THE ROLE OF CAPSAICIN-SENSITIVE NEUROPEPTIDES AND THE INTRINSIC CARDIAC GANGLIA ON CARDIODYNAMICS AND ATRIAL NATRIURETIC PEPTIDE (ANP) RELEASE

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ROBERTA PATRICIA POWER

THE ROLE OF CAPSAICIN-SENSITIVE NEUROPEPTIDES AND THE INTRINSIC CARDIAC GANGLIA ON CARDIODYNAMICS AND ATRIAL NATRIURETIC PEPTIDE (ANP) RELEASE

BY

© ROBERTA PATRICIA POWER

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

Faculty of Medicine
Memorial University of Newfoundland
1993

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ISBN 0-315-82652-5

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ABSTRACT

Atrial natriuretic peptide (ANP) is a circulating hormone, released from the heart, whose natriuretic and diuretic actions play an important role in cardiovascular homeostasis. It is stored as a prohormone in cardiac myocytes and converted to a circulating form upon release from the myocytes. Numerous factors have been shown to modulate ANP release, however the principal stimulus for release is believed to be stretch of the atrial wall.

Recently it has been demonstrated that the capsaicin-sensitive peptidergic innervation of the rat heart, namely the substance P (SP) and calcitonin gene-related peptide (CGRP) fibers, is a component modulating the release of immunoreactiveANP (irANP). This research project initially investigated the role of the intrinsic cardiac ganglia on cardiodynamics and irANP release. In Sprague-Dawley rats, *in vitro* electrical stimulation of epicardial regions dense with cardiac ganglia (50Hz, 15-20μs, 4μA delivered in 5s trains), referred to as 'ganglionic' stimulation, resulted in a dramatic decrease in heart rate followed by a transient period of tachycardia. Cardiodynamic effects were accompanied by a significant increase in irANP release. Results from stimulation of epicardial regions lacking the presence of cardiac ganglia, termed 'non-ganglionic' stimulation indicated that heart rate changes were attributable to excitation of particular components of the ganglia at the site of stimulation, and not simply to spread of an electrical current over the epicardium.

Also investigated were the effects of neonatal capsaicin treatment (capsaicin is a chemical that abolishes SP and CGRP cardiac innervation in rats when administered neonatally) on irANP release in the model of cardiac ganglia stimulation and in other

models previously demonstrated to release irANP, such as stretch of the right atrium. In ganglionic stimulation experiments, release of irANP was independent of changes in heart rate. The presence of innervation by immunoreactive SP and CGRP was shown to be imperative for significant increase in irANP release after ganglionic stimulation and after stretch of the right atrium.

Pharmacological studies were carried out to gain insight into the role of acetylcholine and noradrenaline in the biphasic change in heart rate and release of irANP after ganglionic stimulation. The initial decrease in heart rate was shown to be associated with parasympathetic innervation and similarly, tachycardia was associated with sympathetic innervation. However, release of irANP was demonstrated to be independent from either decreases or increases in heart rate. It is hypothesized that if SP and CGRP innervation are involved in irANP release, and if capsaicin depletes these peptides, then an acute treatment of capsaicin should release stores of SP and CGRP and yield an increased release of irANP. This was the case when vehicle-treated animals were administered an acute dose of capsaicin, however the tissues from animals treated neonatally with capsaicin showed no release of irANP. Acute capsaicin administration had no effect on heart rate in either group.

Finally, Northern blot analysis of ANP messenger ribonucleic acid (mRNA) production was performed to ascertain if neonatal capsaicin treatment was affecting synthesis of ANP and if levels of mRNA expression were equivalent in vehicle- and capsaicin-treated animals.

It was concluded that electrical stimulation of the intrinsic cardiac ganglia results in a biphasic change in heart rate associated with the sympathetic and parasympathetic cardiac innervation. Also, in the models used here, the SP and CGRP cardiac innervation

seem responsible for the modulation of the release of irANP. Some preliminary data also suggest that SP and CGRP may be modulating irANP release in *in vivo* models of atrial pacing as well. Further studies are necessary before conclusions can be drawn regarding the effects of capsaicin on ANP synthesis.

KEY WORDS and PHRASES: atrial natriuretic peptide, electrical stimulation of cardiac ganglia, atrial stretch, atrial pacing, substance P, calcitonin gene-related peptide, mRNA for ANP

ACKNOWLEDGMENTS

I will always be indebted to Dr. Andrew Rankin, not only for giving me an opportunity to study with him, but also for his unfailing guidance, encouragement and friendship. I could always count on him to be accessible and helpful at any time. When frustrating situations arose I could depend on him to be supportive and to help me regain the confidence to keep pushing forward. I feel that Dr. Rankin, and my experience as a graduate student in his laboratory, have taught me the true value of perseverance. I am also deeply grateful to the other two members of my supervisory committee, Drs. James Reynolds and Donald McKay, for their patient assistance as well as their insightful ideas and critical assessment of this manuscript.

I would also like to acknowledge the technical assistance of Fred Swift and Anne Lockwood. Anne had the pleasure of teaching me the basics of molecular biology in three weeks! She is a great teacher, I respect her tremendously and I am very grateful for all of her help.

Special thanks also are extended to Dr. Jon Church for allowing me to perform the molecular biology portion of this project in his lab as well as for his expert opinion during our discussions. Similarly I would like to thank Dr. Tom Scott and Sue Ellen Maher for teaching me the skills required to carry out immunohistochemical staining. I deeply appreciate all the time they both devoted to me. Dr. Detlef Bieger was also very helpful and I availed of his expertise many times. I extend my appreciation to Dr. Bieger as well.

Finally I would like to thank Dan for his constant love, patience and support (and his computer!). This work was funded by The Heart and Stroke Foundation of Newfoundland and The Faculty of Medicine, Memorial University of Newfoundland.

DEDICATION

As acknowledgment and thanks for all their love, support and encouragement throughout all my years, I would like to dedicate this thesis to my mother and father.

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LIST OF ABBREVIATIONS

ANP	Atrial Natriuretic Peptide
ANF	Atrial Natriuretic Factor
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic Guanosine Monophosphate
CHF	Congestive heart failure
CNP	'C' natriuretic peptide
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
EH	Essential hypertension
HPLC	High Phase Liquid Chromatography
irANP	Immunoreactive Atrial Natriuretic Peptide
mRNA	Messenger ribonucleic acid
NEP	Neutral endopeptidase 24.11
PBS	Phosphate buffered saline
Pro-ANP	Pro-atrial natriuretic peptide
PKC	Protein kinase C
RNA	Ribonucleic acid
RPM	Revolutions per minute
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
UV	Ultraviolet

CHAPTER 1

1.0. INTRODUCTION

1.1. Atrial Natriuretic Peptide (ANP)- Background Information

While searching for evidence of the location of receptors influencing urine flow, Henry *et al.* (1956) postulated that increased atrial pressure was associated with diuresis and natriuresis *in vivo*. In 1964 Jamieson and Palade proposed a secretory role for atrial muscle cells as granules present in the atria closely resembled granules of endocrine cells. Then, in 1979 de Bold demonstrated that the population of granules in the rat atria decreased in response to increased salt and water intake and further demonstrated in 1981 that intravenous infusions of atrial extracts caused rapid and significant diuresis, natriuresis and hypotension. All of these observations suggested that the atria may be producing a natriuretic hormone that affects extracellular fluid homeostasis. The biochemical characterization of such a natriuretic and diuretic hormone, referred to as atrial natriuretic peptide (ANP) or atrial natriuretic factor (ANF) was initially carried out by Flynn in 1983 (Flynn *et al.*, 1983). ANP is a 17-member amino acid ring formed by a disulfide bond and has a carboxyl and amino acid extension to the molecule.

Following the discovery of ANP it was speculated that atrial distention, caused by acute hypervolemia, might release ANP. The resulting increase in salt and water excretion could then restore both vascular volume and atrial pressure to normal (de Bold *et al.*, 1981). To investigate this hypothesis, Ledsome *et al.* (1986) carried out experiments in anesthetized dogs. They distended the left atria and increased left atrial pressure by obstructing the mitral orifice with a balloon. ANP measurements during

distention demonstrated that increased left atrial pressure could indeed release ANP. Such a protocol could have also stimulated the atrial volume receptors which may themselves have stimulated ANP release. Therefore, mitral obstruction was repeated after bilateral cervical vagotomy which interrupted the afferent pathway from the atrial receptors. Changes in plasma immunoreactive-ANP (irANP) were the same following vagotomy suggesting that atrial distention released ANP by a local stretch effect rather than through atrial volume receptors (Rankin, 1987). *In vivo* studies of cardiac denervation and *in vitro* experiments in animals, as well as water immersion experiments in man, confirmed that neither afferent nor efferent nerves were essential to the release of ANP during atrial distention (Goetz *et al.*, 1986; Dietz, 1984; Lang *et al.*, 1985; Katsube *et al.*, 1985).

Elevated levels of ANP had been noted in patients with essential hypertension (Sagnella *et al.*, 1986) which was attributed to elevated arterial blood pressure (Manning *et al.*, 1985) or to factors associated with increased myocardial work (Rankin, 1987). Paroxysmal tachycardia has been associated with diuresis and natriuresis in humans and also leads to increased atrial volume and distention. Therefore, arterial blood pressure and heart rate have been implicated in the release of ANP.

These studies reflect an intensive decade of researching a peptide which, by its natriuretic and diuretic properties, had the potential to play a key role in our understanding of hemodynamic homeostasis.

1.2. Stimulation of ANP Release

Many researchers now believe that stretch of the atrial wall is the primary stimulus for release of ANP into the circulation. IrANP release showed an increase within 2 minutes of atrial distention in anesthetized dogs and the half-life of the circulating

molecule was shown to be 4.5 minutes (Ledsome *et al.*, 1986). To determine the involvement of intra-atrial pressure in stretch induced ANP release, Edwards *et al.* (1988) studied the effects of acute cardiac tamponade in the intact dog. Cardiac tamponade is a unique condition in which atrial pressure is acutely elevated. However, because of a balanced increase in both intra-atrial and pericardial pressures, transmural atrial pressure does not increase. By controlling transmural atrial pressure, stretch of the atria is inhibited. When the results from this model were compared to great artery constriction, which results in increases of intra-atrial and transmural pressure, it was demonstrated that an increase in atrial transmural pressure with associated atrial stretch, not increased intra-atrial pressure, acts as the principal mechanism controlling release of ANP.

Studies with low-dose infusions of synthetic ANP in dogs (Bie *et al.*, 1988 and Pichet *et al.*, 1989) and humans (Cuneo *et al.*, 1987; Richards *et al.*, 1988) have demonstrated that renal function, aldosterone secretion, circulating blood volume and arterial pressure may be influenced even in the physiological range of circulating plasma levels. In this range, though the effects of ANP were easily detectable, they were quantitatively rather modest (Cernacek and Levy, 1991). However, the same plasma concentration of ANP was much more effective during volume expansion (Metzler and Ramsay, 1989), suggesting that ANP participated not only in day to day homeostasis of blood volume, but also during increased volume in the vascular compartment. Cernacek and Levy (1991) suggested that secretion of ANP from the atria had to be positively related to volume of the circulating blood to fulfill this role and therefore investigated why plasma levels of ANP do not always respond as a simple function of volume status. In anesthetized dogs, they studied the relationship between irANP release and different volume-expansion protocols concluding that in normal dogs subjected to various types of

intravascular volume expansion, the primary determinant of ANP release is the increase in **central blood volume** translated to an increase in atrial pressure. They implied that in pathophysiological conditions characterized by an expanded blood volume, plasma irANP may not be increased if the extra volume is not proportionately centralized and therefore does not elicit an elevation of pressure. Atrial dimensions were not measured in this study.

Tachycardia in intact animals and isolated hearts has been associated with significant increases in irANP (Rankin *et al.*, 1986; King and Ledsome, 1991; Bilder *et al.*, 1989; Schiebinger and Linden, 1986; King and Ledsome, 1990). In 1986, Rankin and his colleagues demonstrated that electrical pacing of the right atria of anesthetized rabbits could increase irANP release, however these findings were confounded by increases in right atrial pressure and decreases in blood pressure. The results of this study did suggest that ANP release did not involve a classical sympathetic or parasympathetic neural reflex since release was the same after administration of atenolol (a competitive antagonist of noradrenaline at $beta_1$-adrenergic receptors), atropine (a competitive antagonist of acetylcholine at muscarinic cholinergic receptors) and hexamethonium (a ganglionic blocker which inhibits activation of both sympathetic and parasympathetic ganglia). When isoproterenol (a positive inotropic and chronotropic agent that increases myocardial work through activation of beta adrenergic receptors) was given to anesthetized rabbits, ANP was not released, suggesting release of ANP is independent from stimulation of efferent adrenergic fibers innervating the atria; however ANP may be released as a result of changes in myocardial work and oxygen consumption (Rankin *et al.*, 1987a). However, King and Ledsome (1990) found no significant change in right atrial dimensions during tachycardia suggesting that release of ANP in response to tachycardia was not due to

simple mechanical stretch of the atria but perhaps is due to atrial wall stress, particularly systolic right atrial wall stress. Elevated levels of ANP during tachycardia in the presence of constant atrial dimensions has been supported by others (Riddervold *et al.*, 1991). Portaluppi *et al.* (1990) also reported that atrial strain, not intra-atrial pressure in itself nor heart rate, was the main determinant of acute release of ANP. It has also been postulated that the reduction of atrial distention is the stimulus for ANP release. Cho *et al.* (1991) reported that the direct and principal stimulus for irANP secretion in response to atrial pacing was the length shortening of atrial myocytes and that the response of irANP secretion to pacing is the result of an increase in frequency of length shortening of atrial myocytes.

Although results of early studies suggested that an atrial reflex was not involved in release of ANP during atrial distention, those studies had not ruled out neurally mediated release of ANP (Rankin, 1987). To investigate whether a sympathetic component could modulate irANP release, studies were carried out in anesthetized dogs and rabbits. Efferent sympathetic stimulation resulted in increased heart rate but no changes in irANP release ensued; there were also no accompanying changes in arterial or left atrial pressure (Ledsome *et al.*, 1986; Rankin *et al.*, 1987b). Similarly, efferent vagal stimulation in anesthetized rabbits did not cause any accompanying increases in ir-ANP release (Rankin *et al.*, 1987b).

Early studies also addressed the notion that humoral factors may be acting directly on the atrial myocytes or indirectly through hemodynamic changes to release ANP from the atria. Findings of numerous *in vivo* and *in vitro* studies were conflicting as to a role for humoral involvement in ANP release (Sonnenberg *et al.*, 1984; Sonnenberg and Veress, 1984; Lahance *et al.*, 1986; Manning *et al.*, 1985; Katsube *et al.*, 1985; Rankin *et*

al., 1987b). However, some agonists such as catecholamines, vasopressin and angiotensin II could have been causing release of ANP into circulation secondary to their effects on hemodynamic variables, rather than directly on the myocytes.

To date a variety of factors have been shown to influence ANP release including: hypoxia (Baertschi *et al.*, 1988 and Winter *et al.*, 1989), hypercapnia (Clozel *et al.*, 1989), glucocorticoids and mineralocorticoids (Garcia *et al.*, 1985), vasopressin and angiotensin II (Manning *et al.*, 1985), increased sodium concentration and osmolality (Arjamaa and Vuolteenaho., 1985), endothelin (Garcia *et al.*, 1990; Schiebinger and Gomez-Sanchez, 1990), decrease in temperature and increases in rate and extent of atrial stretch (Bilder *et al.*, 1986), cholinergic agents (Sonnenberg *et al.*, 1984), and adrenergic agents (Onwochie and Rapp, 1988; Sonnenberg *et al.*, 1984).

1.3. Cellular Regulation of ANP Release

The cellular processes involved in linking mechanical distention to ANP release have yet to be elucidated. Calcium ionophores, calcium agonists and increasing extracellular calcium concentrations have been shown to increase ANP release in several models (Ruskoaho *et al.*, 1985; Bloch *et al.*, 1988; LaPointe *et al.*, 1990; Iida and Page, 1988; Yamamoto *et al.*, 1988). ANP release stimulated by calcium channel agonists or tachycardia was inhibited by calcium channel antagonists (LaPointe *et al.*, 1990, Saito *et al.*, 1986b, Doubell *et al.*, 1989). As well, sodium-potassium ATPase inhibitors which raise intracellular free calcium stimulated ANP release from isolated rat atria (Schiebinger and Santora, 1989), cultured neonatal myocytes (Bloch *et al.*, 1988), and the *in vivo* heart (Yamamoto *et al.*, 1988). These studies all support an important contribution of calcium in ANP release. In contrast, some researchers have shown that extracellular calcium has

no effect on ANP release or that calcium is an inhibitory regulator. Ito *et al.* (1988) demonstrated that absence of calcium from the perfusion medium has no effect on total ANP release in the perfused rat heart. Moreover, deBold and deBold (1989) demonstrated that increasing the extracellular calcium concentration of the medium inhibits ANP release from isolated atria. The discrepancies between reports of the involvement of extracellular calcium have been regularly attributed to be differences in basal contractility or technique of preparation (Sonnenberg *et al.*, 1989).

Protein kinase C (PKC) has been purified from the heart (Kuo *et al.*, 1984). In 1991 Ruskoaho *et al.* reported that phorbol esters, which mimic the action of diacylglycerol by acting directly on protein kinase C, and the calcium ionophore A23187, which introduces free calcium into the cell, both increased basal ANP secretion in the isolated perfused rat heart. Phorbol ester also increased responsiveness to calcium channel agonists such as Bay k8644 and to agents that increase cAMP, such as forskolin. In cultured neonatal rat atrial myocytes, PKC activation by 12-O-tetradecanoylphorbol 13-acetate (a phorbol ester which is structurally similar to diacylglycerol) stimulated ANP secretion, whereas the release was unresponsive to changes in intracellular calcium. Endothelin, which stimulates phospholipase C-mediated hydrolysis of phosphoinositides and activates PKC, increased both basal and atrial stretch-induced ANP secretion from isolated perfused hearts (Mantymaa *et al.*, 1990) . Similarly, phorbol ester enhanced atrial stretch-stimulated ANP secretion while an increase in intracellular calcium appeared to be negatively coupled to stretch-induced ANP release. This indicates that PKC activity may play an important role in regulation of basal ANP secretion and that stretch of the atrial myocytes appears to be positively modulated by phorbol esters (Ruskoaho *et al.*, 1991).

Inoue *et al.* (1988) did not find any effect of forskolin and dibutyryl-cAMP on ANP release from isolated atria, and the cAMP based regulatory system seemed to be inhibitory in some myocyte culture models. Morgan (1991) suggested that cAMP may affect ANP secretion by modulating the calcium signaling system of the myocardium, including calcium channels, calcium pumps of the sarcoplasmic reticulum and the troponin-tropomyosin complex.

Little information exists on the role of cGMP in the regulation of ANP secretion. Experiments with endothelial-derived relaxing factors (EDRF) suggested that ANP release was inhibited by cGMP-dependent processes (Sanchez-Ferrer *et al.*, 1990). Administration of 8-bromo-cGMP and sodium nitroprusside, which increased cGMP formation, delayed the release induced by phorbol esters from the perfused rat heart (Ruskoaho *et al.*, 1986). Similarly, cGMP appeared to be an inhibitory regulator of ANP release in non-contracting cultured atrial myocytes (Iida and Page, 1988).

In 1990, Gardner and Schultz examined the effects of several prostaglandins on synthesis and release of ANP *in vivo* and *in vitro*. Prostaglandin ($PGF_{2\alpha}$) infusion in anesthetized rats resulted in a significant increase in irANP levels *in vivo* despite effecting only modest changes in hemodynamics. $PGF_{2\alpha}$ administration was also effective at promoting irANP secretion in primary cultures of neonatal rat atrial and ventricular myocytes. Prostaglandin treatment also increased ANP mRNA levels in these cells, suggesting that these agents exert a major effect on the synthesis as well as the secretion of the hormone. Treatment with a cyclooxygenase inhibitor resulted in significant suppression of ANP release *in vitro* and *in vivo*. A follow-up to this study revealed that the prostaglandins were the predominant group of arachidonate metabolites stimulating ANP release (Kovacic-Milivojevic *et al.*, 1991).

1.4. ANP Processing

It is now well accepted that the cardiac myocytes possess granules which contain the 128-amino acid precursor of ANP which serves as a substrate for carboxypeptidase H (Seidman *et al.*, 1984). This enzyme, found in secretory granules of most neuroendocrine cells, including those in atrial myocytes, cleaves the two C-terminal arginine residues from ANP-(1-128) (Lynch *et al.*, 1988). The resulting 126-amino acid peptide, called pro-ANP, serves as the precursor to the bioactive form of the hormone, ANP-(99-126) (Schwartz *et al.*, 1985; Thibault *et al.*, 1985) (See Figure 1).

Figure 1. Schematic diagram of rat pro-ANP and processing by the myocardial cells and heart tissue. (Adapted from Sei *et al.*, 1992).

The peptide derived from the N-terminal of pro-ANP upon cleavage, ANP-(1-98) has been identified in plasma (Thibault *et al.*, 1988), however its biological activities,

if any, have not yet been clearly elucidated (*Sei et al.*, 1992). Studies using both the isolated perfused heart and primary atrial myocyte model systems have verified that only pro-ANP is stored in the atrial myocytes, while product peptides accumulate in the release medium (Lang *et al.*, 1985; Shields and Glembotski, 1988; Ito *et al.*, 1988). Until very recently it was not known whether activation of ANP occurred in or on the myocyte concurrently with secretion, or whether cleavage takes place after release on either the myocyte surface or the surface of a nonmuscle cardiac cell. However in 1992, Sei *et al.* using primary cultures and reversed-phase and ion-exchange high performance liquid chromatography (HPLC), coupled with immunoprecipitation of biosynthetically labeled ANP, demonstrated that the myocyte was the cell type responsible for pro-ANP maturation and that this cleavage event takes place simultaneously with secretion of the pro-ANP. In 1991, Gilloteaux *et al.*, without addressing the exact point at which pro-ANP is transformed into the bioactive form, described an ultrastructural immunolocalization of the ANP pathways in fetal, neonatal and adult Syrian hamsters. In the three stages of cardiac development, they localized and confirmed irANP production, packaging in atrial granules and sarcoplasmic transport by cytoskeletal elements and described a situation in which irANP material reached the sarcolemma where it was released by exocytosis. Diffusing through the endomysium (a cellular matrix between the myocyte and the endothelium), irANP material in the form of pro-ANP was then bound and specifically taken up by the endocardial and epicardial endothelial cells as well as by endothelial cells of blood vessels. Pro-ANP was then activated into ANP and transported transcellularly by receptor-mediated endocytosis before being released into the circulatory system by exocytosis. The integrity of the endothelium was essential in maintaining control for activation and release of ANP into the circulation in response to an appropriate

stimulus. The fact that endothelin, a newly discovered hormone which is secreted by endothelial cells, has been demonstrated to be a very potent stimulus for ANP release (Garcia *et al.*, 1990; Schiebinger and Gomez-Sanchez, 1990), supports a role for the endothelium in regulating ANP release.

The atrial appendages are the major source of circulating ANP (Seul *et al.*, 1992). However there is evidence supporting the importance of alternative sources. For example, Hoffman *et al.* (1990), demonstrated that in atrial appendectomized conscious rats, administration of deoxycorticosterone acetate (DOCA), a mineralocorticoid known to stimulate ANP release in rats, (Ballerman *et al.*, 1986; Metzler *et al.*, 1987) and congestive heart failure could still increase ANP levels and cause modulation of renal sodium handling. The ventricles have proven to be one alternative source of ANP. Ventricular cardiocytes in culture secrete ANP into the incubation medium or into the perfusion fluid (Bloch *et al.*, 1986). In models of ventricular hypertrophy, ANP synthesis, storage and release is augmented (Kinnunen *et al.*, 1992) and the amount of release depends on the degree of ventricular hypertrophy (Ruskoaho *et al.*, 1989). Under certain conditions, then, sources of ANP other than the atrial appendages, such as the ventricles, appear to be important for natriuresis, diuresis and hypotension.

In recent years, other natriuretic peptides have been discovered. The first to be identified and sequenced was a 26-amino acid peptide from the porcine brain, termed 'BNP' (Sudoh *et al.*, 1988). Another member, termed 'CNP', which shows a striking sequence homology with the other peptides, was then isolated from porcine brain (Rosenzweig and Seidman, 1991). Urodilatin, identified in human urine, consists of the amino acid structure of ANP extended at the amino acid terminal by four residues (Rosenzweig and Seidman, 1991). However, while the whole family of natriuretic

peptides have similar natriuretic properties, more work is necessary to identify the specific physiological significance of each peptide (See figure 2).

Figure 2. A schematic representation of Atrial Natriuretic Peptide. Amino acids within the 17-member ring that are identical in all natriuretic peptides are shaded. (Adapted from Rosenzweig and Seidman, 1991).

1.5. ANP Receptors

As with other peptide hormones, ANP exerts its effects by binding to specific membrane-bound receptors in target tissues. To date, three types of ANP-specific receptors have been identified: two different guanylate cyclase-linked receptors that appear to mediate most of ANP's biological activities and a clearance receptor which may play a role in clearing ANP from the circulation (Rosenzweig and Seidman, 1991). BNP and CNP interact with ANP receptors on bovine aortic smooth muscle cells (Song *et al.*, 1988), cultured rat vascular smooth muscle cells (Hirata *et al.*, 1988), rat kidney (Oehlenschlager *et al.*, 1989), and bovine adrenal cortex (Hashigushi *et al.*, 1988). There

is little evidence to suggest that these peptides bind to distinct receptor subpopulations (Fethiere and De Lean, 1991).

1.6. Physiological effects of ANP

Although it is not clear whether all actions of ANP are cGMP-dependent, the increase in plasma, urinary and tissue cGMP often correlates with the effects of ANP (Cusson *et al.*, 1987). In isolated human glomeruli, Ardaillou *et al.* (1985) reported that synthetic ANP could activate guanylate cyclase, an enzyme responsible for the intracellular formation of cGMP, suggesting a role for cGMP as a second messenger of ANP. *In vivo*, ANP administration markedly stimulated glomerular production of cGMP, which coincided with a marked increase in glomerular filtration rate (GFR). Dibutyryl cGMP treatment caused increased GFR, therefore cGMP seemed to be acting as the second messenger of ANP actions in the kidney (Huang *et al.*, 1985).

ANP administration also inhibits aldosterone synthesis and release (Atarashi *et al.*, 1984; Vari *et al.*, 1986), but in contrast to renin administration, cGMP does not seem to be involved in this inhibition (Vari *et al.*, 1986). This effect may be mediated through unknown receptors or through known receptors utilizing different transduction pathways (Rosenzweig and Seidman, 1991). In 1990 McCarthy *et al.* demonstrated that ANP can differentially affect two different populations of calcium channels that may be involved in aldosterone secretion. Thus, ANP-induced inhibition of aldosterone secretion may be partially mediated via a reduction of calcium currents. De Zeeuw *et al.* (1992) have recently summarized the effects of ANP in humans.

Acute administration of exogenous ANP results in natriuresis and diuresis in rats and these effects are mediated by activation of guanylate cyclase and production of

cyclic guanosine monophosphate (cGMP) (Appel *et al.*, 1986). ANP administration causes an increased glomerular filtration rate in dogs (Burnett *et al.*, 1984) and baboons (Bourgoignie *et al.*, 1986). This rate that did not always correlate with diuresis and natriuresis in these animals, suggesting that a renal tubular effect may be involved (Mendez *et al.*, 1986). Results of microperfusion studies in anesthetized rabbits indicates that a distal segment of the kidney may be involved since there was no detection of a direct effect of ANP on proximal tubule transport (Baum and Toto, 1986). Using *in vivo* microcatheterization of the whole medullary collecting system in dogs, Sonnenberg *et al.* (1986) demonstrate an ANP-induced reduction of tubular salt reabsorption compared to results of control experiments, indicating that ANP was an inhibitor of normal sodium reabsorption from the medullary duct, and that such an inhibition was necessary for the natriuretic response.

ANP is a potent vasorelaxant in large arteries (Currie *et al.*, 1983) and this effect is mediated through production of cGMP (Winquist *et al.*, 1984). However, the acute hypotensive response to ANP administration appears to be due to a decreased cardiac output resulting from reduced preload and consequently stroke volume. This effect has been shown in sheep (Parkes *et al.*, 1988) and rats (Lappe *et al.*, 1985). The preload reduction found in rats may be mediated by reduced net capillary absorption rather than an effect on venous capacitance (Trippodo and Barbee, 1987) with ANP altering the extracellular fluid distribution to favor interstitium over plasma (Rosenzweig and Seidman, 1991). However, Woods *et al.* (1989) reported that, in conscious rabbits, ANP lowered mean arterial pressure mainly by decreased atrial filling pressure and cardiac output and by inhibiting neuronal cardiovascular reflex responses. ANP caused reversible loss of fluid from the vascular compartments and these effects were due to direct circulatory actions of

the peptide that did not include generalized vasodilation. These studies suggest that a decrease in intravascular volume due to efflux of fluid from the capillaries was not responsible for the fall in cardiac output following ANP infusions.

ANP also interacts with renin and aldosterone and thus has the potential to play a major role in hemodynamic homeostasis. ANP inhibits renin release from juxtaglomerular cells (Kurtz *et al.*, 1986; Scheuer *et al.*, 1987). This inhibition correlates with increases in cGMP and it is believed that the inhibition occurs by a cGMP-mediated pathway via a guanylate-cyclase associated receptor for ANP (Kurtz *et al.*, 1986; Rosenzweig and Seidman, 1991).

1.7. ANP and Cardiovascular Diseases

It is estimated that more than 90% of hypertensive patients are afflicted by a disease of an unknown etiology, referred to as 'essential hypertension'. Theories about the causes of hypertension include: a renal defect in sodium excretion, increased sympathetic nerve activity, inappropriately elevated aldosterone production, disordered calcium homeostasis, altered vascular compliance, enhanced sensitivity to vaspressor mechanisms and a relative lack of vasodilatory substances (Hollister and Inagami, 1991). All of these theories could be associated with ANP actions. ANP seems to be able to antagonize many mechanisms proposed as causes of EH. The smooth muscle relaxing properties of ANP counteract enhanced arteriolar vasopressor effects leading to dilatation and antagonism of an increase in central blood volume. Natriuretic and diuretic actions of ANP may reverse a defect in sodium excretion and an ANP-mediated inhibition of renin release and aldosterone synthesis. ANP release may, then, antagonize vasopressor and volume expansion mechanisms for elevation of blood pressure. Decreased activity in the central

nervous system by peripheral (Nakamura and Inagami, 1986) or central mechanisms (Floras, 1990) or by an interaction with the cardiopulmonary and arterial baroreceptor mechanisms (Takeshita *et al.*, 1987) may also represent means by which ANP could act as an antihypertensive agent.

ANP has antihypertensive actions, however it is uncertain whether abnormal ANP levels contribute to hypertension. Hollister and Inagami (1991) summarized the findings of 37 studies involving plasma levels of ANP in hypertensive and normal patients, concluding that after age-, sodium intake-, and posture-adjusted elevations in plasma ANP, elevated concentrations of ANP were associated with essential hypertension with end organ damage or volume dependent, secondary forms of hypertension. In a study involving the offspring of hypertensives, Ferrari *et al.* (1990), indicated that both acute and chronic sodium loading produces less of a rise in plasma ANP concentration in the normotensive offspring of hypertensive patients than in age-matched offspring of normotensive subjects.

It is also possible that hypertension is related to resistance to ANP actions or to enhanced ANP clearance. Essential hypertensive patients require higher renal perfusion pressures to excrete sodium, suggesting a reduced renal response to ANP (Hollister and Inagami, 1991). It has also been reported that sympathetic nerve activity may be increased in hypertension (Dibona, 1989) and renal sympathetic nerve activity can antagonize the natriuretic and diuretic responses to ANP (Morgan *et al.*, 1989), further supporting the resistance hypothesis. Alternatively, accelerated clearance of ANP could result in expanded plasma volume, a defect in the excretion of salt load, and less antagonism of vasoconstrictor mechanisms. However, it is important to note that a role for accelerated

ANP clearance in essential hypertension has not yet been established (Hollister and Inagami, 1991).

Bolus as well as short and long term infusions of ANP have been reported to lower blood pressure (Cusson *et al.*, 1987; Cusson *et al.*, 1990; Hollister and Inagami, 1991). However the half-life of ANP is very short (4-5 minutes) and studies utilizing alternative routes of administration or ANP analogs with longer half lives have not been very successful (Kreiter *et al.*, 1989).

Significantly higher levels of ANP have been demonstrated in congestive heart failure (CHF) and valvular diseases. These high levels of ANP were suggested to arise from chronic elevation of left and/or right atrial pressure (Bates *et al.*, 1986) and could be considered an appropriate response to maintain circulatory homeostasis (Dussaule and Ardaillou, 1990). Other stimuli may be involved, however, since in mitral stenosis, atrial fibrillation influenced ANP release independently of atrial pressure or tension (Dussaule *et al.*, 1988). As Perrella *et al.* (1992) reported, in acute CHF, the increase in plasma ANP is regulated by release of stored peptide, and in chronic CHF, elevation of ANP is maintained by an increase in atrial synthesis. This finding conflicts with that of Moe *et al.* (1991) who indicated that ventricular ANP production may serve to sustain the higher plasma ANP concentrations in CHF.

Plasma levels of ANP can be up to 50 times higher than normal in patients with chronic renal failure (Dussaule and Ardaillou, 1990). Elevations in plasma ANP levels in diabetes mellitus have also been implicated in the mechanism of glomerular hyperfiltration which occurs at the early stage of the disease (Dussaule and Ardaillou, 1990). In rats, streptozotocin-induced diabetes-dependent increases in ANP, associated with increased filtration, were inhibited by anti-ANP antibodies (Ortola *et al.*, 1987).

Although there are still many missing links, the evidence suggesting ANP is playing a role in cardiovascular diseases is abundant.

1.8. Future Directions of ANP Research

To gain more insight into the role of ANP in hypertension and cardiovascular diseases, research will likely focus on the use of synthetic ANP or analogs of ANP which degrade or are metabolized less quickly. Similarly, work with neutral endopeptidase has proved very informative. Neutral endopeptidase 24.11 (NEP) is the major ANP degrading enzyme. Inhibiting this enzyme, and thus potentiating the effects of endogenous and exogenous ANP, (Murohara and Johnston, 1992) could have important experimental and therapeutic implications. In rats with CHF, administratration of a new NEP inhibitor caused natriuresis and diuresis and sustained effects on hemodynamics, possibly through potentiation of biological activity of ANP (Murohara and Johnston, 1992). Devising a new therapeutic model for treating CHF is important because the short half-life of ANP, and its degradation by the gastrointestinal system, complicates infusion procedures. Other exciting areas in ANP research involve studies with transgenic mice which overproduce ANP, enzymes which interfere with cleavage of ANP, guanylate cyclase antagonists, and the development of competitive ligands for the ANP clearance receptor.

1.9. Cardiac Ganglia

The presence of autonomic ganglion cells in or on the mammalian heart was first established almost 90 years ago (Meiklejohn and Walker, 1914). These cells are found in the subepicardial tissue of the atria and auricular appendages, around the roots of the great vessels, along the interatrial and atrioventricular sulci, in the interatrial septum

and in the vicinity of the sino-atrial and atrio-ventricular nodes (Mitchell, 1956). Historically, cardiac neurons have been considered to be primarily efferent postganglionic cholinergic parasympathetic neurons (Moravec *et al.*, 1986; Blomquist *et al.*, 1987; Ardell and Randall, 1986). It is now well accepted that the mammalian heart is innervated by both divisions of the autonomic nervous system. Individual neurons receive input from efferent parasympathetic fibers originating in the medulla oblongata, acting as the major mediator of cardiovascular reflexes (Seabrook *et al.*, 1990). Intrinsic cardiac neurons also receive physiological input from efferent parasympathetic preganglionic neurons and from sympathetic postganglionic efferent neurons and afferent axons arising from cardiac and pulmonary mechanoreceptors (Armour and Hopkins, 1990; Gagliardi *et al.*, 1988). Ardell *et al.*, (1991), recorded the responses of 175 spontaneously active neurons from ventral right atrial and ventral intraventricular ganglionated plexuses in anesthetized dogs whose hearts were chronically decentralized i.e., whose afferent and efferent nerve fiber connections were severed from the central and peripheral nervous systems. Spontaneous activity was correlated with the cardiac cycle in 57% of atrial and 62% of ventricular neurons. Cardiac ganglia can generate spontaneous activity that is also modified by cardiac mechanoreceptors indicating that these neurons receive afferent inputs from cardiac mechanoreceptors and thus may be involved in intrinsic modulation. The cardiac ganglia, then, consist of afferent, efferent and local circuit neurons, all capable of interactive control of cardiac function, with the role of the intrinsic cardiac ganglia on ANP release to be elucidated.

1.10. Cardiac Neuropeptides

Nerve fibres immunoreactive to neuropeptide Y, vasoactive intestinal polypeptide, somatostatin, substance P (SP), and calcitonin gene-related peptide (CGRP) have been demonstrated in or on the heart of several species (Wharton *et al.*, 1981; Weihe and Reinecke, 1981; Mulderrry *et al.*, 1985 and Moravec *et al.*, 1990). CGRP- and SP-containing nerve fibers have been identified around the coronary vessels, close to myocardial cells, as well as under and within the pericardia and endocardia (Mulderry *et al.*, 1985; Franco-Cereceda *et al.*, 1987). These fibers are also associated with the sinoatrial node and cardiac valves (Ganten *et al.*, 1991). In the ventricles, CGRP- and SP-like immunoreactive nerves are also mainly associated with blood vessels and the endocardia and pericardia (Ganten *et al.*, 1991). In addition, varicose CGRP- and SP-immunoreactive nerve terminals can be observed around local parasympathetic ganglion cells associated with the atria of the heart, as well as close to some sympathetic ganglion cells in the stellate ganglia (Ganten *et al.*, 1991). Some intrinsic cell bodies have also been demonstrated to be immunoreactive for substance P (Baluk and Gabella, 1989), despite the fact that these sensory fibres have been considered to be exclusively of extrinsic origin from the dorsal root ganglia (Urban and Papka, 1985). Functional studies with peptides have demonstrated an important role for the peptidergic innervation of the heart. In guinea-pig atria treated with adrenergic and cholinergic blockers, transmural stimulation still caused a positive chronotropic response which was inhibited by tetrodotoxin (Saito *et al.*, 1986a). This effect was abolished by capsaicin and mimicked by CGRP application. Capsaicin selectively abolishes SP and CGRP innervation in rats if administered neonatally (Nagy *et al.*, 1981). Rankin and Scott (1990), demonstrated that ablation of SP and CGRP innervation in the rat heart could significantly decrease ANP release *in vitro* and *in*

vivo and many studies have demonstrated the cardiodynamic effects of injecting peptides into the cardiovascular system (Armour, 1989; Thom *et al.*, 1987; Lappe *et al.*, 1987). In the guinea-pig, bolus injections of SP resulted in bradycardia followed by a tachycardia in some preparations (Hoover and Hancock, 1988). In the rat, SP administration resulted in significant reductions in coronary blood flow (Kulakowski *et al.*, 1983). Exogenously applied CGRP exerted a positive chronotropic effect on the isolated rat atrium in a dose-dependent manner (Saito *et al.*, 1986a). Gennari *et al.* (1991) suggested that CGRP stimulated secretion of ANP leading to a vasodilatory and hypotensive effect in humans. Thus, neuropeptides are present in the heart and are capable of modulating cardiac activity.

1.11. Aims of the study

One of the major focuses of this project was to investigate the role of capsaicin-sensitive peptidergic fibres in ANP release. Capsaicin is a potent depletor of Substance P and CGRP peptidergic fibres when administered neonatally (Nagy *et al.*, 1981). Therefore capsaicin may be used to study the role of these peptidergic fibres on cardiodynamics and ANP release. Furthermore, the role of the intrinsic cardiac ganglia on ANP release had not been previously assessed. Therefore the effect of electrical stimulation of the intrinsic cardiac ganglia on ANP release in the *in vitro* isolated perfused heart (Langendorff model) was investigated. More specifically, the aims of this study were:

1) To clarify the previously postulated role of SP and CGRP in ANP release (Rankin and Scott, 1990).

2) To confirm the ability of neonatal capsaicin-treatment to abolish SP and CGRP innervation in rat hearts.

3) To investigate the role of the intrinsic cardiac ganglia on irANP release and cardiodynamics by electrically stimulating a previously identified ganglion-dense (termed 'ganglionic') region of the epicardium of the isolated perfused rat heart.

   3.1. To compare irANP release and cardiodynamics during ganglionic stimulation with irANP release during stimulation of an epicardial region of the isolated perfused rat heart that was not dense with ganglia (termed 'non-ganglionic').

   3.2. To investigate the role of SP and CGRP in irANP release during stimulation of 'ganglionic' and 'non-ganglionic' regions of the epicardium of the isolated perfused rat heart.

   3.3. To investigate whether irANP release during electrical stimulation of the intrinsic cardiac ganglia is linked to the cardiodynamic changes that ensue upon stimulation.

   3.4. To investigate whether irANP release during stimulation of the intrinsic cardiac ganglia is linked to the simultaneous stimulation of cardiac adrenergic or cholinergic nerve fibres.

4) To determine if SP and CGRP have a role in modulating irANP release during:

   - *in vivo* right atrial pacing in the anesthetized rat (a preliminary study)
   - *in vitro* right atrial pacing in the isolated perfused rat heart

6) As irANP release during electrical pacing has been reported to be enhanced during volume expansion (Bilder *et al.*, 1989), to determine if SP and CGRP

have a role in modulating irANP release during *in vitro* right atrial volume expansion accompanied by electrical stimulation of the right atria in the isolated perfused rat heart

6) As the storage of irANP in the cardiac myocytes seems to be unaffected by neonatal treatment with capsaicin (Rankin and Scott, 1990), to carry out RNA isolation and probing for ANP mRNA to determine whether or not capsaicin affects irANP synthesis.

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1 Animals

For experimental procedures described in sections 2.3, 2.4.1 and 2.5-2.6, pregnant Sprague-Dawley female rats were purchased from Canadian Hybrid Farms, Hall's Harbour, Nova Scotia, Canada. Male and female pups were treated as per section 2.3. Male Sprague-Dawley rats (killed at 300-400 grams), for experimental procedures described in section 2.4.2 and 2.4.3, were purchased from Charles River Inc., Montreal, Canada. The source of the rats changed because Canadian Hybrid Farms discontinued its supply.

Rats were maintained under standard light and dark temperature conditions (lights on at 0800h and off at 2000h) at 20-22 °C and 40-60% humidity. Housing was provided in rectangular shoe box cages made of polycarbonate plastic, with a detachable metal rod lid (2-3 adult rats per cage). Bedding for the cages consisted of Beta-chip wood chip bedding purchased from Charles River Inc. Diet consisted of free access to Purina Rat Chow #T-5012 (Ralston Purina Company) and tap water.

2.2 Capsaicin treatment

Pregnant females were observed several times every day. The day the rat pups were discovered was considered the first day of life. Neonatal rats were treated with capsaicin, (50mg/kg s.c.-Sigma 98%; Lot# 59F7080) at 2,3,4 and 7 days, and 2 and 4

weeks after birth and used at approximately 12 weeks of age (250-400g). Capsaicin was administered subcutaneously at the back of the neck with a small gauged needle (30G1/2; Becton, Dickenson). After administration of the drug, pinching the skin at the point of needle insertion kept drug leakage at a minimum. All pups were subjected to the same treatment schedule. For comparison, a control group was treated with the vehicle (10% ethanol and 10% Tween 80 in 0.9% saline) alone.

2.3 Cardiac ganglia studies

2.3.1 Experimental apparatus for *in vitro* isolated perfused rat heart

Initial studies showed no significant differences in irANP release between male and female rats , therefore unless otherwise stated, both sexes were used throughout the studies. Capsaicin- or vehicle-treated rats of approximately 12 weeks of age (ranging from 350-450g for male rats and 250-280g for female rats) were anesthetized with 60mg/kg sodium pentobarbital administered intraperitoneally. The ascending aorta was isolated and catheterized with polyethylene 160 tubing (inner diameter 1.14 mm, outer diameter 1.57 mm; Clay Adams). The heart was flushed with chilled and heparinized (50 IU/100g) Krebs buffer solution, pH=7.4 (See Appendix A for details). All vessels attached to the heart were excised and the heart was mounted on a modified Langendorff apparatus. The entire protocol was completed in less than 2 minutes. The mounted heart was perfused with gassed Krebs (95%0_2/5%CO_2) at a flow rate of 5 mls/min (Gilson Minipuls 2) and allowed to equilibrate for 40 minutes prior to carrying out the protocols; buffer was maintained at 37.5° C with a heated water jacket. A cotton suture (#4.0, Ethicon) was inserted through the apex of the heart and attached to a Grass FTO3 force tranducer to record ventricular force of contraction. Resting tension

of the tissue was set at 0.5g. Perfusion pressure was monitored by a Trantec 60-800 transducer. Force and pressure were amplified and recorded with a Beckman R611 Physiograph. The height of the contractions were measured (in millimeters from the baseline) to assess the inotropic state of the isolated heart. Figure 3 is a diagrammatic representation of the experimental apparatus of the isolated perfused rat heart preparation.

Figure 3. Diagrammatical representation of the experimental apparatus for the isolated perfused rat heart preparation.

2.3.2 Electrical stimulation of the cardiac ganglia.

Nine capsaicin, seven vehicle-treated and eight untreated animals were used in this investigation. A previous anatomical study in our lab (Power *et al.*, abstract in press) demonstrated the location of the epicardial ganglionic plexuses of the rat. Electrical stimulation of these 'ganglionic' regions consisted of square wave pulses (50Hz, 15-20μs, 4μA delivered in 5s trains at variable intervals) delivered through a bipolar Tungsten electrode driven by a Grass S48 stimulator. Regions of the rat epicardia in which no ganglionic plexuses were identified, designated 'non-ganglionic', were stimulated under identical electrical parameters as a control (See Figure 4 for location of 'ganglionic' and 'non-ganglionic' stimulation sites). The initial site of stimulation was alternated from experiment to experiment (i.e. in one experiment the ganglionic site was stimulated first and in the next experiment the non-ganglionic site was stimulated first). A forty minute equilibration period was allowed before the stimulation of the second site in every experiment. Perfusate was collected in chilled 5 ml polypropylene test tubes (Sarstedt) containing 100 μl 1M HCl over 20 second intervals at 4 and 2 minutes prior to electrical stimulation and at 0.3, 0.6, 1, 1.3 and 2.3 minutes after stimulation. Samples were stored at -20° C until they were assayed for irANP.

Figure 4. Schematic diagram of the dorsal view of an isolated rat heart showing ganglionic (g) and non-ganglionic regions (ng) of electrical stimulation.

2.4 Pharmacological studies

2.4.1 Capsaicin

Five capsaicin- and six vehicle-treated animals were used for this investigation. The *in vitro* isolated rat heart preparation was the same as in section 2.3.1. Electrical stimulation of ganglionic region of the epicardium consisted of square wave pulses (50Hz, 15-20μs, 4μA delivered in 5s trains at variable intervals) delivered through a bipolar Tungsten electrode driven by a Grass S48 stimulator. Perfusate was collected in chilled 5 ml polypropylene test tubes (Sarstedt) containing 100 μl 1M HCl over 20 second intervals at -4, -2, 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 14.5, 15, 15.5, 16, and 17 minutes where ganglionic stimulation occurred at 0 and 14 minutes, respectively; tissues were perfused with a 10^{-6}M capsaicin (Sigma Chemicals) solution between 4 and 9 minutes. Capsaicin solution was made with Krebs buffer. Samples were stored at -20° C until they were assayed for irANP.

2.4.2 Atropine

Adult male rats at approximately 350 g (Charles River Inc.) were used for this part of the study (n=6). The *in vitro* isolated rat heart preparation was the same as in 2.3.1. Electrical stimulation of ganglionic region of the epicardium consisted of square wave pulses (50Hz, 15-20μs, 4μA delivered in 5s trains at variable intervals) delivered through a bipolar Tungsten electrode driven by a Grass S48 stimulator. Perfusate was collected over 20 second intervals in chilled 5 ml polypropylene test tubes (Sarstedt) containing 100 μl 1M HCl at -4, -2, 0, 0.3, 0.6, 1 and 2 minutes where ganglionic stimulation occurred at 0 minutes. Tissues were then given a 20 minute equilibration

period and perfusate was sampled at 22, 24, 25, 25.3, 25.6, 26 and 27 minutes where ganglionic stimulation occurred at 25 minutes and perfusion of 10^{-8}M atropine (Sigma Chemicals) began at 17 minutes. Capsaicin solution was made with Krebs buffer. Samples were stored at -20°C until they were assayed for irANP.

2.4.3. Guanethidine

Adult male rats at approximately 350 g (Charles River Inc.) were used for this part of the study (n=6). The *in vitro* isolated rat heart preparation was the same as in 2.3.1. Electrical stimulation of ganglionic region of the epicardium consisted of square wave pulses (50Hz, 15-20μs, 4μA delivered in 5s trains at variable intervals) delivered through a bipolar Tungsten electrode driven by a Grass S48 stimulator. Perfusate was collected over 20 second intervals in chilled 5 ml polypropylene test tubes (Sarstedt) containing 100 μl 1M HCl at -4, -2, 0, 0.3, 0.6, 1 and 2 minutes where ganglionic stimulation occurred at 0 minutes. Tissues were then given a 20 minute equilibration period and samples of perfusate were taken at 22, 24, 25, 25.3, 25.6, 26 and 27 minutes where a second ganglionic stimulation occurred at 25 minutes and perfusion of 10^{-6} M guanethidine (Sigma Chemicals) solution began at 17 minutes. Guanethidine solution was made with Krebs buffer. Samples were stored at -20° C until they were assayed for irANP.

2.5 Cardiac pacing studies

2.5.1. *In vivo* cardiac pacing (a preliminary investigation)

Three capsaicin- and four vehicle-treated rats were used for this study. Rats were anesthetized with 60mg/kg sodium pentobarbital administered intraperitoneally. The

right carotid artery was catheterized with polyethylene 90 tubing (inner diameter 0.86 mm, outer diameter 1.27 mm; Clay Adams) and blood pressure was monitored by a Trantec 60-800 transducer. Through the left superior vena cava, one pole of a bipolar Tungsten electrode was positioned within the right atria. The other pole was attached to the wall of the chest cavity. Cardiac pacing (40% higher than basal heart rate) consisted of square wave pulses (3.5-8.68 Hz, 5ms, 0.85μA) delivered through the Tungsten electrode driven by a Grass S48 stimulator. Blood pressure and heart rate, derived from the pulse pressure signal, were amplified and recorded with a Beckman R611 Physiograph. Experimental procedures began 40 minutes after catheter placements. Blood samples (2 ml/sample) via the carotid artery were collected in chilled 5 ml EDTA test tubes (Vacutainer, Becton, Dickinson) at 4 and 2 minute prior to pacing and at 2 and 4 minutes after pacing. An equal volume of donor blood from adult Sprague Dawley rats (Canadian Hybrid Farms) was added to the circulation immediately after blood was sampled. Samples (including a sample of donor blood) were centrifuged for 10 minutes at 2500Xg and 4°C. Plasma was separated from blood and stored at -20°C until assayed for irANP.

2.5.2 *In vitro* atrial pacing

Six vehicle-treated rats were used in this part of the study. The experimental preparation was the same as the isolated perfused heart protocol described in section 2.3.1. Electrical pacing (60% higher than basal heart rate) of the atria consisted of square wave pulses (3.84-5.12 Hz, 5ms, 3.5μA) delivered through a Tungsten electrode placed on the right atrial wall. Perfusate was sampled in chilled 5ml polypropylene test tubes (Sarstedt) containing 100μl 1M HCl over 20 second intervals at -4, -2, 0, 0.3, 0.6, 1.0,

1.3 and 2.3 where onset of pacing occurred at 0 minutes. Samples were stored at -20°C until they were assayed for irANP.

2.6. *In vitro* atrial stretch and pacing

Six capsaicin and six vehicle-treated rats were used for this part of the study. The experimental preparation was the same as the isolated perfused heart protocol described in section 2.3.1. However, for these experiments, following cannulation of the ascending thoracic aorta, a balloon tipped Fogerty Arterial Embolectomy Catheter (#12-080-3F, Baxter) was inserted into the right atrium via the right external jugular vein. Otherwise, the Langendorff setup was unchanged. Electrical pacing (60% basal heart rate) of the atria consisted of square wave pulses (3.84-5.12 Hz, 5ms, 3.5μA) delivered through a Tungsten electrode placed on the right atrial wall. Perfusate was collected in chilled 5ml polypropylene test tubes (Sarstedt) containing 100μl 1M HCl over 20 second intervals at -4, -2, 0, .3, .6, 1, 1.3, 2.3 and 5 minutes where atrial stretch occurred at 0 minutes. Tissues were given a 20 minute equilibration period (while an expanded atrial state was maintained) and perfusate sampled again at 25, 27, 28, 28.3, 28.6, 29, 29.3, 30.3 and 33 minutes where onset of atrial pacing, in the presence of atrial stretch, occurred at 28 minutes. Samples were stored at -20°C until they were processed for irANP.

2.7. Radioimmunoassay for atrial natriuretic peptide.

IrANP was measured in perfusate based on a radioimmunoassay method previously described (Wilson *et al.*, 1986) using commercially available rabbit anti-α-atrial natriuretic polypeptide serum (RAS8798, Peninsula Laboratories, Belmont, CA); referred

to as ANP antibody in this text. This antibody is not cross reactive with other ANP fractions. A portion of the assays were prepared with the Morich monoclonal antibody (John *et al.*, 1986) donated from N. Wilson, University of British Columbia, Canada. Briefly, on day 1, experimental samples were thawed immediately prior to assay preparation. A standard stock of Atriopeptin III (0.5μg/ml, #8799, Peninsula Laboratories, Belmont, California) was diluted to 1, 2.5, 5, 10, 25, 50 and 100 pg/100μl and used as the standard curve to compare the experimental samples. Test tubes were arranged so that a damage (i.e., non-specific binding) and maximum binding sample was followed by the standard curve (all in triplicate). Damage samples contained 200 μl of 1X phosphate buffer (see Appendix B for details). Maximum binding samples contained 100 μl 1X phosphate buffer and 100 μl of ANP antibody. Standards contained 100 μl of diluted standard stock and 100 μl of ANP antibody. Each experimental sample being assayed was prepared as a non-specific-binding (in duplicate) and specific-binding (in triplicate) sample. Therefore, each experimental sample has 5 test tubes assigned to it: 2 for the non-specific binding and 3 for the specific binding. Non-specific-binding samples contained 100 μl of the thawed experimental sample and 100 μl of 1X phosphate buffer. Specific-binding samples contained 100 μl of the thawed experimental sample and 100μl of ANP antibody. One quality control sample was prepared in the same manner as the experimental samples, however 100 μl of rabbit serum was substituted for 100μl of the experimental sample. All test tubes were agitated on a test tube vortexer and stored at 4° C for 72 hours. On day three ^{125}I-ANP was diluted in 1X phosphate buffer to yield approximately 2500 counts per minute/100μl. 100μl of ^{125}I-ANP was added to every test tube in the assay. All tubes were agitated and stored at 4°C for 24 hours. On day four, 100μl of goat anti-rat IgG serum (GARGG-500, Peninsula Laboratories, Belmont, Ca.)

followed by 600 µl of polyethylene glycol 8000 (100 grams polyethylene glycol+100grams distilled water, Fisher Scientific, Nepean, Ontario) was added to each tube. Again, tubes were agitated on a vortexer. All tubes were then centrifuged at 5°C, 2500Xg for 30 minutes. Each individual test tube yielded a precipitate (bound ^{125}I-ANP) and a supernatant (free ^{125}I-ANP). A computer program linked to a gamma counter calculated ratios of cpm from nonspecific:specific binding to a value which can be compared to the cpm from the standard curve dilutions, therefore yielding the final concentration of irANP in each experimental sample. All samples for a particular experiment were included within the one assay and all assays were conducted as described above. For the *in vivo* experiments, plasma was not extracted prior to the assay. All measurements were corrected for nonspecific binding and the inter- and intra-assay coefficients of variation were 8.9% (n=36) and 3.9% (n=11), respectively. The 80 and 50% binding (where 100% is the maximum binding in the absence of standard) of ^{125}I-ANP were 2.5± 0.86 (n=36, mean±s.e.) and 6.4±1.1 (n=36, mean±s.e.) pg/tube, respectively.

2.8 Immunohistochemistry.

At the conclusion of the cardiac ganglia studies hearts were fixed in Zamboni's fixative (See Appendix C for details). The fatty areas overlying the dorsal surface of the atria, excluding the two auricular appendages were washed in phosphate buffered saline (PBS, See Appendix D for details) for 10 minutes at room temperature. Tissues were then incubated in 10% normal goat serum and 30% H_2O_2 for 45-60 minutes at room temperature followed by incubation in primary antibodies to SP (Immunonuclear, 1:2000) and CGRP (Amersham, 1:5000) for 48 hours at 4°C. Tissues were washed four times for 15 minutes in PBS at room temperature and then incubated in goat anti-rabbit linking

antibody (Sternberger) for 3 hours at room temperature. Tissues were washed again 4 times for 15 minutes in PBS at room temperature and incubated with peroxidase anti-peroxidase (Sternberger) for three hours at room temperature. Tissues were washed 4 times for 15 minutes in PBS and nerves were visualized with diaminobenzidine. Tissues were then dehydrated in successively higher concentrations of ethanol (25%-2 minutes, 50%-2 minutes, 75%-2 minutes, 100%-10 minutes). Finally tissues were washed with xylene, compressed between two slides, and prepared as whole mounts.

2.9 Determination of mRNA for ANP

2.9.1 RNA Extraction

Untreated (n=5) and capsaicin-treated (n=5) adult rats were killed with a blow to the head and then exanguinated. RNA was extracted by the lithium chloride/urea extraction method as previously described (Goldin, 1991). Briefly, the thoracic cavity was opened following a midline incision of the chest wall. Hearts were removed and flushed with saline. Segments of the atrial appendages, ventricle tissue and liver tissue were removed and placed in a denaturing solution (3M LiCl/6M Urea, 10 mM NaOAc (pH=7.5), 0.1% sodium dodecyl sulfate (SDS), 0.5% β mercaptoethanol); 1 ml/100 mg of tissue. Tissues of the same type were pooled (i.e., all atrial tissues were pooled together, etc.), homogenized with a glass-Teflon homogenizer, and transferred to a 10 ml polypropylene test tube. For each tissue type, the homogenate was sonicated (Sonic Cell Disrupter; Model 16-850, Vertis Co.) on medium power three times for 30 seconds (on ice) to shear any DNA present and stored overnight at 4°C. The following day the homogenate was centrifuged at 26,500Xg for 30 minutes at 4°C after which the supernatant was discarded. The pellet was resuspended in 200μl of a 0.3M NaOAc

(pH=7.5)/0.5%SDS solution by vortexing. The homogenate was then extracted with an equal volume of phenol/chloroform (50/50:Volume/Volume). The aqueous phase was retained and the interphase and organic layer were re-extracted with an equal volume of 0.3M NaOAc and 0.5% SDS. The two aqueous layers were pooled and re-extracted with an equal volume of RNAse-free distilled water (diethylpyrocarbonate-treated distilled water). The pooled aqueous phase was extracted with an equal volume of chloroform. The RNA in the final aqueous phase was then precipitated with 0.1 times the volume of 3M NaOAc (pH=5.2) and 2.5 times the volume of 95% ethanol at -20°C overnight. The following day, the RNA was collected by centrifugation at 25,500Xg for 15 minutes and was rinsed of excess salt with 70% ethanol. Pellets were dried carefully under vacuum and resuspended in a small volume of RNAse-free distilled water.

2.9.2 Analysis of ANP mRNA

Total RNA was determined by UV absorption at 260nm. To detect the expression of ANP mRNA, Northern blot analysis was performed (Sambrook *et al.*, 1989). For each tissue type, 10μg of RNA was denatured in formaldehyde gel loading buffer (50% glycerol/1mm EDTA (pH 8.0)/0.25% bromophenol blue/0.25% xylene cyanol FF) at 65°C for 5 minutes. The RNA samples were immediately loaded on a 1% agarose gel and fractionated by horizontal electrophoresis. The RNA was then transferred to nylon-supported nitrocellulose (Nytran; Schliecher and Schuell) overnight by capillary transfer. RNA was linked to the filter using the automatic UV-setting of a Statslinker (Stratagene), and then air-dried completely. The filter was prewashed at 65°C in a 0.1X standard saline citrate (SSC)/0.5%SDS solution [1X SSC=150 mM NaCl/15mM Na citrate (pH 7.0)] for 60 minutes. The filter was prehybridized overnight at 65°C in a

solution containing 6X SSC, 10X Denhardt's Solution, 50mM Tris HCl (pH=7.4), 500μg/ml sheared salmon sperm DNA, and 0.1% SDS. Hybridization was performed overnight in a solution maintained at 65°C containing 6X SSC, 10% dextran sulfate, 50mM Tris HCl (pH=7.4), 100μg/ml sheared salmon sperm DNA, and 1% SDS in the presence of a ^{32}P-labeled gene-specific oligonucleotide probe for ANP (Clontech cat#9010-1, lot# 86-4, Bio/Can Sci. Inc.). The ANP probe was radioactively labeled using a 3' end label kit (Dupont, cat#NEP-100). The following day the filter was subjected to four 3 minute washes in a 2X SSC/0.1% SDS solution at room temperature, and one 30 minute wash in the same solution at 65°C. The filter was autoradiographed for 24 hours at -70 °C.

2.10. Analysis of data.

Values of irANP levels did not fall into a normal distribution, therefore for the purposes of statistical calculations, values of irANP levels were transformed to their natural logarithms (to normalize data). Since heart rate data fell into a normal distribution transformation of these data was not necessary. All data is expressed as percentage change from basal levels or rate (basal level of irANP or basal heart rate =0%). Since standard error values would be uninformative when data is expressed as a percentage of change, standard error bars do not appear on the figures. Statistical comparison of changes in heart rate and perfusate irANP were carried out using Analysis of Variance and the Dunnet's post hoc test of significance.

CHAPTER 3

3.0. RESULTS

3.1 Electrical stimulation of the ganglia.

Figure 5 shows a typical polygraph trace of a heart whose ganglion-dense region was stimulated under the electrical parameters previously described. In this particular tissue the 5 second stimulus train resulted in cardiac arrest; heart rate returned to a pre-stimulus rate and then a transient period of tachycardia ensued. Heart rate typically returned to basal levels 2 minutes after simulation. Stimulation of a non-ganglionated region of the epicardia did not lead to a cardiodynamic response.

Figure 5. Polygraph trace of an isolated perfused rat heart before and after electrical stimulation of a ganglionic region of the epicardia.

Figure 6 shows changes in heart rate and release of irANP upon stimulation of ganglionic and non-ganglionic sites in 8 untreated animals. Mean basal heart rate was 112 ± 9.6 beats per minute; mean basal level of irANP release was 8± 1.3 pg/100μl. Electrical stimulation (50Hz, 15-20μs, 4μA delivered in 5s trains) of the epicardial ganglia resulted in a biphasic chronotropic response with an immediate 70% ($p \leq 0.001$) decrease in heart rate followed by a rebound 23% ($p \leq 0.05$) increase 0.6 minutes after stimulation. Release of irANP was increased by 209% ($p \leq 0.05$) 1 minute after stimulation when compared to the basal levels. Electrical stimulation of non-ganglionic sites did not result in any changes in heart rate or changes in irANP release. Changes in inotropism and perfusion pressure were not statistically significant.

Ganglionic stimulation of 8 vehicle-treated animals resulted in similar effects. Mean basal heart rate was 116± 11 beats per minute; mean basal irANP level was 12.2± 3.8 pg/100μl. Heart rate decreased by 71%($p \leq 0.001$) upon stimulation of the ganglionic site, followed by a 20% ($p \leq 0.05$) increase in heart rate at 0.6 minutes after stimulation. Release of irANP was significantly increased by 203% ($p \leq 0.05$) 1 minute after stimulation. In 7 animals treated neonatally with capsaicin the mean basal heart rate was 114 ± 6.8 beats per minute and basal irANP release was 8.1± 3.6 pg/100μl. Again, heart rate was decreased by 59%($p \leq 0.001$) upon stimulation of the ganglionic site followed by a 21± 5%($p \leq 0.05$) increase 0.6 minutes after stimulation, however in this group the increase in irANP release was abolished. Differences in basal levels of irANP were not statistically significant. Inotropic changes and changes in perfusion pressure were not significant. Figure 7 shows these changes in heart rate and release of irANP in capsaicin and vehicle-treated animals. Immunohistochemical analysis confirmed the presence of innervation by

Figure 6. Time course of changes in heart rate (A) and levels of irANP (B) in untreated isolated perfused rat hearts following electrical stimulation of 8 epicardial ganglionic and 8 epicardial non-ganglionic sites. Onset of stimulation occurred at 0 min. ** $p \leq 0.001$, * $p \leq 0.05$

Figure 7. Time course of changes in heart rate (A) and levels of irANP (B) in 7 vehicle-treated and 9 capsaicin-treated isolated perfused rat hearts following electrical stimulation of epicardial ganglionic sites. Onset of stimulation occurred at 0 min.
** $p \leq 0.001$, * $p \leq 0.05$

irSP and irCGRP fibers in the 4 vehicle-treated tissues examined, and absence of innervation by irSP and irCGRP fibres in the 4 capsaicin-treated tissues examined (Figure 8a,8b).

A

B

Figure 8a. (A) Light micrograph of CGRP immunoreactive nerve fibres in the ganglionic region of a vehicle-treated rat epicardia; magnification 480X. (B) No CGRP immunoreactive nerve fibers could be demonstrated in the light micrograph of a ganglionic region of a capsaicin-treated rat epicardia; magnification 480X

A

B

Figure 8b. (A) Light micrograph of SP immunoreactive nerve fibres in the ganglionic region of a vehicle-treated rat epicardia; magnification 480X. (B) No SP immunoreactive nerve fibres could be demonstrated in the light micrograph of a ganglionic region of a capsaicin-treated rat epicardia; magnification 480X

3.2. Pharmacological studies

3.2.1 Capsaicin

Mean basal heart rates were 123±7.7 and 118±8.6 beats per minute and mean basal release of irANP was 14.6± 2.1 and 11.1±3.2 pg/100μl for vehicle- and capsaicin-treated animals, respectively. There were no statistical differences in basal values between treatment groups. Electrical stimulation of the 'ganglionic' region of 6 vehicle-treated rat epicardia (50Hz, 15-20μs, 4μA delivered in 5s trains) resulted in a 70% ($p \leq 0.001$) decrease in heart rate followed by a 53%($p \leq 0.001$) increase 0.6 minutes after stimulation. In 5 capsaicin treated animals stimulation of the epicardial ganglia led to an 81% ($p \leq 0.001$) decrease in heart rate followed by a 41%($p \leq 0.001$) increase 1 minute after stimulation. Release of irANP was increased by 29%($p \leq 0.05$) at 0.3 minutes after stimulation in the vehicle-treated group, but capsaicin-treated animals showed no significant increases with ganglionic stimulation (See figure 9). In the absence of ganglionic stimulation, perfusion of the tissues with a 10^{-6}M capsaicin solution had no significant effect on heart rate in vehicle- or capsaicin-treated groups. IrANP levels of the capsaicin-treated group were not significantly changed, however the vehicle-treated group showed a 51%($p \leq 0.05$) increase 1 minute after perfusion. Reperfusion of the tissues with normal Krebs buffer had no significant effect on heart rate or irANP in either group. When the ganglionic region was subsequently stimulated under the same stimulus parameters, a significant change in heart rate was seen in the capsaicin-treated groups, however no change was observed in the vehicle-treated group. Capsaicin perfusion and ganglionic stimulation had no significant effect on irANP release in the capsaicin-treated group. The vehicle-treated group, however, showed a significant 55% ($p \leq 0.05$) increase

Figure 9. Time course of changes in heart rate (A) and levels of irANP (B) in 6 vehicle-treated and 5 capsaicin-treated isolated perfused rat hearts following electrical stimulation of epicardial ganglionic sites at 0 and 14 min, respectively. Between 4 and 9 min tissues were perfused with 10^{-6}M capsaicin. ** $p \leq 0.001$, * $p \leq 0.05$

in release of irANP. No significant differences in perfusion pressure or inotropism were observed.

3.2.2 Atropine

Mean basal heart rate of 6 untreated isolated rat hearts was 115±6.1 beats per minute and mean release of irANP was 10.8±3.6pg/100 µl. Electrical stimulation of the 'ganglionic' region (50Hz, 15-20µs, 4µA delivered in 5s trains) resulted in an immediate 82% ($p \leq 0.001$) decrease in heart rate followed by an 19%($p \leq 0.05$) increase 0.6 minutes after stimulation. Release of irANP increased by 53%($p \leq 0.01$) 1 minute after ganglionic stimulation. Following a period of perfusion with 10^{-8}M atropine, ganglionic stimulation resulted in a 20% increase in heart rate, however the initial decrease in heart rate was abolished. During subsequent atropine administration a 38%($p \leq 0.05$) increase in release of irANP occurred 0.6 minutes after stimulation. Figure 10 shows changes in heart rate and irANP release after cardiac ganglia stimulation and atropine perfusion. No significant differences in perfusion pressure or inotropism were observed.

3.2.3 Guanethidine

Mean basal heart rate of 6 untreated isolated rat hearts was 121±5.9 beats per minute and mean release of irANP was 15.1±4.8pg/100 µl. Electrical stimulation of the 'ganglionic' region (50Hz, 15-20µs, 4µA delivered in 5s trains) resulted in a 96%($p \leq 0.05$) decrease in heart rate followed by a 17 % increase 0.6 minutes after stimulation. Release of irANP increased by 36% 0.6 minutes after stimulation when compared to basal values. After perfusion of the tissues with 10^{-6}M guanethidine, heart rate again decreased by 95± 4% ($p \leq 0.001$) and there was no rebound tachycardia, however irANP release increased by 35%($p \leq 0.05$) 1 minute after stimulation. No significant differences in perfusion pressure

Figure 10. Time course of changes in heart rate (A) and levels of irANP (B) in 6 untreated isolated perfused rat hearts following electrical stimulation of epicardial ganglionic sites. Onset of stimulation occurred at 0 and 25 min, respectively. Perfusion with 10^{-8}M atropine began at 17 min. ***$p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

or inotropism were observed in this study. Figure 11 shows changes in heart rate and irANP release after cardiac ganglia stimulation and guanethidine perfusion.

3.3 Cardiac Pacing Studies

3.3.1 *In vivo* atrial pacing

This is a preliminary study. The sample number is very low thus statistical analysis could not be performed. Mean basal heart rates were 204±7.8 and 216± 9.9 beats per minute for 4 vehicle- and 3 capsaicin-treated animals, respectively. Mean basal irANP release was 4.2± 2 and 3.9±1.6 pg/100μl, for vehicle- and capsaicin-treated rats, respectively. *In vivo* electrical pacing of the right atria resulted in 42% increase in heart rate in vehicle-treated animals and a 40% increase in capsaicin-treated animals. IrANP increased by 29% and 23%, 2 and 4 minutes after onset of pacing, respectively. Capsaicin-treated animals showed no increases in irANP release after pacing. There were no significant changes in blood pressure upon pacing. Immunohistochemical analysis confirmed the presence of innervation by irSP and irCGRP fibres in the 3 vehicle-treated tissues investigated. The absence of innervation by irSP and irCGRP fibres in the 3 capsaicin-treated tissues investigated, was also demonstrated. Figure 12 shows the percentage change in irANP release 2 and 4 minutes after cardiac pacing.

Figure 11. Time course of changes in heart rate (A) and levels of irANP (B) in 6 untreated isolated perfused rat hearts following electrical stimulation of epicardial ganglionic sites. Onset of stimulation occurred at 0 and 25 min, respectively. Perfusion with 10^{-6}M guanethidine began at 17 min.***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$

Figure 12. Changes in irANP in 4 vehicle-treated and 3 capsaicin-treated rats after electrical pacing of the right atria

3.3.2 *In vitro* atrial pacing

Mean basal heart rate of 7 vehicle-treated isolated perfused rat hearts was 114±5.4 beats per minute and mean release of irANP was 9.5±3.2 pg/100μl. Electrical pacing of the right atria resulted in a 60% ($p \leq 0.001$) increase in heart rate. Pacing did not yield any significant changes in irANP release. This protocol was not repeated with capsaicin-treated hearts since the vehicle-treated group was unresponsive. Immunohistochemical analysis confirmed the presence of innervation by irSP and irCGRP fibers in the 3 vehicle-treated tissues investigated. No significant changes in perfusion pressure were observed. Inotropism decreased by 18%($p \leq 0.05$). Figure 13 shows the effects of *in vitro* atrial pacing on heart rate and irANP release.

3.4. *In vitro* atrial stretch and pacing

Mean basal heart rate was 1[illegible]8±9.1 for 6 vehicle-treated and 119±6.7 for 6 capsaicin-treated isolated perfused rat hearts. Mean basal release of irANP was 12.9 ±3 pg/100μl and 14.1±2.9 pg/100μl for vehicle- and capsaicin-treated animals, respectively. Differences in basal values were not statistically significant between treatment groups. Stretching of the right atrial cavity by 0.5 ml in the *in vitro* isolated perfused rat heart did not result in significant changes in heart rate in vehicle- or capsaicin-treated hearts. IrANP was not significantly changed after stretch of the atrial cavity in capsaicin-treated animals, however in vehicle-treated animals, irANP release was increased by 26%($p \leq 0.05$) 2.3 minutes after stretch. In a stretched state, electrical pacing of the right atria resulted in a 78%($p \leq 0.001$) increase in heart rate in vehicle-treated animals and a 81% ($p \leq 0.001$)

Figure 13. Time course of changes in heart rate (A) and levels of irANP (B) in 7 vehicle-treated isolated perfused hearts following electrical stimulation of the right atria. Onset of pacing occurred at 0 min. **$p \leq 0.001$

increase in capsaicin-treated animals. Onset of atrial pacing during atrial stretch did not significantly change irANP release in vehicle- or capsaicin-treated animals. No significant differences in perfusion pressure were observed, however the inotropic state of the tissues decreased by 16% ($p \leq 0.05$). Immunohistochemical analysis confirmed the presence of innervation by irSP and irCGRP fibres in the 3 vehicle-treated tissues observed and absence of innervation by irSP or irCGRP fibers in the 3 capsaicin-treated tissues observed. Figure 14 shows the percentage changes in heart rate and irANP release after atrial stretch, and pacing with atrial stretch, in vehicle- and capsaicin-treated animals.

3.5 ANP mRNA

Northern blot hybridization showed three separate bands for atrial ANP mRNA in the vehicle- and capsaicin-treated groups. Qualitative observation of total mRNA expression demonstrated equivalent levels of expression for the vehicle- and capsaicin-treated groups (Figure 15).

Figure 14. Time course of changes in heart rate (A) and levels of irANP (B) in 6 vehicle-treated and 6 capsaicin-treated isolated perfused rat hearts following a 0.5 ml stretch of the right atrial cavity followed by electrical pacing of the right atria. Onset of atrial stretch occurred at 0 min and onset of atrial pacing occurred at 28 min. ** $p \leq 0.001$, * $p \leq 0.05$

Figure 15. Northern blot analysis of pooled atria of 5 vehicle-treated (V) and 5 capsaicin-treated (C) animals qualitatively demonstrated equivalent levels of ANP mRNA in both groups.

CHAPTER 4

DISCUSSION

This research project has provided new insight into the role of the intrinsic cardiac ganglia and the involvement of capsaicin-sensitive peptidergic neurotransmission in the release of irANP. It was discovered that electrical stimulation of ganglionic sites on untreated rat epicardia led to a dramatic biphasic change in heart rate. To attribute this response to excitation of a particular component of the cardiac innervation is difficult as a detailed anatomical study at the exact point of stimulation has not been carried out yet. What is clear however, is that the heart rate response was not the result of direct action of the cardiac muscle as stimulation of a non-ganglionic site did not lead to a cardiodynamic response. Therefore, particular components of the ganglionic stimulation site were responsible for the biphasic change in heart rate. It is possible that one or more ganglia were the origin of post-ganglionic fibers to the effector tissues of the heart. Another possibility is that cholinergic or adrenergic pre-ganglionic fibers were being stimulated simultaneously, resulting in the biphasic response. The release of neuropeptides prevalent in cardiac innervation may also be responsible for the chronotropic responses upon stimulation of the ganglia. Hoover and Hancock (1988) reported that bolus injections of SP into the isolated perfused guinea-pig heart produced a biphasic effect on heart rate. They described that the initial and more prominent phase was a decrease in rate followed by a second period of tachycardia of lesser magnitude. It was suggested that this response could be a consequence of SP binding to receptors in the cardiac parasympathetic ganglia and the subsequent release of acetylcholine. Exogenously applied CGRP has also been

shown to exert a positive chronotropic effect on the isolated rat atrium in a dose-dependent manner (Saito *et al.*, 1986a).

In untreated animals, release of irANP increased significantly upon stimulation of the ganglionic site, however at this stage it was difficult to assess whether the increase in release was simply a result of the ensuing tachycardia. The increase in heart rate upon stimulation peaked at 0.6 minutes, whereas the increase in irANP release peaked at 1 minute. IrANP release could have been independent of heart rate or it may have been dependent on heart rate and the delayed release attributable to freeing of the prohormone has to be freed from the cardiac myocytes, and the conversion to the circulating form, before being detected in the perfusate.

When animals had been treated neonatally with a vehicle or capsaicin solution, electrical stimulation of the ganglionic regions exhibited a similar heart rate response. The consistency of the chronotropic effects suggested that the capsaicin-sensitive CGRP and SP innervation was not influencing heart rate changes. However, a study in which guinea-pig atria were treated with adrenergic and cholinergic blockers, demonstrated that transmural stimulation still caused a positive chronotropic response which was inhibited by tetrodotoxin and that this effect was abolished by capsaicin and mimicked by CGRP application (Saito *et al.*, 1986a). It is possible that the discrepancies lie in the fact that different sites and parameters were used in the electrical stimulations. Also, the Saito study used isolated guinea-pig atria as opposed to the isolated perfused rat hearts used in this study. In terms of irANP release upon electrical stimulation, the capsaicin-treated group did not demonstrate an increase in release while the vehicle-treated group showed a significant increase. The fact that cardiodynamic effects were the same in the two groups, but irANP release was dramatically different, suggested that either changes in irANP

release were independent of heart rate changes or that some local components of the ganglionic network, which were affected by neonatal capsaicin treatment, were capable of modulating irANP release. Since cardiac ganglia themselves respond to cardiac mechanoreceptors (Ardell *et al.*, 1991), it is possible that cardiac volume changes result in a sensory afferent-mediated activation of peptidergic ganglia which in turn modulate irANP release appropriately. This may explain why Rankin and Scott (1991) found stretch-mediated release of irANP from isolated rat heart was abolished by neonatal capsaicin treatment. The SP and CGRP cardiac innervation, when intact, may be modulating the release of irANP upon stimulation as it is these sensory fibers that are selectively and permanently ablated by neonatal capsaicin treatment (Nagy *et al.*, 1981). These data do not contradict the general hypothesis that atrial stretch is the primary stimulus of ANP release, but suggest that other modulating factors might be involved in the hormonal response.

From these cardiac ganglia studies it was concluded that components of the epicardial ganglia nerve network of the rat heart can mediate a biphasic change in heart rate and release irANP upon electrical stimulation. Furthermore, capsaicin-sensitive structures are necessary for release of irANP during ganglionic stimulation.

Rankin and Scott (1990) demonstrated that neonatal capsaicin administration not only destroyed SP and CGRP innervation in the rat heart but also showed that these capsaicin-sensitive nerves influence the release of irANP upon onset of a stimulus such as atrial stretch. There is also evidence confirming that administration of capsaicin to adult rats can result in a certain degree of SP depletion (Buck and Burks, 1986). Therefore, to gain further insight into the role of neonatal capsaicin-treatment on irANP release, vehicle-

and capsaicin-treated perfused rat hearts were administered an acute dose of 10^{-6}M capsaicin. One minute after onset of capsaicin perfusion in the vehicle-treated group, irANP release had increased by 51%. Five minutes after capsaicin perfusion, tissues could still significantly release irANP upon ganglionic stimulation, indicating that prohormone stores had not been depleted or that additional synthesis had occurred within the 5 minutes. No changes in heart rate occurred during capsaicin administration but ganglionic stimulation resulted in a biphasic change in heart rate. This suggests that acute capsaicin administration to the vehicle-treated animals resulted in a dramatic release of stored SP and CGRP which brought about increased levels of irANP release by some as yet undetermined cellular mechanism. Interestingly, the group of animals which had been treated neonatally with capsaicin showed no increase in irANP release upon acute capsaicin administration but showed the same biphasic change in heart rate upon ganglionic stimulation. In these tissues the irSP and irCGRP fibers had been ablated and I believe acute capsaicin treatment did not yield irANP release because there was no SP or CGRP present to trigger the release.

It has been shown that cholinergic (Sonnenberg *et al.*, 1984; Matsubara *et al.*, 1988) and adrenergic (Onwochei and Rapp, 1988; Sonnenberg *et al.*, 1984) agents can cause ANP release, although extrinsic innervation of the heart does not seem to be necessary (Rankin, 1987). To investigate whether there was cholinergic involvement in the irANP release and the cardiodynamic response upon ganglionic stimulation, experiments were carried out using atropine, which is a competitive antagonist of acetylcholine at muscarinic receptors. Isolated perfused rat hearts that received acute treatment with 10^{-8}M atropine did not display the characteristic decrease in heart rate upon ganglionic stimulation, suggesting that parasympathetic postganglionic fibers were

responsible for the bradycardia. However, irANP release was significantly increased upon ganglionic stimulation despite the absence of significant bradycardia, demonstrating that irANP release was independent of the decrease in heart rate brought about by the neurotransmitter acetylcholine.

A latent tachycardia still ensued after ganglionic stimulation of perfused rat hearts in the presence of atropine. Therefore, to evaluate the role of noradrenergic postganglionic fibers on cardiodynamics and irANP release, perfusion of untreated tissues with guanethidine, a drug which inhibits the release of noradrenaline from sympathetic nerve terminals, was carried out using the same protocol as the previous experiment. The cardiodynamic effects of ganglionic stimulation in the presence of 10^{-6}M guanethidine yielded the reciprocal effects of the atropine experiments; the typical bradycardia occurred but the tachycardia was abolished, suggesting that sympathetic postganglionic fibers were responsible for the tachycardia. IrANP release increased by 35% upon stimulation indicating that irANP release was also independent of tachycardia brought about by the neurotransmitter noradrenaline.

These pharmacological studies suggested that a cardiac network other than the classical sympathetic and parasympathetic systems was modulating irANP release and gave further credence to the involvement of SP and CGRP in irANP release. The data also confirm that the release can occur independently of an increase or decrease in heart rate upon ganglionic stimulation.

Cardiac pacing has been shown to be an effective stimulus of irANP release (Rankin *et al.*, 1986; King and Ledsome, 1991; Bilder *et al.*, 1989; Schiebinger and Linden, 1986; King and Ledsome, 1990). The results of a small pilot study of *in vivo*

pacing showed increased irANP release in vehicle-treated rats. Capsaicin-treated animals did not increase irANP release upon pacing. These findings, albeit preliminary in nature, are consistent with the hypothesis that SP and CGRP innervation have a role in the release of irANP.

Another protocol was devised to use the Langendorff model of *in vitro* atrial pacing with vehicle- and capsaicin-treated rats. However, despite consistent cardiac pacing,, no release of irANP was detected. The discrepancy from the results of others (Bilder *et al.*, 1989; Schiebinger and Linden, 1986) may be attributed to the fact that most of the cardiac pacing studies in the past used a design other than the Langendorff.

Release of irANP during pacing of the right atrium has been shown to be enhanced if the atrium is also in a volume-expanded or stretched state (Bilder *et al.*, 1989). Initial stretching of the right atrium caused significant release of irANP in the vehicle-treated rats, but no such change was seen in capsaicin-treated animals. This suggested that capsaicin-sensitive fibers might also be involved in irANP release during atrial stretch and supported the findings of Rankin and Scott (1990). However, pacing of the right atrium in addition to inflation of the balloon catheter, did not release irANP in either group. Therefore, at this time, no correlation can be made between the SP and CGRP peptidergic neurotransmission and irANP release during pacing of the right atria in the isolated perfused rat heart. Future experiments will investigate stretching and pacing of the isolated left rat atria using techniques which involve perfusion of the endocardium and epicardium with physiological saline (Deng and Kaufman, abstract in press) as it is possible that the Langendorff technique did not adequately supply the myocardium with the nutrients and oxygen it needed to elicit ANP release through tachycardia.

Rankin and Scott (1990) demonstrated that the storage of irANP prohormone appeared to be unaffected by neonatal capsaicin treatment, as the prevalence and conformation of storage granules within the myocardium looked the same as in vehicle-treated animals. That study also demonstrated, as did this one, that basal levels of irANP release were not significantly different in vehicle- and capsaicin-treated animals. To gain further insight into the possible effects of capsaicin on synthesis of ANP, qualitative assessment of levels of ANP mRNA were carried out by Northern blot analysis. Levels of ANP mRNA appeared to be the same in vehicle- and capsaicin-treated animals. Since storage granules of pro-ANP and basal release of the hormone appeared to be the same in vehicle- and capsaicin-treated tissues it is possible that the basal synthesis and storage was not affected by neonatal capsaicin treatment. Another possibility may be that since ANP mRNA isolation and immunohistochemical procedures were not carried out while the tissues were in a 'stimulated state', i.e., during stimulation of the cardiac ganglia or stretching of the right atria, the SP and CGRP innervation might not have been activated to initiate enhanced synthesis of mRNA or enhanced storage of pro-ANP. This would explain why only basal rates of ANP synthesis and storage were detected. If the peptidergic fibers were not being stimulated to release SP and CGRP, their presence within the tissues might be unimportant and the basal states of synthesis and storage may be the same whether the SP and CGRP innervation was intact or whether it had been abolished neonatally. Evidence of release of vesicular stores of pro-ANP have only been demonstrated after physiological stimuli (Gilloteaux *et al.*, 1991), therefore studies will need to be carried out on stimulated release of vesicular stores following neonatal capsaicin treatment to confirm that SP and CGRP can affect storage. Measurements of irANP release in vehicle and capsaicin-treated animals likely demonstrated differences

because perfusate was sampled while the CGRP and SP peptides were being stimulated (by direct stimulation of the ganglia or indirectly via atrial stretch) to effect ANP release. In addition to the immunohistochemical studies, a future experiment will be to measure mRNA for ANP when tissues are in a stimulated state . For example, beginning an RNA extraction procedure 1 minute (the point at which irANP levels are at their peak) after a ganglionic site has been stimulated to demonstrate if the presence of SP and CGRP immunoreactivity effects synthesis of ANP mRNA during a known stimulus. If neither synthesis of ANP mRNA nor storage of irANP seem to be affected by neonatal capsaicin treatment, it can be postulated that the capsaicin-sensitive neurotransmission throughout the heart is affecting the mechanism or rate of biosynthesis from prohormone to the circulating hormone.

In summary, electrical stimulation of the ganglionic-dense regions of the rat atrial epicardium resulted in a biphasic change in heart rate attributable to interactions between the sympathetic and parasympathetic innervation of the heart. The increases in irANP release upon electrical stimulation seemed to be independent of the effects of noradrenergic or cholinergic effects on heart rate, however release of irANP should be examined in the face of constant heart rate to confirm this fact. Studies utilizing rats neonatally-treated with capsaicin, demonstrated that the presence of a capsaicin-sensitive peptidergic innervation, namely SP and CGRP innervation, is necessary to bring about a significant release in irANP upon stimulation of the cardiac ganglia. In addition to the model of ganglionic stimulation, SP and CGRP innervation was confirmed as a necessary factor in releasing irANP during *in vitro* atrial stretch. To further support the role of peptidergic innervation in irANP release, acute capsaicin administration was shown to

significantly release irANP in vehicle- but not capsaicin-treated animals, demonstrating that capsaicin-induced release of SP and CGRP, when present, will initiate release of irANP. Although this study did not demonstrate differences in ANP mRNA levels in vehicle- and capsaicin-treated animals, it would be premature to conclude that synthesis of ANP is equivalent in vehicle- and capsaicin-treated groups. Noteworthy is that these results do not contradict the widely held view that atrial stretch is the primary stimulus for release of ANP. However, the data do indicate that peptidergic nerves and cardiac ganglia may have an intermediary role in eliciting release as a result of atrial stretch.

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APPENDIX A

Preparation of Krebs buffer

To yield 1 liter:

To 500 mls of distilled water add seqentially:

50 mls NaCl, 118mM

10 mls KCl, 4.7mM

10 mls $CaCl_2$, 2.5mM

10 mls KH_2PO_4,, 1.2mM

10 mls $MgSO_4$, 1.2mM

100 mls $NaHCO_3$, 12.5mM

5 mls Dextrose, 11.1mM

Bring up to 1liter with distilled water and mix thoroughly

Maintain at pH=7.4 with 95% O_2: 5% CO_2

APPENDIX B

Preparation of 1X Phosphate Buffer

Buffer is initially made up at twice the concentration, i.e., '2X Phosphate Buffer'

Titrate the following to a final pH=7.4, using approximately a 1:1 volume of:

5.24 g/L NaH_2PO_4 H_2O (monobasic)

23.0 g/L Na_2HPO_4 (dibasic)

To the above add (for 1 liter):

5.84g NaCl

0.2g Bovine Serum Albumin

0.2g NaN_3

2.0 mls Triton X-100

Store stock of '2X Buffer' at 4°C. Dilute 100 mls of '2X' with 97 mls distilled water and 3 mls of aprotinin (Sigma Chemicals, St. Louis, Missouri) to yield '1X buffer'. Store at 4°C.

APPENDIX C

Preparation of Zamboni's Fixative

This procedure was carried out in a fume hood

To yield 1 liter:

Dissolve 20g of paraformaldehyde in 200 mls of distilled water maintained at 60°C

Add a few drops of 1M NaOH if paraformaldehyde will not dissolve

Add 500 mls of 0.2 M Phosphate Buffer, pH=7.4 (See Appendix E for recipe)

Add 150 mls of 2M picric acid

Bring solution up to 1 liter with distilled water (room temperature)

Mix thoroughly

APPENDIX D

Preparation of Phosphate Buffered Saline

Add 13g of sodium phosphate dibasic, heptahydrate and 8g of sodium chloride to 900 mls of distilled water

Stir until dissolved

Obtain a pH of 7.4 by slowly adding 2M HCl to the mixture

Bring to final volume of 1 liter with distilled water

Store at 4°C

APPENDIX E

Preparation of 0.2 M Phosphate Buffer

Add 8.06g of potassium phosphate and 37.75g sodium phosphate, heptahydrate to 900 ml distilled water

Stir until dissolved

Adjust with 0.2M HCl or 2M NaOH until a pH of 7.4 is reached

Bring to a final volume of 1 liter with distilled water

Store at 4°C