THE UNIVERSITY OF CALGARY

THE CARDIOVASCULAR PHYSIOLOGY OF ATRIAL NATRIURETIC FACTOR

EXPERIMENTAL STUDIES ON ATRIAL NATRIURETIC FACTOR (ATRIOPEPTIN) WITH PARTICULAR EMPHASIS ON MECHANISMS OF RELEASE FROM ATRIAL TISSUE, AND ITS EFFECT ON CARDIAC MUSCLE FUNCTION

by

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ABSTRACT

Atrial natriuretic factor is a polypeptide hormone primarily synthesized and secreted by atrial cardiocytes. Although a definitive physiological role for atrial natriuretic factor remains to be elucidated, it has been shown to affect function in the brain, liver, lungs, adrenals, kidneys, vascular tree, and the heart. The present series of studies was undertaken to more clearly define the physiology of atrial natriuretic factor with respect to mechanisms of release from, and its functional effect on, cardiac muscle.

Right atrial pressure has been identified as a major stimulus for atrial natriuretic factor release. However, transmural atrial pressure, calculated as the difference between intracavitary pressure and pericardial pressure, more accurately describes the true distending pressure within the atrium and as such, may better predict plasma levels of atrial natriuretic factor. Pericardial pressure was found to significantly attenuate the release of atrial natriuretic factor from canine hearts. During acute volume expansion with the pericardium intact, plasma atrial natriuretic factor failed to increase despite significant increases in right atrial pressure. However, following removal of the pericardium (when, by definition, pericardial pressure is zero and intracavitary pressure equals transmural pressure), plasma atrial natriuretic factor levels increased significantly from 76 ± 17 to 136 ± 41 $pM.L^{-1}$. Therefore, transmural atrial pressure represents the true distending pressure acting on

cardiac tissue and as such is a primary stimulus for stretch-mediated release of atrial natriuretic factor (Chapter III).

Superfusion of isolated rat cardiac muscle with concentrations of atrial natriuretic factor up to 10^5 times greater than those normally measured in the systemic circulation, resulted in a significant reduction (33 ± 6%) in force development. This effect was associated with a reduction in the time to peak tension but had no effect on the time to fifty percent relaxation (Chapter IV). Atrial natriuretic factor also reduced the positive inotropic effect of isoproterenol on rat trabeculae but failed to completely abolish this effect. When post-extrasystolic potentiation was utilized to indirectly measure calcium recirculation within the sarcoplasmic reticulum, superfusion of the rat trabeculae with atrial natriuretic factor did not significantly alter calcium recirculation within the muscle.

These studies confirm atrial transmural pressure to be a major determinant of stretch-mediated release of atrial natriuretic factor. The negative inotropic effect of atrial natriuretic factor was demonstrated in these studies, but at such high concentrations that the physiologic relevance of this phenomena remains to be established. Further study is required to identify a plausible site of action for atrial natriuretic factor in cardiac muscle and the molecular basis for stretch-induced atrial natriuretic factor release from cardiac tissue.

ACKNOWLEDGMENTS

It is difficult for me, in retrospect, to remember the myriad of reasons I had for undertaking a Doctor of Philosophy in the basic sciences. I felt quite strongly it would make me a better physician. I also hoped it would impact on my ability to undertake relevant, logical, and meaningful clinical research. Time will determine my success regarding the attainment of the latter objective. The assessment of my abilities as a physician I will leave to my patients and peers.

The completion of this thesis was made possible through the support and assistance of an outstanding collection of individuals. My supervisor, Dr. John V. Tyberg, consistently showed himself to be a gentleman of scientific genius, subtle wit, and seemingly endless patience. The privilege of learning from and studying with John is an opportunity every individual interested in cardiovascular physiology should be afforded. Any thanks I can express here, in these few words, cannot do justice to the the deep sense of gratitude and appreciation I feel for all of his efforts on my behalf.

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DEDICATION

My own father died prematurely from coronary artery disease. My step father has recently undergone open-heart surgery for repair of a rheumatic mitral valve. My mother has steadfastly endured the stress and worry attendant with both their diseases. It is to these three people, and the millions of other persons who are afflicted by heart disease, that I dedicate this thesis. If any portion of it helps to reduce the suffering of these patients and their families, the investment of time will be paid back with interest.

... We do not do what we want and yet we are responsible for what we are, that is the fact.

John Paul Sartre (1939)

The truth is, the science of Nature has already too long made only a work of the brain and the fancy: It is now high time that it should return to the plainness and soundness of observations on material and obvious things.

Robert Hooke (1665)

CHAPTER I

THE CARDIOVASCULAR PHYSIOLOGY OF ATRIAL NATRIURETIC FACTOR

INTRODUCTION

The publication of a report detailing the natriuretic properties of a tissue extract, purified from rat atrium, by Adolfo de Bold and associates in 1981 represented the culmination of close to thirteen years of intensive, dedicated research [de Bold et al., 1981]. As a direct consequence of this historic discovery, a virtual explosion of scientific interest and investigation has produced a plethora of information with regard to the molecular, genetic, biochemical, and physiologic properties of this hormone. Since 1981, more than two thousand articles on atrial natriuretic factor (ANF), atrial natriuretic peptide, atriopeptin, auriculin, atrin, cardionatrin, or cardiodilatin, have appeared in peer-reviewed journals.

The scope of this review will be limited mainly to the cardiovascular aspects of ANF physiology. It will touch briefly on the historical development of ANF, its biochemistry and molecular biology. Only a short survey of the many proposed sites of ANF activity will be presented. The main thrust of this review will be an examination of those areas of primary concern to the studies presented within this dissertation. Thus, mechanisms of ANF secretion and the cardiovascular effects of ANF will receive the most extensive evaluation. More detailed documentation of the various properties of ANF

not covered in this review can be found in a number of previously published articles [Anderson and Bloom, 1986; Atlas and Laragh, 1987; Ballerman and Brenner, 1986; Cantin and Genest, 1987, 1986, 1985; Genest, 1986; Goetz, 1988b; Inagami, 1989; Kramer and Lichardus, 1986; Maack et al., 1985; Sonnenberg, 1987a,b; Trippodo et al., 1987a,b].

HISTORICAL BACKGROUND

The history of atrial natriuretic factor centers around the parallel development of a theory regarding the presence of a natriuretic hormone and the investigation of atrial granules. Although it was a number of years before a link between atrial granules and natriuresis would be proposed, investigators were working in both areas simultaneously.

The Natriuretic Hormone

In a series of experiments in which normal (0.9 %) saline was infused into dogs, de Wardener and co-workers found an increase in sodium and water excretion [de Wardener et al., 1961]. This increase was independent of any changes in inulin, creatinine or para-aminohippurate (PAH) clearance and the diuresis was found to take place even if the kidneys were denervated or when there was a fall in renal blood flow. Although they were unable to determine if the observed natriuresis and diuresis was the result of an increase or a

decrease in the circulating concentration of a humoral agent, the authors hypothesized that the excretion of sodium which accompanied the saline infusion was due to a change in the concentration of a humoral agent, other than aldosterone. This putative hormone eventually became known as the 'Third Factor' [Smith, 1957] or 'Natriuretic Hormone' [de Wardener, 1977].

A recent, exhaustive article on natriuretic hormone [de Wardener and Clarkson, 1985], reviewed the relevant scientific literature. The majority of evidence from both humans and animal models supports the observation that acute intravascular volume expansion confers on plasma three properties. These are: 1) the ability of plasma to produce a natriuresis when injected into other animals [Knock, 1980]; 2) the ability to inhibit the sodium-potassium ATP-ase transport mechanism in various tissues [Nutbourne et al., 1970]; and, 3) the ability to increase vascular reactivity [Jandhyala and Hom, 1983]. At present, there is no reason to assume that only a single hormone is responsible for all three activities.

The discovery of ANF immediately implicated it as the elusive natriuretic hormone [Cowley et al., 1988]. However, plasma from volume expanded animals, in addition to producing natriuresis, is capable of inhibiting sodium transport and increasing vascular reactivity [de Wardener and Clarkson, 1985]. Atrial natriuretic factor, on the other hand, does not inhibit sodium-potassium ATP-ase derived from rat kidney [Pollock et al., 1983] and may actually decrease vascular smooth muscle activity via sodium-potassium ATP-ase [Sybertz and Desiderio, 1985]. Thus, although ANF has natriuretic properties,

it seems unlikely, at present, that it is the elusive hormone proposed by de Wardener almost thirty years ago. It represents a unique hormone whose existence had not been hypothesized prior to its discovery.

Atrial Granules

Early in the relative infancy of electron microscopy, morphological observations of mammalian atrial cells documented the presence of granules, similar to secretory granules found in other tissues, within atrial cardiocytes [Kisch, 1956; Jamieson and Palade, 1964]. The granules were most frequently located at paranuclear sarcoplasmic sites within the atrial cells [de Bold, 1978]. For a number of years, it was postulated that these granules contained cardiac catecholamines [de Bold, 1987a]. Indeed, studies on rat atrial cells demonstrated that exogenous administration of noradrenaline increased the number of granules present in the cells [Seiden, 1979]. However, incubation of the cells with reserpine, which should theoretically deplete the granule population, if they contained catecholamines (either through blockage of synthesis or impaired granule formation [Goodman and Gillman, 1970]), failed to produce any detectable change in the number of granules observed [de Bold, 1987a]. It was known that atrial granules were present in a number of mammalian species [Cantin et al., 1979] and that they ranged in size from 350 to 500 nm [de Bold, 1987a]. In addition, the number of granules per cell in small rodents, was inversely proportional to the size of the animal studied [de Bold, 1987a] and

increased with the age and body weight of the animal [Chang and Benscome, 1969]. Subsequent studies [Huet and Cantin, 1974a,b] demonstrated that the atrial granules contained mostly protein but also a small amount of complex carbohydrate. It was hypothesized that the carbohydrate might function in the secretion of the proteinacious material resident within the granules [Huet and Cantin, 1974b].

In 1973, de Bold and Benscome demonstrated that the granules did not contain significant amounts of catecholamines, specifically no noradrenaline [de Bold and Benscome, 1973]. They subsequently were able to show that the atrial granules rapidly accumulated new protein and the kinetics of the Golgi apparatus, in these cells, bore a striking similarity to that seen in known endocrine and exocrine cells [de Bold, 1987a]. The 'discovery' of ANF was first alluded to in 1979 [de Bold, 1979], when de Bold found that water deprivation and sodium restriction significantly increased the number of atrial granules. Conversely, deoxycorticosterone and sodium chloride administration decreased the number of granules. On the basis of these and previous observations, de Bold hypothesized a relationship may exist between atrial-specific granules and fluid and electrolyte balance. With the assistance and cooperation of Dr. Harald Sonnenberg, de Bold subsequently injected a crude atrial muscle extract into rats and found it produced a massive diuresis and a prominent hypotensive effect [de Bold et al., 1981].

BIOCHEMISTRY AND MOLECULAR BIOLOGY

Structure

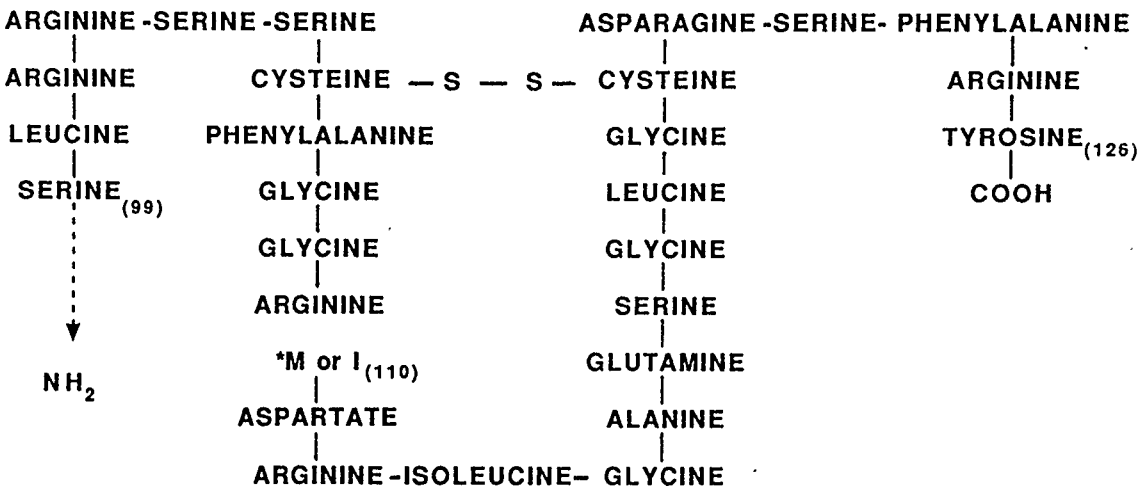
A little more than two years after its initial extraction from rat atrial tissue, the amino acid sequencing of a natriuretic peptide derived from rat atrium was successfully completed [Flynn et al., 1983]. The polypeptide isolated was labelled rat $ANF_{(99-126)}$ or Cardionatrin I (see Figure 1.1). However, this particular polypeptide structure did not turn out to be unique, and several, similar compounds were purified and sequenced [Thibault et al., 1987]. Each of these peptides possesses variable natriuretic and vasorelaxant properties (see Table 1.1), but in each molecule the disulfide bond is critical for receptor binding [Hirata et al., 1985]. Reducing the number of residues at the carboxy-terminal end or replacing methionine with n-leucine significantly reduces the peptide's spasmolytic activity without changing its natriuretic activity [Watanabe et al., 1988]. Additional studies have pinpointed the phenylalanine-arginine sequence at the carboxy-terminus as being the component most necessary for vasorelaxant activity [Thibault et al., 1987; Needleman, 1986].

A total of seven major atrial natriuretic peptides have been purified and sequenced [Cantin and Genest, 1985] (see Figure 1.2). The human and dog hormones are identical [Trippodo et al., 1987a]. Rat ANF differs by only one amino acid at position 12, where methionine is replaced by isoleucine. [Flynn, 1987].

FIGURE 1.1

AMINO ACID SEQUENCE OF ATRIAL NATRIURETIC FACTOR

Amino acid sequence of rat and human atrial natriuretic factor [Flynn et al., 1983; Genest, 1986.]



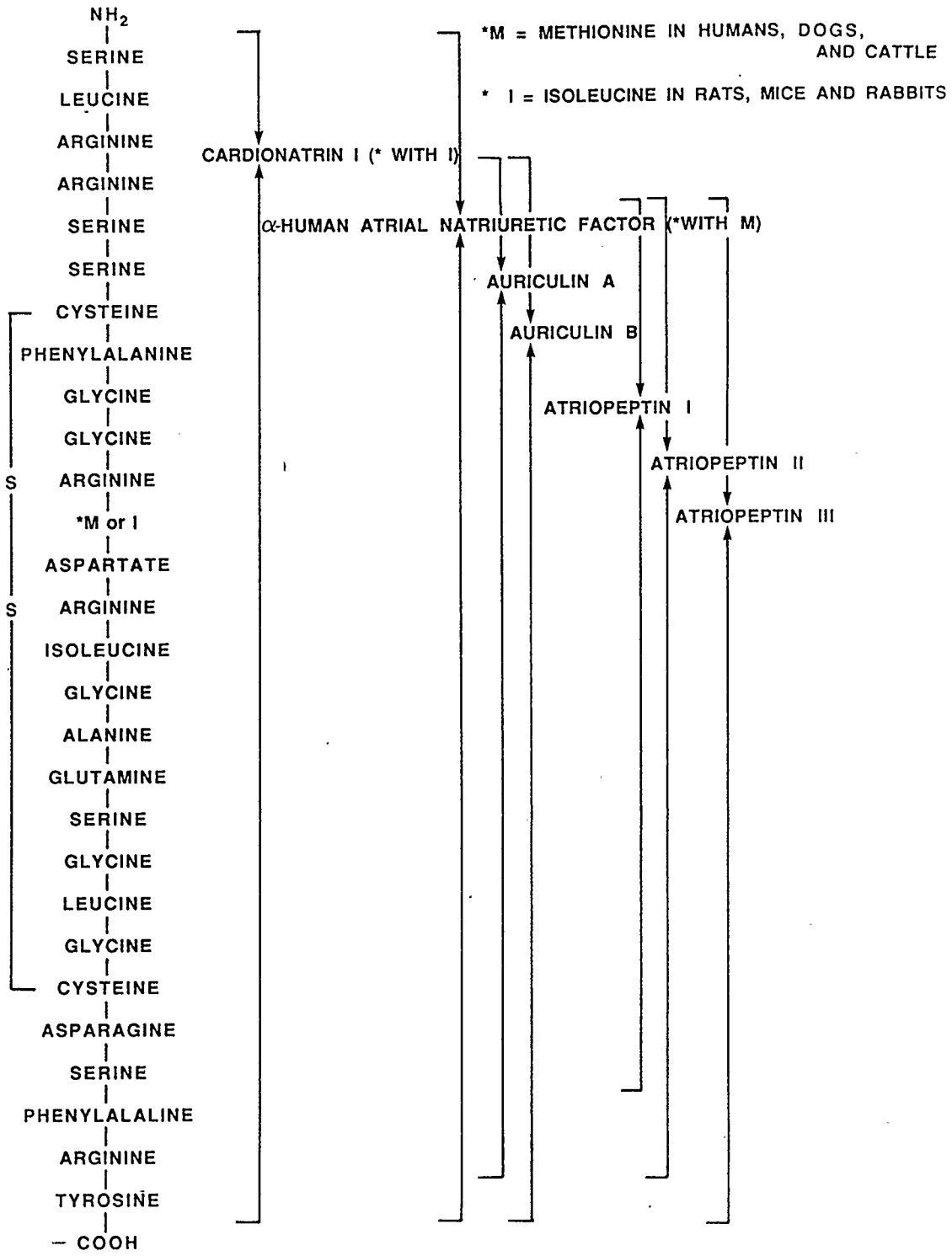
* M = METHIONINE - IN HUMANS, DOGS AND CATTLE

I = ISOLEUCINE - IN RATS, MICE AND RABBITS

FIGURE 1.2

AMINO ACID SEQUENCE FOR THE MAJOR ANALOGUES OF ATRIAL NATRIURETIC FACTOR

The polypeptide molecules identified are Cardionatrin I [Flynn et al., 1983], α-Human Atrial Natriuretic Factor [Ballerman and Brenner, 1986], Auriculin A and B [Atlas et al., 1984], Atriopeptin I and II [Currie et al., 1984], Atriopeptin III [Schwartz et al., 1985]


NH2
SERINE
LEUCINE
ARGININE
ARGININE
SERINE
SERINE
CYSTEINE
PHENYLALANINE
GLYCINE
GLYCINE
S ARGININE
*M or I
ASPARTATE
S ARGININE
ISOLEUCINE
GLYCINE
ALANINE
GLUTAMINE
SERINE
GLYCINE
LEUCINE
GLYCINE
CYSTEINE
ASPARAGINE
SERINE
PHENYLALALINE
ARGININE
TYROSINE
– COOH
*M = METHIONINE IN HUMANS, DOGS, AND CATTLE
* I = ISOLEUCINE IN RATS, MICE AND RABBITS
CARDIONATRIN I (* WITH I)
α-HUMAN ATRIAL NATRIURETIC FACTOR (*WITH M)
AURICULIN A
AURICULIN B
ATRIOPEPTIN I
ATRIOPEPTIN II
ATRIOPEPTIN III

Genetics

The biosynthetic pathway for atrial natriuretic factor in humans is depicted in Figure 1.3. A similar pathway has also been documented for rats [Flynn, 1987]. Using DNA sequencing of cDNA clones, investigations quickly revealed the presence of both a pro- and a pre-pro-ANF molecule [Ballerman and Brenner, 1986]. In addition to gene sequencing, the human ANF gene has been localized to the 1P36 band of the distal short arm of chromosome 1 [Yang-Feng et al., 1985] and the ANF gene has been used as a tool in cloning studies to transmit tumour oncogenes [Field, 1988]. Furthermore, ANF has been implicated as a possible constituent in the development of isolated atrial amyloidosis [Linke et al., 1988].

Recently, an IgG_1 monoclonal antibody to α-hANF has been synthesized [Mukoyama, 1988] and it will undoubtedly prove to be a useful tool in the investigation of ANF physiology.

Molecular Forms

The realization that each of the peptides sequenced was essentially an amino terminal extension of the basic ANF structure, in conjunction with information derived from ANF cDNA cloning studies, led to the hypothesis that the disulfide-bonded ANF loop was the carboxy-terminal end of a much larger molecule, i.e. a pro-ANF type molecule [Flynn, 1987]. Indeed, a number of studies [Bloch et

FIGURE 1.3

THE BIOSYNTHETIC PATHWAY OF ATRIAL NATRIURETIC FACTOR AND ITS PRECURSORS

The biosynthetic pathway, and the sequences of the human pre-pro-ANF gene, pro-ANF and ANF_{1-28}. ANF_{1-28} is numbered from amino- to carboxy-terminus. Specific amino acids in the pro-ANF and ANF sequences are given by single letter code:

alanine=A	arginine=R
asparagine=N	aspartic acid=D
cysteine=C	glutamine=Q
glutamic acid=E	glycine=G
histidine=H	isoleucine=I
leucine=L	lysine=K
methionine=M	phenylalanine=F
proline=P	serine=S
threonine=T	tryptophan=W
tyrosine=Y	valine=V

From Ballerman and Brenner, [1986]. Reproduced with the permission of the American Heart Association.

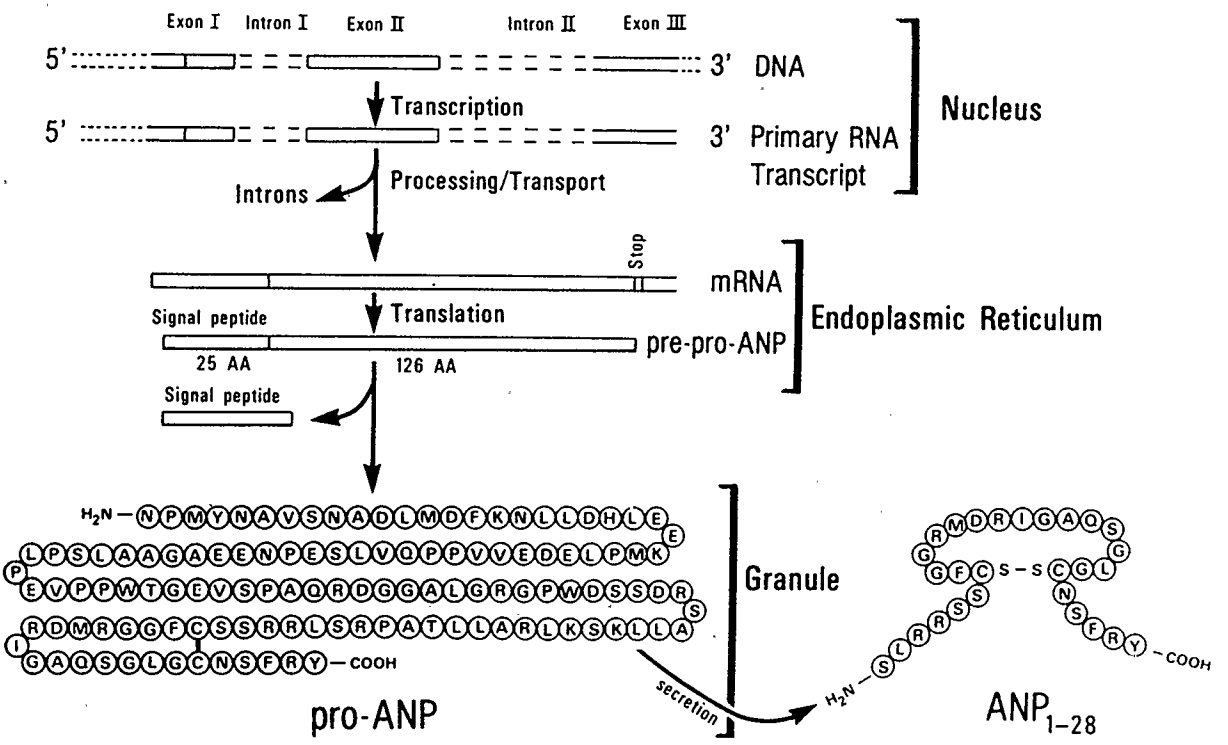

Exon I
Intron I
Exon II
Intron II
Exon III
5'
3'
DNA
Transcription
Primary RNA Transcript
Nucleus
Introns
Processing/Transport
Stop
mRNA
Signal peptide
Translation
pre-pro-ANP
Endoplasmic Reticulum
25 AA
126 AA
Signal peptide
Granule
pro-ANP
secretion
ANP1–28

TABLE 1.1*

THE RELATIVE EFFECT OF ATRIAL NATRIURETIC FACTOR AND ITS DELETION ANALOGUES ON SODIUM EXCRETION, SMOOTH MUSCLE RELAXATION, AND INHIBITION OF ALDOSTERONE SECRETION

*Adapted from Thibault et al., 1987.

ANF Peptides	Sodium Excretion	Relaxation		Aldosterone Inhibition	
		Chick Rectum	Rabbit Aorta	Cow Adrenal Cells	Rat Adrenal Cells
54-126	44	---	0.2	63	---
96-126	57	---	32	253	---
99-126[1]	---	---	---	---	30
99-126[2]	51	122	127	100	100
101-126*	100	100	100	100	100
102-126[3]	133	36	100	100	---
103-123[4]	22	5	0.3	0.3	5
103-125[5]	42	9	0.6	63	20
103-126[6]	95	15	10	100	20
104-126	86	76	8	32	---
105-126	143	162	32	32	---
101-125	116	74	4	1000	150
101-124	75	---	0.4	---	---
101-123	21	43	0.2	3	5
101-121	3	21	0.1	1	---
106-125	---	0.5	0.004	0.1	---

1. α-Human Atrial Natriuretic Factor (Met^{110})
2. Cardionatrin I
3. Auriculin B
4. Atriopeptin I
5. Atriopeptin II
6. Atriopeptin III

* All values expressed as percentage effects relative to $ANF_{(101-126)}$

al., 1985; Lewicki et al., 1986; Wildey et al., 1988; Zisfein et al., 1986;] have identified a 126 amino acid prohormone of ANF in which the last 8 amino acids are identical to the circulating compound. This prohormone has been confirmed as the major storage form of ANF [Zisfein et al., 1986]. Under normal circumstances it is barely detectable in the peripheral circulation, but in pathophysiological states such as congestive heart failure (CHF), elevated plasma levels of pro-ANF have been measured [Arendt et al., 1987].

The circulating form of ANF, $ANF_{(99-126)}$, has subsequently been labelled α-ANF. It has been identified in a number of mammalian and non-mammalian species [Cantin and Genest, 1986; Kramer and Lichardus, 1986] and has a molecular weight of approximately 3000 [Goetz, 1988b]. Two further subtypes of ANF have also been identified in atrial tissue [Akimoto et al., 1988]. These are γANF, the major type of ANF present within atrial tissue in patients under twenty years of age, and βANF, the frequency of which tends to increase with age [Akimoto et al., 1988]. β-human ANF is a 56 amino acid, anti-parallel dimer of α-human ANF. It has also been identified in increased quanities in patients with severe CHF and has been found to decrease following treatment of CHF [Marumo et al., 1988]. At the present time, its function in the circulation is unknown but it appears to be unique to humans [Akimoto et al., 1988; Marumo et al., 1988].

Peptide Cleavage and Activation

The prohormome was relatively inactive when injected into an isolated rat kidney or on exposure to a smooth muscle preparation [Trippodo et al., 1986]. Intravenous injection, on the other hand, caused marked dilatation of the renal artery, suggesting that pro-ANF can become activated in the systemic circulation. Incubation of pro-ANF with blood or platelets subsequently produced relaxation in aortic smooth muscle tissue [Trippodo et al., 1986], and whole serum is capable of cleaving pro-ANF [Fischman et al., 1989]. The site of cleavage of pro-ANF to ANF has yet to be identified.

Using a polyclonal antiserum which recognises $ANF_{(99-126)}$, Wildey and associates were not able to identify the circulating form of the hormone within atrial myocytes and concluded that the proteolytic processing of ANF was not an intracardiocytic event [Wildey at al., 1988]. Other investigators have succeeded in identifying and purifying a protease that is capable of converting pro-ANF to the circulating form but this enzyme is not present in atrial granules [Imada et al., 1988]. Kallikrein converts pro-ANF to $ANF_{(103-126)}$ by cleaving it at a dibasic site and thrombin converts pro-ANF to its true circulating form, $ANF_{(99-126)}$ at a monobasic site [Fischman et al., 1989]. Heparin has been found to markedly decrease the diuretic and hypotensive effects of ANF in rats. The large, highly charged heparin moities may modify the physical and/or chemical properties of ANF, thereby interfering with its biologic activity [Wei et al., 1987].

Receptors

Binding sites for ANF have been identified in numerous tissues and will be discussed below. These receptors exhibit a relatively high degree of specificity in that unrelated polypeptides such as angiotensin II, somatostatin, parathyroid hormone, and glucagon do not compete with ANF for receptor binding [Genest, 1986]. Studies in rat kidney and adrenal tissue have identified three polypeptides which are capable of binding ANF [Vandlen, 1986]. These peptides have molecular weights of 60, 70, and 120 kilodaltons. Two ANF binding sites have been identified in bovine aortic smooth muscle [Fuller et al., 1988]. The 130 kilodalton binding site appears to be coupled to guanylate cyclase. The smaller binding protein, 60 kilodaltons, had a high affinity for truncated and deletion analogs of ANF but was not found to be induce guanylate cyclase activity. The 60 kilodalton binding protein may serve as a clearance site for removal of unwanted forms of ANF from the circulation [Maack et al., 1987].

Intracardiac Disposition of Atrial Natriuretic Factor

The maximal concentration of ANF has been found in the atrial appendages of a number of animals [Cernacek et al., 1988]. In small rodents such as the rat, the concentration of ANF appears to be greater in the right atrial appendage. However, in larger mammals such as the dog [Cernacek et al., 1988] and the rabbit [Synhorst and Gutkowska, 1988], the situation is opposite and ANF concentration in

the left atrial appendage is higher than on the right. A similar situation may occur in humans [Rodeheffer et al., 1986]. Identification of the pre-pro-ANF peptide, as a marker of ANF, has demonstrated a relative abundance in the rat as follows; atrium 1.0, ventricle 0.01, lung, 0.001, pituitary 0.001, and hypothalamus 0.00025 [Lewicki et al., 1986]]. Levels of ANF messanger RNA (ANF-mRNA) are 150 times higher in the atria than in the ventricles of rats. ANF concentration itself is 1000 times higher in the atria [Nemer et al., 1986].

Pharmacokinetics

Using a two-compartment open model, Nakao and coworkers analysed the pharmacokinetics of α-human ANF in healthy volunteers [Nakao et al., 1986]. The half-life of ANF in the plasma was found to be 1.7 ± 0.1 minutes for the fast elimination component and 13.3 ± 1.7 minutes for the slow component. These results are similar to those of Tonolo et al. [1988], who found a plasma half-life of 3.5 minutes in healthy humans. The presence of end-stage renal disease has been shown to prolong the plasma half-life of ANF by approximately 30 % [Tonolo et al., 1988]. Plasma half-lives for ANF in rats [Vari et al., 1986] and dogs [Verburg et al., 1987] have been estimated at 3.4 ± 1.2 minutes and 14.3 ± 2.0 minutes, respectively. The model used in determining these clearance times was not specified.

MECHANISMS OF SECRETION

The secretion of atrial natriuretic factor from cardiac tissue is likely mediated by two primary, possibly interdependent processes [Veress et al., 1988]. Release of ANF from atrial granules was initially believed to be mediated primarily by stretch of atrial myocytes [Lang et al., 1987]. However, there is now increasing evidence that ANF can be released from myocytes via a receptor mechanism which is independent of alterations in the stress/strain relationships of atrial tissue [Veress et al., 1988].

In view of the relatively short half-life af ANF, the circulating plasma concentration of atrial natriuretic factor likely reflects its secretion rate [Hirata et al., 1988]. Secretion of ANF appears to be mainly into the capillary circulation surrounding myocytes, rather than directly into the cardiac chambers. This hypothesis is supported by experiments demonstrating plasma ANF levels in coronary sinus effluent are 4-20 times higher than levels measured in the peripheral arterial or venous circulation [Bates et al., 1989; Crozier et al., 1986; Fitzpatrick et al., 1989; Gines et al., 1988; Hirata et al., 1988]. In addition, the intravenous infusion of ANF has been shown to significantly decrease the secretion rate of the peptide into the coronary sinus and this may reflect the presence of a negative feedback mechanism regulating ANF secretion [Hirata et al., 1988].

Receptor Mediated Release of ANF

Release of ANF from cardiac tissue may occur independently of interventions designed to change atrial stress/strain relations [Hayashi et al., 1988; Rankin, 1987; Veress et al., 1988]. Humoral factors [Ballerman and Brenner, 1986; Genest, 1986; Goetz, 1988b; Ruskoaho et al., 1989; Shields and Glembotski, 1988], nervous system signals [Ballerman and Brenner, 1986; Genest, 1986; Lang et al., 1987; Nishida et al., 1988; Rankin, 1987; Schiebinger and Santora, 1989], fluctuations in intracellular messengers; i.e. activation of the polyphosphoinositide system [Ishida et al., 1988; Ruskoado et al., 1985], intracellular calcium [Saito et al., 1986], increases in extracellular ionic concentrations of sodium [Amadieu-Farmakis et al., 1989], potassium and glucose [Gibbs, 1987], calcium [Iida and Page, 1989], and hypoxemia [Baertschi et al., 1988, 1986] have all been shown to increase secretion of atrial natriuretic factor from cardiac myocytes. Conversely, administration of calcium channel antagonists has been associated with a reduction in circulating ANF concentrations [Haufe et al., 1988a]. It is likely that intracellular messengers represent the final common pathway of receptor-mediated ANF release, regardless the origin of the initiating stimulus [Lang et al., 1987; Loeb and Gear, 1988; Veress et al., 1988].

Receptor-mediated ANF release is not specifically studied in the experiments which make up this dissertation and thus further exploration of this aspect of ANF physiology is extraneous to the present review. The interested reader is referred elsewhere for a

more in-depth treatment of this area [Catt et al., 1979; Goldberg, 1974; Insel and Ransnas, 1988; Rasmussen, 1986].

Interventions Altering Atrial Stress/Strain Relations

Attempts at altering the intrinsic morphologic architecture of atrial myocytes to induce secretion of atrial natriuretic factor have utilized a variety of techniques including intravascular volume expansion, atrial balloons, intracardiac pacing, constriction of the great vessels, and pericardial tamponade (see Table 1.2).

Intravascular volume expansion. Verburg and associates demonstrated that the infusion of isotonic saline (2.5 % of body weight) significantly increased the femoral arterial concentration of ANF [Verburg et al., 1986]. Conversely, a return to baseline (diuresis following the administration of ethacrynic acid, [Verberg et al., 1986]) or below baseline (acute hemorrhage [Carlson et al., 1989]) caused a reduction in the circulating level of ANF. In a dog model utilizing a high-fidelity pressure transducer and left atrial appendage sonomicrometer crystals, the infusion of one liter of normal saline over 5 minutes, significantly increased left atrial pressure and diameter, and plasma ANF levels [Hintze et al., 1989]. Since the atria were stretched only during the "v" wave of the atrial cycle (passive stretch), the use of atrial "v" wave pressure recording to calculate atrial wall stress had a closer correlation with plasma ANF levels than atrial stress calculated using either "a"

TABLE 1.2

THE EFFECT OF ATRIAL MANIPULATION ON PLASMA LEVELS OF ATRIAL NATRIURETIC FACTOR

REFERENCE	INCREASE IN PLASMA LEVEL OF ANF
Intravascular Volume Expansion	
Akabane et al., 1987	40 ± 3 to 101 ± 25 $pM.L^{-1}$
Anderson et al., 1986b	5 ± 1 to 17 ± 4 $pM.L^{-1}$
Cowley et al., 1988	17 ± 3 to 27 ± 7 $pM.L^{-1}$
Dietz, 1987	29 ± 6 to 250 ± 51 $pM.L^{-1}$
Hintze et al., 1989	15 ± 3 to 112 ± 39 $pM.L^{-1}$
Salazar et al., 1986b	15 ± 2 to 40 ± 8 $pM.L^{-1}$
Vergurg et al., 1986	19 ± 2 to 46 ± 12 $pM.L^{-1}$
Balloon Distention of Atria	
Akabane et al., 1988	70 ± 15 to 134 ± 25 $pM.L^{-1}$
Bilder et al., 1986	2000 ± 300 to 3700 ± 300 $pM.L^{-1}$
Ledsome et al., 1985	32 ± 3 to 45 ± 5 $pM.L^{-1}$
Release of Cardiac Tamponade/Great Vessel Constriction	
Edwards et al., 1988	25 ± 5 to 85 ± 18 $pM.L^{-1}$
Mancini et al., 1987	19 ± 12 to 22 ± 18 $pM.L^{-1}$
Negative Pressure Breathing	
Anderson et al., 1989	5 ± 1 to 17 ± 4 $pM.L^{-1}$
Cardiac Pacing	
Haufe et al., 1988b	26 ± 10 to 86 ± 33 $pM.L^{-1}$
Walsh et al., 1988a	116 ± 55 to 1126 ± 226 $pM.L^{-1}$
Walsh et al., 1987	12 ± 3 to 146 ± 41 $pM.L^{-1}$

wave atrial pressure or mean atrial pressure. The"a" wave component of atrial pressure may be of importance with respect to ANF secretion in conditions such as atrio-ventricular dissociation, where the atria may be contracting against a closed atrio-ventricular valve [Vardas et al., 1989]. Under these circumstances, the rapid increase in atrial pressure may be an important factor in the release of ANF. The relation between plasma ANF levels and right atrial pressure is shifted to the left by vasopressin administration in rats [Ruskoaho et al., 1989]. Thus, for a given increase in right atrial pressure more ANF is released. Selective β_1 and non-selective β-blockade inhibited basal release of ANF in healthy male volunteers, but the increase in ANF associated with acute volume expansion was not prevented [Nguyen et al., 1988].

Salazar and associates [Salazar et al., 1986b] found that acute saline loading (5 % of body weight) in conscious dogs, resulted in a significant increase in plasma ANF levels. However, with chronic saline infusion over seven days, plasma ANF levels were unchanged despite increasing sodium intake from 5 to 300 $meq.day^{-1}$. Thus, in chronic volume overload states, a major trigger for ANF release, i.e. increased atrial pressure, may not be activated [Salazar et al., 1986b]. Similar results regarding the effect of acute volume expansion on ANF levels have been found in isolated rat heart(-lung) preparations [Anderson and Bloom, 1986a; Dietz, 1984, 1987; Kabayama et al., 1986; Lang et al., 1985] and in anesthetized dogs [Akabane et al., 1987].

Although alterations in atrial stress/strain relationships may affect plasma ANF levels (pANF), it is interesting to note that bilateral atrial resection (~60 % of total atrial tissue) may not affect resting plasma levels of ANF [Cowley et al., 1988]. Furthermore, volume expansion under these circumstances, still elicited an increase in pANF. These observations may be secondary to a chronic increase in ANF secretion from remaining, damaged atrial cells and/or altered stress/strain responses of residual atrial myocytes [Cowley et al., 1988].

Plasma levels of atrial natriuretic factor in congestive heart failure will be reviewed below. However, the development of congestive heart failure and the attendant increase in central venous pressure was associated with increased circulating levels of ANF compared to controls [Chien et al., 1988]. It is possible that chronic elevation of atrial pressures and ANF levels might lead to depletion on ANF-containing atrial granules secondary to prolonged secretion. However, acute intravascular volume expansion (6 % of body weight) in the presence of chronic heart failure, resulted in similar changes in circulating ANF to those derived from control animals. Thus, the ability of cardiac cells to secrete ANF is likely preserved in chronic, stable congestive heart failure [Chien et al., 1988].

Mechanical Distension of Atrial Tissue. Increasing atrial pressure by inflation of a balloon situated in the left atrium significantly increased the level of ANF measured in coronary sinus

effluent [Akabane et al., 1988]. This effect was found to reach a maximum after 15 minutes of balloon inflation and then decreased, despite the maintenance of increased atrial pressures. Balloon obstruction of the mitral valve orifice significantly increased both left atrial pressure and plasma ANF levels [Ledsome et al., 1985]. Distension of the pulmonary veins failed to produce any significant alteration in ANF concentrations. The increase in plasma ANF produced by elevation of left atrial pressures was not affected by either β-blockade or bilateral vagotomy [Ledsome et al., 1985]. In perfused isolated rabbit hearts, the slope of the regression curve relating ANF release to atrial distending pressure was steeper for the left atrium than the right atrium. Thus, at the same level of intracavitary pressure, the left atrium releases more ANF than does the right [Synhorst and Gutkowska, 1988].

The local etiology of stretch-induced ANF release has been demonstrated in rats with chronically implanted, small inflatable balloons at the junction of the superior vena cava (SVC) and the right atrium [Meikle and Kaufman, 1988]. Following inflation of the balloons for sixty minutes, the animals were sacrificed and an extract of atrial tissue was prepared. Compared to control animals, in which balloon implantation but not inflation took place, the natriuretic potency of right atrial extract was reduced following balloon inflation. Balloon inflation had no effect on the natriuretic potency of left atrial extract, hence, the effect of balloon inflation on the natriuretic activity of atrial extract would seem to be locally mediated.

Further demonstration of stretch-sensitive release of ANF is found in studies by Agnoletti et al. [1987]. Using isolated rat atria, these investigators found that mechanical stretch of spontaneously beating atrial tissue produced a significant increase in the concentration of ANF measured in effluent from the perfusion bath in which the atria were situated. This increase reached a peak in five minutes and then declined until a second stretch induced a second, smaller increase in ANF. However, the diminished effect of the second stretch was not due to a decrease in the cellular content of ANF and therefore may represent an attenuation in the response of the stretch-sensitive release mechanism to continued stretch. Of further interest in this study was the observation that stretch of the atrial tissue did not significantly alter release of neurotensin or bombesin and thus atrial stretch, as a mechanism of hormone release, would seem to be specific for ANF release [Agnoletti et al., 1987].

Clinically, the effect of atrial distension on ANF secretion is demonstrated in a study by Hung et al. [1989]. These authors report a dramatic decrease in left atrial pressure and plasma ANF levels following successful balloon valvuloplasty for mitral stenosis. This effect occurred within fifteen minutes of the procedure and plateaued at approximately sixty minutes.

Effects of Cardiac Tamponade and Other Manoeuvres. In an effort to directly examine the effects of atrial pressure on ANF release, as opposed to changes in atrial distension, a number of

experimenters have studied the effects of cardiac tamponade on ANF release [Edwards et al., 1988; Mancini et al., 1987; Zioris et al., 1989]. In open-chested dogs, volume expansion with one liter of Ringer's solution was associated with a significant increase in plasma ANF levels [Mancini et al., 1987]. The induction of cardiac tamponade, during the hypervolemic state, increased right atrial pressure but plasma levels of ANF actually decreased. Volume loading in the presence of tamponade, failed to produce any significant changes in plasma ANF levels. Zioris and co-workers [1989] found no effect of cardiac tamponade on plasma ANF levels in dogs. Using a Foley catheter to measure pericardial pressure, they found that plasma ANF levels did not change during pericardial tamponade, despite an increase in atrial pressure of 6 mmHg. Measured transmural atrial pressure decreased by 4 mmHg during the same time period, most likely as a consequence of increased pericardial pressure. Decompression of the cardiac chambers following release of tamponade did not result in any significant change in plasma ANF (pANF) levels.

Pericardial tamponade produced an increase in atrial pressure, decreased atrial transmural pressure, and had no effect on plasma ANF levels [Edwards et al., 1988]. Following completion of the tamponade portion of the experiments, the animals underwent pericardiectomy effectively rendering atrial pressure equal to transmural pressure (see Chapter 2). Simultaneous constriction of the aorta and the pulmonary artery resulted in an increase in right atrial pressure and no change in pericardial pressure. The increase in atrial transmural

pressure was associated with an increase in pANF [Edwards et al., 1988].

Volpe et al. [1988b] have found that lower body negative pressure lowers plasma levels of ANF (28 ± 6 $pM.L^{-1}$ to 20 ± 6 $pM.L^{-1}$) and this observation is associated with a 2 mmHg reduction in mean right atrial pressure.

In an extensive review, Epstein et al. [1989] reported that water immersion to the neck, in both humans and canines, was consistently associated with an increase in plasma ANF levels during the first hour of water immersion. The zenith for plasma ANF levels occurred in the second or third hour of immersion and represented a 2 to 3-fold increase from baseline. At the conclusion of water immersion, ANF levels quickly returned to normal levels.

Cardiac Pacing. Rapid pacing (250 bpm) of the right atrial appendage in anesthetized dogs for 60 minutes significantly increased both arterial and coronary sinus concentrations of ANF [Walsh et al., 1987]. During the pacing interval intra-atrial pressure increased by 4 mmHg. In an attempt to determine if the increase in ANF release was secondary to the rapid heart rate or a consequence of the increase in atrial pressure, Walsh and collegues [Walsh et al., 1988a] studied the effect of incremental pacing in dogs whose atrial pressures were kept low by inferior vena cava (IVC) occlusion. During pacing from 200 to 300 bpm, without IVC occlusion, plasma ANF levels rose in conjunction with right atrial pressures at each pacing level.

However, when the IVC was occluded, right atrial pressure and plasma ANF levels failed to increase with pacing. Hence, in this study, the release of ANF was primarily associated with increased atrial pressure and not atrial rate. These results differ from those recorded by Schiebinger and Linden [1986a] in isolated rat atria. These authors found that increasing the stimulation rate of the atria significantly increased the release of ANF from the preparation but had no effect on the amount of resting tension present within the preparation. Thus, atrial rate and not tension seemed to mediate ANF release. In another study, these same authors found that increases in tension of the atrial preparation, with or without pacing, enhanced release of ANF [Schiebinger and Linden, 1986b]. Thus, both contraction rate and atrial stretch, as interdependent modifiers of atrial stress and strain, may affect release of ANF from cardiocytes.

Right ventricular pacing in humans (150 bpm) for only 4 minutes was found to significantly increase plasma ANF levels [Haufe et al., 1988b]. After a fifteen minute pause, a further 4 minutes of atrial pacing resulted in a similar increase in right atrial pressure (+ 3 mmHg) but was associated with augmented release of ANF, i.e. plasma ANF during the second period of stimulation was significantly higher than during the first pacing period. The 'priming effect' on ANF release may have been secondary to propagation of granular exocytosis, increased intracellular ANF synthesis, or increased conversion of pre-pro- and pro-hormone to ANF [Haufe et al., 1988b]. The latter possibility is supported by studies in dogs which demonstrated that right ventricular pacing (at 125, 150, and 180 bpm)

significantly increased plasma levels of two ANF pro-hormones, $ANF_{(1-30)}$ and $ANF_{(31-67)}$, and α-ANF [Ngo et al., 1989].

Effect of Anesthetics and Temperature on ANF Secretion

The use of anesthetics in studies of atrial natriuretic factor was called into question by Horký et al. [1985]. General anesthesia in rats, with morphine or diethyl-ether, was associated with a significant increase in plasma ANF levels compared to controls or animals anesthetised with sodium pentobarbital. A similar effect of morphine sulphate on plasma ANF levels, in concert with increases in plasma levels of epinephrine and norepinephrine has been described [Crum and Brown, 1988]. Intra-cerebroventricular administration of morphine produced an increment in plasma ANF levels one order of magnitude higher than those which resulted from peripheral intravenous administration of morphine. However, the intravenous administration of opioid peptides (met-enkephalin) failed to produce any change in the systemic concentrations of ANF measured in humans [Borges et al., 1988]. Thus, morphine sulphate may act within the central nervous system to produce ANF secretion [Crum and Brown, 1988] but this observation may simply represent a non-specific effect of high-dose morphine rather than a direct effect [Borges et al., 1988].

In contrast to the above observations, narcotic anesthesia, in dogs, had no effect on plasma ANF concentrations [Edwards et al., 1988] and barbiturates (pento-barbital) have been previously utilized

as anesthetic agents in ANF related experiments [Edwards et al., 1988]. Blood samples drawn in our laboratory following induction of anesthesia with sodium thiopental (a barbiturate anesthetic agent differing from pento-barbital by only one methyl group), failed to demonstrate a significant elevation of plasma ANF levels outside the normal range. In addition, further blood samples obtained from two dogs after subsequent administration of α-chloralose anesthesia, did not show any significant change in plasma ANF levels compared to the values obtained following thiopental induction [Stone, unpublished results].

Temperature reduction, from 37°C to 21.5°C, produced a decrease in basal and mechanically stimulated secretion of ANF [94]. The temperature dependence of ANF release in vivo may be related to changes in spontaneous heart rate, membrane and cytoplasmic fluidity, ion fluxes, rate of synthesis, and/or granule fusion with other membrane surfaces [Bilder et al., 1986].

SITES OF ACTION AND EXTRA-CARDIOVASCULAR EFFECTS OF ANF

The following section will briefly examine the organ systems in which the weight of scientific evidence supports a plausible physiologic role for atrial natriuretic factor.

Localization of ANF Binding Sites

Autoradiographic detection of atrial natriuretic factor binding sites has identified numerous ANF receptors on the endothelial cells of the endocardium, and on the endothelial lining of vessels in the kidney, adrenal, lung, and liver [Bianchi et al., 1985]. A lower density population of ANF receptors has also been detected on epithelial cells of the small intestine and on the surface of smooth muscle cells of the muscularis mucosa of the colon [Bianchi et al., 1985]. A high density of ANF binding sites is also found within the central nervous system of rats [Quirion et al., 1988, 1984; Saavedra et al., 1986a,b; Samson, 1988;]. Significant numbers of ANF binding sites have not been detected in the aorta, thymus, ovary, uterus, stomach, pancreas, urinary bladder, and rectum [Bianchi et al., 1985].

Lung. Although large numbers of ANF receptors have been identified in lung tissue [Bianchi et al., 1985], there are conflicting results in the literature concerning the extraction of ANF by the pulmonary bed. Bates and associates [Bates et al., 1989] determined that the decrease in plasma ANF concentration across the lung in samples drawn from the pulmonary artery, pulmonary capillary wedge position, pulmonary vein, and the left atrium, was 17.7 ± 4.1 $pM.L^{-1}$. Utilizing a bioassay model, Wesselcouch et al. [1985], found only a 25 % reduction in the vascular relaxing activity of a crude atrial extract after passage through isolated, perfused guinea pig lungs. This apparent discrepancy may be explained by the relative insensitivity of the latter technique in detecting true variations in plasma concentration of ANF. ANF can induce dilatation in pulmonary

arteries [Winquist et al., 1984] and the effect is greater than the vasorelaxant effect produced in omnental arteries [Lindberg and Andersson, 1988]. Although ANF relaxes bovine and guinea pig tracheal muscle, in vitro [Hamel and Ford-Hutchinson, 1986; Ishii and Murad, 1989], aerosolized ANF, at a concentration of 10^8 pM, had no effect on bronchial resistance in spontaneously breathing guinea pigs [Hamel and Ford-Hutchinson, 1986]. Interestingly, α-hANF was found to reduce pulmonary oedema, independent of its natriuretic and diuretic effects, caused by various chemicals in an isolated, perfused guinea pig lung system [Imamura et al., 1988].

Adrenals. Atrial natriuretic factor decreases the adrenal production of aldosterone, both in vitro [Atarashi et al., 1985, 1984; Campbell et al., 1985; Chartier et al., 1984; de Léan et al., 1984b; Kudo and Baird, 1984] and in humans [Waldhausal et al., 1986; Weidmann et al., 1986]. The inhibitory effect of ANF was most pronounced following pre-stimulation of the adrenal cortex by the aldosterone secretagogues angiotensin II, potassium and adrenocorticotrophic hormone [Mulrow et al., 1987]. Atrial natriuretic factor-specific receptors are found in the surface membranes of cells from the zona glomerulosa of the adrenals [de Léan et al., 1984a; Kramer and Lichardus, 1986] and the inhibition of aldosterone production, in the presence of ANF, is associated with an increase in cyclic GMP (cGMP) [Matsuoka et al., 1985]. A recent study by Takagi and co-workers in adrenal glomerulosa cells [Takagi et al., 1988], has demonstrated that ANF did not affect calcium influx or efflux. The inhibitory effect of ANF on aldosterone production was not caused

by interference with the intracellular calcium messenger signal. Thus, the inhibitory effect of ANF occurred in a pathway or process distal to the generation of the calcium signal [Takagi et al., 1988].

Central Nervous System. The demonstration of ANF receptors in the cerebral cortex, thalamic nuclei, amygdala, hippocampus, cerebellum, subfornical organ, area postrema, choroid plexus, empendyma [Quirion et al., 1988, 1984; Saavedra et al., 1986a,b; Samson, 1988;], and on the blood-brain barrier [Chabrier et al., 1988], suggests that some of the physiologic effects of ANF may be mediated through the central nervous system. The central nervous system may also synthesize a unique twenty-six amino acid form of ANF [Sudoh et al., 1988].

Administration of atriopeptin III (6700 $pM.kg^{-1}$) into the fourth ventricle of conscious, spontaneously hypertensive and normotensive rats [Levin et al., 1988], resulted in reductions of mean arterial pressure of 13 ± 1 and 9 ± 2 mmHg, respectively. The hypotensive effect was blocked by α_2 blockade (yohimbine) or central catecholamine depletion (following intracerebroventricular (ICV) injection of 6-OH dopamine). In a similar rat model, ICV administration of ANF increased urinary volume and sodium excretion over a six hour period [Israel et al., 1988]. This effect was still present following sympathectomy and adrenalectomy. ICV injection of angiotensin II in rats potentiated cardiac ANF secretion induced by volume loading, but had no effect on basal levels of plasma ANF [Itoh et al., 1988]. McKitrick and Calaresu [1988] have shown that

microinjection of ANF (10^5 pM) into the nucleus tractus solitarius (NTS) of rats produced a significant decrease in heart rate and mean arterial blood pressure. As an extension of this study, Ermirio et al. [1989] found that the microinjection of ANF into the NTS excited neurons which were also excited by activation of arterial baroreceptors and inhibited by baroreceptor unloading. Intravenous ANF infusion into humans [Ebert, 1988b,c] and rabbits [Hirooka et al., 1988; Volpe et al., 1987a] have demonstrated that ANF may alter the normal baroreceptor-mediated tachycardia induced by systemic hypotension.

In an elegant series of studies designed to assess ANF disposition in rat anterior pituitary gonadotrophs, Morel et al. [1989] observed radioactive ANF labelling of the plasma membrane, cytoplasmic matrix, secretory granules, mitochondrial components and the Golgi apparatus within one minute of intravenous injection. The nuclear and cytoplasmic components of the gonadotrophs contained significant amounts of ANF-mRNA. These findings suggested that rat anterior pituitary gonadotrophs can both synthesize and internalize extracellular ANF. Finally, Saavedra and collagues [Saavedra et al., 1986b] found a decrease in the numbers and affinity of ANF receptors present in the subfornical organ of spontaneously hypertensive rats.

Liver. Hepatic extraction of atrial natriuretic factor does take place [Crozier et al., 1986; Gines et al., 1988] and patients with liver cirrhosis and moderate ascites show increased plasma levels of ANF [Hedner et al., 1988]. The significance of ANF uptake

within the liver and the question of whether hepatic metabolism of ANF represents the major route of degradation remains to be determined.

Kidney. The physiologic (and pharmacologic) effects of ANF on renal function are many and varied. Although early studies of ANF and renal function are of questionable physiological significance in view of the pharmacologic levels of ANF administered [Laragh, 1985], more recent studies have shown that the administration of ANF to animals [Brands and Freeman, 1988] and healthy volunteers [Cuneo et al., 1987; McMurray and Struthers, 1989; Oelkers et al., 1988; Williams et al., 1988], in doses which produced serum levels observed under physiologic conditions (30-90 $pM.L^{-1}$), significantly decreased plasma renin activity and serum aldosterone levels.

The renal effect of ANF most frequently described, and the one for which it was named, is the promotion of natriuresis and diuresis. Soejima and collagues [Soejima et al., 1988] infused synthetic ANF into normotensive rats in an attempt to determine the minimum infusion rate and plasma concentration of ANF at which alterations in renal function could first be appreciated. They found that significant natriuresis and diuresis began when the infusion rate was approximately 7 $pM.kg^{-1}.min^{-1}$ and the plasma ANF concentration was 279 ± 20 $pM.L^{-1}$ versus 158 ± 12 $pM.L^{-1}$ in the control rats. Changes in potassium secretion, glomerular filtration rate, and mean arterial blood pressure did not occur until plasma ANF levels reached approximately 850 $pM.L^{-1}$, 1000 $pM.L^{-1}$, and 3600 $pM.L^{-1}$, respectively.

A similar response with respect to natriuresis and diuresis, at a plasma ANF concentration of 67 $pM.L^{-1}$, was reported by Banks [1988]. Hintze [1988] has also reported a natriuresis and diuresis during ANF infusion. However, measured plasma ANF levels in this study exceeded 14,333 $pM.L^{-1}$ and therefore, the physiologic relevance of this study is doubtful.

The question of whether left atrial distension, per se, or the release of ANF during distension, and its subsequent effect on the kidney, is the major factor responsible for ANF associated natriuresis and diuresis remains to be determined. Goetz and co-workers [Goetz et al., 1988a, 1986a,b], Hartupee et al. [1989], and Myers et al. [1988] have concluded that much of the natriuresis and diuresis observed during ANF infusion or left atrial distension may be mediated by changes in renal sympathetic nerve activity. Indeed, Goetz [1986a] has shown that left atrial distension and the consequent increase in plasma ANF levels caused no change in urine flow or sodium excretion in cardiac denervated dogs. Zimmerman et al. [1987b] have shown that although plasma ANF parallels increases in right atrial pressure during a period of acute volume expansion, during a maintained hypervolemic state (infusion rate=urine outflow), both atrial pressure and plasma ANF fell but the fractional excretion of sodium remained elevated. These findings suggest that a non-ANF mechanism may be of importance in maintaining natriuresis during stable volume expansion [Zimmerman et al., 1987b].

The effects of ANF on sodium balance and urine flow may be mediated via cyclic GMP [Leung et al., 1987; Náray-Fejes-Tóth et al.,

1988], although some authors [Blaine et al, 1986] have reported a dissociation between urinary cGMP levels and natriuresis during ANF infusion. Redistribution of renal blood flow to the deep cortex during ANF infusion has been attributed to prostaglandins [Salazar et al., 1988].

Studies examining direct tubular effects of ANF have been conflicting [Leckie, 1987]. Peterson et al. [1987] found no significant peritubular effect of ANF on the thick ascending Loop of Henle sodium chloride permeability in the outer cortical nephrons of rats. However, Fried and collegues showed that ANF infusion at 167 $pM.kg^{-1}.min^{-1}$ significantly increased the delivery of chloride to the base and tip of the papillary collecting ducts [Fried et al., 1988]. They also determined that ANF inhibited resorption of chloride between the late distal tubule and the base of the papillary collecting duct.

Tsunoda and associates [Tsunoda et al., 1988] have demonstrated a reduction in the number of ANF binding sites present in the inner medulla of rat kidneys following the development of congestive heart failure in acutely infarcted rats. This observation in concert with the findings of Mendez et al. [1986] that peritubular capillary hydraulic and oncotic pressures modulate the natriuretic and diuretic effects of ANF, may partially explain the blunted natriuretic response to ANF observed in congestive heart failure [Riegger et al., 1988, Molina et al., 1988]. However, the possibility that ANF may still have some beneficial role in heart failure is suggested by the observation that the bolus infusion of

atriopeptin III (4000 $pM.kg^{-1}$) into rats, significantly reduced the number of aldosterone receptors in rat kidney cytosol [Horiuchi et al., 1989].

THE CARDIOVASCULAR EFFECTS OF ATRIAL NATRIURETIC FACTOR

The cardiovascular effects of ANF can be divided into two main groups, those which are primarily involved with cardiac muscle function itself and those which involve the vascular tree. This section will focus initially on the cardiac effects of ANF and then on its vascular effects, both in the coronary and peripheral arterial beds.

Cardiac Effects. The ability of atrial natriuretic factor to modify cardiac muscle function is greatly dependent on the particular model chosen for experimental examination. As a direct consequence of the myriad of animal models and human data present in the literature, studies can be divided into those in which ANF administration: 1) had no effect on cardiac performance [Faison et al., 1985; Hiwatari et al., 1986; Iwanaga et al., 1988; Natsume et al., 1986]; 2) was associated with a possible reduction in myocardial contractile function [Ackermann, 1984,1986; Bergey and Kotler, 1985; Breuhaus et al., 1985; Seymour et al., 1987; Volpe et al., 1987b]; and 3) actually seemed to improve cardiac function [Indolfi et al., 1989; Kleinert et al., 1986].

Hiwatari and collagues found that synthetic $ANF_{(8-33)}$ in concentrations up to 3×10^5 pM had little effect on the contractile force development in guinea pig papillary muscle [Hiwatari et al., 1986]. The infusion of whole blood into three groups of rats, each receiving either no injection, Atriopeptin III at 3300 $pM.kg^{-1}$, or Atriopeptin III (3300 $pM.kg^{-1}$) and a continuous infusion of phenylephrine to prevent the ANF induced decline in blood pressure, failed to alter the cardiac performance curves of the groups with respect to each other [Natsume et al., 1986].

In four awake, chronically instrumented dogs, a 1000 $pM.kg^{-1}$ bolus followed by a 1000 $pM.kg^{-1}.min^{-1}$ infusion of Auriculin A, mean arterial blood pressure decreased significantly (125 ± 4 to 108 ± 5 mmHg), cardiac output fell significantly (2.66 ± .60 to 2.18 ± .60 $L.min^{-1}$) and maximum dp/dt decreased from 2475 ± 200 to 2088 ± 216 [Kleinert et al., 1986]. The authors concluded that ANF did not have a negative inotropic effect and when dp/dt was corrected for developed left ventricular systolic pressure (dp/dt/P), ANF infusion appeared to actually improve cardiac function. A similar effect of α-hANF on dp/dt has been reported in humans undergoing cardiac catheterization [Indolfi et al., 1989]. In this study the infusion of ANF, sufficient to raise plasma concentrations of ANF to 630 ± 141 $pM.L^{-1}$, slightly reduced mean arterial blood pressure and increased ejection fraction (72 ± 5 to 77 ± 4 %) and dp/dt (1270 ± 91 to 1375 ± 99 $mmHg.sec^{-1}$).

Studies documenting the apparent negative inotropic effects of ANF are somewhat obscure and difficult to interpret. Volpe and co-workers [Volpe et al., 1987b], in patients with essential hypertension, administered a bolus of α-hANF (167 $pM.kg^{-1}$) followed by an ANF infusion of 17 or 34 $pM.kg^{-1}.min^{-1}$. No significant hemodynamic effects were noted at the lower infusion rate. However, at the higher infusion rate, ANF administration was associated with a decrease in mean arterial blood pressure (133 ± 5 to 119 ± 5 mmHg), a fall in cardiac output (3.7 ± 0.2 to 3.3 ± 0.2 $L.min^{-1}$), and a reduction in stroke volume index (46 ± 4 to 39 ± 3 $ml.m^{-2}$). There was no change in total peripheral resistance and dp/dt/P was not affected. Although cardiac output and stroke volume index were reduced in this study, the parameter selected as a measure of cardiac contractility (dp/dt/P) was unchanged. Concurrent reductions in ventricular preload and impedence in this study, make interpretation of relative contractile performance in the myocardium almost impossible. A significant reduction in dp/dt_{50} (dp/dt measured at a left ventricular systolic pressure of 50 mmHg), during ANF infusion in rats, has been reported [Seymour et al., 1987]. Unfortunately, definitive interpretation is again obscured by concurrent variations in ventricular preload, afterload and the use of a synthetic, non-physiologic forms of ANF.

Force development in cardiac muscle is related to cellular calcium concentration and the length-dependent calcium sensitivity of the contractile proteins [Kentish et al., 1986]. If ANF does exert a negative inotropic effect on cardiac muscle it is reasonable to postulate that it may affect calcium kinetics. Gisbert and

Fischmeister [1988], using the whole cell patch clamp technique in frog ventricular cells, have found that Atriopeptin III and rat ANF ($\sim 10^5$ pM) had no effect on basal calcium currents. However, if the cells were pre-stimulated with isoproterenol (ISO), ANF decreased the ISO-elevated calcium currents by an average of 33 %.

Coronary Circulation. The vast majority of studies present in the literature have documented a vasodilator effect of atrial natriuretic factor, both in vitro [Rapoport et al., 1986; Yanagisawa and Lefer, 1988] and in vivo [Bache et al., 1988; Bauman et al., 1987; Chu et al., 1989; Chu and Cobb, 1987; Laxson et al., 1988]. Atypically, a vasoconstrictor effect of ANF has been described [Wangler et al., 1985].

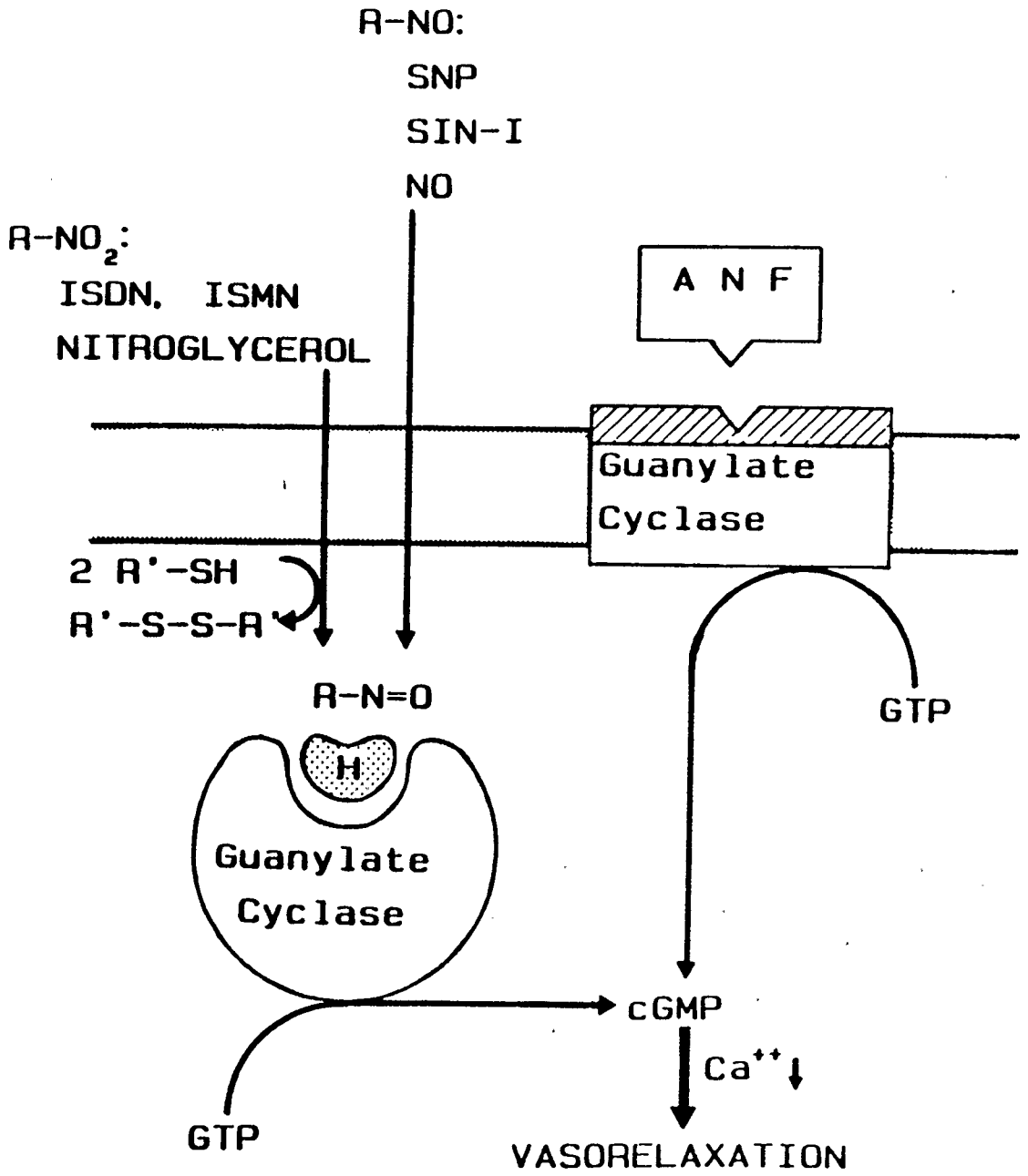
Rapaport et al. [1986] have reported that atriopeptins I, II, and III all induced relaxation in strips of human coronary artery. An increase in cGMP production was associated with atriopeptin administration and may have mediated its vasodilator effect (see Figure 1.4). More recently, Yanagisawa and Lefer [1988] found that synthetic $ANF_{(8-33)}$ produced a concentration dependent reduction in perfusion pressure, indicative of vasodilation, when feline arteries were infused with ANF containing solutions (ANF at 10^{-9} to 10^{-7} M). This effect was endothelial independent and was not blocked by methylene blue, a soluble guanylate cyclase inhibitor.

Bolus injection of α-hANF and atriopeptin II into the left circumflex arteries of awake dogs (3300 $pM.kg^{-1}$), produced a 61 %

FIGURE 1.4

REGULATION OF CYCLIC GMP FORMATION BY ATRIAL NATRIURETIC FACTOR AND NITROVASODILATORS

Atrial natriuretic factor activates the membrane-bound particulate guanylate cyclase. This enzyme is a glycoprotein. Nitrovasodilators activate soluble guanylate cyclase. These enzymes differ in various aspects and their actions are independently regulated. In vascular smooth muscle cells, increased formation of cGMP by either enzyme induces vasorelaxation. NO_2-containing compounds ($R\text{-}NO_2$); isosorbide dinitrate (ISDN); isosorbide mononitrate, ISMN; NO-containing compounds (R-NO); sodium nitroprusside (SNP); bioactive metabolite of molsidomine (SIN-1); heme of soluble guanylate cyclase (H). From Gerzer et al., [1987]. Reproduced with the permission of Springer-Verlag.


R-NO:
SNP
SIN-I
NO
R-NO2:
ISDN, ISMN
NITROGLYCEROL
A N F
Guanylate
Cyclase
2 R'-SH
R'-S-S-R'
R-N=O
H
Guanylate
Cyclase
GTP
cGMP
Ca++
GTP
VASORELAXATION

increase in peak coronary blood flow [Laxson et al, 1988]. The increase in coronary blood flow was dose-dependent and was not altered by adenosine receptor blockade with 8-phenyltheophylline, or by cyclo-oxygenase inhibition with indomethacin. In a similar study performed in α-chloralose anesthetized dogs, α-hANF (660 $pM.kg^{-1}$) caused a 27 ± 4 % reduction in coronary resistance [Bache et al., 1988]. Again, indomethacin and 8-phenyltheophylline, in addition to propranolol, had no effect on the vasodilator effect of ANF.

Using radionuclide-labelled microspheres to assess regional transmural blood flow, Bauman et al. [1987] found that bolus infusion of ANF (830 pM and 1660 pM) into the left circumflex coronary artery of anesthetized dogs, increased coronary blood flow by 41 %. The endocardial/epicardial blood flow ratio remained constant during ANF infusion indicating that the observed vasodilatation occurred uniformly throughout the ventricular wall. In the presence of a flow-limiting coronary stenosis in dog hearts, sub-endocardial blood flow decreased and sub-epicardial blood flow increased when blood flow was compared to a non-ischemic region [Chu et al., 1989]. The sub-endocardial/sub-epicardial blood flow ratio in the ischemic region was 0.56 ± 0.03 compared to 1.18 ± 0.04 in the non-ischemic region. Following pre-treatment of the preparation with a bolus infusion of α-hANF (1000 $pM.kg^{-1}$), sub-endocardial blood flow to the ischemic region increased significantly. The sub-endocardial/sub-epicardial blood flow ratio increased to 0.91 ± 0.10. Thus, in the presence of a flow limiting coronary stenosis, pre-treatment with ANF favourably redistributed coronary blood flow to the sub-endocardium,

supporting a vasodilator role for ANF in the intramural arteries [Chu et al., 1989].

Coronary vasoconstriction in conjunction with ANF administration was demonstrated in isolated, Langendorff perfused guinea-pig hearts [Wangler et al., 1985]. Atriopeptin II in concentrations of 10^3 to 10^5 pM, administered via coronary artery bolus, produced a dose-dependent decrease in coronary blood flow. As stated previously, this result represents an anomaly in the vasodilator literature. It is difficult to easily reconcile two such diametrically opposed effects. Experimental differences in animal models, perfusion systems, types and quantities of ANF utilized, acute versus chronic studies, and differences in routes of administration all combine to render meaningful interpretation of these differing effects virtually impossible. It is likely of significance, however, that additional studies, documenting this vasoconstrictor effect of ANF, have failed to appear in the literature.

Peripheral Vascular Effects. The effects of atrial natriuretic factor in the peripheral circulation, for the most part, mirror those of the coronary circulation. ANF infusion into healthy, human volunteers [Ebert, 1988; Roy et al., 1989; Tikkanen et al., 1985a], sheep [Parkes et al., 1988], dogs [Bie et al., 1988; Lee and Goldman, 1989], and rabbits [Volpe et al., 1988] has consistently resulted in a reduction in mean arterial blood pressure. Although part of this effect may be secondary to renal artery dilatation and an increase in urine flow [Dunn et al., 1986], Volpe et al. [1988]

found that ANF infusion in anephric rabbits also produced a significant decrease in mean arterial blood pressure (97 ± 4 to 90 ± 2 mmHg) and a reduction in circulating blood volume. The reduction in circulating blood volume has been associated with an increase in haematocrit secondary to a shift of plasma water across the vessel walls into the interstitium [Williamson et al., 1989]. The hypotensive response to ANF infusion has been observed at ANF concentrations (75 to 167 $pM.L^{-1}$) known to occur in volume overload states such as congestive heart failure [Bie et al., 1988]. The decrease in cardiac output observed during ANF infusion in these studies, has been attributed to a decrease in cardiac filling pressures [Bie et al., 1988; Ebert, 1988; Lee and Goldman, 1989]. The decline in filling pressures may be related to a decrease in total blood volume [Lee and Goldman, 1989; Parkes et al., 1988] or ANF induced venodilatation [Roy et al., 1989]. Finally, Faber and associates have demonstrated, in rat cremaster skeletal muscle, that ANF exhibits a high potency and selectivity for reversal of α_1-adrenoreceptor-mediated constriction of large arterioles and venules. Thus, the ability of ANF to alter resistance in the microvasculature may depend on the relative contribution of α_1-, α_2-, and intrinsic vasospastic elements in determining the prevailing level of smooth muscle tone [Faber et al., 1988].

CLINICAL PATHOLOGICAL CORRELATES AND ATRIAL NATRIURETIC FACTOR

Increases in plasma ANF levels have been described in a myriad of medical conditions (see Table 1.3). Of direct interest to the

present group of studies, is the effect of myocardial infarction on plasma ANF levels.

Tomoda [1988] has reported that acute myocardial infarction in humans elevates plasma ANF levels, and this elevation is correlated with pulmonary capillary wedge pressure (PCWP). In this study, ANF levels appeared to have some of prognostic significance in that the two patients with the highest levels died within one week of their infarct. However, patients were not screened to exclude baseline elevation of ANF levels prior to infarction. A similar correlation between plasma ANF levels and PCWP was reported by Matsubara and co-workers in patients undergoing right-heart catheterization within ten hours after the onset of infarction [Matsubara et al., 1987].

Rector et al. [1985], reported that left coronary artery ligation in rats decreased the diuretic-natriuretic potency of atrial extracts. In this model, chronic left atrial distension secondary to elevated left ventricular filling pressures may have depleted atrial stores of ANF. In a similar rat model, however, Wilkes et al. [1987] found that congestive heart failure secondary to left coronary artery ligation, was associated with increased plasma levels of ANF. The contradictory results of these two studies are impossible to resolve in retrospect but may relate to differences in sampling intervals, assay techniques, or type of rat utilized.

TABLE 1.3

CLINICAL CONDITIONS ASSOCIATED WITH ELEVATED PLASMA LEVELS OF ATRIAL NATRIURETIC FACTOR

Congestive Heart Failure

Anderson et al., 1988
Burnett et al., 1986
Creager et al., 1988
Drexler et al., 1989
Fyhrquist and Tikkanen, 1988
Franch et al., 1988
Moe et al., 1989
Molina et al., 1988
Tsutamoto et al., 1989
Webster et al., 1989

Myocardial Infarction

Matsubara et al., 1987
Tomoda, 1988

Congenital Heart Disease

Matusoka et al., 1988
Ross et al., 1988

Cardiac Dysrhythmias (pacing induced)

Crozier et al., 1989
Ellenbogen et al., 1988a,b
Nicklas et al., 1989
Roy et al., 1987
Tikkanen et al., 1985b
Tsai et al., 1988

Exercise

Donckier et al., 1988
Richards et al., 1987a

Carcinoid Heart Disease

Lundin et al., 1989

Hypertension

Sagnella et al., 1986

SUMMARY

The discovery of an endocrine function within cardiac tissue has had a vast ripple effect throughout the scientific community. Atrial natriuretic factor is a peptide hormone whose twenty-eight amino acid structure has been precisely determined, as has the gene structure for ANF and its complete transcription process. Receptors for ANF, and a possible physiologic role, are found in relatively high numbers in the heart, lung, liver, kidney, adrenals, and central nervous system. Formation of ANF takes place primarily in atrial tissue, although under certain circumstances, ventricular tissue is capable of dramatically increasing its ANF production.

Release of ANF from cardiac tissue is likely both receptor mediated and secondary to alterations in the atrial (and possibly ventricular) stress/strain relationship. Intravascular volume expansion, mechanical distension of the atria, and cardiac pacing may all cause ANF release. In addition, increased plasma levels of ANF have been identified in congestive heart failure, hypertension, congenital heart disease, carcinoid heart disease, during strenuous exercise and cardiac dysrhythmias, and following myocardial infarction.

Of specific interest with regard to the present group of studies is the relationship between atrial pressure and ANF release and the inotropic effect of ANF on cardiac tissue. A brief review of the relevant literature concerning these topics is presented with each chapter. In general terms, however, it is important to focus the previous review on the experiments which follow.

The studies contained within this dissertation were designed to answer questions regarding the mechanisms of ANF release and possible effects on cardiac muscle function following release. Although the literature indicates a likely relationship between atrial pressure and ANF release, difficulties in correctly assessing pericardial pressure have led to confusion regarding the true effect of atrial pressure on ANF release. By measuring pericardial pressure with a flat, liquid-containing balloon, the initial study focuses on the experimentally determined transmural pressure and its effect with regard to release of ANF. In the second study, the effect of ANF on force development in cardiac muscle is investigated. In order to remove confounding variables such as changes in preload and afterload, this study was performed in isolated rat cardiac trabeculae. Thus, these studies were undertaken in an attempt to further define important variables controlling the release of ANF from cardiac tissue and, once released, whether ANF is likely to have any significant effect on cardiac muscle function.

CHAPTER II

METHODOLOGY

An outline of the general methods is presented with the individual experiments. However, three of the measurement methods utilized in the current series of studies are outside the mainstream of standard laboratory techniques. In an effort to facilitate reader understanding and appreciation, it is the intent of this section to provide an introduction to these measurement methods.

RADIOIMMUNOASSAY FOR ATRIAL NATRIURETIC FACTOR

Measurement Principle and Background

Blood plasma is separated from whole blood by centrifugation. ANF is then pre-extracted from plasma using C_{18} reverse phase columns (Sep-Pak cartridges) or assayed directly. (Extracted ANF values are not reported in these experiments). Dried ANF extract is redissolved in assay buffer prior to radioimmunoassay (RIA). After a twenty-four hour preincubation with the first antibody (raised against atrial natriuretic factor in a rabbit), a set of standards, quality controls, and unknown sera are incubated with a constant amount of ^{125}I-ANF. This complex is then precipitated with a goat antirabbit IgG antiserum. The competition between labelled and unlabelled hormone for binding sites on the antibody results in a progressive inhibition of

the binding of labelled hormone as the concentration of unlabelled hormone is increased. The concentration of hormone in the unknown sample is determined by comparing the degree of inhibition observed in the sample with that produced by standard solution containing a known concentration.

Radioimmunoassays for ANF have also been described in the literature [Richards et al., 1987b] and in 1987 an international standard for ANF was established with the authorization of the Expert Committee on Biological Standardization of the World Health Organization [Poole et al., 1988]. The following protocol was developed by Dr. Paul Keane and Ms. Teresa Wong, Endocrinology Laboratory, Foothills Provincial Hospital, Calgary, Alberta.

Normal Ranges

The normal value for unextracted plasma ANF in healthy volunteers, in this laboratory, is 55 ± 10 $pM.L^{-1}$. Normal values for extracted samples have yet to be determined.

Factors Influencing Accuracy of the ANF Assay

Control studies performed in the Endocrinology Laboratory, Foothills Provincial Hospital, have demonstrated that plasma ANF concentrations measured by the above radioimmunoassay technique

decrease after plasma is heat treated at 37°C for two hours or allowed to sit at room temperature for two hours, prior to sample purification by C_{18} extraction cartridges. Following plasma extraction by C_{18} cartridges, ANF is stable for up to sixteen hours at room temperature and for up to two weeks in a - 20°C freezer. Prolonged storage (four weeks) of unpurified ANF at - 80°C has been reported to decrease plasma ANF concentrations by as much as 20 % [Lijnen et al., 1988]. Hemolysis was found to lower the measured value of ANF and this finding is in agreement with the literature [Lijnen et al., 1988]. In our laboratory, the addition of aprotinin (50 $KIU.ml^{-1}$ of plasma) did not alter the stability of ANF at room temperature before sample purification.

During the initial development of an ANF assay, determination of ANF levels was made directly from blood plasma. In the last few years however, an increasing number of laboratories now utilize a technique in which plasma for ANF assay is "filtered" through a set of extraction or separation cartridges in an effort to obtain a relatively pure product on which to perform the radioimmunoassay. Although the exact mechanism by which plasma extraction produces its effect is presently unknown, it has been speculated that extraction may remove ANF prescursors or other plasma proteins of similar molecular weight from the blood sample, thereby reducing their interference with the RIA [Yandle et al., 1986]. The net effect of this extraction process is to increase the sensitivity of the assay, and this step is of particular importance at plasma levels of ANF below 30 $pM.L^{-1}$ [Gutkowska et al., 1986]. At the plasma concentrations of

ANF measured in the present series of studies (approximately 70 to 140 $pM.l^{-1}$), there is virtual linearity between the value of ANF measured by unextracted or extracted techniques [Gutkowska et al., 1986]. Conversion from unextracted to extracted values is possible by applying their regression equation ($y = 1.14x + 32$) to obtain approximate extracted values for ANF in $pM.L^{-1}$ [Gutkowska et al., 1986].

Effect of Sampling Site on Measured Plasma Level of ANF

The ANF levels measured in plasma are, to some extent, dependent on the site selected for sample collection. ANF levels are usually highest when measured in samples obtained from coronary sinus effluent [Bates et al., 1989; Fitzpatrick et al., 1989]. The level decreases markedly in the right atrium and pulmonary artery as cardiac venous drainage mixes with venous blood from the whole body [Ballerman and Brenner, 1986]. There is a slight decrease in the level of ANF as it passes through the pulmonary circulation and then the level increases in the left side of the heart and the arterial circulation [Bates et al, 1989]. Peripherally, tissue extraction and/or receptor binding of ANF results in an arterio-venous ANF gradient. The ANF content of venous blood may be as little as 50 % when compared to arterial blood [Goetz, 1988b] and there is considerably more variability present in ANF levels measured in venous rather than arterial blood samples [Schutten et al., 1987].

In the studies presented in Chapter III, the femoral artery was selected as the ANF sampling site. This site was selected for three major reasons: 1) Ease of catheter placement and collection; 2) Blood levels of ANF are maximal on the arterial side of the circulation (apart from coronary sinus blood), and therefore, any changes in cardiac secretion of ANF during the experiments should be maximized; and, 3) Measurement of plasma ANF levels on the arterial side of the circulation should minimize any variations present as a consequence of altered tissue metabolism and/or extraction of ANF.

Equipment Required to Perform Assay

1. 12x75 mm polystyrene tubes
2. 12x75 mm disposable borosilicate glass tubes
3. 13x1090 mm disposable borosilicate glass tubes
4. Disposable plastic syringes - 3 ml, 5 ml, and 30 ml
5. Precision Glide needle - 18G 1 1/2
6. Micro-line tubings which fit 18-20 gauge needles
7. A rack to hold C_{18} cartridges
8. MLA pipettes - 100 µL, 300 µL, and 1000µL
9. Eppendorf repeater pipettes - 100 µL and 250 µL
10. Ice bath
11. 4 ^{o}C centrifuge
12. Gamma counter

Reagent

Reagents are usually ordered in bulk. Individual suppliers for the performance of this assay are listed under each reagent.

A. Composition of Phospho-saline Buffer, pH 7.4, 100 $mM.L^{-1}$

sodium phosphate monobasic	($NaH_2PO_4.H_2O$)	19 $mM.L^{-1}$	2.62 g
sodium phosphate dibasic	(NaH_2PO_4)	81 $mM.L^{-1}$	12.63 g
sodium chloride	(NaCl)	40 $mM.L^{-1}$	2.92 g

1. Dissolve in 900 ml of doubled distilled deionized water.
2. Make up to one litre.
3. Check the pH of the buffer. It should be 7.4 ± 0.05.
4. Store at 4°C.
5. Make buffer every six months.

B. α-Human-Atrial Natriuretic Polypeptide (ANF 1-28)

M.W. 3080.95 $g.M^{-1}$

1. Obtained from Peninsula Laboratories Inc., catalogue number 8798 (current lot number - 010207).
2. Contains 0.2 mg lyophilized standard per vial.
3. Stock standard - 162.288 $\mu M.L^{-1}$
   a. Dissolve 0.2 mg lyophilized ANF in 0.4 ml of acetic acid (0.1 $M.L^{-1}$).
   b. Store 0.15 ml aliquots in - 70° C freezer.
4. Working standard - 1622.88 $nM.L^{-1}$
   a. Dilute 0.1 ml of stock standard with 9.9 ml of phosphate buffer solution (1 $g.L^{-1}$).

b. Store 0.3 ml aliquots in - 70°C freezer.

5. Check standard concentration of each new lot number by comparing values with the previous lot number.

C. Rabbit Anti - Atrial Natriuretic Polypeptide Serum (RAS-hANF)

1. Obtained from Peninsula Laboratories Inc., catalogue number RAS 8798 (current lot number 009325-7).
2. Stock antiserum
   a. Reconstitute vial with 25 ml of double distilled deionized water (D^3H_2O).
   b. Store 1 ml aliquots in - 70°C freezer.
3. Working antiserum
   a. Dilute 1 ml of antiserum with 1 ml of Triton X-100 (0.2 $ml.100^{-1}$ ml d-ddH_2O) and 1 ml of assay buffer.
   b. Prepare working antiserum fresh for each essay.

D. Normal Rabbit Serum (NRS-500)

1. Obtained from Peninsula Laboratories Inc., catalogue number NRS-500 (current lot number 011534).
2. Reconstitute vial with 50 ml of assay buffer.
3. Store 10 ml aliquots at 4°C.
4. Aliquots may be stored for up to six months.

E. Goat Anti - Rabbit IgG Serum (GARGG-500)

1. Obtained from Peninsula Laboratories Inc., catalogue number GARGG-500 (current lot number 012159).
2. Reconstitute vial in 50 ml of assay buffer.
3. Store 10 ml aliquots at 4°C.

4. Aliquots may be stored for up to six months.

F. Human (3-[^{125}I]iodotyrosyl^{28})-Atrial Natriuretic Factor, ^{125}I-hANF

1. Obtained from Amersham Corporation, catalogue number IM 187.
2. Contains 5 µCi per vial.
3. Specific activity is ~2000 Ci.mM^{-1}.
4. Stock ^{125}I-hANF
   a. Reconstitute vial with 1 ml of D^3-H_2O.
   b. Aliquot in 0.25 ml immediately after reconstitution (~5 minutes) and store in - 70°C freezer.
   c. Aliquots may be stored for up to two months after date of analysis by Amersham.
5. Working ^{125}I-hANF
   a. Prepare fresh for each assay.
   b. Dilute stock ^{125}I-hANF with assay buffer to give approximately 10,000 cpm per 0.1 ml.

G. Composition of assay buffer

Phospho-saline buffer solution - 100 mM.L^{-1}	200 ml
Triton X-100	0.2 ml
Bovine serum albumin	0.2 g

1. Allow Triton X-100 and bovine serum albumin to dissolve in the phospho-saline buffer.
2. Mix well.
3. Prepare fresh for each assay.

H. BSA/PSB ($1\ g.L^{-1}$)

1. Dissolve 0.1 g Bovine serum albumin in 100 ml phospho-saline buffer (PSB).

I. BSA

1. Obtained form Sigma Chemical Company, catalogue number A-7888 (current lot number 62F-00771).

J. Acetic Acid - $100\ mM.L^{-1}$

1. Dilute 5.7 ml of glacial acetic acid to one litre with D^3-H_2O.

K. Hydrochloric Acid - $2mM.L^{-1}$

1. Dissolve 33.2 ml concentrated hydrochloric acid to 200 ml with D^3-H_2O.
2. Remake every six months.

L. Extraction Cartridges

1. C_{18} separation cartridges obtained from Waters Associates, catalogue number 51910 (current lot number P7215A1)
2. Cartridges can be used a total of three times.

M. Trifluoroacetic Acid

1. Obtained form Pierce Chemical Company, catalogue number 28902 (current lot number 870113089).
2. HPLC/spectro grade
3. Contains one gram per vial.
4. Store at room temperature.

5. Unopened vial is good indefinitely.
6. Working TFA - 0.1 % (V/V)
   a. Dissolve one ml of stock Trifluoroacetic acid in approximately 500 ml of D^3-H_2O.
   b. Make up to one litre.
   c. Prepare fresh for each assay.

N. Acetonitrile

1. Obtained from Fisher Scientific Company, catalogue number A-988.
2. HPLC grade.

O. Methanol

1. Obtained from Fisher Scientific Company, catalogue number A-408B
2. Certified A.C.S. - spectroanalyzed.

P. Acetonitrile/Trifluoroacetic Acid (TFA) Mixture (60:40)

1. Mix 60 ml of acetonitrile with 40 ml of working TFA (0.1%).
2. Prepare fresh for each assay.

Specimen Requirement

Collection Volumes

1. Adult collection volume: 3 ml - 2 runs
   1.5 ml - 1 run
2. Micro collection volume: 1.5 ml - 2 runs
   0.8 ml - 1 run

Specimen Collection Procedure

1. Draw blood into a chilled 7 ml EDTA vacutainer.
2. Keep blood on ice after collection and during transit.
3. Add 20 ml of aprotinin (10,000 $KIU.ml^{-1}$) to 7 ml of EDTA blood.
4. Mix by gentle inversion.
5. Centifuge blood using 1500g at 4° C for thirty minutes.
6. Aliquot 1.5 ml of plasma in duplicates and freeze in - 70° C freezer.

Sample Extraction and Purification Procedures

A. Sample Purification by C_{18} cartridges

1. Regeneration of C_{18} cartridges prior to sample application (if required).
   a. Wash with 5 ml of methanol.
   b. Wash with 5 ml of D^3-H_2O.

2. Plasma acidification: **Note:** The following procedures are done in an ice bath.

   a. Pipette 1 ml of cold plasma into 12, 75 mm disposable borosilicate glass tubes with an MLA pipette.

   b. Acidify with 0.25 ml of hydrochloric acid (2 $mM.L^{-1}$)

   c. Vortex

   d. Centrifuge at 4 °C for ten minutes at 3500 rpm.

   e. Decant into clean 12x75 mm disposable borosilicate glass tubes.

3. Sample purification

   a. Apply each sample onto a regenerated C_{18} cartridge.

   b. Reapply the discard sample through the cartridge two more times.

   c. Wash with 15 ml of TFA (0.1 % V/V).

   d. Elute sample with 4 ml of acetonitrile/TFA mixture (60:40) and collect into a 13x100 mm disposable borosilicate glass tube.

   e. Dry samples at room temperature (drying time approximately 5-6 hours).

   f. Reconstitute each sample with 0.3 ml of cold assay buffer.

   g. Store sample at 4 °C prior to assaying.

Radioimmunoassay

Radioimmunoassay is set in a ice bath.

1. Construct a standard curve according to the following dilution scheme:

Cold Assay Buffer Volume (ml)	Standard Volume (ml)	Standard Concentration
0.9	0.1	162.29 $nM.L^{-1}$
0.9	0.1	16.229 $nM.L^{-1}$
1.8	0.2	1.6229 $nM.L^{-1}$
1.2	0.8	649.2 $pM.L^{-1}$
1.0	1.0	324.2 $pM.L^{-1}$
0.2	0.8	259.7 $pM.L^{-1}$
0.4	0.6	194.8 $pM.L^{-1}$
0.5	0.5	128.8 $pM.L^{-1}$
0.5	0.5	64.9 $pM.L^{-1}$
0.5	0.5	32.5 $pM.L^{-1}$
0.5	0.5	16.2 $pM.L^{-1}$
0.5	0.5	8.1 $pM.L^{-1}$
0.5		0

2. Pipette 0.1 ml in duplicate for each of the standards, extracted quality controls and extracted unknown samples into appropriate 12x75 mm polystyrene tubes with a pipette.
3. Pipette 0.2 ml of assay buffer, in duplicate, into "NSB" tubes.
4. Dispense 0.1 mls of working RAS-hANF to all tubes except "TC" and "NSB" tubes with an Eppendorf repeater pipette.
5. Shake racks.

6. Incubate assay at 4°C for 20-24 hours.
7. Dispense 0.1 ml of working ^{125}I-hANF (~10,000 CPM) to all tubes with an Eppendorf repeater pipette.
8. Shake racks.
9. Incubate assay at 4°C for an additional 24 hours.
10. Dispense 0.1 ml of working NRS-500 to all tubes except "TC" tubes with an Eppendorf repeater pipette.
11. Dispense 0.1 ml of working GARGG-500 to all tubes except "TC" tubes with an Eppendorf repeater pipette.
12. Shake racks.
13. Incubate assay at room temperature for 2 hours.
14. Dispense 0.5 ml of assay buffer to all tubes except "TC" with an Eppendorf repeater pipette.
15. Immediately centrifuge assay at 4°C for 12 minutes at 4500 rpm in a refrigerated centrifuge.

Calculation of ANF PLasma Concentration

A. Automatic Calculation

1. Count on Capintec CAP RIA 16 gamma counter using 4 parameter reduction program.
2. Correct for extraction volume:

   $pM.L^{-1}$ ANF from printout x 0.3 ml/1.0 ml

   0.3 ml = specimen volume used in extraction

   1.0 ml = volume of assay buffer used to dissolve dried extract

3. Diluted sample will be corrected for the appropriate dilution factors.

B. Manual Calculation

1. Calculate % B.
2. Plot % B versus standard concentration for each standard.
3. Read ANF in $pM.L^{-1}$ off graph.
4. Correct for extraction volume:

   $pM.L^{-1}$ ANF from printout x 0.3 ml/1.0 ml

   0.3 ml = specimen volume used in extraction

   1.0 ml = volume of assay buffer used to dissolve dried extract

3. Diluted sample will be corrected for the appropriate dilution factors.

MEASUREMENT OF MUSCLE SARCOMERE LENGTH

Sarcomere length was measured by laser diffraction techniques which have been described previously [de Tombe, 1989; Iwazumi and Pollack, 1979; Kentish et al., 1986; Krueger and Pollack, 1975; Pollack and Krueger, 1976; ter Keurs et al., 1980]. The pattern of striations present in a cardiac muscle trabecula will act as an optical diffraction grating when illuminated (i.e., with laser light). The trabecula diffracts the laser beam into a zero order band and multiple, spatially symmetrical, higher order band pairs [Krueger and Pollack, 1975; Pollack and Krueger, 1976]. The sine of

the angle between the zero order band and the first order diffraction band is inversely proportional to the sarcomere length of the muscle and the wavelength of laser light. By projecting one of the first order diffraction bands onto a scanning, photodiode array, this angle can be determined.

The median sarcomere length of the preparation is considered to be reflected by the position of the first order intensity distribution. This was determined by an analog circuit, after correction for the contribution of the zero order diffraction band and scatter [Iwazumi and Pollack, 1979]. This correction is normally quite small provided only thin muscle preparations are studied. The median position of the intensity distribution was determined by comparing the integral of the intensity distribution within one observation window to the integral of the intensity distribution from the previous window. When the current integral was equal to 50 % of the previous scan, the position of the median arc of intensity of the first order diffraction pattern was stored in a sample and hold circuit. The output of the sample and hold circuit was converted into a voltage, proportional to sarcomere length, by means of a non-linear amplifier. The transfer function of this amplifier was calibrated by laser illumination of glass gratings, with a standardized grid pattern, placed in the same position as the muscle preparation. One sarcomere length was measured and recorded every 500 µsec [de Tombe, 1989].

MEASUREMENT OF PERICARDIAL PRESSURE

It can be reasoned that at end-diastole, cardiac intracavitary pressure must exactly equal the pressure present within the walls of the myocardium (transmural pressure, P_{tm}) plus the pressure exerted by any external constraints (in this case the pericardium) (see Figure 2.1). Thus, pericardial pressure can be defined as intracavitary pressure minus the transmural pressure and can be calculated by determining intracavitary pressure when the pericardium is closed and intracavitary pressure, at the same cardiac chamber volume, when the pericardium has been removed [Smiseth et al., 1987b] (see Figure 2.2).

Traditionally, pressure in the pericardial space has been determined with an open-ended, fluid-filled catheter [Kenner and Wood, 1966]. This measurement technique assumes that pressure within the pericardium, i.e., liquid pressure, obeys Pascal's Law and therefore, is exerted equally in all directions [Smiseth et al., 1987a]. Although this technique is probably valid when there is a substantial amount of fluid present in the pericardial sac [Smiseth et al., 1985; Smith et al., 1987], it may be void under physiologic conditions. Normally, there is only a thin, lubricating layer of hypotonic, plasma-like fluid present within the pericardium [Shabetai, 1985]. Under these conditions, the pressure exerted on the cardiac surface by the pericardium is analogous, anatomically, to the pressure developed within the pleural space. Agostoni [1986] has coined the term "surface pressure" to describe pleural pressure. It

FIGURE 2.1

THE STATIC EQUILIBRIUM OF FORCES WITHIN THE MYOCARDIUM AT END-DIASTOLE

Schematic illustration of the static equilibrium of forces applied to a unit area (i.e., stresses) within the heart. Intracavitary pressure is exactly opposed by the transmural pressure that the ventricle can sustain at that volume plus the equivalent pressure of the pericardium (force.unit^{-1} area). When the pericardium is excised and the lungs are retracted, intracavitary pressure is equal to transmural pressure because pericardial pressure is zero. From Tyberg and Smith, [1987]. Reproduced with the permission of Martinus Nijhoff Inc.

$P_{P(R)}$ = Right ventricular pericardial pressure

$P_{RV(TM)}$ = Right ventricular transmural pressure

P_{RV} = Right ventricular pressure

P_{TS} = Transeptal pressure

P_{LV} = Left ventricular pressure

$P_{LV(TM)}$ = Left ventricular transmural pressure

$P_{P(L)}$ = Left ventricular pericardial pressure

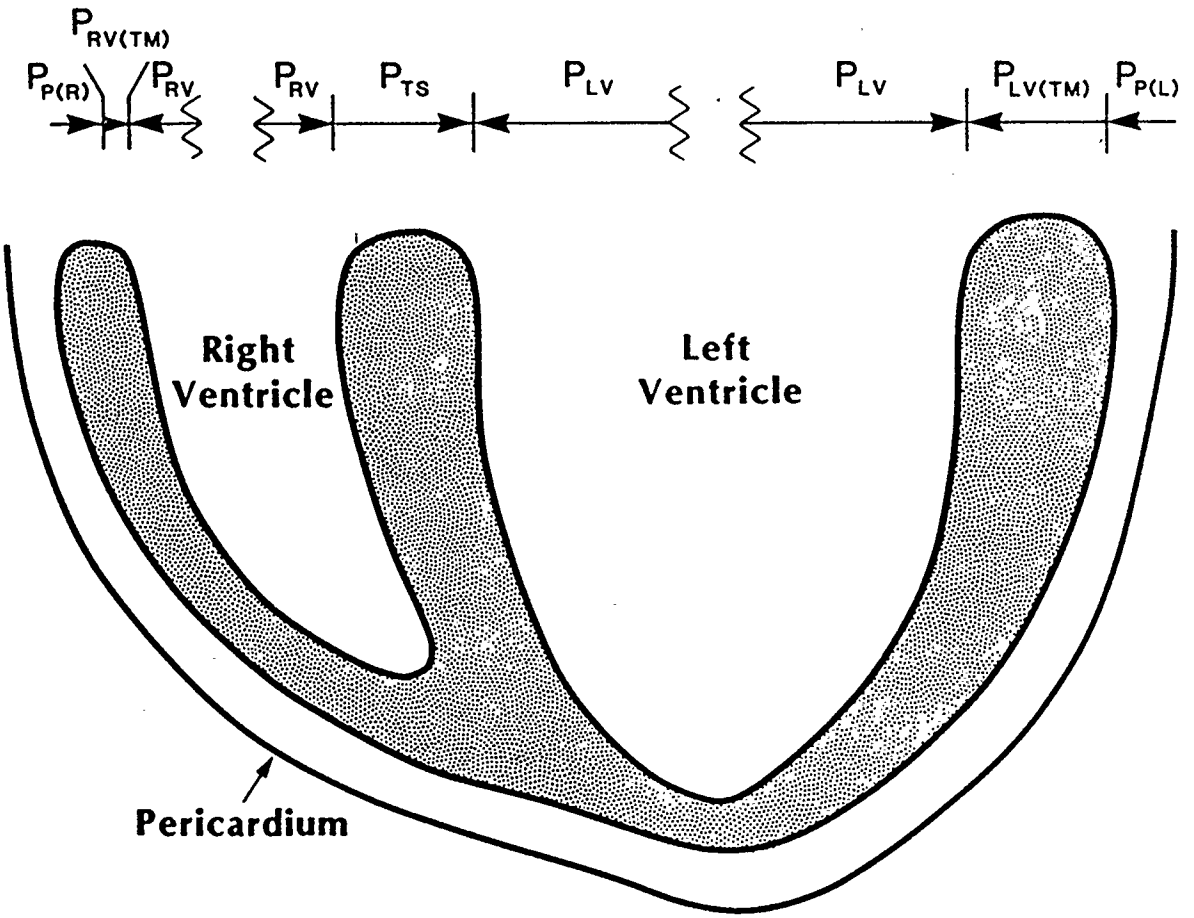
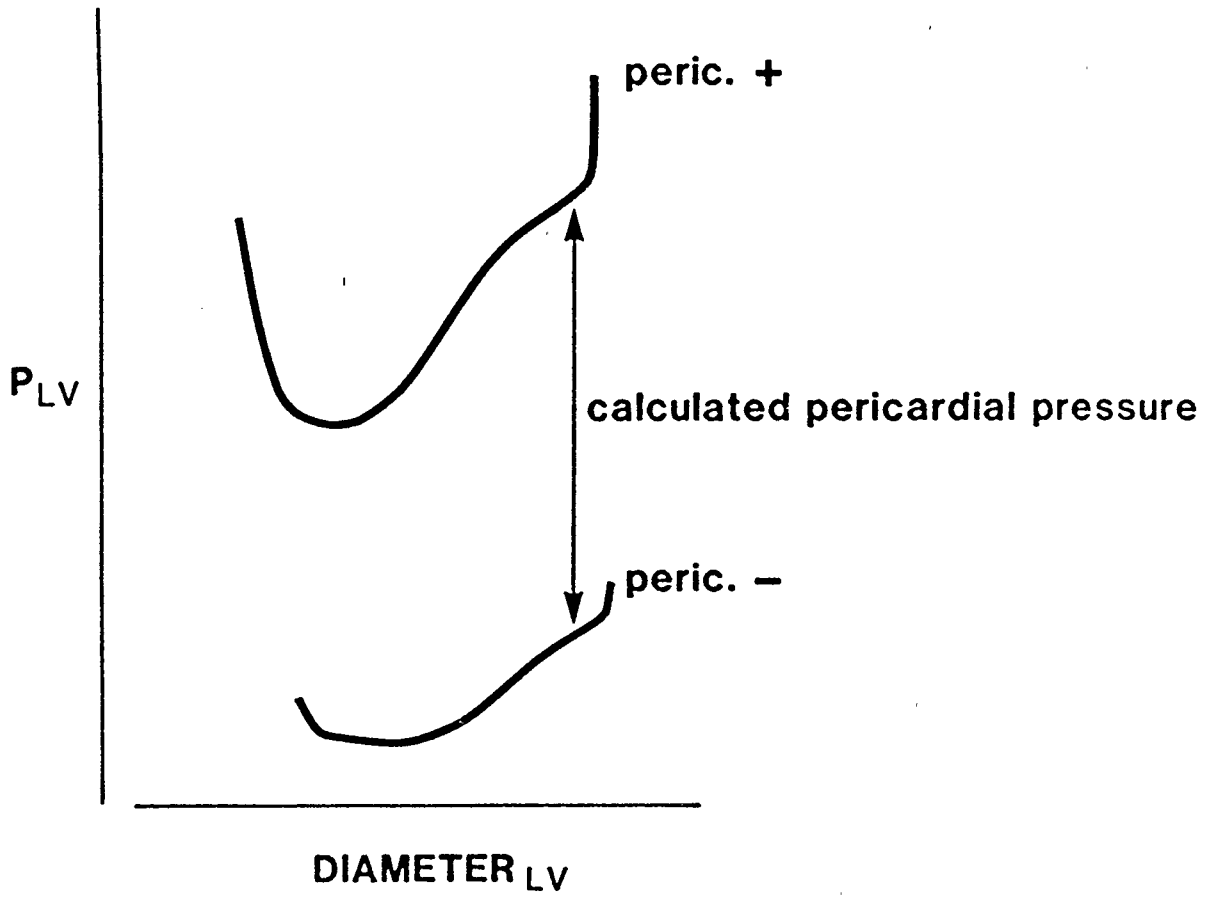

$P_{RV(TM)}$
$P_{P(R)}$
P_{RV}
P_{RV}
P_{TS}
P_{LV}
P_{LV}
$P_{LV(TM)}$
$P_{P(L)}$
Right Ventricle
Left Ventricle
Pericardium

FIGURE 2.2

DEFINITION OF CALCULATED ("TRUE") PERICARDIAL PRESSURE

Schematically, the upper curve represents left ventricular diastolic pressure vs diameter with the pericardium closed and the lower curve that after the pericardium has been removed. The lower curve therefore represents the directly measured left ventricular transmural pressure and the upper curve represents the sum of the left ventricular transmural pressure and a pressure caused by the presence of the pericardium. The decrease in left ventricular pressure (at a given diameter) after removal of the pericardium is defined as the calculated pericardial pressure. P_{LV} = left ventricular pressure. From Smiseth et al. [1985]. Reproduced with the permission of the American Heart Association.


peric. +
P_{LV}
calculated pericardial pressure
peric. –
DIAMETER $_{LV}$

is defined as the force per unit area exerted by one surface on another surface. Surface pressure is not a "pure" pressure, in the Pascal sense, and according to the Agostini interpretation represents the sum of the liquid pressure and the contact stress (the pressure generated by opposition of the pericardial surface with the cardiac surface) [Smiseth et al., 1987]. Consequently, a transducer capable of measuring both contact stress and liquid pressure should provide the most complete estimate of surface pressure.

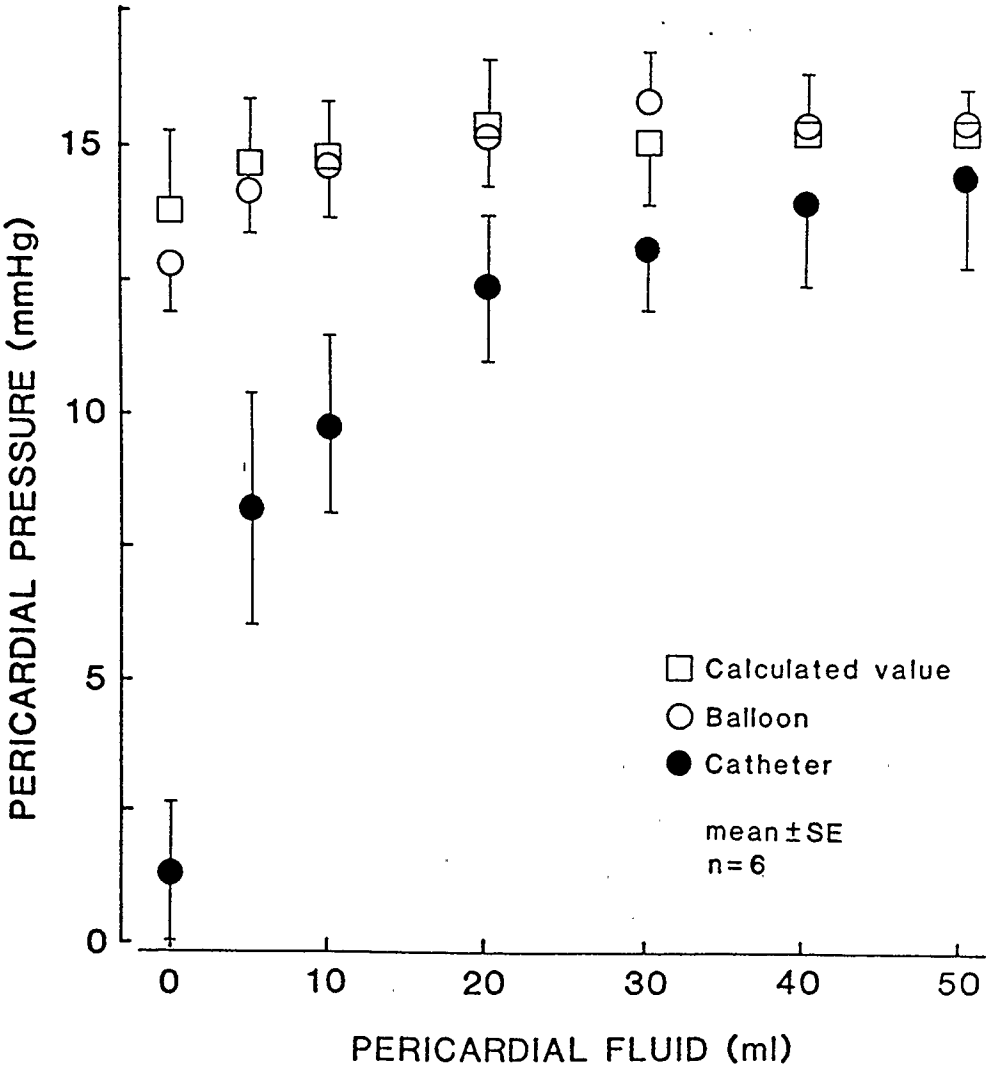
In Chapter III, a flat, liquid-containing, silastic balloon is used to measure pericardial pressure. Smiseth et al. [1985] have shown this type of pericardial pressure transducer faithfully tracks calculated pericardial pressure (see Figure 2.3) and is superior to an open-ended catheter when there is less than 30 ml of fluid present in the pericardial space. The balloon itself is composed of silastic, maintains a relatively flat profile when filled with fluid, and is approximately 9 cm^2 in area.

Calibration of the balloon is relatively simple. After ensuring that the balloon and its attached catheter are completely free of bubbles, the balloon is then placed onto a deformable diaphragm and secured within a plastic calibration chamber. The balloon catheter is then attached to a pressure transducer. Pressure within the chamber is varied with a mercury manometer, usually over a range of 0 to 30 mmHg, and fluid is added to or withdrawn from the balloon until there is a linear relationship between the pressure measured within the calibration chamber, as determined by the balloon

FIGURE 2.3

MEASURED AND CALCULATED PERICARDIAL PRESSURES
AT VARIOUS PERICARDIAL FLUID VOLUMES

Measured and calculated pericardial pressure at various pericardial fluid volumes. Note that the balloon (open circles) measures pressures that approximate the calculated values (open squares). The open-ended catheter (closed circles), however, measures pressures substantially below the calculated value unless the pericardium contains a significant volume of fluid. From Smiseth et al. [1985]. Reproduced with the permission of the American Heart Association.


PERICARDIAL PRESSURE (mmHg)
15
10
5
0
0
10
20
30
40
50
PERICARDIAL FLUID (ml)
Calculated value
Balloon
Catheter
mean ± SE
n = 6

and the pressure measured by a fluid-filled catheter in the chamber. At this point the balloon is ready for placement within the pericardium (see Chapter III).

CHAPTER III

PERICARDIAL PRESSURE ATTENUATES RELEASE OF ATRIOPEPTIN IN VOLUME-EXPANDED DOGS*

ABSTRACT

The role of the pericardium in the release of atriopeptin (AP) was examined, utilizing two separate protocols, in α-chloralose-anesthetized dogs. Protocol I consisted of an experimental group (9 dogs), in which the pericardium was incised to allow instrumentation and reapproximated, and a control group (6 dogs), in which the pericardium was left undisturbed. In the experimental group, mean right atrial pressure (P_{ra}) was elevated from a control value of 1.8 ± 0.9 mmHg (mean ± SD) to 8.3 ± 0.8 mmHg for 40 minutes by volume expansion with isoncotic, lactated Ringer's solution. After this period of volume expansion, the pericardium was removed while holding P_{ra} at 8 mmHg. During volume expansion, arterial blood samples for AP analysis were taken at 5, 10, 15, 20, 30, and 40 min, pre- and postpericardiectomy. A similar protocol was followed in the control group. At a P_{ra} of 8 mmHg prepericardiectomy, the plasma AP concentration was 76 ± 17 $pM.L^{-1}$ and 74 ± 38 $pM.L^{-1}$ in the

* This chapter was previously published in the American Journal of Physiology 1989;256:H648-H654. It is reproduced with the permission of the American Physiological Society.

experimental and control groups, respectively. However, after pericardiectomy, AP levels increased significantly in both the experimental group (136 ± 41 $pM.L^{-1}$; $P < 0.001$) and the control group (107 ± 53 $pM.L^{-1}$; $P < 0.025$). In Protocol II (6 dogs), the pericardium was removed before volume expansion, and P_{ra} was elevated by 2- to 3-mmHg increments and maintained for periods of 13 min at each pressure. AP concentration did not increase until P_{ra} reached 3-4 mmHg. These results indicate that the pericardium offers a substantial constraining influence on the cardiac chambers, which attenuates atrial distension and thus release of AP, when intracavitary pressure is increased. Manipulation and instrumentation of the pericardium did not significantly alter plasma AP levels.

INTRODUCTION

It is now well established that release of atriopeptin (AP) from cardiac tissue is related to mechanical distension of the atria [Agnoletti et al., 1987; Akabane et al., 1988; Anderson and Bloom, 1987; Bilder et al., 1986; de Bold and de Bold, 1987; Edwards et al., 1988, 1986; Goetz et al., 1986b; Mancini et al., 1987; Raine et al., 1986]. Although a number of studies imply that atrial distension results simply from an increase in intracavitary atrial pressure [Akabane et al., 1987; Burnett et al., 1986; Dietz, 1987; Goetz et al., 1986a; Ledsome et al., 1985; Raine et al., 1986; Rodeheffer et al., 1986; Shenker et al., 1985], fundamental physical principles dictate that distension of the atria can only occur as a result of increased transmural pressure, not intracavitary pressure per se.

Transmural pressure (P_{tm}) is the pressure difference across the wall of a cardiac chamber, i.e. the intracavitary pressure minus external pressure. When the pericardium is removed, external pressure is zero and transmural pressure is, by definition, equal to intracavitary pressure. However, in anesthetized dogs where the pericardium and chests were resealed, Smiseth et al. [1985] demonstrated that the pericardium offers a substantial constraining influence on the cardiac chambers and that only a transducer such as a liquid containing balloon can accurately measure this constraint. Other studies have shown that pericardial constraint (expressed as an equivalent pressure) is similar in magnitude to mean right atrial pressure [Tyberg et al., 1986]. In consideration of these physical principles, a simple, linear relation between right atrial intracavitary pressure and atrial distension, and thus AP release, should not be expected. We hypothesized that the presence of the pericardium would result in a significant attenuation of the release of AP for a given rise in atrial intracavitary pressure.

The purpose of the present study was to examine the effect of the pericardium on the release of AP from the atria of volume-expanded anesthetized dogs.

METHODS

Surgical Preparation for Protocols I and II

Two experimental protocols were carried out in 21 mongrel dogs (both male and female) weighing 20 to 25 kg. The animals were not salt restricted prior to the study and they had free access to water until two hours before the study. All animals in Protocol I (consisting of two groups, A and B) and Protocol II were anesthetized initially with sodium thiopental (approximately 25 $mg.kg^{-1}$ i.v.) and were then maintained on α-chloralose (maximum dosage was approximately 60 $mg.kg^{-1}$). The effects of these anesthetic agents on plasma ANF levels are discussed in Chapter I. Dogs were placed in the supine position on a heated surgical table (to maintain body temperature between 37 and 38° C) and ventilated by a constant-volume respirator (Model 607, Harvard Apparatus Co., Inc.; Millis, MA). Each dog was prepared with femoral arterial and venous catheters for blood sampling and delivery of anesthetic respectively. In addition, left carotid arterial and right jugular venous catheters for arterial and right atrial pressure measurements were inserted. A large bore catheter connected to an open infusate reservoir was inserted into the left jugular vein to control right atrial pressure (P_{ra}). Left and right ventricular pressures were measured with 8F micromanometer-tipped catheters with reference lumens (Model PC-480, Millar Instruments, Houston, TX). Fluid filled catheters were connected to pressure transducers (Model P23IB, Statham-Gould, Oxnard, CA). The dogs were placed on their left side (to avoid undue compression of the right atrium), and 15-20 minutes was allowed to elapse before an

initial arterial blood sample (see Chapter II for rationale concerning arterial rather than venous AP samples) for AP analysis was obtained. Following this initial AP sample, the heart was exposed through a right, fifth intercostal incision. The right lung was retracted so as not to impose any additional constraint on the right atrium. Blood gases were monitored in order to ensure adequate oxygenation.

Further surgical preparations were dependent on the particular experimental protocol. In Protocol I, the animals in Group A (9 dogs) had ultrasonic crystals (for diameter measurements) sutured to the anterior and posterior surfaces of the right atrial appendage, as close to the atrial chamber as possible. This site was chosen because it provided an area where accurate atrial dimensions could be measured with a minimum of surgical trauma required to suture the crystals in place. Suturing the ultrasonic crystals to the anterior and posterior surfaces of the right atrium proper would have required major dissection around the atrium and may have seriously damaged atrial tissue. Currently, there are no data available directly comparing the effect of ultrasonic crystal placement on assessment of atrial appendage diameter as an indicator of atrial diameter. However, the crystals were placed in the same relative position in each animal and thus any effects placement may have had on the measured atrial diameter were likely consistent within each animal studied.

Pericardial pressure was measured with a 3 x 3 cm flat liquid-containing silastic rubber balloon calibrated as described by Smiseth

et al. [1985]. The balloon was placed over the right atrium and the pericardium re-approximated. Care was taken to avoid compromising the pericardial volume. These procedures added approximately 15 to 20 minutes to the preparation of animals in Group A of Protocol I. In order to ascertain whether instrumentation of the heart and manipulation of the pericardium had any deleterious affects on results, a second group of 6 dogs (Group B), in which the heart was instrumented only with intracavitary pressure catheters and the pericardium was left intact, was included in Protocol I. In Protocol II, the pericardium was removed completely and ultrasonic crystals were placed on the right atrial appendage as outlined for Group A animals.

Experimental Protocols

Group A. After pericardial instrumentation, a 15-20 minute equilibration period was followed by collection of control samples for AP. P_{ra} was then increased to 8 mmHg (over 2-3 minutes), with warmed, isoncotic lactated Ringer's solution. This pressure was selected because we felt that subsequent to pericardiectomy, a transmural atrial pressure of 8 mmHg would represent a substantial stimulus to atrial stretch and ANF release. The pressure of 8 mmHg was maintained for 40 minutes by adjusting the height of an open fluid reservoir. After this period of volume expansion with an intact pericardium (VE1), a rapid increase in transmural pressure was effected by pericardiectomy. P_{ra} (now equal to P_{tm}) was maintained at 8 mmHg by continuous volume infusion. This second period of

volume expansion (VE2), without the pericardium, was also maintained for 40 minutes. Arterial blood samples for AP analysis were taken during a control period prior to volume expansion and at 5, 10, 15, 20, 30 and 40 minutes during VE1 and at the same time intervals following pericardiectomy (VE2). The total duration for each of the experiments in Group A, including surgical preparation and an experimental protocol of 90 minutes, was approximately 4 hours.

Group B. In order to ensure that incision and instrumentation of the pericardium did not artefactually alter plasma AP values, a series of 6 control experiments were performed. These animals were prepared as described above except that the pericardium was not disturbed. After recording initial pressures and obtaining a blood sample for AP determination, P_{ra} was increased to 8 mmHg (over a 2-3 minute period) with warmed, lactated Ringer's solution (VE1). Arterial blood samples for AP analysis were obtained at 5, 10, 15, and 20 minutes after P_{ra} reached 8 mmHg. The pericardium was then removed rapidly rendering P_{ra} equal to P_{tm}. P_{ra} was maintained at 8 mmHg with volume infusion (VE2) and blood samples for AP were obtained at 5, 10, 15, and 20 minutes post-pericardiectomy. Results from Group A, as well as from Agnoletti et al. [1987], indicated that maximum circulating AP concentrations were obtained 5-10 minutes following an increase in atrial distension. Therefore, the collection of data was continued for only 20 minutes post-volume load. Total experimental duration for this part of Protocol 1 was approximately 3 hours.

Protocol II (6 dogs) was designed to examine the release of AP as a continuous function of atrial transmural pressure and distension. In these animals the pericardium was completely removed prior to volume expansion so that pericardial pressure was equal to atmospheric pressure throughout the experiment. Otherwise the instrumentation and positioning of the animals was the same as Protocol I. P_{ra} was increased by 2-3 mmHg increments from an initial value of approximately 1 mmHg to a final pressure of approximately 8 mmHg. Each sampling period lasted 13 minutes. P_{ra} was held at each pressure increment for 10 minutes (sufficient time for stabilization of plasma ANF levels, i.e. 5 times the plasma half-life of ANF, ~2 minutes), prior to obtaining three sequential arterial samples at 1 minute intervals for AP analysis. Hemodynamic data and AP values obtained at each pressure increment were averaged to obtain a single value for each 13 minute sample period.

Analytical Methods

Pressures, diameters and ECG were recorded on an 8-channel chart recorder (Gould, Model 17-6500, Cleveland, OH). Data were also recorded in digitized form on a Minc-23 (Digital Equipment Corp., Maynard, MA) data acquisition system. Data grabs of 1 minute duration were collected at the time of each blood sample. Data were subsequently analyzed on a VAX/VMS system (Digital Equipment Corp., Maynard, MA). Only sinus initiated cardiac cycles at end expiration were analyzed.

AP was measured by a commercial heterologous radio-immunoassay (RIA) (Peninsula Laboratories, Belmont, CA) on cold, coded, unextracted plasma samples drawn from the femoral artery. See Chapter II for specific details of the assay. In this RIA, the minimal detectable quantity of α-hANP was less than 2.5 $pM.L^{-1}$; intra- and inter-assay variability for multiple measurements were 6.0 and 8.0 % respectively; the recovery of cold α-hANP (100 $ng.ml^{-1}$) added to the plasma bank solution was 93 %. The assay is not affected by pH variations within the physiologic range.

Statistics

Repeated measures analysis of variance (ANOVA) was used to test for significant trends within Groups A and B of Protocol I. Individual significant differences between control values and the values obtained immediately before and 5 minutes after pericardiectomy within groups in Protocol I, were determined by paired t-tests. To account for multiple comparisons, the significance levels for t-testing were adjusted according to the Bonferroni inequality [Glantz, 1987]. Differences between groups A and B in Protocol I were analyzed by one-way analysis of variance and Student-Newman-Keuls test for multiple comparisons between groups. All results are reported as the mean ± one standard deviation.

RESULTS

Protocol I

There was no difference in circulating arterial levels of AP in samples taken prior to opening the chest (74 ± 3 $pM.L^{-1}$) and samples taken during the control period after thoracotomy and instrumentation of the heart (73 ± 10 $pM.L^{-1}$). Figure 3.1 displays the overall trends in the cardiovascular parameters measured for Protocol I. Control values, the values obtained at the end of VE1, and the values obtained 5 minutes into VE2 of Protocol I are shown in Figure 3.2. Mean right atrial pressure was elevated significantly ($p<0.001$) from initial values of 1.8 ± 0.9 mmHg and 1.2 ± 1.1 mmHg in Groups A and B respectively, to 8.3 ± 0.8 mmHg and 8.3 ± 0.3 mmHg during VE1 immediately prior to removal of the pericardium. There was also a significant increase ($p<0.01$) in transmural pressure from 0.3 ± 0.7 mmHg during the control period to 1.9 ± 1.7 mmHg during VE1 in Group A. As a consequence of this increase in transmural pressure, a small, statistically significant increase ($p<0.01$) in atrial appendage diameter was also observed while the pericardium was intact. Following pericardiectomy, P_{ra} (now equal to P_{tm}) was held at 8.2 ± 0.5 mmHg (Group A) and 8.3 ± 0.2 mmHg (Group B). On removal of the constraining influence of the pericardium, appendage diameter in Group A increased significantly from 12.2 ± 2.8 mm during VE1 to 16.7 ± 4.6 mm during VE2 ($p<0.01$).

The plasma AP levels for Protocol I are shown in Figure 3.1 and Figure 3.2. During VE1, there was no significant change in the

FIGURE 3.1

TEMPORAL CHANGES IN DATA FROM PROTOCOL I

Data from Protocol I for right atrial diameter (D_{ra}), transmural pressure (P_{tm}), right atrial pressure (P_{ra}), plasma atriopeptin (AP), and heart rate (HR). Control values were obtained ~ 20 min after completion of surgical instrumentation, before volume loading. Other values were obtained at 5, 10, 15, and 20 min of volume expansion with pericardium intact (VE_1) and the pericardium off (VE_2). P_{tm} and D_{ra} were not measured in group B. (**** $P<0.001$; *** $P<0.01$; ** $P<0.05$). (From Stone et al. [1989]. Reproduced with the permission of the American Physiological Society.

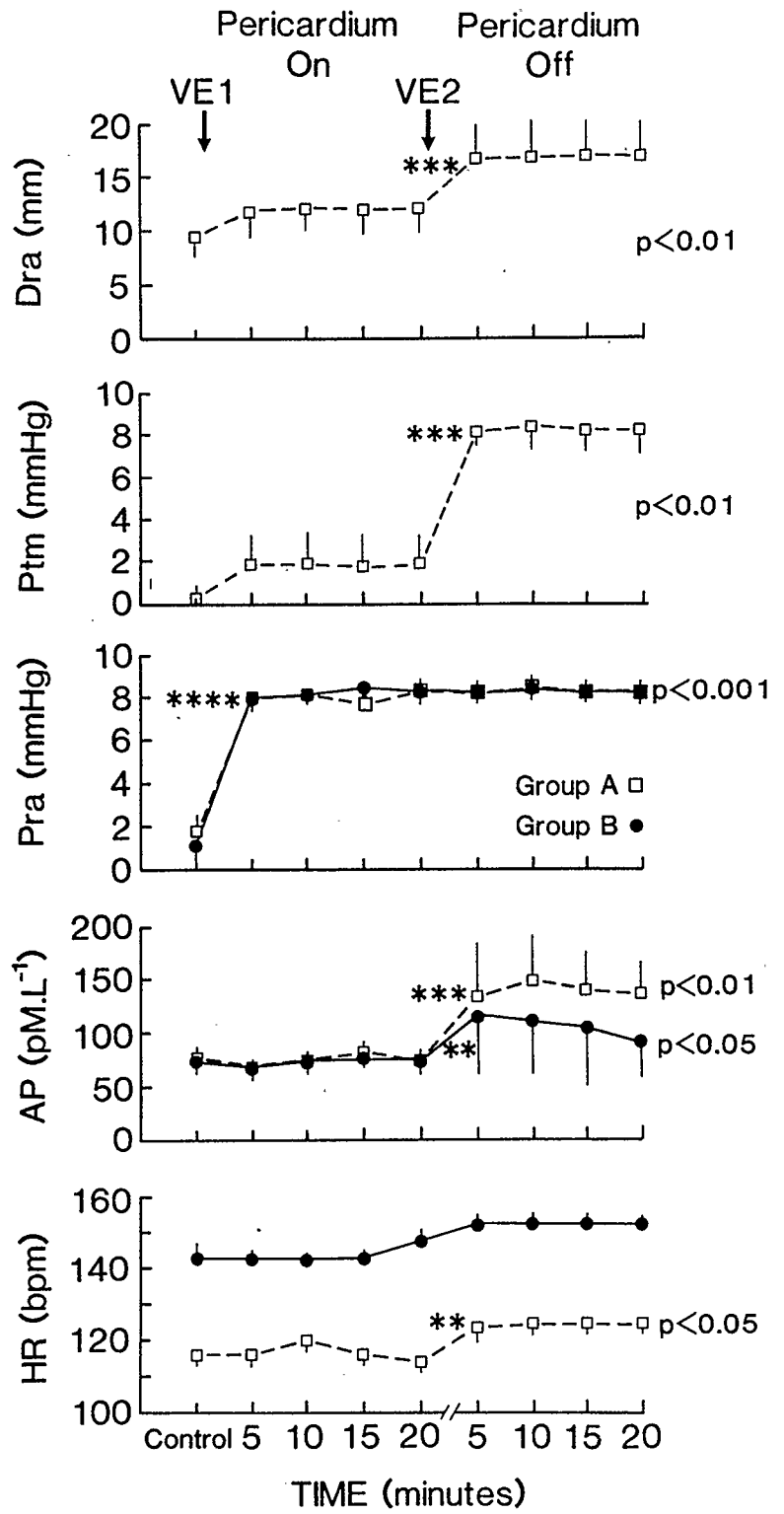
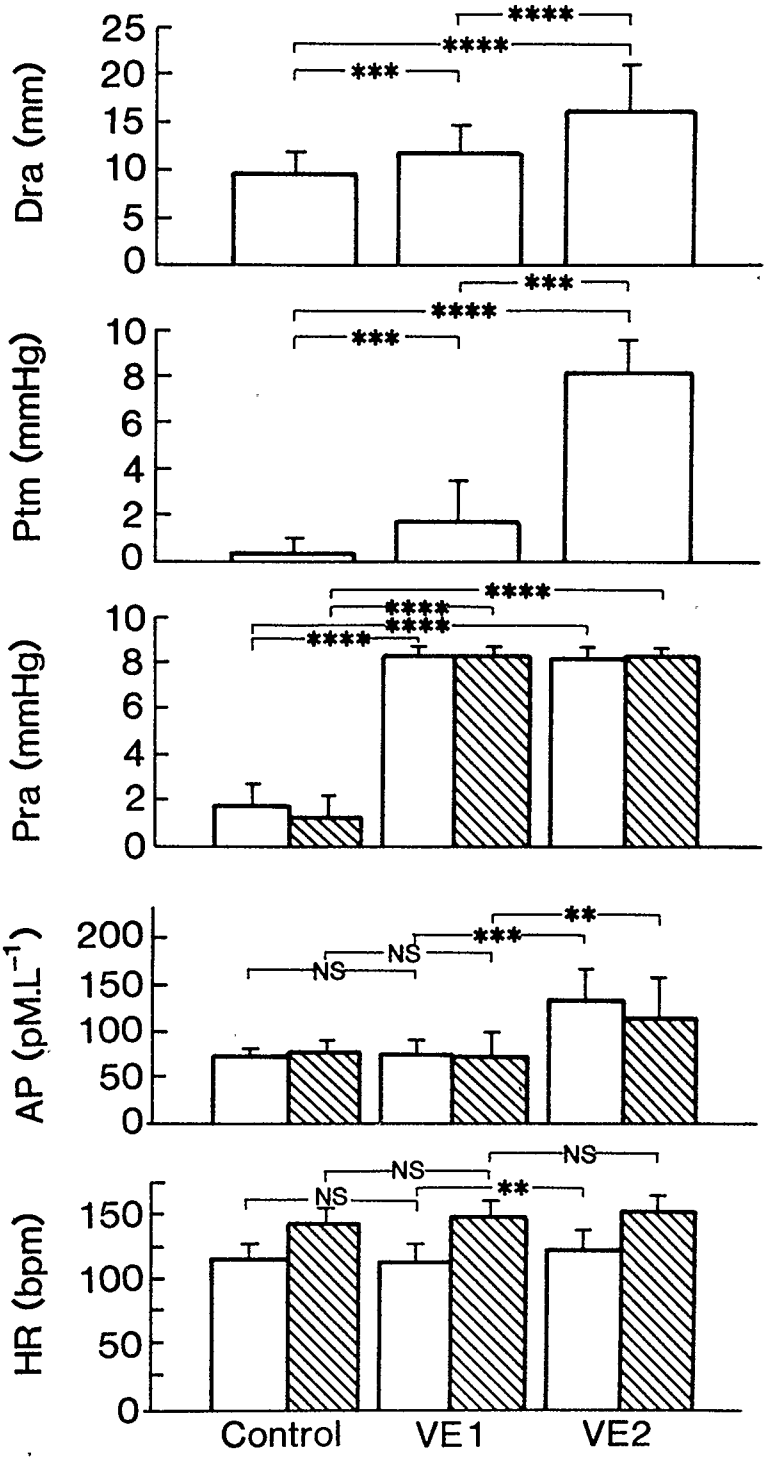

Pericardium On
Pericardium Off
VE1
VE2
Dra (mm)
20
15
10
5
0
***
p<0.01
Ptm (mmHg)
10
8
6
4
2
0
***
p<0.01
Pra (mmHg)
10
8
6
4
2
0
****
p<0.001
Group A □
Group B ●
AP (pM.L-1)
200
150
100
50
0
***
p<0.01
**
p<0.05
HR (bpm)
160
140
120
100
**
p<0.05
Control 5 10 15 20 5 10 15 20
TIME (minutes)

FIGURE 3.2

MEAN VALUES FOR DATA FROM PROTOCOL I

Mean values from protocol I (Group A, open bars; Group B, shaded bars) for right atrial diameter (D_{ra}), transmural pressure (P_{tm}), right atrial pressure (P_{ra}), plasma atriopeptin concentration (AP), and heart rate (HR). Control values were obtained ~ 20 min after completion of surgical instrumentation, before volume loading. Values for VE_1 were measured 20 min after start of volume load, immediately before pericardiectomy. Values for VE_2 were obtained 5 min after removal of pericardium. P_{tm} and D_{ra} were not measured in group B. (**** $P<0.001$; *** $P<0.01$; ** $P<0.05$; NS, not significant). (From Stone et al. [1989]. Reproduced with the permission of the American Physiological Society.


Dra (mm)
25
20
15
10
5
0
****
****
***
Ptm (mmHg)
10
8
6
4
2
0
***
****
***
Pra (mmHg)
10
8
6
4
2
0
****
****
****
****
AP (pM.L^{-1})
200
150
100
50
0
**
***
NS
NS
HR (bpm)
150
100
50
0
NS
NS
NS
**
Control
VE1
VE2

AP concentration from control, in either Group A (76 ± 17 $pM.L^{-1}$) or Group B (74 ± 38 $pM.L^{-1}$). Following pericardiectomy, the plasma AP concentration in both groups increased significantly to 134 ± 58 $pM.L^{-1}$ ($p<0.01$) and 117 ± 65 $pM.L^{-1}$ ($p<0.05$) for groups A and B, respectively. There were no statistically significant differences in the mean plasma AP levels between groups A and B.

Heart rate in Group A, during the control period (116 ± 15 bpm), did not increase during VE1 (114 ± 11 bpm). It did rise significantly ($p<0.05$) during VE2 to 124 ± 17 bpm. In Group B, the heart rate changes from an initial value of 143 ± 21 bpm to 148 ± 14 bpm during VE1, and 153 ± 14 bpm during VE2, were not statistically significant.

Protocol II

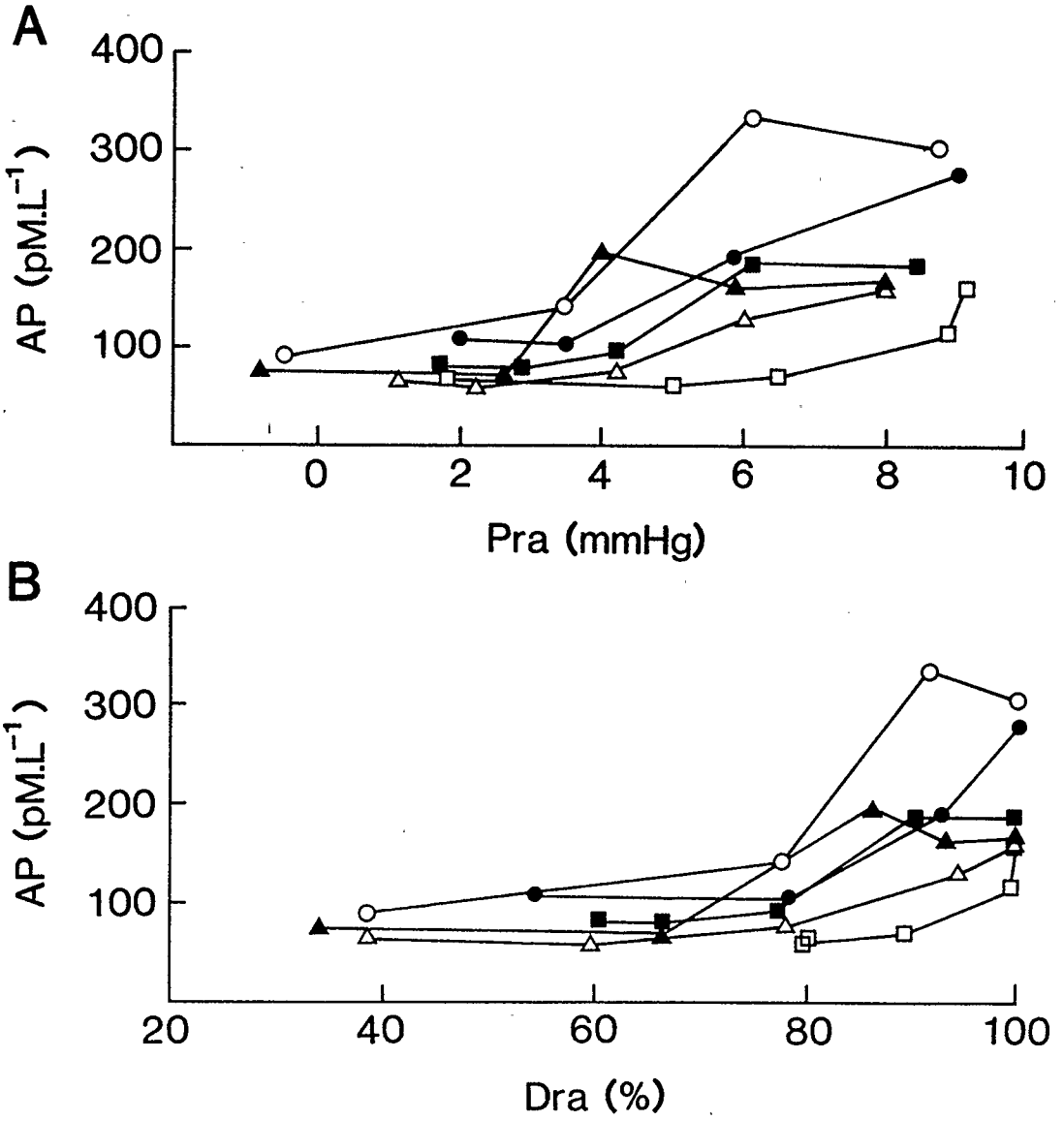
In Protocol II, the pericardium was completely removed at the beginning of the experiment and P_{ra} was increased by 2-3 mmHg increments for intervals of 13 minutes at each pressure. Since there was no external constraint on the atrium, intracavitary and transmural pressures were equal. Results (Figure 3.3) are reported as the average of three sequential measurements of P_{ra}, appendage diameter and AP recorded over the final three minutes of each time interval.

It is apparent from Figure 3.3A that the AP concentration did not begin to increase until a transmural pressure of 3-4 mmHg was

FIGURE 3.3

RELATIONSHIP BETWEEN RIGHT ATRIAL PRESSURE, RIGHT ATRIAL DIAMETER, AND PLASMA LEVELS OF ATRIAL NATRIURETIC FACTOR

A: Circulating atriopeptin (AP) concentration as a function of mean right atrial pressure (P_{ra}) for protocol II. Because the pericardium was not present, intracavitary and transmural pressures are equivalent. B: Circulating AP concentration as a function of normalized appendage diameters. Maximum diameter, i.e. the largest atrial diameter recorded for each individual animal, was set at 100 %. Each symbol in A and B represents data from an individual dog used in protocol II. D_{ra}, right atrial diameter. (From Stone et al. [1989]. Reproduced with the permission of the American Physiological Society.


A
400
300
200
100
AP (pM.L^{-1})
0
2
4
6
8
10
Pra (mmHg)
B
400
300
200
100
AP (pM.L^{-1})
20
40
60
80
100
Dra (%)

attained. Figure 3.3B shows the data for right atrial diameter normalized to the maximum value attained. Atrial diameter increased to almost 80 % of its maximal diameter (i.e., the diameter achieved at a transmural pressure of approximately 8 mmHg) without any appreciable increase in plasma AP levels. Only when the atrium was distended to more than 80 % of is maximal diameter, were substantial increases in plasma AP levels observed.

There were no significant changes in heart rate observed during this protocol.

DISCUSSION

The results of this study demonstrate that the release of atriopeptin, in the anesthetized, ventilated dog, is dependent on atrial distension and not on intracavitary pressure per se. This is so because the pericardium impedes the distension of the atrium, despite increased intracavitary pressure. This effectively elevates pericardial pressure and renders transmural pressure less than intracavitary pressure. Elevation of intracavitary right atrial pressure in Group A of Protocol I from 1.8 ± 0.7 mmHg to 8.2 ± 0.7 mmHg during VE1 was associated with an increase in atrial transmural pressure only from 0.3 ± 0.7 mmHg to 1.9 ± 1.7 mmHg. Despite this significant increase in transmural pressure, no increases in plasma AP levels were observed either when the pericardium was instrumented and reapproximated (Group A) or left intact (Group B).

Although instrumentation and reapproximation of the pericardium in Group A could potentially prevent a rise in plasma AP during the initial volume loading period (VE1), this is not supported by the results obtained from Group B. Plasma AP levels did not change significantly, as a consequence of volume load alone, in either group. It is possible, however, that a modest increase in plasma AP concentration, during initial volume loading (VE1), may have been obscured by the use of unextracted plasma samples. Extraction of sample plasma, prior to AP determination, increases the sensitivity of the assay, particularly at relatively low circulating AP concentrations. However, results obtained from the utilization of plasma samples with high circulating AP levels, i.e. after removal of the pericardium, are less likely to be affected by the extraction procedure. Thus, the present observation of a large increase in plasma AP levels following removal of the pericardium, would likely not be altered by measuring AP concentration in extracted plasma. Therefore, regardless of the method used to measure plasma AP levels, transmural atrial pressure, as the primary determinant of atrial dimension, remains one of the major stimuli for AP release from canine atria.

No data are available on the specific effect of body position in ventilated, anesthetized dogs with respect to its influence on plasma ANF levels. In the present experiments, the animals were studied in the left lateral position because direct observation of the right atrium and right atrial appendage, during pilot studies, provided visual evidence of possible right heart compression and/or restriction to chamber expansion, in the supine position. Actual

comparison of plasma ANF levels in the two experimental conditions was not carried out. However, all blood samples for ANF analysis were drawn with the animals in the left lateral position and therefore, any effect of this body position on ANF levels was presumed to be constant. Similarly, the effect of positive pressure ventilation on plasma ANF levels was not addressed. Studies have indicated that positive pressure breathing may decrease plasma ANF levels compared to those measured in spontaneously breathing patients [Pacher et al., 1986]. Again, however, all ANF samples were drawn while the animals were intubated and ventilated, thus minimizing the relative effects of this intervention.

Although the increase in heart rate from VE1 to VE2 during Protocol I in Group A was statistically significant, the difference between the two groups (10 bpm) was not felt to be clinically important. Thus, it is unlikely this heart rate increase accounted for increased plasma AP levels. Similarly, the differences in heart rates between Groups A and B ($p<0.05$) of approximately 25 bpm were not felt to be of importance. It is possible that differences in vagal tone or sympathetic activation, secondary to manipulation of the atria during surgical preparation, may have accounted for the discordant results in heart rate. However, the higher heart rates recorded in Group B have been associated with increased plasma AP levels [Roy et al., 1987] rather than the lower levels observed in this study, and thus it is difficult in this instance to directly implicate differences in heart rate as an important variable in the determination of circulating plasma AP levels. Furthermore, no

statistically significant differences were noted between plasma AP levels in Groups A and B in Protocol I.

The effect of anesthesia on plasma ANF levels has been reviewed in Chapter I. In the present study, the animals in Group A of Protocol I were anesthetized for approximately four hours while those in group B were anesthetized for three hours. It is possible that this added anesthetic time had an effect on plasma ANF levels, but given the observation that ANF levels in both Groups A and B declined throughout the course of the second volume expansion, it is difficult to imagine that the additional anesthesia adversely affected the animals in Group A compared to those in Group B, i.e. although the absolute values in the two groups were dissimilar (but not significantly different), the decline in plasma ANF levels with time was the same in each group.

Numerous other studies have been performed in dogs to examine the relation between right and/or left atrial intracavitary pressure and release of AP. Akabane et al. [1988, 1987] and Zimmerman et al. [1987a] noted an increase in circulating AP levels in association with increased right atrial pressure and pulmonary capillary wedge pressure following graded volume expansion. Ledsome et al. [1985] and Goetz et al. [1986a] increased atrial pressures by mitral valve obstruction in chloralose-anesthetized and conscious dogs, respectively. Although increased levels of AP were associated with elevation of atrial pressures in each of these studies, methodological differences make it difficult to compare results quantitatively.

Both investigators [Goetz et al., 1986b; Ledsome et al., 1985] related the increase in AP concentration to relative increments in atrial pressures rather than to absolute values. In addition, the extremely high atrial pressures (greater than 15 mmHg) observed in some of these experiments [Akabane et al., 1988; Ledsome et al., 1985] makes it difficult to compare results to the relatively modest pressure increases obtained in the present study. However, of greater importance than the absolute pressures attained in these studies, is the possibility that the pressures recorded in some experiments may have actually been transmural pressures rather than intracavitary pressures. The surgical preparation of the animals in these experiments may have necessitated either complete pericardiectomy or at least extensive incision of the pericardium. As a consequence, the animals may have been effectively pericardiectomized. Therefore, the increases in AP at lower atrial pressures may have resulted because of high atrial transmural pressures.

The observation that transmural atrial pressure, rather than absolute intracavitary pressure, is a principal determinant of AP release is not particularly surprising, given that atrial transmural pressure determines the degree of atrial stretch. What is perhaps surprising in the present results is the observation that AP release is relatively insensitive to relatively small changes in transmural atrial pressure. As shown in Figure 3A, significant release of AP did not occur until transmural atrial pressure had reached approximately 4 mmHg. We are not certain what intracavitary pressure would correlate with a transmural atrial pressure of 4mmHg when the pericardium is intact. However, in this study a transmural atrial

pressure of 2 mmHg was associated with a P_{ra} of 8 mmHg. Thus, an intracavitary pressure considerably higher than 8 mmHg may be required to effect significant release of AP. Indeed, other workers have noted that atrial pressures in excess of 10 mmHg are often required to induce significant changes in AP concentration [Akabane et al., 1987; Edwards et al., 1988; Goetz et al., 1986a; Zimmerman et al., 1987a].

The time course of these experiments is also of interest. Whereas an intracavitary pressure of 8 mmHg sustained for less than an hour produces a transmural pressure of approximately 2 mmHg, it is possible that a similar intracavitary pressure, if sustained over a longer period, would be associated with the development of increased transmural pressure (i.e, a lower pericardial pressure secondary to stretch of the pericardium). This rise in P_{tm}, with a more profound atrial distension, may be due to plastic deformation and/or growth of the pericardium. Such a possibility is supported by the studies of LeWinter and Pavalec [1982]. These authors found that pericardiectomy had a negligible effect on the left ventricular pressure-dimension relationship after a sustained volume load lasting 4 to 5 weeks. The sustained volume load apparently resulted in pericardial enlargement and lower pericardial pressure. In addition, these observations may help to explain the high levels of circulating AP observed in patients with chronic congestive heart failure [Raine et al., 1986]. Sustained elevation of atrial pressures in these patients may produce pericardial enlargement resulting in reduced pericardial pressure over the atria. This would effectively increase P_{tm} and thereby facilitate the release of AP.

Results from Protocol II of the present study demonstrate that transmural pressure must increase to 3-4 mmHg before an increase in circulating levels of AP is detected (Figure 3A). Normalization of diameter measurements demonstrates that this transmural pressure correlates with approximately 80% of the diameter achieved at a transmural pressure of 8 mmHg (Figure 3B). However, this relationship is far from absolute and there is a considerable degree of non-uniformity. These data do suggest that the right atrium can be subjected to considerable pressure before major increases in plasma AP levels are detected. This possibility is strengthened by the findings of Edwards et al. [1988]. In experiments performed on dogs in which the pericardium was widely incised, the authors undertook a series of great vessel occlusions in order to raise right and left atrial pressures. The results demonstrated a linear relationship between plasma AP levels and transmural atrial pressure. However, of the 33 values reported for transmural right atrial pressure and plasma AP concentrations, only 5 plasma AP measurements were made at atrial transmural pressures exceeding 4 mmHg. Without these 5 values, there is no relationship between transmural atrial pressure and plasma AP levels. A similar argument can be applied to the findings for the left atrial transmural pressure/AP relationship. Thus, in agreement with our data, these authors found no relationship between atrial transmural pressures and plasma AP levels at transmural atrial pressures less than 4 mmHg.

The baseline value of the AP vs transmural pressure curve likely reflects those factors which determine chronic circulating hormone levels. Numerous studies involving healthy human subjects

[Luft et al., 1986; Sagnella et al., 1986; Shenker et al., 1985; Solomon et al., 1987], and rats [Tanaka et al, 1984] have indicated that steady state AP levels are related to dietary sodium intake. However, the connection between dietary sodium and AP is by no means universally observed (Luft et al. [1986] in rats and Salazar et al. [1986a] in dogs). Indeed, Buhler et al. [1987] have reported that although acute saline infusions and sodium loading over 3 to 5 days with high salt diets produced increases in plasma AP, more prolonged sodium administration resulted in AP levels which were not significantly different from control. Possibly the release and/or response of AP to a given stimulus is quantitatively dependent upon the volume status prior to the stimulus. Thus, a blunted release or response to AP might be expected in subjects who are sodium avid prior to a volume load. Conversely, subjects in a sodium expanded state might have an exaggerated response to the same volume stimulus. It would be of interest to examine changes in AP release and end organ sensitivity in response to volume changes in disease states known to be associated with higher levels of AP such as congestive heart failure [Burnett et al., 1986; Raine et al., 1986; Shenker et al., 1985], supraventricular tachycardia [Roy et al., 1987] or essential hypertension [Sagnella et al., 1986; Sugimoto et al., 1986].

Although our results demonstrate that atrial intracavitary pressure is not the stimulus responsible for release of AP, it should not therefore be concluded that transmural pressure (or atrial strain) directly determines AP release. It is not possible from our results to differentiate between the effects of an increase in distension (strain) or tangential stress as being the responsible

stimulus. The observation that plasma AP concentration may increase during pacing-induced tachycardia, without a change in P_{ra}, suggests that atrial stress may also be a determining factor [Nishimura et al., 1987; Walsh et al., 1987]. Thus, it seems likely that AP release is sensitive to both atrial wall stress and strain.

The findings of the present study are also of some clinical significance. In a recent study of a patient with cardiac tamponade, Koller et al. [1987] reported that a "paradoxical" increase in AP occurred with a decrease in right atrial pressure following pericardiocentesis. Since removal of the effusate almost certainly increased transmural pressure and thus allowed distension of the atria, the observed increase in AP actually represents the predicted rather than a paradoxical finding. This conclusion is further supported by Mancini et al. [1987]. In a recent study of cardiac tamponade performed in anesthetized dogs, these authors observed that increasing right atrial pressure by volume loading (presumably increasing transmural atrial pressure) produced a significant increase in circulating AP. However, a similar elevation in P_{ra} induced by tamponade (with no change or a reduction in transmural atrial pressure) produced a slight decrease in plasma AP levels.

In conclusion, our results demonstrate that the pericardium (or other external constraint) can significantly attenuate the release of atriopeptin in volume expanded, anesthetized dogs. An increase in intracavitary (right) atrial pressure per se is not necessarily responsible for release of atriopeptin. Hormone release is a function of either atrial distension (resulting from an increase

in transmural pressure) or tangential stress (related to transmural pressure by the law of LaPlace) or both.

CHAPTER IV

THE EFFECT OF ATRIAL NATRIURETIC FACTOR ON FORCE DEVELOPMENT IN RAT CARDIAC TRABECULAE

ABSTRACT

Studies in single cardiac muscle cells have demonstrated that atrial natriuretic factor (ANF) decreases the surface membrane L-type calcium current. Recent investigations in human atrial cells have also demonstrated that ANF causes a voltage-dependent reduction in sodium channel activity and thus may reduce intracellular calcium via decreased activity of the sodium-calcium exchange mechanism. By reducing intracellular calcium, ANF may have a negative inotropic effect on cardiac muscle. In order to characterize the effect of ANF on the development of force, we studied the force-sarcomere length relationship in 11 right ventricular rat trabeculae, both before and after exposure of the muscles to increasing concentrations of ANF. Sarcomere length was measured by laser diffraction techniques and controlled by a servomotor system. The addition of ANF to the superfusion solution, at concentrations of 10^{-9} to 10^{-7} M ($10^{3}$to 10^{5} pM), produced a dose-dependent reduction in end-systolic peak force development. Incubation of muscle preparations with concentrations of ANF below 10^{-9} M (10^{3} pM) has no effect on force generation. In addition, the twitch characteristics of the muscles were altered with ANF inducing a reduction in the time to peak muscle tension. The results of this study demonstrate, for the first time, the negative

inotropic effect of ANF in association with a change in the shape of the force-sarcomere length relationship, and suggest that this effect may be mediated by a reduction in intracellular calcium. The negative inotropic effect of ANF may have important consequences in clinical situations such as congestive heart failure.

INTRODUCTION

The length dependence and calcium concentration dependence of force development in cardiac muscle has been recognised for almost one hundred years. Landmark studies by Frank [Chapman,1959], Ringer [1883], Starling [Patterson et al., 1914], and Berglund and Sarnoff [1954] demonstrated a close relationship between the force produced by cardiac muscle and the length of the muscle prior to the onset of contraction. In the latter half of this century, we have come to appreciate that this length dependence is also closely related to end-systolic muscle length [Sagawa, 1981]. In addition, studies by ter Keurs and associates [Kentish et al., 1986] have demonstrated that force development in cardiac trabeculae is determined by the length dependent sensitivity of the contractile system to calcium ions.

The impetus for the present study derives from investigations demonstrating atrial natriuretic factor (ANF) reduces force development in vascular smooth muscle strips and decreases intracellular calcium levels in myocardial cells. Studies have shown that ANF is capable of inducing smooth muscle relaxation in both arterial [Bache

et al., 1988; Chu and Cobb, 1987; Winquist, 1985] and venous preparations [Winquist et al., 1984]. As well as these in vitro studies, the vasodilator effect of ANF has been shown following infusion of ANF in both animals [Bie et al., 1988; Parkes et al., 1988] and humans [Bolli et al., 1987]. This vasorelaxant effect of ANF is most likely secondary to a reduction in intracellular calcium and ANF has been shown to inhibit intracellular calcium release in vascular smooth muscle [Fujji et al., 1986; Meisheri et al., 1986]. In addition, activity in the slow inward calcium current of myocardial cell membranes is inhibited by ANF [Gisbert and Fischmeister, 1988] and this effect may be mediated by cyclic AMP hydrolysis via an ANF-induced cyclic GMP-stimulated cyclic nucleotide phosphodiasterase [Anand-Srivastava and Cantin, 1986; Hartzell and Fischmeister, 1986]. Thus, ANF appears to reduce intracellular calcium and may demonstrate a negative inotropic effect when exposed to cardiac muscle.

Therefore, the purpose of the present study was twofold: 1) to determine if ANF was capable of inhibiting force development in cardiac muscle; and, 2) to determine if ANF altered myocardial twitch kinetics.

METHODS

Preparation and Mounting Procedures

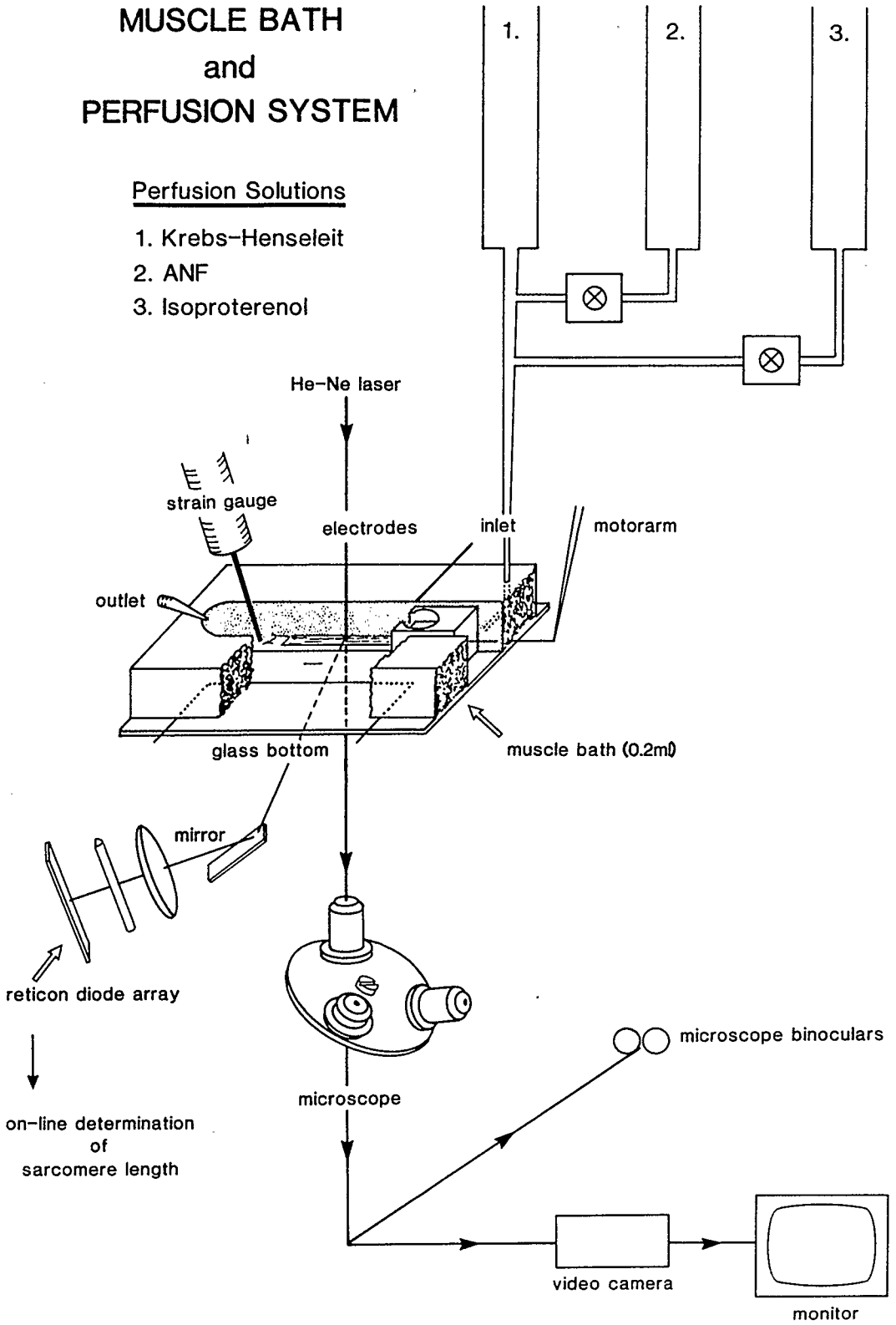
Twelve-week-old Wistar rats were anesthetized with ether. The heart was rapidly removed and transferred to a dissection chamber.

The aorta was cannulated and perfused with an oxygenated physiological salt solution at a flow rate of 5 ml/min. The right ventricle and atrium were opened exposing the free wall of the right ventricle. The papillary muscles were removed. Trabeculae attached to the atrioventricular (AV) ring and the free wall of the right ventricle were selected. One or two suitable trabeculae could be found in approximately half of the hearts examined. Ventricular and atrial tissues were dissected carefully from the AV ring, thus freeing a part of the tricuspid valve, the AV ring, and the trabecula. The portion of the ventricular free wall attached to the trabecula was also dissected. The trabecula was immediately transported to the experimental chamber which was mounted on the stage of an inverted Nikon (Diaphot) microscope (Figure 4.1). A hook on the motor arm was passed through the valve ring. The section of ventricular free wall, at the other end of the trabeculae, was passed through a stainless steel loop attached to the force transducer. Thus, the trabecula itself was left relatively undamaged. The specimen was remounted repeatedly such that all cell strands throughout the cross-section of the muscle had equal lengths at all muscle lengths. Consequently, the preparation, which usually had the shape of a ribbon, did not move in the horizontal plane but bent uniformly downward during passive shortening below slack length (preparation moved with motor control arm).

FIGURE 4.1

EXPERIMENTAL APPARATUS

Schematic diagram of muscle bath for trabeculae, the perfusion system used in these experiments, and the lightpath of a helium-neon laser (He-Ne, 15 mW laser, Spectro-Physics, model 106-2, Eugene, OR) used for measuring sarcomere length.


MUSCLE BATH
and
PERFUSION SYSTEM
Perfusion Solutions
1. Krebs-Henseleit
2. ANF
3. Isoproterenol
1.
2.
3.
He-Ne laser
strain gauge
electrodes
inlet
motorarm
outlet
glass bottom
muscle bath (0.2ml)
mirror
reticon diode array
on-line determination
of
sarcomere length
microscope
microscope binoculars
video camera
monitor

Perfusion Solutions

The perfusing solution was modified Krebs-Henseleit (K-H) solution with a calcium concentration of 0.5 mM. The solutions, K-H, and K-H with either isoproterenol (ISO) or atrial natriuretic factor added, were equilibrated with 95% O_2 and 5% CO_2; the pH in the perfusion chamber was 7.41; PCO_2, 5.3 kPa; PO_2, 80 kPa. The volume of the chamber was 2 ml and the flow-trough rate varied between 2 and 10 ml/min, depending on the concentration of ANF or ISO in the perfusing solution. Temperature of the fluid in the chamber was kept constant at 26.0°C. Under these conditions, stable responses were observed for experiments lasting 6 hours and longer.

Apparatus

Sarcomere length in the non-translating part of the muscle was measured by laser diffraction (Figure 4.1). Briefly, the intensity distribution of the first order diffraction pattern was monitored by a photodiode array (Reticon 256 EC). The median sarcomere length (SL) was computed electronically after a correction had been made for the contribution of light scattered from zero order (see Chapter II). SL could usually be measured to a resolution of 0.02 μm. Muscle length was measured and controlled with a servo motor (Cambridge Technology 300 Dual Mode Servo) with a capacitive length transducer (overall compliance of motor+arm = 0.6 μm/mN). The force transducer was a semiconductor strain gauge (AE801, AME) with a short carbon

extension arm (sensitivity = 1.5 mV/mN, compliance = 1 μm/mN, natural frequency = 2.9 kHz).

Experimental Protocol

After the muscle had been mounted, a region that did not move longitudinally was selected for diffraction study. The trabeculae were stimulated at a rate of 1.0 Hz by two platinum electrodes placed parallel to the muscle. Stimulus intensity was carefully adjusted to 20% above threshold, and the stimulus duration was 2 msec.

The data were first obtained from a series of contractions in a medium which contained no ANF. The muscle was kept at reference length (~SL = 1.9 μm) for 5-10 contractions between each series of three or four test contractions. Peak force for relations between force development and SL was derived from the final contraction of each test series. Test lengths were chosen in random order. Perfusion was then changed to solutions containing ANF (rat α-ANF, Bio-Mega Laboratories, Montreal, Quebec) in concentrations ranging from 10^{-12} M to 2.5 X 10^{-7} M or isoproterenol (ISO), alone or in combination with ANF, at concentrations of 10^{-11} to 10^{-8} M (10 to 10^{4} pM). Each series of test contractions was performed approximately 10-15 minutes after a change in solution. Studies were conducted serially with increasing ANF concentrations. At the conclusion of four of the nine experiments, the muscle bath was washed three times with ANF-free K-H perfusing solution. This was done prior to the

measurement of force development post-ANF. In a fifth experiment, the muscle bath was rinsed with ANF-free K-H solution only once.

In two additional experiments, the effect of ANF on force development in trabeculae contracting at a constant muscle length was observed. Atrial natriuretic factor, at a concentration of 10^{-7} M (10^{5} pM), was added to the superfusion medium and the effect on force development was observed.

Statistics

Force data were normalized for the maximum force obtained for each trabecula and were expressed as a percentage of the maximum control force at each sarcomere length. A non-linear regression analysis between force and sarcomere length was then performed at each ANF concentration [Kentish et al., 1986]. The recirculation fraction and the the twitch characteristics of the trabeculae were analysed by linear regression analysis [Kleinbaum and Kupper, 1978]. The regression equations for the recirculation fraction, the time to peak tension, and the time to 50% relaxation, in addition to the value of the exponent in the non-linear equations, were compared by an unpaired t-test and the significance levels adjusted through the Bonferroni inequality to account for multiple comparisons [Glantz, 1981]. The effect of ANF on force development at a SL of 1.85 μm was evaluated by one way analysis of variance and Student-Newman-Keuls test for multiple comparisons between groups [Kleinbaum and Kupper, 1978].

RESULTS

Force Development

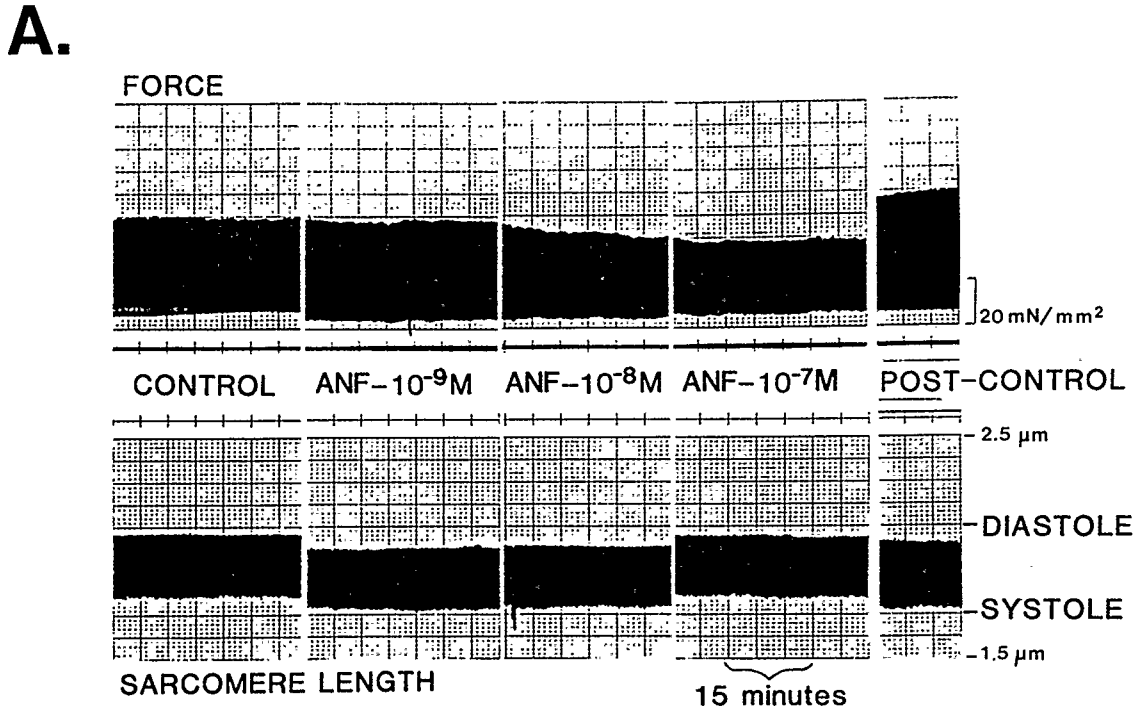
At a sarcomere length of 1.85 μm, the addition of ANF to the superfusion medium resulted in a significant ($p<0.05$) decrease in force development at ANF concentrations of 10^{-8} and 10^{-7} M (10^{4} and 10^{5} pM). Stimulus threshold of the trabeculae increased consistently by approximately 20-40 % at ANF concentrations above 10^{-9} M (10^{3} pM). The reduction in force development is shown for one typical trabecula in Figure 4.2. Comparison of peak tension at a constant systolic SL clearly demonstrates reduction of force development with increasing concentrations of ANF in the medium. The ANF concentration-force relation is demonstrated in Figure 4.3 where the data have been fitted to a Hill equation.

This effect was further evaluated in five muscles, and to ensure that it was not related to a deterioration of force generating capacity of the muscles over time, a series of contractions was analysed after return to the control solution. In four of the five experiments, force completely recovered. In one preparation, where the muscle bath had only been rinsed once with ANF-free K-H solution, force recovered to only 60% of control. This observation appeared to be secondary to the presence of residual ANF bound to structures in the muscle bath as a result of incomplete ANF washout and not secondary to muscle fatigue. Consequently, the results from this preparation were included in the post-ANF control data.

FIGURE 4.2

THE EFFECT OF ATRIAL NATRIURETIC FACTOR ON FORCE DEVELOPMENT AND TWITCH CHARACTERISTICS

A. Strip chart record of force and sarcomere length in one trabecula. When compared at a similar sarcomere length (~1.80 μm) force development in the trabecula decreased as the atrial natriuretic factor (ANF) concentration was increased. Following exposure of the trabecula to ANF, return of the muscle to control conditions resulted in a return of force to normal levels. **B.** The twitch characteristics for a single cardiac trabecula, at similar levels of developed force, are displayed. ANF, at a concentration of 10^{-7} M (10^{5} pM), produced a decrease in the time to peak tension.


A.
FORCE
20 mN/mm²
CONTROL
ANF–10⁻⁹M
ANF–10⁻⁸M
ANF–10⁻⁷M
POST–CONTROL
2.5 µm
DIASTOLE
SYSTOLE
1.5 µm
SARCOMERE LENGTH
15 minutes

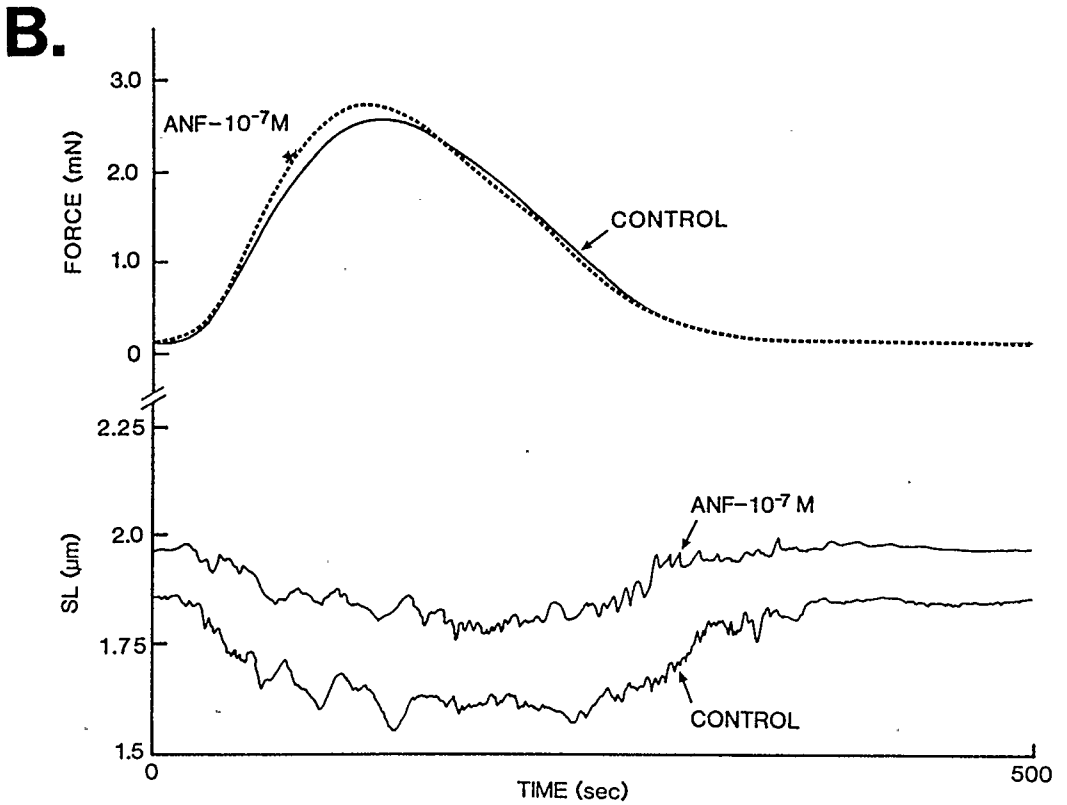

B.
FORCE (mN)
3.0
2.0
1.0
0
ANF–10⁻⁷M
CONTROL
2.25
2.0
1.75
1.5
SL (µm)
ANF–10⁻⁷ M
CONTROL
0
500
TIME (sec)

FIGURE 4.3

DOSE-RESPONSE CURVE FOR ATRIAL NATRIURETIC FACTOR

Dose-response curve for atrial natriuretic factor (ANF) concentration in the superfusion medium and the percentage reduction in force. Data were fitted to a modified Hill equation, $y = \frac{a \times c^n}{(k^n + c^n)}$. In the present data set, a = 37.3, k = 3.08, n = 0.72. Values are expressed as mean ± S.E., except below 10^{-9} M (10^3 pM) and above 10^{-7} M (10^5 pM), where only single data points were available.

* = significantly different from control and post-control, $P < 0.05$.

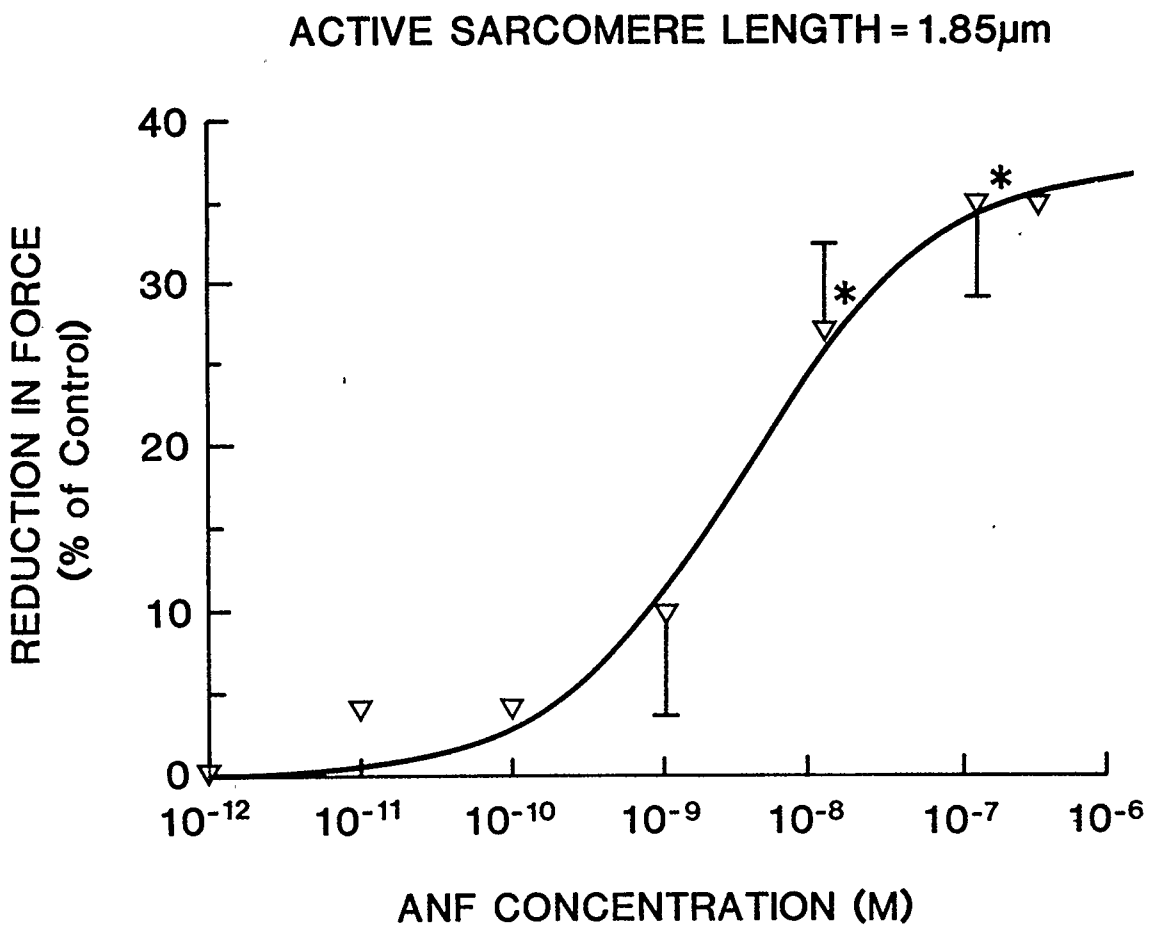

ACTIVE SARCOMERE LENGTH = 1.85µm
REDUCTION IN FORCE
(% of Control)
40
30
20
10
0
*
*
10-12
10-11
10-10
10-9
10-8
10-7
10-6
ANF CONCENTRATION (M)

Figure 4.4 depicts the relationship between force and sarcomere length for a single preparation. It can be seen that superfusion with ANF, at increasing concentrations, produced a progressive reduction in force development at all sarcomere lengths studied. Panel B of Figure 4.4 illustrates the results of non-linear regression analysis for peak force development and sarcomere length in the 10 preparations. ANF administration was associated with a non-significant change in the shape of the derived equations from concave to the abcissa for the control preparations to convex to the abcissa at concentrations of 10^{-8} M (10^{4} pM) or greater (see Table 4.1). The same change of the force-sarcomere length relation was observed when the $[Ca^{2+}]_o$ is lowered (results not shown here) [ter Keurs et al., 1980; Kentish et al., 1986]

When atrial natriuretic factor was added to two preparations contracting at constant muscle length, with no intervening pertubations of the muscle in the form of repeated force-sarcomere length determinations, the observed effect on force development was a 10 % reduction on one occasion and apparently no effect in the second instance. During a subsequent rat bioassay of the biological integrity of this ANF, intravenous infusion of high dose ANF (3000 pM) failed to produce the expected increase in urinary flow rate. Thus, the biochemical activity of this particular ANF was suspect and may account for the relative lack of effect observed in these two preparations.

FIGURE 4.4

THE EFFECT OF ATRIAL NATRIURETIC FACTOR ON THE FORCE-SARCOMERE LENGTH RELATION

A. The force-sarcomere length relationship for a single cardiac trabeculae. **B.** Results of non-linear regression analysis for nine trabeculae. Force-sarcomere relations were fitted to the equation $F = a(SL - SL_o)^c$ [Kentish et al., 1986]. See Table 4.1 for values in equation.

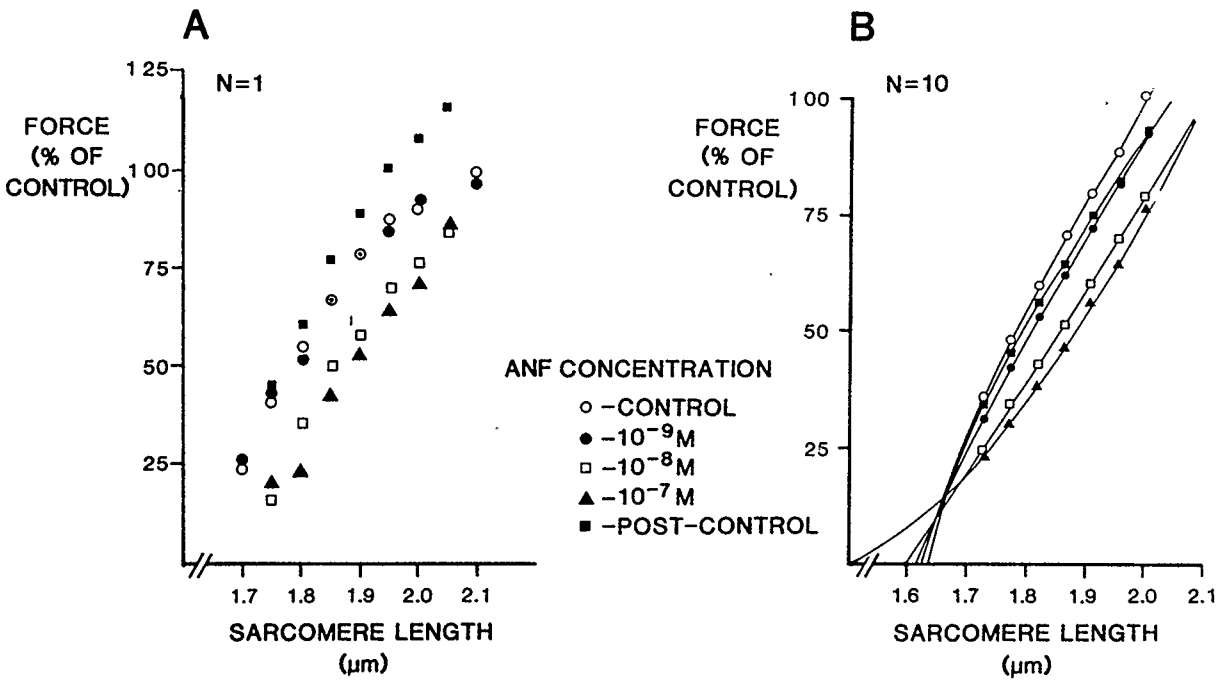

A
N=1
FORCE
(% OF
CONTROL)
125
100
75
50
25
1.7
1.8
1.9
2.0
2.1
SARCOMERE LENGTH
(μm)
ANF CONCENTRATION
○ -CONTROL
● -10⁻⁹M
□ -10⁻⁸M
▲ -10⁻⁷M
■ -POST-CONTROL
B
N=10
FORCE
(% OF
CONTROL)
100
75
50
25
1.6
1.7
1.8
1.9
2.0
2.1
SARCOMERE LENGTH
(μm)

TABLE 4.1

VALUES IN EQUATION FOR FORCE-SARCOMERE LENGTH RELATION

[ANF]	SL_o (μm)	c	r
Control	1.64 ± .04	.77 ± .16	.96
10^{-9} M (10^3 pM)	1.61 ± .07	.95 ± .20	.89
10^{-8} M (10^4 pM)	1.60 ± .13	1.09 ± .52	.87
10^{-7} M (10^5 pM)	1.60 ± .20	1.03 ± .70	.80
Post-Control	1.63 ± .03	.81 ± .10	.74

Data (mean ± SD) are from nine trabeculae. Force-sarcomere length relations were fitted by non-linear regression analysis to the equation $F = a(SL - SL_o)^c$, where SL_o is the intercept with the abscissa, $c > 1$ indicates the regression line is convex with respect to the abscissa, $c < 1$ indicates the regression line is concave with respect to the abscissa, and r is the correlation co-efficient.

Twitch Characteristics

The twitch characteristics of a single trabecula are shown in Figure 4.2. The time to peak tension and the relaxation time were analysed with respect to normalized force. At a concentration of 10^{-7} M (10^{5} pM), ANF significantly ($p<0.05$) reduced time to peak tension (see Figure 4.5).

Bustamante [1989] and Gisbert and Fischmeister [1988] have shown that both Na^{+} and Ca^{2+} currents in myocardium decrease due to exposure to ANF. This might explain the reduction of force observed in the present study. In addition, force may have been reduced due to an effect of ANF on calcium uptake by the sarcoplasmic reticulum (SR) or due to enhanced calcium extrusion through the sarcolemma. The latter should be reflected by accelerated relaxation, while a change in the contribution of calcium sequestration by the SR versus extrusion through the cell membrane is expected to affect the rate of decay of post-extraxsystolic potentiation [Bridge et al., 1988; Schouten et al., 1987].

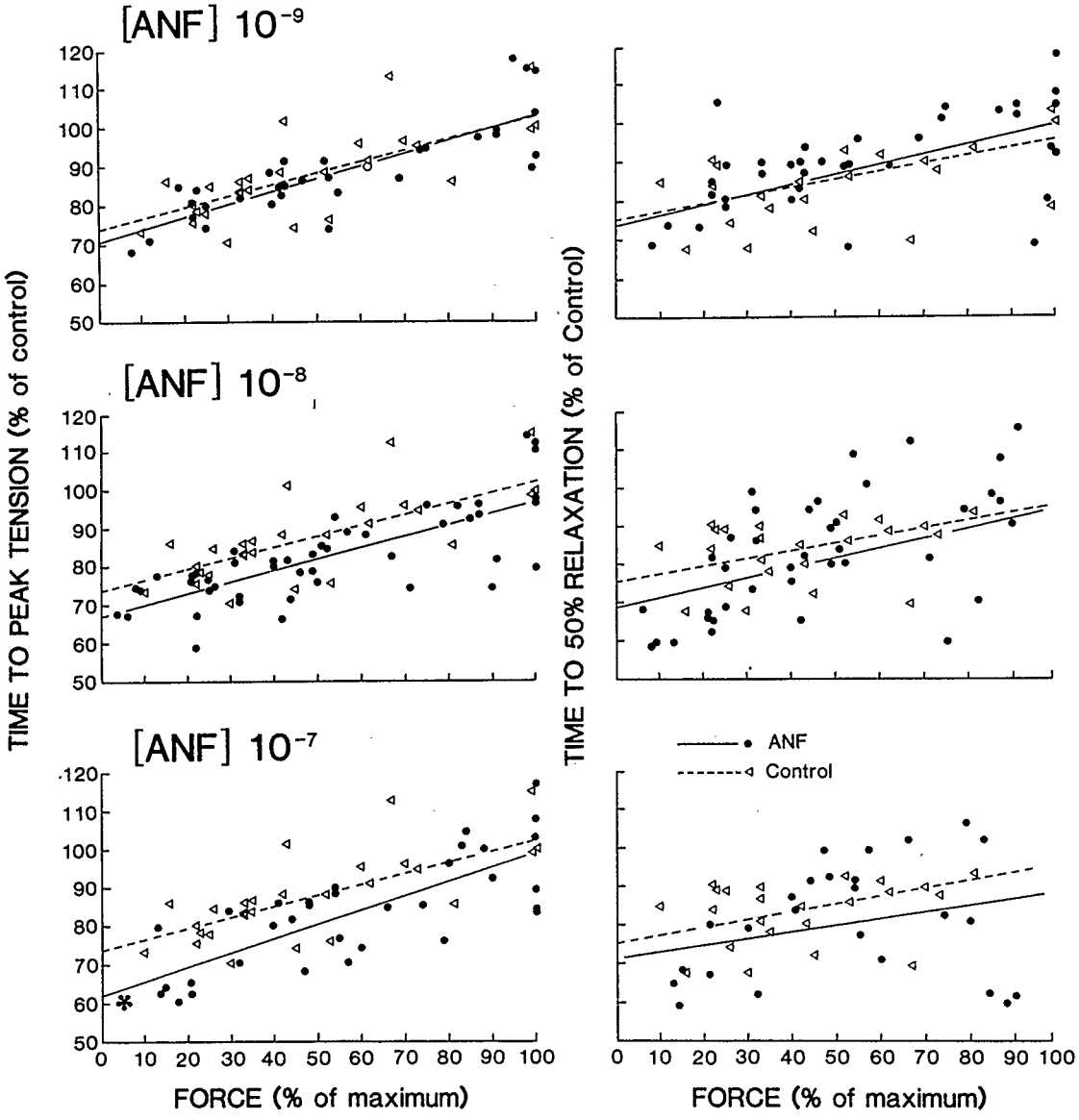
The modest negative inotropic effect was accompanied by a non-significant change in the relaxation time (see Figure 4.5). Only a trend toward a positive lusitropic effect was observed when the relaxation parameters were corrected for dependence on twitch force [Bucx et al., 1986; Van Heuningen et al., 1982].

FIGURE 4.5

THE EFFECT OF ATRIAL NATRIURETIC FACTOR ON TIME TO PEAK TENSION AND TIME TO FIFTY PERCENT RELAXATION

Time to peak tension (TPT) and time to 50 % relaxation (RT50) in a typical trabecula. Atrial natriuretic factor (ANF) at 10^{-7} M (10^{5} pM) (closed circles and solid regression line) significantly ($p<0.05$) reduced the value of TPT compared to control (open triangles and dashed regression line).

* = significantly different from control, $P < 0.05$.


[ANF] 10⁻⁹
[ANF] 10⁻⁸
[ANF] 10⁻⁷
TIME TO PEAK TENSION (% of control)
TIME TO 50% RELAXATION (% of Control)
ANF
Control
FORCE (% of maximum)
FORCE (% of maximum)

The relative amount of calcium recirculating within the SR was estimated by comparing the force of subsequent potentiated contractions [Schouten et al., 1987] (see Figure 4.6). This relation appeared to be linear as has been described before [Schouten et al., 1987]. ANF did not alter the rate of decay of potentiation (Figure 4.6), while ISO slightly but significantly ($p<0.01$) accelerated the rate of decay of potentiation. In the presence of both ISO and ANF, the recirculation fraction returned to almost the same value as obtained under control conditions.

DISCUSSION

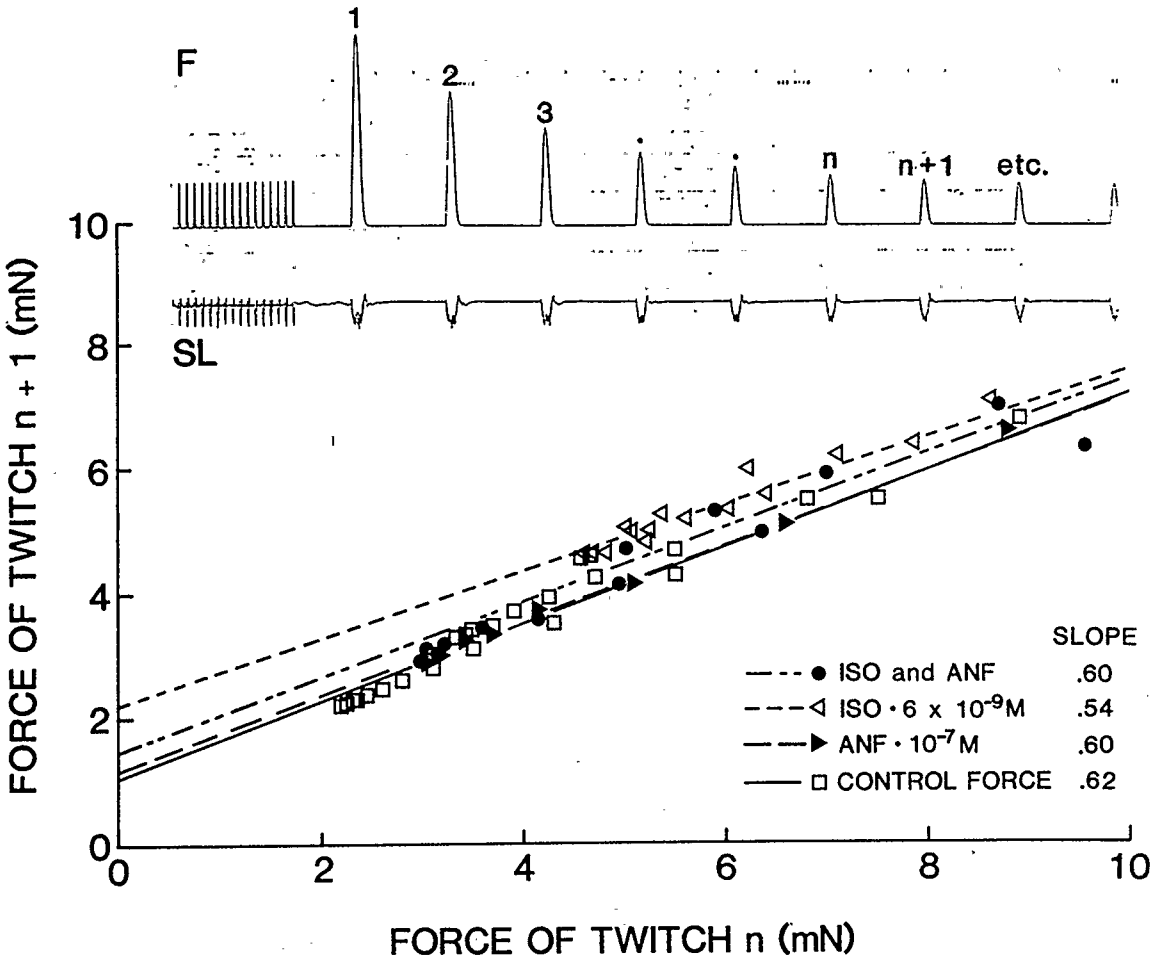
The results of this study document, for the first time, the negative inotropic effect of atrial natriuretic factor on rat cardiac trabeculae and the associated change in the shape of the force-sarcomere length relationship. The negative inotropic effect of ANF is in agreement with previous studies in intact animals which demonstrated a reduction in myocardial contractility (as measured by dp/dt) during systemic infusion of ANF [Dunn et al., 1986; Seymour et al., 1987]. In the present study, the addition of ANF to the muscle bath resulted in a dose-dependent reduction in the development of force at all sarcomere lengths studied and at ANF concentrations close to those measured in coronary sinus blood [Hirata et al., 1988; Thomassen et al., 1987; Walsh et al., 1988b]. These findings are in conflict with only one study in which ANF administation at a plasma level of approximately 6×10^{-9} M (6×10^{3} pM) in humans was associated with an increase in dp/dt [Indolfi et al., 1989].

FIGURE 4.6

THE EFFECT OF ATRIAL NATRIURETIC FACTOR ON CALCIUM RECIRCULATION

The slope of the relationship between force in one potentiated beat and force in the succeeding beat was taken as an estimate of the recirculation fraction of calcium. Slopes were determined by linear regression analysis. The addition of atrial natriuretic factor (ANF) to the muscle bath did not change the calcium recirculation fraction. However, isoproterenol (ISO) significantly reduced the calcium recirculation fraction. * = significantly different from control, $P < 0.01$.

Inset: Example of typical force decay during post-extrasystolic potentiation.


F
SL
1
2
3
n
n+1
etc.
FORCE OF TWITCH n + 1 (mN)
FORCE OF TWITCH n (mN)
0
2
4
6
8
10
SLOPE
ISO and ANF .60
ISO • 6 x 10⁻⁹M .54
ANF • 10⁻⁷M .60
CONTROL FORCE .62

The increase in dp/dt and apparent improvement in cardiac performance may have been related to afterload reduction secondary to ANF-induced vasodilatation.

The fall in force related to the administration of ANF may have been secondary to an absolute reduction in intracellular calcium or decreased calcium sensitivity of the myofibrils. Gisbert and Fischmeister [1988] demonstrated in isolated frog cardiac cells, that atrial natriuretic factor (atriopeptin $III_{(5-28)}$), in concentrations similar to those utilized in the present study, significantly reduced the calcium current. Furthermore, a recent study of human atrial myocytes by Bustamante [1989], has shown that ANF depressed the sodium current. The increase of stimulus threshold, during ANF administration, observed in the present study (20-40 %) is consistent with a reduction in the sodium current. A reduction in intracellular sodium concentration would produce a secondary reduction in intracellular calcium as a result of decreased sodium-calcium exchange. The observation that ANF changed the shape of the force-sarcomere relationship in a manner consistent with a reduction in intracellular calcium [Kentish et al., 1986] also supports the hypothesis that the decrease in force development may well have resulted from diminished intra-cellular calcium levels. However, the maximum negative inotropic effect of ANF occurred at an activating calcium concentration of 0.5 mM, i.e. an activating calcium concentration found on the steepest portion of the force-calcium concentration curve. The observed effect may be considerably more variable at higher or lower calcium concentrations and it may also be length dependent. In addition, the negative inotropic effect of ANF may be species

specific, i.e. greater in rats, but the inherent problems of harvesting trabeculae (success rate of ~50 %) renders the use of larger species such as dogs inpractical. Consequently, direct comparison of the results obtained in this study with those from the previous study are not possible.

Our results also showed that ANF significantly ($p<0.05$), although marginally, reduced the time to peak tension, had a small and variable effect on relaxation time and produced no change in the rate of decay of post-extrasystolic potentiation. Acceleration of cardiac muscle relaxation by ANF has been described previously [Meulemans et al., 1988]. The effect found here after correction for dependence of the relaxation rate on the total force [Bucx et al., 1986; Van Heuningen et al., 1982] was small and not significant. In addition, we observed that the rate of decay of potentiation (see Figure 4.6) was unaffected by ANF. This would suggest that the rate of calcium sequestration by the SR and calcium extrusion through the sarcolemma, as well as their relative contributions to relaxation which is reflected by the slope of the relation between force of subsequent beats during decay of potentiation [Bridge et al., 1988; Schouten et al., 1987], have been little changed by ANF.

It must be emphasized that these studies involved the use of ANF concentrations in the range of 10^{-7} to 10^{-9} M (10^{5} pM to 10^{3} pM) while those usually measured in the <u>systemic</u> circulation of healthy individuals are 10^{-9} to 10^{-12} M (10^{3} pM to 1 pM) [Raine et al., 1986]. In the present study, the experimental concentrations of ANF were selected on the basis of studies documenting the vasorelaxant

effect of ANF in smooth muscle [Yanagisawa and Lefer, 1988]. The need for higher concentrations of ANF may be, in part, related to restricted diffusion of the ANF peptide through endocardium surrounding the muscle. It has consistently been demonstrated [Hirata et al., 1988; Thomassen et al., 1987; Walsh et al., 1988b] that levels of ANF measured in coronary sinus blood are from 4-20 times higher than systemic levels, 10^{-9} to 10^{-10} M (10^{3} to 10^{2} pM). Thus, as ANF is secreted into the coronary capillary bed, the concentration of ANF around ventricular myocytes may be much higher than measured systemically, and as a consequence, the negative inotropic effect of ANF may be much more important than is currently appreciated. Furthermore, following development of congestive heart failure, ventricular tissue is capable of dramatically increasing ANF production [Drexler et al., 1989], and even the circulating level of ANF may approach nanomolar concentrations [Bates et al., 1987]. ANF elevations of this magnitude would correlate with the concentrations at which we noted a negative inotropic effect of ANF in the present study. However, without actual cardiac tissue levels of ANF available for analysis, the above arguments remain purely speculative and are of importance only as an impetus for further research.

In summary, an increase of the threshold for action potential generation and a decrease in force generation in response to atrial natriuretic factor, was demonstrated at ANF concentrations approximately 10^{5} times higher than those usually measured in the systemic circulation. Whether these observations will be of any physiological or clinical relevance depends upon further experimental

assessment of these effects and direct measurement of capillary ANF concentrations.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

Atrial natriuretic factor is a recently discovered hormone of cardiac origin with a complex and multidimensional physiologic profile. The (patho-)physiologic effects of ANF have been demonstrated in numerous organ systems including the lungs, liver, kidneys, brain, adrenals, peripheral circulation, and the heart. The majority of studies in the literature have concentrated on the natriuretic and diuretic properties of this cardiac hormone, although more recent studies have begun to explore the vasoactive properties of ANF.

The present dissertation has focused mainly on the cardiovascular physiology of ANF and its primary purpose was to further our current understanding of this hormone. In Chapter III, it was hypothesized that atrial transmural pressure, as the true cardiac distending pressure, and not intracavitary pressure should logically be the major determinant of plasma ANF levels. Using a flat, liquid-containing balloon to measure pericardial pressure, it was demonstrated that plasma levels of ANF did not increase during acute intravascular volume expansion in dogs, despite a significant increase in atrial intracavitary pressure. Following removal of the pericardium, atrial transmural pressure increased dramatically as did

plasma ANF levels. These observations were unaltered if the pericardium was not violated prior to the volume loading portion of the protocol and thus, served to validate our investigative hypothesis that atrial transmural pressure represents a primary stimulus for stretch-mediated release of atrial natriuretic factor.

The possibility that atrial natriuretic factor might function as a negative cardiac inotrope was initially suggested by studies in cardiac myocytes demonstrating that ANF inhibited surface membrane calcium channel activity. Corroborative evidence for the calcium channel activity of ANF was found in studies of vascular tissues where ANF produced smooth muscle relaxation and vasodilatation. Thus, the final study of this dissertation was an examination of the inotropic effects of atrial natriuretic factor on isolated rat cardiac muscle. The use of an isolated preparation excluded confounding variables such as preload and afterload and facilitated a more direct interpretation of the observations. In the presence of atrial natriuretic factor, rat cardiac muscle force development decreased significantly and ANF induced a partial change in the shape of the force-sarcomere length relationship consistent with a reduction in intracellular calcium. The decrease in intracellular availability of calcium could explain the negative inotropic effect of ANF. Finally, ANF had no effect on calcium recirculation within the preparation suggesting that the rate of calcium sequestration by the sarcoplasmic reticulum and the rate of cellular calcium extrusion were little affected by ANF.

Conclusions

1. A primary stimulus for stretch-mediated release of atrial natriuretic factor from cardiac tissue is transmural pressure. It remains to be determined whether alterations in atrial stress and/or strain are of greater importance in determining the release of ANF.

2. Force development in rat cardiac trabeculae was significantly reduced by superfusion of the muscle with atrial natriuretic factor. The observed effect was consistent with a reduction in intracellular calcium levels. However, the magnitude of the negative inotropic effect was relatively small (~30 %) and occurred at a very steep portion of the force-calcium concentration relation. The inhibitory effect of ANF on cardiac muscle may vary at greater or lesser calcium concentrations but this remains to be documented.

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