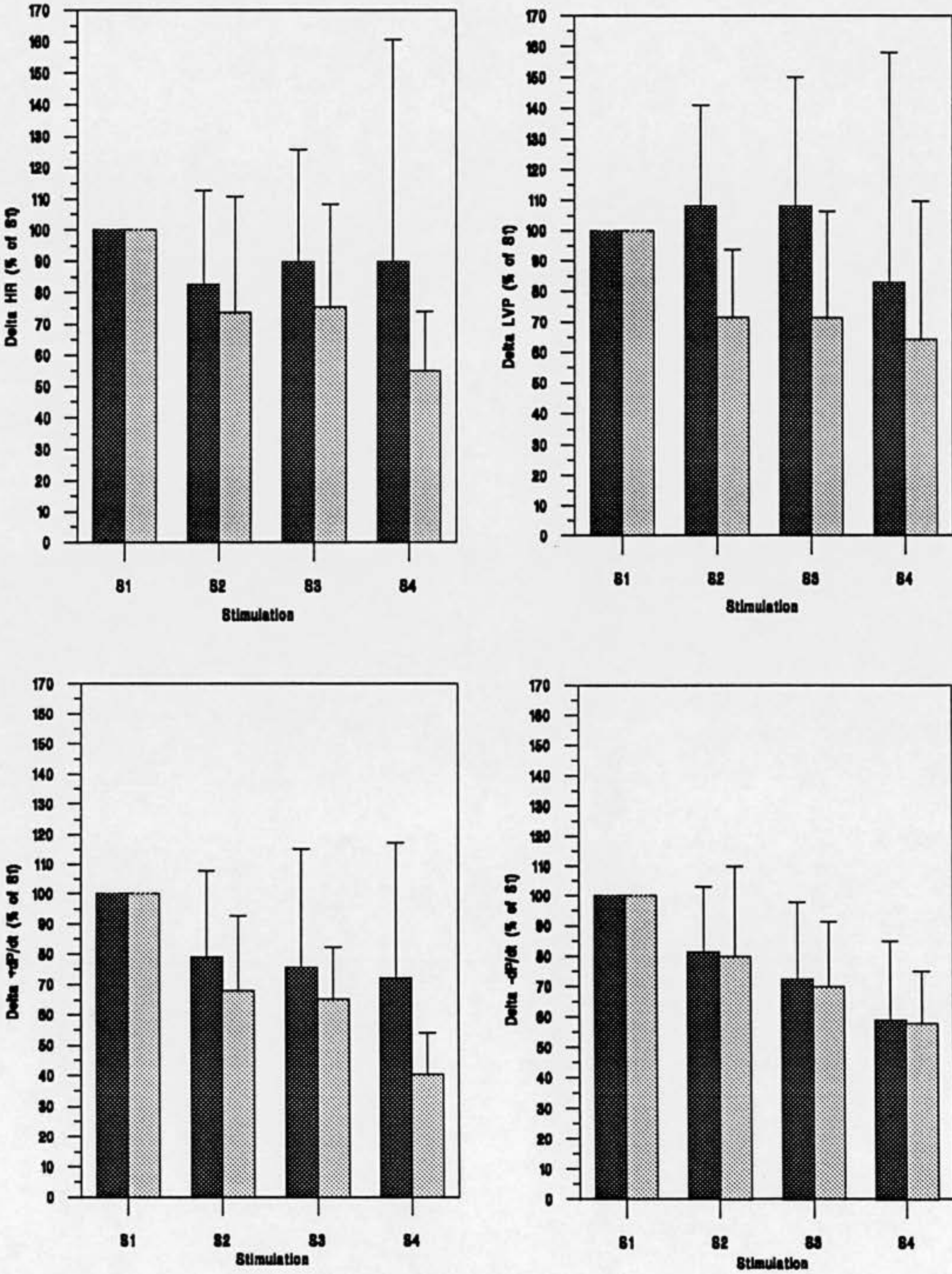
Group A_1 (60 s ganglion stimulation), is represented by dark grey bars.
Group A_2 (30 s ganglion stimulation), is represented by light grey bars.
Each bar is the mean of 6 experiments.
For statistical analysis see Table 4.3.2.

• *Haemodynamic function*

The first left stellate ganglion stimulation increased heart rate, left ventricular pressure and dP/dt (Table 4.3.3). The magnitude of the S1 haemodynamic responses to ganglion stimulation were not significantly effected by the duration of the stimulation.

Although there was a trend for heart rate and left ventricular pressure responses to decrease over the course of the four ganglion stimulations, these changes failed to reach significance owing to the large variability. Variability was higher in the group stimulated for 60 s (A_1), and tended to increase from S1 to S4 (Figure 4.2). Stimulus duration did not have a significant effect on the heart rate or left ventricular pressure responses.

Figure 4.2 *The effect of stimulus duration (60 or 30 s) on the haemodynamic response to four consecutive ganglion stimulations*



Dark grey bars represent group A_1 (60 s ganglion stimulation).
Pale grey bars represent group A_2 (30 s ganglion stimulation).
Each bar is the mean of 6 experiments.
Heart rate and left ventricular pressure responses were not significantly altered during S1 to S4. The magnitude of the +dP/dt and -dP/dt responses declined significantly during S1 to S4 (2 way ANOVA, $p < 0.01$). Stimulus duration had no effect on the rate of this decline.

Left ventricular contractility did, however, demonstrate a significant decline during S1- S4 ($p < 0.01$ for both +dP/dt and -dP/dt; Figure 4.2). Stimulus duration had no effect on the rate of this decline.

Table 4.3.3 *Haemodynamic results from first ganglion stimulation (S1). Mean ± SD values are shown*

Exp. Group	Flow Rate #	Stim Dur (s)	Delta HR (Beats min^{-1})	Delta LVP (mm Hg)	Delta +dP/dt (mm Hg s^{-1})	Delta -dP/dt (mm Hg s^{-1})
A_1 (n = 6)	5	60	63 ± 24	12 ± 5	717 ± 223	808 ± 265
A_2 (n = 6)	5	30	79 ± 13	12 ± 5	883 ± 169	825 ± 204
B_1 (n = 6)	10	30	*40 ± 11	12 ± 5	*425 ± 167	792 ± 169

Units of ml g^{-1} min^{-1}.
Group A_1 was compared to group A_2, and group A_2 was compared to group B_1 using unpaired T-tests.
* Significantly lower than group A_2 (unpaired T-test, $p < 0.001$).

• *Lactate overflow*

Lactate overflows measured during ganglion stimulation at S1 were 2.6 ± 1.2 and 2.5 ± 1.2 μmol g^{-1} min^{-1} in groups A_1 and A_2 respectively, and were not significantly different (Table 4.3.4). Although lactate overflow during ganglion stimulation tended to decline from S1 to S4, this trend was not significant. Stimulus duration did not affect the amount of lactate overflow during stimulation (2 way ANOVA, p = NS; Table 4.3.4).

Table 4.3.4 *The effect of stimulus duration on the lactate overflow (μmol g^{-1} min^{-1}) during repetitive ganglion stimulation*

Exp Group	*Stim Dur (s)	LACTATE OVERFLOW (μmol g^{-1} min^{-1})			
		S1	S2	S3	S4
A_1 (n = 6)	60	2.6 ± 1.2	2.2 ± 1.3	2.2 ± 1.3	2.1 ± 1.1
A_2 (n = 6)	30	2.5 ± 1.2	2.8 ± 1.1	2.3 ± 1.0	1.8 ± 0.7

* Stimulus duration

4.3.3 The effect of perfusion flow rate on the response to repetitive ganglion stimulation.

The effect of flow on the effect of a 30 s ganglion stimulation, repeated 4 times, was further analysed statistically by comparing group A_2 (5 ml g^{-1} min^{-1}) with group B_1 (10 ml g^{-1} min^{-1}). Mean ± SD values were normalized by expressing them as a percentage of S1. The results are summarised in Table 4.3.5. Note that the results for group A2 have already been given in Table 4.3.2. Values of S1 are stated in the text or presented in Table 4.3.3.

Table 4.3.5 *The effect of flow rate on the response to repetitive 30 s ganglion stimulation (data are presented as percent of S1 ± SD (S1 = 100 %).*

Exp. Group	Flow Rate	Stim No.	NA overflow (% of S1)	Delta Heart rate (% of S1)	Delta LVP (% of S1)	Delta +dP/dt (% of S1)	Delta -dP/dt (% of S1)
		S1	100	100	100	100	100
# A_2	5	S2	94 ± 29	74 ± 37	71 ± 22	68 ± 25	80 ± 30
(n = 6)		S3	65 ± 9	75 ± 33	71 ± 35	65 ± 17	70 ± 22
		S4	46 ± 11	55 ± 19	64 ± 45	41 ± 14	58 ± 17
		S1	100	100	100	100	100
B_1	10	S2	93 ± 15	88 ± 25	100 ± 22	110 ± 40	95 ± 29
(n = 6)		S3	95 ± 15	122 ± 25	92 ± 32	94 ± 27	93 ± 24
		S4	93 ± 16	123 ± 25	121 ± 49	100 ± 40	98 ± 19
2 way ANOVA		Flow rate	p < 0.001	p < 0.05	p < 0.05	p < 0.001	p < 0.05
		S1 - S4	p < 0.01	NS	NS	p < 0.01	NS
		Interaction	p < 0.05	NS	NS	p < 0.01	NS

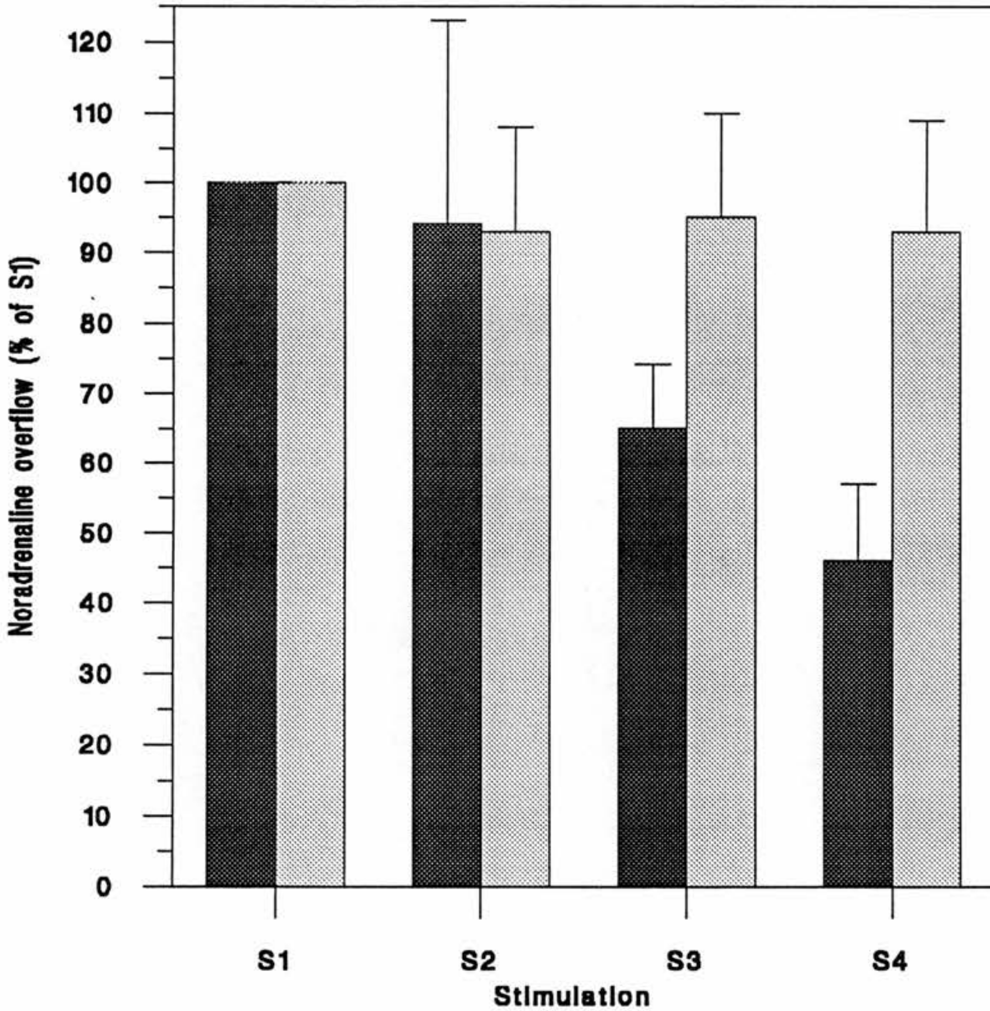
The data of group A2 in this table is identical to that already shown in Table 4.3.2.

• *Noradrenaline overflow*

Left stellate ganglion stimulation evoked a significant noradrenaline overflow during the two minute collection period in all experiments. The amount of noradrenaline overflow during S1 was significantly higher in the group perfused at a flow rate of 10 ml g^{-1} min^{-1} compared to 5 ml g^{-1} min^{-1} (229.5 ± 35.5 and 161.3 ± 39.9 pmoles g^{-1} min^{-1} respectively; $p < 0.05$).

At the lower flow rate (5 ml g^{-1} min^{-1}), noradrenaline overflow decreased significantly during S1 to S4 (ANOVA, $p < 0.001$). When the flow rate was increased to 10 ml g^{-1} min^{-1}, however, the noradrenaline overflow was well maintained throughout all four stimulations.

Figure 4.3 *The effect of coronary flow rate (5 and 10 ml g^{-1} min^{-1}) on the noradrenaline overflow evoked by four left stellate ganglion stimulations*



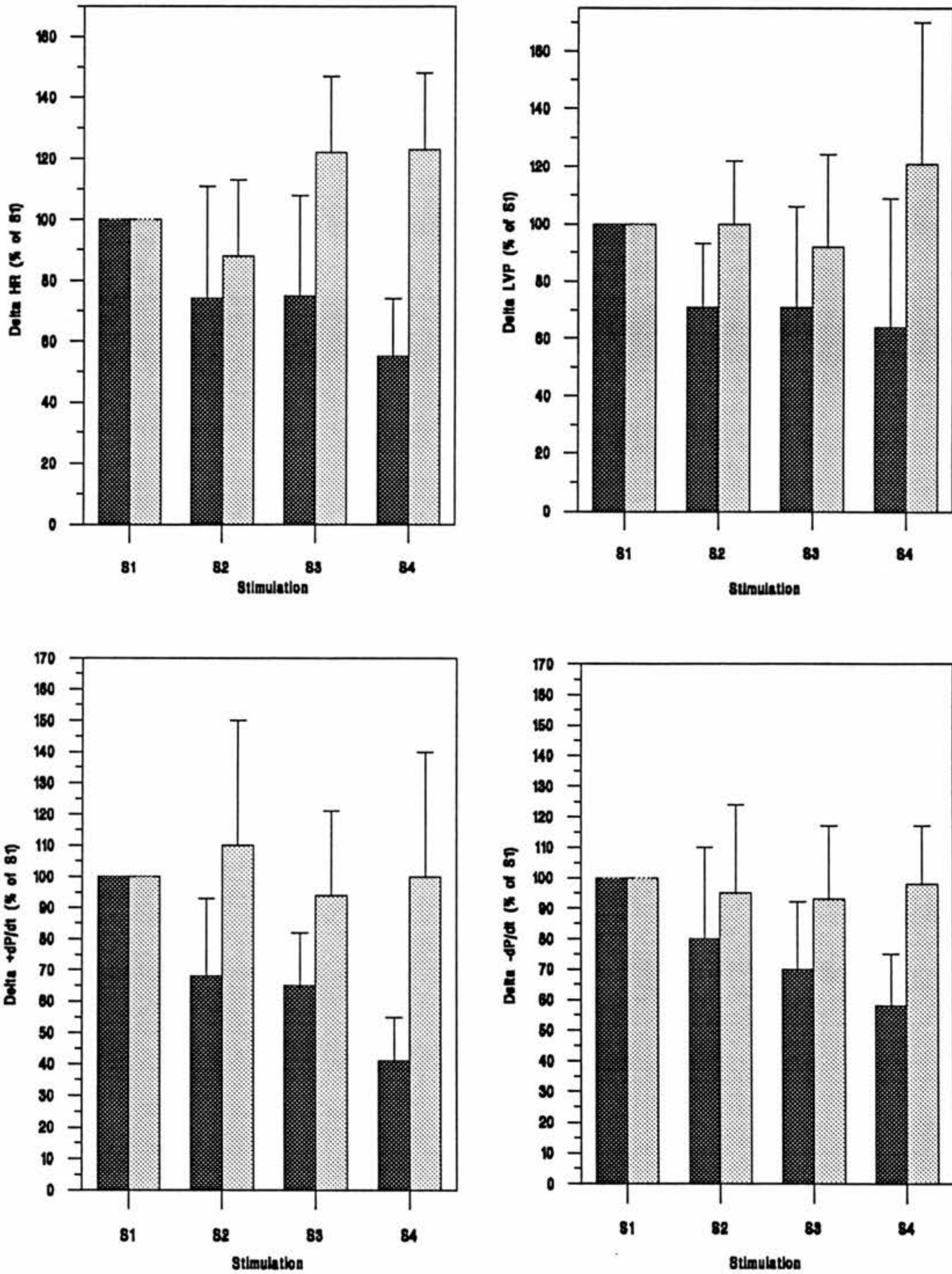
Group A_2, 5 ml g^{-1} min^{-1} is represented by dark grey bars.
Group B_1, 10 ml g^{-1} min^{-1} is represented by pale grey bars.
Each bar represents the mean of 6 observations.
Noradrenaline overflow declined in the group perfused at 5 ml g^{-1} min^{-1}, but was stable in the high flow rate group. For statistical analysis see Table 4.3.5.

• *Haemodynamic function*

Values at S1 are presented in Table 4.3.3. The magnitude of left ventricular pressure and -dP/dt responses were not significantly affected by the flow rate. The magnitude of the heart rate and +dP/dt responses to ganglion stimulation was, however, significantly reduced in group B_1 (10 ml g^{-1} min^{-1}) compared to A_2 (5 ml g^{-1} min^{-1}).

The haemodynamic responses to repetitive nerve stimulation were better maintained in the higher flow rate group (see Figure 4.4).

Figure 4.4 *The effect of flow rate (5 or 10 ml g^{-1} min^{-1}) on the reproducibility of the haemodynamic response to four identical 30 s ganglion stimulations.*



Group A_2 (5 ml g^{-1} min^{-1}) is represented by the dark grey bars.
Group B_1 (10 ml g^{-1} min^{-1}) is represented by the pale grey bars.
Each bar is the mean of 6 experiments.
Haemodynamic responses were all stable in the higher flow rate group.

• *Lactate overflow*

Lactate overflows during nerve stimulation are shown in Table 4.3.6. Lactate overflows tended to be slightly higher in the group perfused at 10 ml g^{-1} min^{-1}, possibly reflecting the higher contractile function, although this difference was not significant (unpaired T-test, p = NS). The lactate overflow was stable throughout the experiment.

Table 4.3.6 *The effect of flow rate on lactate overflow (μmol g^{-1} min^{-1}) during a repeated 30 s ganglion stimulation.*

Exp Group	# Flow Rate	LACTATE OVERFLOW (μmol g^{-1} min^{-1})			
		S1	S2	S3	S4
A_2 (n =6)	5	2.5 ± 1.2	2.8 ± 1.1	2.3 ± 1.0	1.8 ± 0.7
B_1 (n = 6)	10	3.8 ± 3.4	3.5 ± 3.3	3.5 ± 3.3	3.2 ± 3.3
Unpaired T-test		NS	NS	NS	NS

Units of ml g^{-1} min^{-1}.

4.4 Discussion

The results demonstrate that a coronary flow rate of 5 ml g^{-1} min^{-1} is not sufficient to maintain ganglion stimulation-evoked noradrenaline overflow in this model. Even when the duration of the stimulation was reduced, the noradrenaline overflow declined, although the onset was delayed. Interestingly, the haemodynamic responses were not always a good indicator of the failing noradrenaline overflow. Heart rate and left ventricular pressure were particularly poorly related to noradrenaline overflow, although it is possible that with greater numbers, these parameters would have shown a significant decline in their response to repetitive stimulations. When the stimulus duration was longer (60 s), the heart rate and left ventricular pressure changes were better maintained, although the variability was very large. This suggested that perhaps the magnitude of these responses could be maintained in some preparations, but a longer period of nerve stimulation was required to achieve the maximal response. When the flow rate was 5 ml g^{-1} min^{-1}, left ventricular contractility responses were a more sensitive marker of noradrenaline overflow, particularly -dP/dt. It is of interest that Du found a good relationship between neurally mediated noradrenaline overflow and haemodynamic responses in the innervated rat heart perfused at 5 ml g^{-1} min^{-1} (Du, 1991). His experiments were in that case, however, limited to two ganglion stimulations.

Although in the hearts perfused at 5 ml g^{-1} min^{-1} noradrenaline overflow undeniably declined, and was assumed to reflect a corresponding reduction in presynaptic noradrenaline release, it is possible that the failing dP/dt responses at this flow rate were actually due to postsynaptic mechanisms. For example, small changes in intracellular pH could have affected intracellular enzyme activity and the coupling of receptors to second messenger systems. Adrenergic receptor binding characteristics may also have been altered. No attempt was made to differentiate between a pre- and postsynaptic mechanism for this effect, although repeated short noradrenaline or isoprenaline infusions, in place of the ganglion stimulations, could have provided this information.

Increasing the flow rate to 10 ml g^{-1} min^{-1} caused significant increases in all the haemodynamic parameters measured at baseline, confirming that at the lower flow rate, the contractile function of the heart was already limited.

The reduction of the magnitude of the stimulation-evoked heart rate and +dP/dt responses at the higher flow rate (see Table 4.3.3), was interesting. Despite the fact that the magnitude of the stimulation induced increases were reduced, the actual values of heart rate and +dP/dt reached during stimulation, were higher as they were superimposed onto higher baseline values. This result probably reflects that the magnitude of the heart rate and +dP/dt responses were nearing their maximum in the higher flow rate group, and the 'dose-response' curves were no longer linear. Alternatively, the increased rate of washout of noradrenaline from the synaptic cleft at the higher flow rate, reduced the concentration of noradrenaline in contact with the postsynaptic receptors, even though the total amount of noradrenaline released during the stimulation was greater.

Lactate overflow was not significantly different at the two flow rates although the overflow tended to be greater in the hearts perfused at 10 ml g^{-1} min^{-1}. Lactate overflow measured during ganglion stimulation, was not a good indicator that the haemodynamic and nervous function of the heart was compromised at the lower flow rate. It is likely that the increase in lactate overflow lagged behind the haemodynamic response to ganglion stimulation, and was not adequately represented in our 120 s sample. If lactate overflow had been sampled for longer periods following a stimulation, it is possible that it would have resulted in a closer relationship to noradrenaline overflow and haemodynamic function.

The reason that this lack of model stability at flow rates of 5 ml g^{-1} min^{-1} has only been observed now, is difficult to determine. It is hard to compare these results with those of experiments in which the conditions were not identical. It is possible, for example, that the inclusion of pyruvate in the perfusion buffer by groups working in Germany during the 1980s, may have stabilised the model. The use of pyruvate, however, has not been ubiquitous in

experiments performed at a flow rate of 5 ml g^{-1} min^{-1} (Du, 1991; Du and Dart, 1993; Kurz, 1995). One possibility is that the improved sensitivity and precision of the assay procedures used to analyse noradrenaline levels in the coronary effluent in these experiments, led to the discrepancy. The radioenzymatic assay was a notoriously difficult assay to work with and often produced aberrant results. For this reason, samples were run in triplicate and often had to be repeated. Given an unexpectedly low result, it would have been very easy to assume that the assay was at fault and discard or repeat the sample. This may have been even more likely if the heart rate and left ventricular pressure responses were not demonstrating a corresponding fall. Furthermore, if as a result of the exclusion criteria, preparations were discarded if the haemodynamic response to ganglion stimulation began to fail at any point throughout the experiment, the problem would have been exacerbated.

In conclusion, these results demonstrate the need to work with higher coronary flow rates than have been used in the past, to study ganglion stimulation-evoked noradrenaline overflow from the buffer perfused rat heart. A flow rate of 10 ml g^{-1} min^{-1} is sufficient to provide model stability. The results emphasise the importance of measuring neurotransmitter overflow when studying nerve function. Haemodynamic responses alone are not sufficiently sensitive markers of nerve activity.

CHAPTER 5

Noradrenaline and post-ischaemic function

5.1 Introduction

Brief episodes of ischaemia and reperfusion, which themselves do not cause myocardial damage, preceding a longer, sustained period of myocardial ischaemia, delay the onset and decrease the severity of cell injury (Murry *et al.*, 1986). This phenomenon of 'ischaemic preconditioning' has been observed in a wide range of species and models (see Section 1.2), where in addition to its infarct size-limiting effect, it improves the post-ischaemic recovery of contractile function (Cave, 1995) and reduces the incidence of post-ischaemic arrhythmias on reperfusion (Parratt and Vegh, 1994).

The mechanism of ischaemic preconditioning has been extensively investigated (for review, see Cohen and Downey, 1996). Adenosine appears to be a principal mediator of ischaemic preconditioning in the majority of species which have been investigated including the rabbit, dog and human heart (Liu *et al.*, 1991; Grover *et al.*, 1992; Claeys *et al.*, 1996). Adenosine, however, seems not to be involved in the adaptive response to ischaemic preconditioning in the rat heart where the primary mediator remains to be elucidated (Asimakis *et al.*, 1993; Li and Kloner, 1993). A role for catecholamines in the mechanism of ischaemic preconditioning in the rat heart was first investigated by Locke-Winter and co-workers in 1991. They reported that β-adrenoceptor stimulation facilitated ischaemic preconditioning in the protection against contractile dysfunction during post-ischaemic reperfusion in the buffer-perfused rat heart (Locke-Winter *et al.*, 1991). They concluded that this effect was achieved by increasing the presynaptic release of endogenous noradrenaline during the transient preconditioning ischaemia (although noradrenaline release was not measured). The endogenous noradrenaline was then proposed to mediate ischaemic preconditioning through an effect on postsynaptic α_1-adrenoceptors. Subsequently, other evidence supporting a role for catecholamines as mediators of ischaemic preconditioning has been reported. Exogenously infused noradrenaline (10 nmol min^{-1}; 2 min) mimicked ischaemic preconditioning in the globally ischaemic rat heart (Banerjee *et al.*, 1993). The effect was blocked by depletion of endogenous noradrenaline (reserpine), or an α_1-adrenoceptor antagonist.

Although very limited endogenous noradrenaline release may occur during transient global ischaemia in the isolated buffer-perfused heart (Banerjee *et al.*, 1993), the magnitude of

the release is small (approximately 150 pmol g^{-1} min^{-1}). The situation *in vivo* is slightly different. Reflex activation of sympathetic nerves occurs very quickly after the onset of acute ischaemia (Malliani *et al.*, 1969). Even so, this reflex-mediated release is small, and of a local nature, compared to the unphysiologically high levels of exogenous noradrenaline which were infused in the above experiments. The perfused, innervated *in situ* rat heart preparation provides a more suitable model in which to investigate the role of noradrenaline in the preconditioning response.

The following experiments aimed to establish whether direct sympathetic ganglion stimulation could protect against post-ischaemic dysfunction and tissue enzyme depletion in the *in situ*, Langendorff perfused rat heart, and whether this effect differed from that of exogenous noradrenaline infusion.

5.2 Methods

5.2.1 Protocol

The *in situ*, innervated, Langendorff perfused rat heart model was used (as described in Section 2.1.3). Free flow conditions were used and the mean ± SD flow rate was 9.6 ± 1.0 ml g^{-1} min^{-1}. The coronary perfusion pressure was 100 cm H_2O.

Animals were randomly assigned to three groups: A total of 27 animals completed the study (see below). Mean wet heart weights between the groups were not significantly different (1 way ANOVA, p = NS).

Group 1: control group (n = 9); wet heart weight 1.36 ± 0.04g.

Group 2: sympathetic ganglion stimulation (5 Hz, 5 min) (n = 9); 1.35 ± 0.05g.

Group 3: noradrenaline infusion (5 nmol min^{-1}, 5 min) (n = 9); 1.24 ± 0.06g.

Following a 45 min equilibration period, baseline haemodynamic values of heart rate, left ventricular pressure and left ventricular contractility were recorded. Left ventricular developed pressure (LVDP) was defined as the difference between end diastolic and peak systolic pressure. Coronary flow rate, lactate and noradrenaline overflows were also recorded.

Groups 2 and 3 then underwent a 5 min preconditioning intervention. In group 2, this was a period of sympathetic ganglion stimulation (GS). In order to prevent 'nerve fatigue', ganglion stimulations were carried out in 30 s bursts, interspersed with 30 s recovery periods for a total of 5 minutes. Group 3 underwent 5 minutes of exogenous noradrenaline infusion at

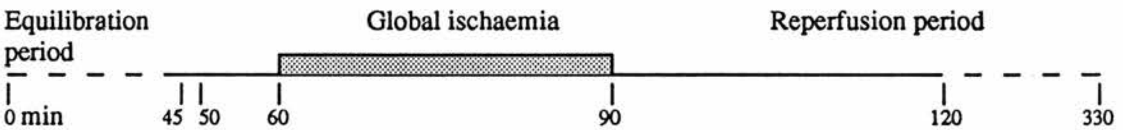
the corresponding time. A 50 μM noradrenaline solution was infused at a rate of 100 μl min^{-1}, (i.e. 5 nmol min^{-1}). Coronary effluent was collected throughout this period for determination of noradrenaline overflow. A total of twelve hearts underwent this procedure. Three of them developed sustained ventricular fibrillation and could not be manually defibrillated. These three preparations were discarded and did not complete the protocol. The remaining nine hearts were all in sinus rhythm at the onset of global ischaemia.

Following the ganglion stimulation or noradrenaline infusion, the hearts were normoxically perfused for a further 10 min, and the parameters were rerecorded immediately before the onset of global ischaemia (BGI). Hearts in group 1 were normoxically perfused for the entire 15 min period.

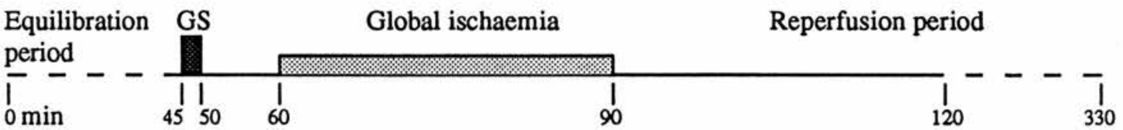
All three groups were then subjected to 30 min global stop-flow ischaemia. During this time, the left ventricular apical cannula was removed and a thermostatic cup was placed over the heart to maintain the temperature at 37 °C. Continuous superfusion with warmed buffer ensured that the preparation remained moist. One minute before the end of the ischaemic episode, the apical cannula was carefully replaced in the ventricle.

Figure 5.1 *Schematic illustration of experimental protocol.*

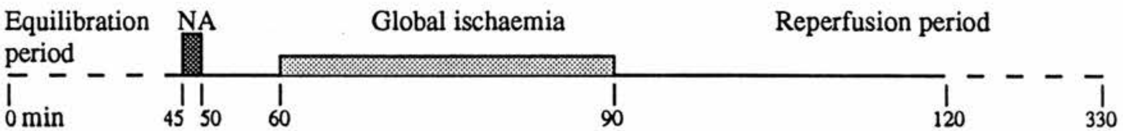
Group 1



Group 2



Group 3



GS represents ganglion stimulation.
NA represents exogenous noradrenaline infusion.
Perfusion was free-flow (100 cm H_2O), except during global stop-flow ischaemia.
Haemodynamic recovery, noradrenaline and lactate overflows were monitored for the first 30 minutes of the reperfusion period. Tissue creatine kinase was measured after 4 hours of reperfusion.

5.2.2 Data collection and processing

The presence of arrhythmias throughout the first twenty minutes of reperfusion made analysis of the progression of haemodynamic recovery impossible. Post-ischaemic recovery of haemodynamic function was assessed after 30 minutes of reperfusion. At this time, most hearts were beating in sinus rhythm and were free of arrhythmias. In 4 of the 27 preparations, sustained, serious ventricular arrhythmias were present and these preparations were excluded from the analysis of haemodynamic functional recovery. They were, however, included in the analysis of post-ischaemic arrhythmias, tissue creatine kinase, lactate and noradrenaline overflow.

Coronary effluent was collected into pre-weighed, chilled tubes for each of the first 5 minutes of the reperfusion period, and for every fifth minute thereafter. Recovery of coronary flow rate was assessed from these samples and lactate and noradrenaline overflows were assayed in the effluent. Lactate and noradrenaline overflows were expressed per gram wet weight of heart tissue.

The presence of ventricular tachyarrhythmias could be identified from the left ventricular pressure recording. Heart rates in excess of 500 beats per minute were classified as tachycardia. All 27 hearts suffered sustained severe ventricular arrhythmias during the early part of the reperfusion period. The incidence of these arrhythmias gradually declined throughout the first 30 minutes of reperfusion. The duration of ventricular tachycardia or fibrillation was measured between 20 and 30 minutes of reperfusion.

Tissue creatine kinase levels, as an indicator of cell damage, were assayed in ventricular homogenate (see section 2.3), at the end of 4 hours of reperfusion.

5.2.3 Statistical analysis

Normally distributed data was analysed using one and two way analysis of variance where appropriate. Where the first test was significant, follow up testing was done with paired and unpaired Student's t-tests. Bonferoni corrections were applied.

Skewed data was analysed using the non-parametric Kruskal-Wallis test. Follow-up testing was done with the Mann-Whitney test and appropriate Bonferoni correction.

5.3 Results

5.3.1 Effect of ganglion stimulation or noradrenaline infusion on pre-ischaemic function

• *Noradrenaline overflow*

Noradrenaline was not detected in any samples of coronary effluent at baseline (< 1 pmol g^{-1} min^{-1} ; Table 5.3.1). During intermittent left stellate ganglion stimulation (group 2), the mean ± SD overflow of noradrenaline in the coronary effluent was 249 ± 92 pmol g^{-1} min^{-1}. In group 3, the concentration of noradrenaline in the effluent during noradrenaline infusion reached 378 ± 16 nM, resulting in an apparent 'overflow' of 3712 ± 92 pmol g^{-1} min^{-1}. This was significantly higher than during ganglion stimulation (unpaired T-test; $p < 0.001$). Noradrenaline overflow was not detected ten minutes after the end of the ganglion stimulation or noradrenaline infusion protocols (Table 5.3.1).

Table 5.3.1 *Coronary flow and washout of lactate and noradrenaline at baseline and immediately before the onset of global ischaemia.*

Treatment Group	Noradrenaline overflow (p mol g^{-1} min^{-1})		
	Baseline	Treatment	BGI
Control (n = 9)	* ND	ND	ND
GS (n = 9)	ND	[a] 249 ± 92	ND
NA (n = 9)	ND	[a b] 3712 ± 92	ND

Treatment Group	Coronary flow rate (ml g^{-1} min^{-1})		Lactate overflow (n moles g^{-1} min^{-1})	
	**Baseline	#BGI	Baseline	BGI
Control (n = 9)	9.3 ± 0.9	9.3 ± 1.7	91 ± 22	102 ± 22
GS (n = 9)	9.7 ± 1.3	9.9 ± 1.5	86 ± 27	70 ± 21
NA (n = 9)	9.8 ± 0.4	10.2 ± 1.0	85 ± 8	78 ± 15
2 way ANOVA				
GS or NA vs control:	NS		NS	
BGI vs baseline:	NS		NS	

** Measured at the end of the 45 minute equilibration period.

BGI represents measurements taken immediately before global ischaemia.

* ND represents not detected (< 1 pmol g^{-1} min^{-1}).

[a] Significantly higher than baseline (paired T-test; $p < 0.001$).

[b] Significantly higher than GS (unpaired T-test; $p < 0.001$).

• *Haemodynamic function*

Baseline values of coronary flow rate did not differ between the three groups (Table 5.3.1). Coronary flow rate measured immediately before the onset of global ischaemia was not different from baseline (2 way ANOVA, p = NS).

Heart rate, left ventricular pressure and dP/dt in groups 2 and 3 at baseline, were not different from control (Table 5.3.2). Intermittent ganglion stimulation (5 Hz) and noradrenaline infusion both caused significant increases in peak heart rate, left ventricular pressure, +dP/dt and -dP/dt compared to baseline (Table 5.3.2). Noradrenaline infusion caused a greater increase in the peak value of -dP/dt than intermittent ganglion stimulation (unpaired T-test, $p < 0.05$). Noradrenaline infusion caused a sustained increase in haemodynamic function during the 5 minute infusion period. Intermittent ganglion stimulation, however, resulted in a cyclical response which corresponded to the periods of stimulation. The haemodynamic response to ganglion stimulation was maintained over the course of all five stimulations. Heart rate, left ventricular pressure and dP/dt had all returned to baseline before the onset of global ischaemia (Table 5.3.2).

• *Lactate overflow*

Baseline lactate overflows did not vary significantly between the groups (Table 5.3.1). Lactate overflow measured immediately before the onset of global ischaemia was not different from baseline.

• *Arrhythmias*

Preparations suffering sustained, serious ventricular arrhythmias during the 60 min pre-ischaemic period were abandoned. A total of three preparations were rejected during the noradrenaline infusion protocol in group 3, after they developed sustained VF from which they could not be manually defibrillated.

5.3.2 Effect of 30 minute, global stop-flow ischaemia on haemodynamic function.

At the onset of global, stop-flow ischaemia, haemodynamic variables declined progressively. All preparations, reached asystole within the first three minutes of global ischaemia, and remained so for the duration of the ischaemic episode.

Table 5.3.2 *The effect of intermittent ganglion stimulation and noradrenaline infusion on baseline haemodynamic function.*

Treatment Group	Heart Rate (beats min^{-1})			*LVDP (mm Hg)			**LVEDP (mm Hg)		
	[c]Baseline	[d]Treatment	[#]BGI	Baseline	Treatment	BGI	Baseline	Treatment	BGI
Control (n=9)	242 ± 34	246 ± 37	241 ± 21	79 ± 8	81 ± 8	78 ± 9	6 ± 1	6 ± 1	6 ± 2
GS (n=9)	247 ± 23	a 314 ± 24	252 ± 29	78 ± 7	a 91 ± 14	75 ± 6	6 ± 1	a 2 ± 2	6 ± 1
NA (n=9)	232 ± 25	a 319 ± 15	234 ± 24	85 ± 6	a 108 ± 11	81 ± 6	5 ± 1	a 2 ± 2	7 ± 3

Treatment Group	+dP/dt (mm Hg s^{-1})			-dP/dt (mm Hg s^{-1})		
	Baseline	Treatment	BGI	Baseline	Treatment	BGI
Control (n=9)	2521 ± 321	2571 ± 280	2495 ± 282	1500 ± 250	1443 ± 215	1555 ± 261
GS (n=9)	2557 ± 172	a 3114 ± 204	2571 ± 251	1557 ± 140	a 2843 ± 341	1407 ± 219
NA (n=9)	2529 ± 206	a 3250 ± 96	2601 ± 304	1543 ± 223	ab 3257 ± 151	1529 ± 250

c Measured at the end of the 45 minute equilibration period.
d Measured during the period when ganglion stimulation or noradrenaline infusion was carried out. Peak values were recorded.
BGI represents measurements taken immediately pre- global ischaemia.
* LVDP represents left ventricular developed pressure.
** LVEDP represents left ventricular end diastolic pressure.
a Significantly different from baseline (paired T-test, $p < 0.05$).
b Significantly different from GS (unpaired T-test, $p < 0.05$).
Haemodynamic variables measured at baseline and BGI in the ganglion stimulation and noradrenaline infusion groups, were not different from control values (2 way ANOVA, p = NS). Values at BGI, immediately before the onset of global ischaemia, were not different from those at baseline (2 way ANOVA, p = NS).

5.3.3 Post-ischaemic reperfusion.

• *Post-ischaemic noradrenaline overflow*

Reperfusion following 30 minutes of global ischaemia caused a significant overflow of noradrenaline in the coronary effluent (Table 5.3.3). Post-ischaemic washout of noradrenaline was complete within the first three minutes of reperfusion. The total noradrenaline recovered in the effluent was not significantly different between the groups (Kruskal-Wallis; p = NS; Table 5.3.3). The variability was high within all three groups, although this was particularly so in the groups which received either ganglion stimulation or noradrenaline infusion in the pre-ischaemic period.

Table 5.3.3 *Total noradrenaline overflow (pmol g^{-1}) and coronary flow rate (% of baseline rate) during post-ischaemic reperfusion.*

TREATMENT GROUP	*NA OVERFLOW (p moles g^{-1})	**FLOW RATE (% of baseline)	
	Early reperfusion	Early reperfusion	Late reperfusion
Control (n = 9)	502 ± 73	68 ± 4	78 ± 5
GS (n = 9)	685 ± 222	82 ± 4	81 ± 5
NA (n = 9)	742 ± 261	[a] 101 ± 5	86 ± 4

[a] Significantly higher than control (1 way ANOVA followed by unpaired T-test; $p < 0.01$).

* Total post-ischaemic washout of noradrenaline (complete within first 3 minutes of reperfusion). This data was not normally distributed and was analysed using the non-parametric Kruskal-Wallis test.

** Values during early reperfusion are the average coronary flow rate during the first 5 minutes of post-ischaemic reperfusion. Values during late reperfusion are coronary flow rate measured in the 30th minute of post-ischaemic reperfusion. Mean ± SD values are expressed as the percentage of baseline flow rate.

• *Post-ischaemic haemodynamic function*

Hearts which received pre-ischaemic noradrenaline infusion, reperfused better than control hearts, demonstrating significantly higher average coronary flow rates during the first 5 minutes of post-ischaemic reperfusion (1 way ANOVA, followed up by unpaired T-test; $p < 0.01$; Table 5.3.3). This effect was lost when coronary flow rates were compared at the end of the 30 minute reperfusion period (Table 5.3.3).

Values of LVDP, +dP/dt and -dP/dt, measured after 30 minutes of post-ischaemic reperfusion, were significantly depressed when compared to baseline (2 way ANOVA, $p < 0.001$; Table 5.4; Figure 5.2). Left ventricular end diastolic pressure measured at the same time, was significantly higher than baseline in all three groups (21 ± 12, 36 ± 16 and 21 ± 18 mm Hg in control, GS and NA group respectively; $p < 0.05$). Neither pre-ischaemic noradrenaline infusion, nor intermittent ganglion stimulation, had any effect on the magnitude

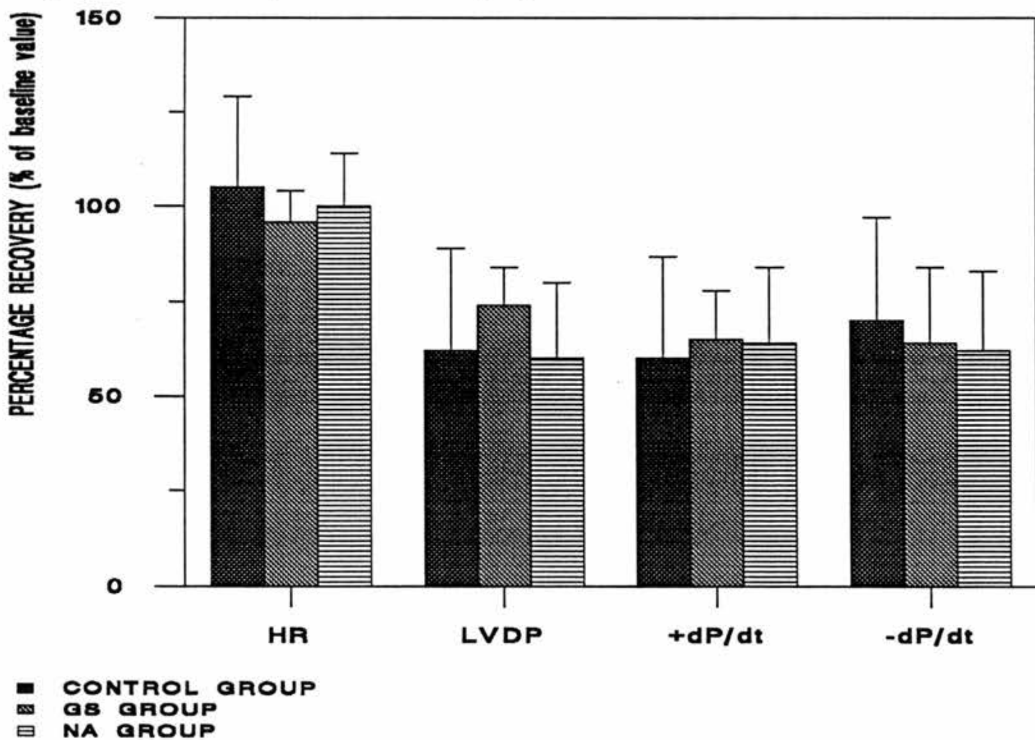
of the changes in post-ischaemic contractile function (2 way ANOVA, p = NS; Figure 5.2). Heart rate measured after 30 minutes of reperfusion was not significantly different from baseline in any of the groups (Table 5.3.4).

Table 5.3.4 *The effect of intermittent ganglion stimulation or exogenous noradrenaline infusion on contractile recovery after 30 minutes of post-ischaemic reperfusion.*

Treatment Group	Heart Rate (% baseline)	LVDP (% baseline)	+dP/dt (% baseline)	-dP/dt (% baseline)
Control (n=7)	105 ± 24	62 ± 27	60 ± 27	70 ± 27
GS (n=8)	96 ± 8	74 ± 10	65 ± 13	63 ± 23
NA (n=8)	100 ± 14	60 ± 20	64 ± 20	62 ± 21
2 way ANOVA				
vs. baseline:	NS	p < 0.001	p < 0.001	p < 0.001
vs. control:	NS	NS	NS	NS

All parameters, except heart rate, were significantly reduced after 30 minutes of post-ischaemic normoxic reperfusion compared to baseline.

Figure 5.2 *The effect of ganglion stimulation or noradrenaline infusion on contractile recovery after 30 minutes post-ischaemic reperfusion.*



For statistical analysis see Table 5.3.4.

• *Lactate overflow*

Lactate overflow after 30 minutes of post-ischaemic reperfusion was significantly higher than baseline values in all three groups (paired T-test, $p < 0.001$). Neither pre-ischaemic ganglion stimulation nor noradrenaline infusion had a significant effect on this value (1 way ANOVA, p = NS; Table 5.3.5).

Table 5.3.5 *The effect of ganglion stimulation or noradrenaline infusion on lactate overflow, incidence of arrhythmias and tissue creatine kinase levels during post-ischaemic reperfusion.*

Treatment Group	*Lactate Overflow (% of baseline)	Duration of VT or VF **(min)	#Creatine Kinase (Units mg^{-1} protein)
Control (n = 9)	[a] 771 ± 127	2.8 ± 0.9	32.2 ± 2.0
GS (n = 9)	[a] 728 ± 109	1.4 ± 1.7	32.9 ± 1.3
NA (n = 9)	[a] 702 ± 138	1.1 ± 2.1	33.0 ± 1.7
1 way ANOVA	NS	NS	NS

* Measured in the 30th minute of reperfusion.
** Measured between 20 and 30 minutes of the reperfusion period.
# Tissue level, measured after 4 hours of reperfusion.
[a] Significantly higher than baseline (paired T-test, $p < 0.001$).

• *Post-ischaemic arrhythmias*

The total time spent in either ventricular tachycardia or ventricular fibrillation, was analysed between twenty and thirty minutes of reperfusion (Table 5.3.5). There was a trend towards fewer post-ischaemic arrhythmias in groups which underwent pre-ischaemic ganglion stimulation or noradrenaline infusion, although there was a very large variability and the trend was not statistically significant.

• *Creatine Kinase*

In control hearts (see Table 5.3.5), the mean tissue creatine kinase level was 32.2 ± 2.0 units-mg^{-1} protein. This was significantly lower than the level observed in a group of 4 hearts perfused with no interventions for 6 hours (38.2 ± 0.8 units mg^{-1} protein, $p < 0.05$, not shown). Neither pre-ischaemic sympathetic ganglion stimulation, nor noradrenaline infusion significantly effected the amount of tissue enzyme depletion after 4 hours of reperfusion (32.9 ± 1.3 and 33.0 ± 1.7 units mg^{-1} protein, respectively).

5.4 Discussion

This is the first study to show that a five minute left stellate ganglion stimulation does not protect the buffer perfused rat heart against the development of post-ischaemic contractile dysfunction following 30 minutes of global ischaemia. This result would indicate that under physiological conditions, endogenous noradrenaline release is unlikely to play an important role in the adaptive response to ischaemic preconditioning in the rat heart. As ever, there are limitations involved in the use of a buffer perfused model. For example, the coronary flow rates are unphysiologically high, resulting in the increased washout of metabolites and neurotransmitters from the heart. The rate of glycolytic flux is also unnaturally high owing to the absence of fatty acids for oxidative phosphorylation. Ideally, experiments should be carried out under more physiological conditions such as the rat heart in vivo preparation to substantiate this conclusion.

We were also unable to demonstrate that pre-ischaemic exogenous noradrenaline infusion caused an improvement in post-ischaemic contractile function. This was unexpected as the protocol described in these experiments was very similar to the one used by Banerjee and co-workers, in which exogenous noradrenaline infusion resulted in a significant improvement in post-ischaemic contractile function (Banerjee *et al.*, 1993). In that study, a 2 minute infusion of exogenous noradrenaline (10 nmol min^{-1}) or phenylephrine, ten minutes before a twenty minute global ischaemia, facilitated the recovery of post-ischaemic contractile function in isolated rat hearts. Preconditioning produced with either noradrenaline infusion or transient ischaemia was abolished with α_1-adrenoceptor blockade. This led them to conclude that ischaemic preconditioning in the isolated rat heart involves sympathetic neurotransmitter release and α_1-adrenoceptor activation. It is possible that slight differences in the methodology may explain why noradrenaline infusion failed to precondition the globally ischaemic rat heart in the experiments described here.

The exact amount of noradrenaline infused during the preconditioning stimulus was not felt to be a critical factor in explaining the lack of any protective effect observed in these experiments. Banerjee used a 2 minute preconditioning period during which noradrenaline was infused at the rate of 10 nmol min^{-1}. Unfortunately, it proved difficult to infuse this concentration of noradrenaline for 5 minutes due to the appearance of many serious ventricular arrhythmias which failed to resolve themselves after 10 minutes of normal perfusion. To overcome this problem, the concentration of infused noradrenaline was reduced to 5 nmol min^{-1}. The 5 minute preconditioning stimulus was, therefore, greater than that in

Banerjee's experiments, but at a lower concentration. It is remarkable that Banerjee *et al.* do not report that they experienced these difficulties. The concentration of potassium in their perfusate was slightly higher than ours (4.7 vs 4.0 mM), although this alone would not seem to be sufficient to explain the difference.

The primary difference in the methodology of the two studies was the severity of the ischaemic insult. Banerjee's group used a twenty minute period of global ischaemia, whereas thirty minutes was used here. The more severe ischaemic episode may have overcome any protection conferred by the noradrenaline infusion. This is not a likely explanation for the discrepancy, however, as a surprising feature of Banerjee's results is the relatively poor recovery of his control group after such a short ischaemic episode. The final recovery of developed pressure after 40 minutes of reperfusion in his control group was 57.4 ± 2.4 % (mean ± s.e.m) of baseline pre-ischaemic function. In the experiments reported here, the recovery of developed pressure in the control group was not different from that result (62 ± 9 % mean ± s.e.m) despite a longer (30 min) ischaemic insult, and a shorter reperfusion period (30 min).

Other investigators were also unable to demonstrate such a magnitude of post-ischaemic contractile dysfunction after only twenty minutes of global ischaemia (Asimakis *et al.*, 1994, Weselcouch *et al.*, 1995). It has been demonstrated that enhanced rates of glucose uptake and glycolysis during reperfusion, improve the post-ischaemic contractile function of the isolated rat heart (Mochizuki and Neely, 1980; Fralix *et al.*, 1992). Banerjee used a lower perfusate glucose concentration (5.5 mM) compared to the glucose concentration reported here, and by Asimakis and Weselcouch in their experiments (11 mM). Thus, a proportion of the depression of post-ischaemic contractile function in Banerjee's experiments may have been ascribed to a limitation of the rate of glucose uptake. Noradrenaline enhances glucose uptake in the rat heart (Clark and Patten, 1984). If this effect had persisted, the improvement that Banerjee observed with noradrenaline infusion, may have been due to improved glucose uptake during reperfusion. Furthermore, Banerjee's hearts were paced during the post-ischaemic reperfusion period. The pacing may have unmasked an effect by forcing the hearts to contract during their recovery period and causing greater metabolic demands. Under these circumstances, any intervention to improve the rate of glucose uptake would have had been expected to result in an even more marked improvement in contractile function. It would be interesting to repeat Banerjee's experimental protocol comparing the effect of the two different concentrations of glucose on the contractile recovery and tissue glycogen level during post-ischaemic reperfusion.

In these experiments, monitoring of the recovery of contractile function was complicated by a high incidence of arrhythmias during the first twenty minutes of post-ischaemic reperfusion in all three groups (not shown). Heart rate, left ventricular pressure and dP/dt were difficult to assess in these circumstances, as a period of at least one minute of sinus rhythm was required to ensure that the contractile function was stable. Post-ischaemic contractile recovery was assessed after 30 minutes of reperfusion. Preparations which still had sustained serious ventricular arrhythmias at this time were excluded from the analysis of contractile recovery. A total of four preparations were excluded in this manner (two from control and one each from the other groups). It could be said that any hearts which did not regain sinus rhythm within 30 minutes of reperfusion recovered less well and that excluding them does not allow for this to be taken into account. In the event that they were included in the analysis, however, they would have been scored as having zero percent recovery, which also biases the data. Pre-ischaemic ganglion stimulation or noradrenaline infusion did not have a significant effect on post-ischaemic contractile recovery if the arrhythmogenic preparations in question were included in the analysis. Banerjee did not report having had problems with arrhythmias, although hearts were defibrillated when necessary. It is not possible to say if this could have been a contributing factor to the difference in the two sets of results. The use of a slightly higher potassium concentration by them (4.7 mM) may have reduced the incidence of arrhythmias and made monitoring of the contractile recovery somewhat easier.

Although Asimakis also reported that a five minute pre-ischaemic noradrenaline infusion (1.5 nmol min^{-1}) could protect the isolated rat heart against post-ischaemic contractile dysfunction, these results were confounded by haemodynamic factors that may have been responsible for the so-called ischaemic preconditioning (Asimakis *et al.*, 1994). A rebound decrease in haemodynamic function, immediately after the noradrenaline infusion, persisted throughout the 5 minute washout period until the start of the 30 minute global ischaemia. At the onset of ischaemia therefore, these hearts had reduced energy requirements which may have led to a less severe metabolic acidosis. In the experiments reported here, a 10 minute post-stimulus washout period was used and the haemodynamic function of the heart at the onset of ischaemia was fully restored to baseline.

Since these experiments were completed, two other groups have reported being unable to effect an improvement in post-ischaemic contractile function in the isolated rat heart with exogenous noradrenaline (Weselcouch *et al.*, 1995), or phenylephrine infusion (Moolman *et al.*, 1996. In the former study, denervation with either 6-hydroxydopamine or reserpine, did not affect the improvement in post-ischaemic contractile recovery observed with ischaemic preconditioning. This led them to conclude that endogenous catecholamine release was not

involved in the adaptive response to ischaemic preconditioning in the isolated rat heart. Moolman and colleagues reported that in their experiments, phenylephrine infused at several concentrations in a repeated fashion during the pre-ischaemic period, was without effect on the contractile recovery following 25 minutes of global ischaemia and 30 minutes reperfusion in the isolated rat heart. They also observed that α_1-adrenoceptor antagonisism was unable to abolish the effect of ischaemic preconditioning on post-ischaemic contractile recovery.

One interesting observation from these experiments was that pre-ischaemic noradrenaline infusion caused significantly improved coronary flow in the early stages of reperfusion. This was presumably as a result of an effect on endothelial function, but of relatively short duration (less than 25 min). Improved perfusion may have been expected to lead to an overall improvement in contractile function. It is possible that the mechanisms behind the preconditioning phenomena of improved post-ischaemic contractile recovery, reduced infarct size and reduced incidence of arrhythmias are not the same. In this case, it cannot be discounted that an improved reperfusion reflow would have caused a reduced infarct size under appropriate conditions. The initial rate of coronary reflow after 30 minutes of global ischaemia is, however, not important in determining the degree of post-ischaemic contractile recovery.

Post-ischaemic washout of noradrenaline has been related to the duration of global ischaemia (Schömig, 1988), and could relate to ischaemic severity. Catecholamines are associated with increased incidence of arrhythmias (Thandroyen 1983, Penny 1985), tissue damage and poor outcome after ischaemia (Nayler 1985). Before the experiment was carried out, it was hypothesised that if there was an effect of either noradrenaline infusion or ganglion stimulation to improve post-ischaemic function, then it might be related to a reduction in post-ischaemic noradrenaline overflow, resulting from a depletion of endogenous noradrenaline stores before the onset of ischaemia. In the experiments reported here, neither of the interventions caused an improvement in post-ischaemic contractile function. Interestingly, however, it has recently been shown that preconditioning with transient ischaemia reduces post-ischaemic noradrenaline overflow (Seyfarth *et al.*, 1996), suggesting that post-ischaemic noradrenaline overflow may, indeed, be another useful marker of preconditioning.

One observation from these experiments is that although post-ischaemic noradrenaline overflow was not significantly different between the groups, the variance was much greater in the noradrenaline infusion and ganglion stimulation groups compared to control. This suggested heterogeneity within the groups that received a preconditioning stimulus. As a result of this heterogeneity, the noradrenaline extraction and analysis procedure was checked. The recovery of the internal standard was not different in those samples where a low or a high

concentration of noradrenaline was present. The post-ischaemic acidosis may have selectively reduced the adsorption of noradrenaline to the alumina. Although the sample pH was not checked, there was nothing to suggest a relationship between high post-ischaemic lactate overflow (as a marker of acidosis) and low noradrenaline overflow. Furthermore, lactate overflow in the treated groups was not different to the control group, even during the first 5 minutes of reperfusion (not shown). The reason for the increased heterogeneity of noradrenaline overflow in the ganglion stimulated and noradrenaline infusion groups in these experiments was not able to be deduced.

In summary, endogenous noradrenaline release during a 5 minute sympathetic ganglion stimulation did not protect the innervated, perfused rat heart against ischaemia-induced contractile dysfunction following 30 minutes of global ischaemia and reperfusion. Nor did exogenous noradrenaline infusion afford protection against the effects of subsequent global ischaemia. Recent publications support the conclusion of this study that adrenergic mechanisms are not involved in the protection afforded by ischaemic preconditioning in the rat heart. The mechanisms involved in the mediation of the ischaemic preconditioning response in the rat heart remain to be elucidated.

CHAPTER 6

General discussion

In recent years, the development of baroreflex sensitivity and heart rate variability testing as reliable, non-invasive techniques, has made the study of cardiac autonomic function *in vivo* that much easier. As a result, the influence of autonomic activity on arrhythmogenesis and the outcome of acute myocardial ischaemia has been brought more sharply into focus. A huge amount is known about the cardiac sympathetic neuroeffector junction and its release of neurotransmitters. Although the results reported here indicate that some of these experiments may have been carried out under less than ideal conditions, the ability to measure noradrenaline overflow in the coronary effluent of isolated hearts has been invaluable in determining the presynaptic mechanisms which control that release. It has also permitted a greater insight into the effect of ischaemia on those presynaptic mechanisms. There is consensus among investigators that endogenous noradrenaline release occurs during acute myocardial ischaemia. That the presence of catecholamines is deleterious to the ischaemic myocardium, increasing the vulnerability to arrhythmias and promoting tissue damage, is also not in dispute.

Two different mechanisms of noradrenaline release in the ischaemic myocardium of the isolated heart have been identified- an early, reflex-mediated exocytotic release, which is followed by a greater, non-exocytotic release after a longer period of ischaemia. Although the reflex-evoked noradrenaline release fails after a relatively short period (1-3 minutes) of stop-flow ischaemia in the isolated rat heart, this may not accurately reflect the situation in other animal species, or in humans *in vivo*, where the reduction of myocardial blood flow within the ischaemic territory is not absolute, owing to the presence of collaterals. During global low flow ischaemia (95 % flow reduction) in the isolated buffer perfused rat heart, the mechanism of exocytotic noradrenaline release may be maintained for long periods (Du, 1991). The physiological significance of the non-exocytotic release has been questioned as it only occurs with long periods of zero-flow ischaemia in isolated preparations. Thus, it is the reflex-evoked noradrenaline release which is believed to increase the vulnerability to arrhythmias during the early stages of acute ischaemia - the most dangerous, in terms of arrhythmogenesis and mortality. It is apparent from studies of baroreflex sensitivity in the dog *in vivo* preparation, that the presence of superior parasympathetic reflexes can protect against these early phase

arrhythmias during acute ischaemia (Schwartz *et al.*, 1988a). Numerous other studies, both *in vivo* and in isolated heart preparations, support the view that parasympathetic nerve activity is antiarrhythmic. Parasympathetic nerve function in the acutely ischaemic state is therefore of great interest and importance. It is striking that there are very few publications in the literature where cardiac acetylcholine overflow has been measured. In view of the difficulties which seem to be associated with measuring such acetylcholine overflows in the mammalian heart, perhaps this is not so surprising.

Negative findings are reported less frequently and may be more difficult to get published than positive ones. It is possible that many other investigators have carried out experiments to measure acetylcholine overflow from the mammalian heart but that their results have not appeared in the literature. There appears to be only one published report where HPLC with electrochemical detection was used to measure cardiac acetylcholine overflow - also, it must be noted, without success. This emphasises the need for negative findings to be reported. In many ways they may be equally as important as positive ones.

The experiments reported here, highlight the importance of measuring transmitter overflow as an endpoint, even though the techniques involved may be difficult and time-consuming to perform. Haemodynamic effects may not be sufficiently sensitive endpoints on their own to reflect nerve activity and transmitter release. There is a great requirement for someone to develop a method for measuring acetylcholine overflow from the perfused mammaliam heart. Only then will it be possible to really understand the interaction of the parasympathetic and sympathetic branches of the autonomic nervous system during ischaemia.

It has been reported that the vagally-mediated reduction in sympathetic stimulation-evoked noradrenaline overflow is abolished very early in an ischaemic episode (Du, 1991). Does this reflect a reduction in acetylcholine release? Alternatively, might ischaemia induce changes in the muscarinic receptor binding or coupling characteristics at the *presynaptic* sympathetic nerve terminal? The reduction of noradrenaline overflow produced by methacholine infusion was also diminished after 10 minutes of low flow ischaemia in that study, suggesting that the latter mechanism might indeed be responsible. The results, however, were inconclusive as the rates of attenuation of the response to vagus stimulation and methacholine infusion were different. The ability to measure stimulation-evoked acetylcholine overflow would help to elucidate the mechanisms opperating under these circumstances.

Other issues which would be of equal interest would be: whether parasympathetic and sympathetic nerve terminals have different sensitivities for the factors associated with ischaemia, such as lowered pH, adenosine release, tissue potassium efflux etc. Do pre- or postsynaptic mechanisms fail first? Without the ability to measure acetylcholine overflow, the answer to these questions pertaining to *presynaptic* effects will remain unanswered.

The HPLC assay for acetylcholine described here would provide a good starting point for anyone who was interested in developing a method. Once set up, it is inexpensive to run, sensitive and the samples are processed very quickly. What is required, however, is a method of concentrating acetylcholine in the samples without concentrating substances such as salts, which interfere with the electrochemical detection. The attempts to concentrate acetylcholine in the samples by evaporation or solvent extraction mentioned previously, were rendered unsuccessful as a result of interference of this nature. It is possible that solid phase extraction might provide a solution, although here again there are likely to be methodological difficulties. One of the main restrictions to developing an extraction method for this assay is that organic solvents cannot be introduced into the HPLC system because they poison the enzymatic reactor column. This obviously limits the choice of solvents available for solid phase extraction procedures. Additionally, the extracted samples must be free of electrochemically active compounds which might interfere with the detection of acetylcholine. Unfortunately, limitations of time prevented the development of such a method for this thesis.

Of course, it cannot be ruled out from these experiments that acetylcholine released in response to vagus stimulation in the rat heart is hydrolysed by acetylcholinesterase before it can wash out of the heart. The results of the experiment in which acetylcholinesterase inhibitors were used in homogenates during the measurement of acetylcholinesterase activity, support the conclusion that the concentration of inhibitor used was sufficient to prevent the significant hydrolysis of acetylcholine. This conclusion, however, is dependent upon the assumption that when the inhibitor was infused at that concentration into an isolated heart, it would have the same efficacy to inhibit acetylcholinesterase activity as in the homogenate, where it presumably has greater access to the membrane bound enzyme. Although the inhibitor did appear to prevent the significant hydrolysis of infused acetylcholine, this was a high concentration of acetylcholine, and it cannot be discounted that small amounts of acetylcholine would have been completely hydrolysed. In most biological systems it is realistically very difficult to ensure that an inhibitor will block 100 % of enzyme activity, particularly when the inhibition is reversible, as in this case. The use of an irreversible inhibitor such as

physostigmine was not possible as it would have rendered the enzymatic reactor column useless.

It is possible that the combination of a relatively small release of acetylcholine in response to nerve activity in the mammalian heart, and an efficient clearance mechanism will be a very hard one to overcome. It appears that evolution has been rigorous in ensuring that a compound such as acetylcholine which is very capable of inducing cardiac arrest is never present in excess. Another option would be to switch to a different model, such as the perfused chicken heart, in which acetylcholine overflow has been demonstrated to be larger, and therefore more easily measurable. The main drawback to this approach is that the physiology of the cardiac autonomic nervous system differs from that present in mammalian species. Mammalian hearts only contain the m_2-muscarinic subtype of acetylcholine receptors. There are now several reports in the literature, however, of the presence of more than one subtype of muscarinic receptor in the chick heart (Tietje *et al.*, 1991; Gadbut and Galper, 1994). In addition, the very thing that may make the model attractive as an alternative to the rat heart, ie. the comparatively large amount of acetylcholine overflow achieved with vagus nerve stimulation, raises questions about how this may affect the interaction of the parasympathetic and sympathetic branches of the autonomic nervous system. Thus, information obtained from the chicken heart may not be readily applicable to the mammalian, and most particularly the human, heart where it is potentially of greatest interest clinically.

The experiments reported in this thesis were performed in buffer-perfused hearts. The importance of a high coronary flow rate in maintaining nerve function was highlighted. This was presumably because the oxygen supply was not adequate. Although buffer perfused heart preparations have been invaluable in the study of all aspects of heart function, there are obvious drawbacks involved with their use. These concern the unphysiological nature of some aspects of the model, in particular, the high oxygen tension, of around 600 mm Hg, which is required to provide an adequate oxygen supply. Even at high flow rates, which themselves are unphysiological, buffer-perfused hearts exhibit reduced contractile function compared to blood-perfused preparations (Masuda *et al.*, 1986). The high flow rates facilitate the wash out metabolites and transmitters much faster than would naturally occur. This may have implications for presynaptic control mechanisms, as substances such as noradrenaline, acetylcholine, adenosine, and NPY have to diffuse over relatively large distances to exert their presynaptic effects. Particularly where trans-synaptic modulation is involved, increased flow rates could result in a markedly reduced modulatory effect. High flow rates are also liable to

cause large sheer stresses on the endothelial walls. It is possible that this may have effects on the production of substances such as nitric oxide, endothelin and prostaglandins some of which may also exert presynaptic effects on autonomic neurotransmission. Finally, buffer-perfused hearts have to generate most of their high energy phosphates from aerobic glycolysis, unlike the situation prevailing *in vivo,* where oxidative phosphorylation of free fatty acids makes by far the biggest contribution to the energy requirements. One of the consequences of this is that the NADH plus H^+ / NAD^+ ratio is reduced when glucose instead of fatty acid is the predominant substrate. If the heart becomes ischaemic, then it will take longer for such an heart to reach a level of acidosis at which the glycolytic pathway is inhibited through inhibition of the pH-sensitive enzyme glyceraldehyde-3-phosphate-dehydrogenase.

Some of the above problems are eliminated if a blood-perfused model is used. Oxygen delivery is improved and so high flow rates are not necessary. In addition, contractile function is enhanced compared to the buffer-perfused model (Masuda *et al.*, 1986). There are at least two methods of carrying out blood perfusions. Although both have advantages over the buffer-perfused model, they also have certain technical difficulties and disadvantages associated with their use. In perhaps the most commonly used method, a genetically similar animal may be used as a recirculating oxygenator or 'support' animal. As the support animal is anaesthetised, endogenous substances or anaesthetic may have effects in the recipient. For example, blood pressure fluctuations in the support animal may lead to reflex adrenaline release which can affect the recipient heart. Another drawback is that the amount of blood is limited and so large samples cannot be taken. If this model were used for nerve stimulation experiments, measurement of transmitter overflow would be complicated by the reduced sample volumes. Even then, a plasma expander needs to be given to avoid the support animal becoming hypovolaemic. Another method is to use washed red blood cells, often bovine in origin, suspended in a physiological salt solution. With this method, larger volumes can be prepared, avoiding the necessity of using a recirculating system. Oxygenation is carried out through the use of a membrane or filament oxygenator. Problems can occur with haemolysis, which allows free haemoglobin to infiltrate the endothelial lining of the blood vessel walls. Free haemoglobin acts as a sponge to mop up nitric oxide, resulting in a profound vasoconstriction. Complement activation can also be a problem in this model, and the failure rate of experimental preparations can be very high. When the method was tested in this laboratory, as many as 50 % of all the preparations failed, despite very careful preparation of the blood cells (R. Riemersma, personal communication).

One approach which might be expected to avoid some of the difficulties associated with the use of the above-mentioned methods is to use an artificial blood solution. These solutions are being developed as an alternative to whole blood for blood transfusions as they have a longer shelf-life, need less exacting storage conditions and do not have the same disease risks as whole blood. During the course of this project, a prototype artificial blood solution became available to us from the Scottish Blood Transfusion Service. The solution was a glycoaldehyde cross-linked human haemoglobin solution formed by combining molecules of free haemoglobin. This process resulted in the production of an oxygen-dissociation curve which, with a p50 value of around 25-30 mm Hg, was very similar to that of red blood cells (MacDonald and Middleton, 1992). The preparation had already been used successfully to perfuse isolated liver preparations. When it was infused into the rat heart preparation in solution with Krebs-Henseleit buffer, the heart progressively stopped beating over the course of approximately 2 to 3 minutes and the coronary perfusion pressure became elevated to over 250 mm Hg, indicating that a profound vasoconstriction had occurred. If normal oxygenated Krebs buffer was then perfused, they recovered completely, The vasoconstriction probably resulted from the effect of free heamoglobin to bind nitric oxide (Marcus and Broekman, 1996). Perhaps in the future, if these molecules could be incorporated into liposomes, this undesirable side-effect might be avoided. In addition, despite the fact that the solution was fully oxygenated before entering the heart, it appeared that the heart could not extract sufficient oxygen to maintain its haemodynamic function, although whether this was as a consequence of severe vasoconstriction preventing nutritive flow was not determined. This effect was not likely to have been so apparent in other isolated organ preparations as they do not have such a large metabolic requirement for oxygen as the heart. It would thus appear that glycoaldehyde cross-linked haemoglobin was not a suitable alternative to either buffer, whole blood or suspensions of washed red blood cells in the perfusion of isolated heart models.

A second type of artificial blood solution - perfluorocarbons - may be of potentially more use in this field. Indeed, they have already been successfully used to perfuse isolated heart preparations (Freeman *et al.*, 1987). Unfortunately, their cost may well prohibit their use for experiments of this kind. The *in situ*, innervated buffer-perfused rat heart model was initially developed as an alternative to *in vivo* nerve stimulation experiments in larger animals such as dogs. Not only was the rat heart preparation substantially cheaper, but it also avoided the complication of anaesthetic effects on the nerves. With the improvement in techniques to quantify noradrenaline release, however, it may be preferable for future experiments to utilise flow rates of 10 ml g^{-1} min^{-1} instead of the more 'traditional' 5 ml g^{-1} min^{-1} . The presence of pyruvate in the perfusate during some of the earlier experiments may have ameliorated the

effect of the low flow rates by stimulating aerobic glycolysis and removing hydrogen ions in the process (Liedke *et al.*, 1976).

Interestingly, the importance of the choice of experimental model is also highlighted in the study of the influence of noradrenaline on post-ischaemic contractile recovery. It appears that one of the principle reasons why the results reported here may differ from those of Banerjee *et al.*, is that different glucose concentrations were used in the two studies. It is possible that Banerjee observed an effect of pre-ischaemic noradrenaline infusion to improve post-ischaemic contractile function because the glucose concentration used in that study was below the optimum level. When noradrenaline was infused, an increase in glucose uptake may have occurred. Pacing of the hearts during the post-ischaemic reperfusion period may have unmasked an effect of improved glycolytic flux in these hearts compared to control. Unlike Banerjee, the results reported here do not support a role for pre-ischaemic noradrenaline in improving the post-ischaemic contractile recovery in the rat heart. This result, and others published since these experiments were completed, would indicate that it is unlikely that noradrenaline is involved in the mechanism of ischaemic preconditioning in the rat heart. Even if the noradrenaline infusions carried out in the published studies had resulted in a reliable improvement in post-ischaemic contractile function, it would have been difficult to relate the result to the situation *in vivo*, owing to the unphysiologically high concentrations of noradrenaline which were infused (up to 10 nmol g^{-1} min^{-1}). This amount of noradrenaline would never be released endogenously from the rat heart. Indeed, during the first ten minutes of acute myocardial ischaemia it is very difficult to demonstrate an enhanced release of endogenous noradrenaline at all, despite evidence that sympathetic reflexes are activated. Infusions of such unphysiologically high concentrations of agonist into any system is liable to result in an increased chance of non-specific effects such as the non-specific binding of agonist to other receptors. Even if the non-specific binding does not result in activation of that receptor, it may still act as a competitive antagonist.

In contrast to the situation in the rat, it appears that pre-ischaemic noradrenaline or tyramine infusion may protect the rabbit heart against infarction. A reduction of infarct size was observed in the rabbit *in vivo* model when a five minute infusion of either tyramine (Thornton *et al.*, 1993; Bankwala *et al.*, 1994) or noradrenaline (Bankwala *et al.*, 1994) was carried out ten minutes before a 30 minute coronary occlusion. The noradrenaline was shown to act via α_1-adrenoceptors, as α-adrenergic blockade abolished the effect in both studies. A non-specific adenosine antagonist also abolished the effect in Thornton's study it is thus

possible that noradrenaline was actually acting on α-adrenergic receptors to cause an increase in myocardial adenosine release (although adenosine levels were not measured). In addition, there may have been a cooperative effect of the tyramine infusion to raise adenosine levels in the synaptic cleft by causing the co-release of purines with the endogenous noradrenaline from the presynaptic vesicles. Adenosine has been repeatedly demonstrated to be involved in the mechanism of ischaemic preconditioning in the rabbit heart (Liu *et al.*, 1991 and 1996; Tsuchida *et al.*, 1994; Armstrong and Ganote, 1994). Further evidence that noradrenaline release was not solely responsible for the preconditioning effect was provided when α-adrenergic blockade failed to abolish preconditioning with transient ischaemia, (Thornton *et al.*, 1993). This inability of adrenergic receptor antagonists to prevent the infarct-limiting effect of ischaemic preconditioning has also been reported by other investigators working with this model (Haessler *et al.*, 1996).

It was always extremely unlikely that endogenous noradrenaline release would turn out to be of primary importance in mediating the ischaemic preconditioning response in any animal model, as the phenomenon of ischaemic preconditioning is clearly demonstrable in isolated hearts. A single episode of 5 minutes of ischaemia, which is sufficient to cause preconditioning, is insufficient to cause substantial noradrenaline overflow in the isolated heart. The duration of global ischaemia would have to be at least 10 to 15 minutes before significant noradrenaline release would occur (Schömig, 1988). It is, however, possible that the situation opperating *in vivo* with reflex sympathetic activation occurring within the first few minutes of ischaemia, may contribute to the mechanism of preconditioning by facilitating the accumulation of purines in the synaptic cleft. The purines which are co-released from presynaptic vesicles with noradrenaline are degraded by ecto-ATPase to form adenosine. In addition, endogenous noradrenaline acting via the α_1- adrenoceptor stimulates haemodynamic function, resulting in the increased use of ATP. Some of the resulting ADP will be degraded to adenosine and will diffuse out of the muscle. Thus, in species such as the rabbit, dog, human and pig, in which adenosine is clearly involved in the mechanism of ischaemic preconditioning, reflex-evoked noradrenaline release could play a facilitatory role in the *in vivo* situation by increasing the ischaemia-evoked adenosine accumulation. It would be interesting to look at the effect of acute and chronic cardiac denervation on adenosine release during transient ischaemia in an *in vivo* model

At the time of writing, the mechanism(s) responsible for producing the beneficial effects of ischaemic preconditioning remain unresolved. It appears, however, that the situation

in the rat heart differs from that of other species such as the dog, pig, rabbit and human, where the involvement of adenosine at some level is not disputed. It may turn out that no single pathway is responsible for the effect, but that multiple pathways are involved and act in a cooperative fashion.

Finally, in relation to the objectives of the study (Introduction, p27), although stimulation of the perfused innervated rat heart had the expected negative chronotropic and inotropic effects, acetylcholine overflow in the coronary effluent was not detected when a novel HPLC method was used to quantify the neurotransmitter. The apparent absence of acetylcholine in the coronary effluent could not be attributed to biodegradation of the acetylcholine by myocardial acetylcholinesterase activity. Bioassay of acetylcholine using the leech dorsal muscle preparation confirmed the lack of detectable acetylcholine in the coronary effluent samples following vagus stimulation. As a consequence, it was not possible to determine either the extent to which the severity and duration of global ischaemia affect the vagally mediated overflow of acetylcholine or the relative importance of the various concomitants of ischaemia on this overflow. Likewise, it was not possible to investigate the effect of the severity and duration of global ischaemia on the presynaptic interaction of the parasympathetic and sympathetic nervous system with respect to the overflow of acetylcholine and noradrenaline. The importance of an adequate coronary flow rate in the maintenance of stimulation-evoked noradrenaline overflow in buffer perfused rat hearts was highlighted. A five minute sympathetic ganglion stimulation or infusion of noradrenaline increased heart rate and contractility. All parameters returned to baseline within ten minutes. These adrenergic stimuli to the normoxic heart did not result in improved haemodynamic recovery following a subsequent thirty minute period of global, stop-flow ischaemia. Other markers of ischaemia - induced damage such as noradrenaline release on reperfusion or myocardial creatine kinase content, were also unaffected by these interventions.

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