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A Study of Stretch-Stimulated ANF Release from the Atrial Myocardium

Harman Mangat

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Abstract

Atrial muscle stretch is widely believed to be the main stimulus for ANF release. However, we demonstrate in this study that although stretch induces an immediate increase in ANF output, this release rapidly decays even though hormone stores are not significantly depleted. In the present work, this phenomenon was studied in an isolated rat atria preparation using double isotope labelling. The tissue was labelled with ^{14}C-leucine for 3 h followed by a 1 h chase, and then with ^{3}H-leucine for 1 h. A final 1 h chase period was conducted with the tissue under basal (0.2 g load) or stretched (5 g load) conditions. During this final chase period, the ^{14}C-ANF represented "older", stored ANF and the ^{3}H-ANF the "newly synthesized" peptide. Following both the ^{14}C- and ^{3}H-leucine pulses, immunoprecipitable (IP) isotope incorporated into ANF appeared in the chase medium within the first 10 min and stabilized to lower levels after 20 min of chase. Stretch resulted in an immediate significant increase in immunoreactive (ir) ANF release and a decrease in the medium ^{14}C-ANF specific activity (S.A). However, no change was observed in the medium ^{3}H-ANF S.A. but the tissue S.A. tended to decrease. It is concluded that, in the basal state, a portion of ANF is immediately and preferentially released upon synthesis, while the remainder is presumably taken up into tissue stores and released from them at a lower rate. The secretory response to stretch was demonstrated to consist of a rapid, short-lived burst of newly synthesized ANF, suggesting an increased translocation of newly synthesized hormone into a stretch-sensitive, rapidly depletable pool. Given the nature of this pool, additional factors yet to be characterized likely come into play to maintain chronically elevated circulating levels of ANF.

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List of Abbreviations

ACN	-	acetonitrile
ACTH	-	adrenocorticotrophic hormone
ANF	-	atrial natriuretic factor
BNP	-	brain natriuretic peptide
cGMP	-	guanosine 3',5'-cyclic monophosphate
CHF	-	congestive heart failure
CNP	-	type C natriuretic peptide
cpm	-	counts per minute
Cys	-	cysteine
DNA	-	deoxyribonucleic acid
DOCA	-	deoxycorticosterone acetate
dpm	-	disintegrations per minute
EDTA	-	ethylenediaminetetraacetic acid
GC	-	guanylate cyclase
GFR	-	glomerular filtration rate
h	-	hour
HT-BSA	-	heat-treated bovine serum albumin
IP	-	immunoprecipitable
ir	-	immunoreactive
KRBB	-	Krebs Ringer bicarbonate buffer
Leu	-	leucine
min	-	minutes

mRNA	-	messenger ribonucleic acid
NRS	-	normal rabbit serum
PCR	-	polymerase chain reaction
PKC	-	protein kinase C
RIA	-	radioimmunoassay
RNA	-	ribonucleic acid
RP-HPLC	-	reverse-phase high performance liquid chromatography
SA	-	specific activity
SAG	-	specific atrial granules
s	-	seconds
SEM	-	standard error of the means
TCA	-	trichloroacetic acid
TFA	-	trifluoroacetic acid
TGN	-	*trans*-Golgi network

Introduction

The Discovery and Isolation of Atrial Natriuretic Factor

In 1956, Kisch reported morphological differences between atrial and ventricular guinea pig cardiocytes. He observed that atrial cardiocytes had morphological features that were characteristic to secretory cells and that these were absent in ventricular cardiocytes. The main difference observed was the presence of membrane-bound storage granules, named specific atrial granules (SAG), which contain an electron-dense core and measure 250 to 500 nanometres. These observations were subsequently confirmed by other investigators (Bencosme and Berger, 1971; Jamieson and Palade,1964). The granules were found to be more concentrated in the central sarcoplasmic core and often were observed to be associated with a prominent Golgi complex from which they arose (Jamieson and Palade, 1964).

Evidence for the dual secretory-contractile function of atrial cardiocytes was provided by de Bold and Benscome in 1975 from results obtained from autoradiographic studies conducted in rat atrial tissue. It was reported by these investigators that the labelling pattern in the atrial granules displayed kinetics similar to that observed in cells producing polypeptide hormones. Further evidence for this was provided histochemically by de Bold (1978, 1979) who developed a procedure to measure the degree of granulation of atrial cardiocytes and used it to demonstrate that experimental procedures which result in changes in water and electrolyte balance cause a significant change in the SAG population. It was these along with other morphological observations (Marie et al., 1976) which led to the hypothesis that the SAG may store a basic polypeptide containing tryptophan and sulphur

amino acids and that it may be involved in the regulation of water and electrolyte balance. This hypothesis was subsequently tested by de Bold et al (1981) who demonstrated that intravenous injection of a crude homogenate of rat atrial tissue resulted in a rapid, short-lived, yet profound natriuretic and diuretic response in rats. This effect was accompanied by a decrease in blood pressure and an increase in haematocrit. This response was not observed following injection of extracts which were obtained from ventricular tissue. The extract was also reported to be heat stable and acid soluble, thereby pointing it to be a small protein or peptide. This proteinaceous factor responsible for inducing these effects was named "Atrial Natriuretic Factor" (de Bold et al., 1981). The relationship between SAG and ANF was demonstrated when, following atrial tissue fractionation, it was only the granule-enriched fraction which demonstrated the natriuretic effects (de Bold 1982; Baines et al., 1983; Garcia et al., 1982).

Reverse phase high performance liquid chromatography (RP-HPLC) of acid extracts of rat atrial tissue was used to isolate and purify ANF (de Bold and Flynn, 1983). From these studies, four fractions which contained natriuretic, diuretic and vasorelaxant activity were isolated and named cardionatrin I, II, III and IV, corresponding to the order of elution. Cardionatrin I was purified to chemical homogeneity and submitted to amino acid sequencing by Edman degradation to demonstrate it to be a 28 amino acid long peptide (ANF_{99-126}) which contained 2 cysteines and no tryptophan (Flynn et al., 1983).

Other investigators continued this work and reported the isolation of several peptides with lengths varying from 21 to 33 amino acids but containing approximately the same core sequence. In 1984, Currie and coworkers reported the sequences of a series of short

bioactive peptides isolated from rat atrial tissue, which shared complete homology with ANF_{99-126}, but lacked several amino acids at the amino terminus. These 21, 23 and 24 amino acid long peptides were referred to as atriopeptin I, II and III respectively. Seidah et al. (1984) isolated from rat atrial tissue a polypeptide which was 33 amino acids long and 3 shorter polypeptides called ANF 1-33, 2-33, 3-33 and 8-33. All four peptides were found to be fully homologous to ANF_{99-126}, but contained additional amino acid residues at the amino terminal end. Atlas et al. (1984) reported the isolation of 2 polypeptides from rat atrial extracts, a 24 amino acid long peptide referred to as auriculin A, and one containing 25 amino acids named auriculin B. These peptides also lack several amino acids at the amino terminal but differ from the atriopeptin family by the presence of an additional arginyl residue at the carboxyl terminus.

In 1984, Kangawa and coworkers isolated three bioactive fractions from human atrial tissue and referred to them as α-, ß- and γ- human atrial natriuretic peptides (hANP). The α-hANP showed strong homology to rat ANF_{99-126}. The human peptide differed from the rat by the replacement of the isoleucine residue 12 by a methionine residue. Further studies by the same group (Kangawa et al., 1985) characterized the ß and γ hANP to be 56 and 126 amino acids long respectively. While γ-hANP, which has the α-hANP sequence at its carboxyl terminus, was identified as the hANP precursor, ß-hANP was reported to be an antiparallel dimer of the α-hANP. The rat and human propeptides exhibited strong homology as they only differed in 17 of the 126 amino acid residues.

ANF Structure and Synthesis

Cloning and characterization of cDNAs from mRNA established that ANF is translated to a prepro form (Nakayama et al., 1984; Seidman et al., 1984; Kennedy et al., 1984; Flynn et al., 1985; Kangawa et al., 1984). These studies have established that the human preproANF sequence consists of 151 amino acids and shares strong homology with the 152 amino acid rat preproANF. The amino terminal methionine, which typically initiates protein sequences in eucaryotes, is followed by a sequence rich in hydrophobic residues, the signal peptide (25 and 24 amino acids long in the human and rat respectively). In the rough endoplasmic reticulum (RER), the signal peptide is cleaved co-translationally to yield a 128 amino acid long precursor in the rat or a 126 amino acid long precursor in the human. Two arginine residues in the rat ANF, Arg^{127}-Arg^{128} are then cleaved, possibly by a carboxypeptidase E-like enzyme (Lynch et al., 1989), to yield proANF, a 126 amino acid peptide which is the main storage form of ANF (ANF_{1-126}) (Flynn et al., 1985). Following appropriate signals for hormone release, proANF is further cleaved into the biologically active hormone, ANF_{99-126} (Voulteenaho et al., 1985), which is the main circulating form of ANF (Flynn et al., 1983; Kangawa et al., 1984), and a cryptic peptide, ANF_{1-98}. The cleavage of proANF appears to occur within the heart, just prior to, or together with, the release of the peptide into the circulation (Dubé and de Bold, 1993; Sei et al., 1992). However, neither the precise site nor enzyme responsible is known to date.

Analysis of the ANF peptides demonstrate a common 17 amino acid central ring structure formed by a disulphide bridge between Cys^{105} and Cys^{121}. Disruption of this ring structure by cleavage of the Cys^{105}-Phe^{106} bond or Leu^{119} to Cys^{121} bonds results in the loss of bioactivity (Misono et al., 1984). The 3 carboxy-terminal residues Phe-Arg-Tyr also

demonstrate importance for biological activity as deletion of these residues results in a marked reduction of natriuretic and vasorelaxant potency (Misono et al., 1985). Deletions or extensions at the amino terminus do not appear to have a critical effect on bioactivity (Misono et al., 1984; 1985).

In addition to atrial cardiocytes, ANF has also been shown to be synthesized and stored in other tissues, although at much lower levels than those found in atria. ANF mRNA or immunoreactive ANF has been detected in ventricles experiencing hemodynamic overload such as in left ventricular hypertrophy and congestive heart failure (Edwards et al., 1988; Gutkowska and Nemer, 1989). It has also been expressed in the lung, anterior pituitary and hypothalamus (Gardner et al., 1986), the kidney (Sakamoto et al., 1985), the adrenals (Ong et al., 1987) and the aortic arch (Gardner et al., 1987).

THE STRUCTURE OF THE ANF GENE AND ITS REGULATION

ANF is coded by a single gene. The human ANF gene has been mapped on chromosome 1 while the mouse gene has been located on chromosome 4 (Yang-Feng et al., 1985). The ANF gene is composed of three exons separated by two introns (Seidman et al., 1984; Nemer et al., 1984; Argentin et al., 1985; Greenberg et al., 1984). The first exon contains the AUG codon, 5′ untranslated sequences, the hydrophobic residue signal peptide and the 16 amino acids following the signal peptidase cleavage site. The second exon contains information for ANF up to the penultimate amino acid in human or up to the third last amino acid in rats. The latter are coded in the third exon located at the 3′ end of the gene, as are the 3′ untranslated sequences and the stop codon. The human gene spans approximately 2 kilobases and has the basic structure of a eukaryotic protein coding gene

(Greenberg et al., 1984; Nemer et al., 1984). A single nucleotide change at the end of the coding sequence of the human with respect to the rat sequence converts an arginine codon (CGA) into a stop codon (UGA) thereby reducing the human peptide length by 2 amino acids (2 Arg). The proximal flanking sequence upstream of the transcription initiation site contains elements characteristic of eukaryotic promoters including a TATAA box which is located approximately 30 bp upstream of the transcription initiation or CAP site (Seidman et al., 1988). This sequence is essential for the transcription of many eukaryotic genes.

The amount of ANF specific mRNA in atria has been estimated to be between 0.5% to 3% of total RNA (Nakayama et al., 1984; Seidman et al., 1984; Needleman et al., 1989). ANF mRNA in tissues other than the atria has been detected at levels of 1% to 2% of those found in atrial tissues (Gardner et al., 1987). Ventricular expression is approximately 100-fold lower than in the atria (Lewicki et al., 1986). ANF expression is strictly modulated during development. Atrial ANF expression continues to rise during fetal and postnatal development. However, ventricular expression is high *in utero* but declines quickly following birth (Zeller et al., 1987). Ventricular expression of ANF mRNA can be induced by a variety of pathologic conditions, most notably cardiac hypertrophy (Day et al., 1987; Izumo et al., 1988; Lee et al., 1988). This induction has been associated with myosin heavy chain isoform switching and proto-oncogene (*c-fos* and *c-myc*) expression (Lee et al., 1988; Izumo et al., 1988).

Water or sodium deprivation results in a reversible 30-70% decrease in ANF mRNA levels (Mukoyama et al., 1990; Takayanagiet al., 1985). Administration of the mineralocorticoid deoxycorticosterone acetate (DOCA) to adrenalectomized animals has led

to an increase of mRNA by 70% (Ballerman et al., 1986). Studies have demonstrated that glucocorticoids and thyroid hormone increase ANF mRNA levels both *in vivo* (Dananberg and Grekin, 1992; Day et al., 1987; Gardner et al., 1986) and in cultured neonatal cardiocytes *in vitro* (Matsubara et al., 1987; Muir et al., 1992; Nemer et al., 1987) and it has been suggested that these responses are due to direct effects on gene transcription or effects on the stability of mRNA. Studies conducted by Gardner and coworkers (1990) have reported that extracellular calcium increases ANF mRNA levels by two- to three-fold in primary neonatal cardiocyte cultures, and that calcium antagonists decreased ANF transcripts. The vasoconstrictor peptide, endothelin has also been shown to increase ANF secretion and mRNA in neonatal cardiocytes (Fukuda et al., 1989). These studies suggest that the changes seen in ANF mRNA may reflect altered rates of biosynthesis which in turn could contribute to the changes observed in circulating ANF levels.

ANF RELEASE

Atrial Distension

With the knowledge that atrial extracts possessed natriuretic properties and that changes in water and electrolyte balance result in a significant change in the SAG population, investigators were led to speculate that ANF release may be responsive to changes in intravascular circulating volume.

In 1984, Dietz et al. demonstrated that the release of ANF was stimulated by an increase of venous return in an isolated rat heart-lung preparation. The first unequivocal demonstration that ANF was released in response to increased circulating volume was provided by Lang et al (1985). These investigators showed that in rats, volume expansion

by infusion of saline resulted in up to a six-fold increase in plasma ANF concentration, in association with right atrial pressure increases and also that an increase in right atrial pressure in a Langendorff-perfused isolated heart resulted in a doubling of ANF release. Volume expansion produced by infusion of fluids or by salt loading has been reported to cause elevated plasma levels of ANF in both humans and animals (Hollister et al., 1989; Gutkowska et al., 1984; Tanaka et al., 1984).

The importance of atrial stretch as a primary stimulus for ANF release was demonstrated *in vitro* by de Bold and co-workers (1986) who developed a perifused, isolated rat atria preparation to demonstrate that upon raising or lowering resting tension, there was a concomitant increase or decrease in ANF release. This "stretch-secretion coupling" (de Bold et al., 1986) was evident in both quiescent and beating atrial preparations. Some reports have correlated ANF release with frequency of atrial pacing (Bilder et al., 1989; Ellenbogen et al., 1988; Riddervold et al., 1991). However, it has also been shown that beating is not necessary for ANF release to occur (de Bold and de Bold, 1991; Vardas et al., 1989). Therefore the results in this area are rather conflicting.

In vitro studies have employed a variety of stretching conditions in atrial tissue in order to study mechanisms of ANF release including changes in linear stretch, stimulus frequency, and osmotic pressure (Bilder et al., 1986; 1989; Schiebinger and Linden, 1986; de Bold et al., 1986; Greenwald et al., 1989). The importance of atrial stretch, not atrial pressure, in ANF release has been the focus of studies in which atrial transmural pressure was maintained constant (Edwards et al., 1988). In this study, it was reported that an increase in atrial transmural pressure with the associated atrial stretch, and not increased

intra-atrial pressure, acts as the stimulus for ANF release. Investigators have measured both atrial pressures and dimensions simultaneously in order to calculate atrial wall stress (which is the product of transmural atrial pressure and dimensions). Christensen et al. (1988) showed that systolic wall stress was an important determinant of ANF release during volume expansion and tachycardia in dogs. Stewart et al. (1991) propose that, physiologically, stretch of the atrial wall results from passive distension during atrial diastole as a result of diastolic intramural wall stress. Moe and co-workers (1991) conducted studies on humans during volume loading and also concluded that atrial stretch, rather than atrial pressure, is the predominant stimulus for ANF secretion.

Not much is known of the subcellular mechanisms which transduce ANF secretion in response to cardiocyte stretch. This stretch-secretion coupling effect is also apparent in the juxtaglomerular cells in the kidney, where stretch results in an increase in renin release in Ca^{2+}-free media (Fray et al., 1987). In addition, alterations in extracellular osmolality and the subsequent changes in cell volume (stretch) have been reported to exert an inverse effect on basal, potassium- and angiotensin- stimulated aldosterone secretion (Wang et al., 1992). The stretch-secretion response has also been demonstrated in the lung, where an increase in lung volume stimulates the epithelial cells to secrete surfactant (Wirtz and Dobbs, 1990). Mechanical stretch has been shown to modify a number of ion channels. These stretch-activated ion channels have been identified in many cell types including fibroblasts, erythrocytes, epithelial cells, aortic endothelium, kidney cells and in skeletal, smooth and cardiac muscle cells (Morris, 1990). However, to date, there is little definitive information regarding the biological processes that may be regulated by, or coupled to, these stretch-activated ion conductances. Sodium and calcium channels have been reported to be

activated by stretch, whereas potassium channels are inhibited (Morris, 1990; Guharay and Sachs, 1984). The changes in ion fluxes induced by stretch result in a depolarizing effect which causes increased pacemaker activity and may be involved in subsequent peptide secretion. Furthermore, the cytoskeleton may also play a regulatory role, as cell elongation and the generation of tension depend on the microfilament system. Ingber (1991) suggests that the mechanical stress imposed on the cell surface is transmitted to the cytoskeleton, and the signal thereafter may initiate the activation of second messenger systems. Alternatively, it has been suggested (Watson, 1991) that the cytoskeleton may form a complex with the membrane proteins such as ion channels, Na^+/H^+ exchanger and adenylate cyclase and in turn regulate their responsiveness to the applied external forces.

In many endocrine and neural cells, Ca^{2+} has been reported to act as a mediator in the process of stimulus-secretion coupling (Penner and Neher, 1988) as well as in muscle excitation-contraction coupling. ANF release has been reported to be stimulated from isolated perfused hearts treated with the calcium ionophore A23187 (Ruskoaho et al., 1986) and also by the calcium channel agonist Bay K 8644 (Saito et al.,1986). Matsubara et al. (1988) also reported that ANF release was dependant on increases in intracellular free calcium concentration. These observations led to the concept that ANF secretion may be regulated by the protein kinase C system. However, de Bold and co-workers (1989) reported that, unlike other endocrine systems, ANF release is negatively modulated by extracellular calcium, so that removal of Ca^{2+} from the media resulted in a significant increase in the rate of basal immunoreactive ANF release. Stretch-induced ANF release was also found to be independent of extracellular calcium and took place in the presence of EGTA (de Bold and de Bold, 1989). These results have been confirmed by other investigators (Greenwald et al.,

1989; Iida and Page, 1989), and provided more evidence supporting the notion that atrial beating is not necessary for ANF release. Furthermore, studies (de Bold and de Bold, 1991) in which extracellular Ca^{2+} was replaced by Ba^{2+}, Sr^{2+} or La^{3+}, and intracellular Ca^{2+} was modified by ryanodine, caffeine and chemical depolarization, revealed that intracellular Ca^{2+} transients, although essential for excitation-contraction coupling, are not required for basal ANF release. However, the same study revealed that the ANF release elicited by stretch was partly dependent on a ryanodine-sensitive compartment. Therefore, there appears to be a dissociation between stretch-secretion coupling and contraction, which may allow atrial cardiocytes to accomplish their dual function of contraction and secretion.

It has been established that, in the acute situation, ANF secretion is proportional to the degree of atrial distension. Physiological situations in which this stretch-induced ANF release has been demonstrated are conditions in which there are acute increases in central venous pressure such as in thermoneutral head-out water immersion and acute volume load (Pendergast et al., 1987; Miki et al., 1986; Lang et al., 1985). In contrast, the relationship between atrial distension and ANF release under conditions associated with long term atrial distension is more controversial. There have been a number of studies conducted in states of chronic atrial pressure overload, including heart failure, in which elevated levels of ANF have correlated with either atrial pressure or the severity of heart disease (Bates et al., 1986; Dietz et al., 1986; Drexler et al., 1986; Mendez et al., 1987). However, there have also been reports in animals with chronic heart failure which demonstrate that ANF secretion is inappropriately low for the rise in atrial pressure (Moe et al., 1989; Redfield et al., 1989; Ding et al., 1987). In addition, several investigators (Drexler et al., 1989; Redfield et al., 1989; Raine et al., 1896), though not all (Dietz et al., 1986; Chien et al., 1988), have found

that experimental animals or patients exhibiting heart failure had an attenuated secretion of ANF in response to further increases in atrial pressure induced by either volume expansion or exercise. The natriuresis present in supraventricular tachycardia has also been correlated with ANF release (Nicklas et al., 1986), and distension of the atria is a correlate of the increased atrial pressure present in this condition. Chronic renal failure also results in elevated ANF release and is associated with atrial distension secondary to increased preload (Rascher et al., 1985). Furthermore, although chronic atrial distension does appear to enhance atrial ANF synthesis, tissue stores have been shown to be decreased during chronic stimulation (Brenner et al., 1990). Therefore, the mechanisms responsible for these sustained, elevated ANF levels have still yet to be clearly demonstrated.

Neurohumoral Modulation of ANF Release

It has been suggested that neural and humoral factors may modulate ANF release but the results are rather conflicting and vary with the experimental model used (Ruskoaho et al., 1991). Early work (Sonnenberg and Veress, 1984) showed that adrenaline and acetylcholine increased ANF release (as assessed by bioassay) in an *in vitro* preparation, and this effect was confirmed by investigators in later years using the perfused heart model (Ruskoaho et al., 1986; Toth et al., 1986) and the isolated atria (Wong et al., 1988; Inoue et al., 1988). Other investigators (Naruse et al., 1986; Lachance et al., 1986) observed no effect of adrenaline, noradrenaline or isoprenaline on ANF release (as detected by radioimmunoassay) in an *in vitro* atrial tissue model, but both Arg-vasopressin and acetylcholine were shown to increase ANF release in a dose-dependent manner (Naruse et al., 1986). Some investigators have found no effect of either adrenergic or cholinergic agonists (Garcia et al., 1986; de Bold et al., 1991) on ANF release. Schiebinger et al. (1986)

demonstrated that ANF release following increases in tension or contraction frequency was not blocked by propranolol, phentolamine and atropine in high concentrations, and therefore concluded that these tension and rate mediated effects were not mediated by endogenous adrenergic or cholinergic neurotransmitters. However, the same investigators (Schiebinger et al., 1987) went on to demonstrate a 2.5-fold increase in ANF release after the application of noradrenaline and isoprenaline to isolated rat left atria. Methacholine (a cholinergic agonist) was found to exert no effect on the basal release of ANF, but it did attenuate noradrenaline-stimulated secretion. It was concluded that ANF release was increased by cardiac ß-adrenergic activation but was reduced by parasympathetic activation.

Some investigators have reported that ANF release is not altered after sympathetic stimulation *in vivo* (Ledsome et al., 1986) but increases following acetylcholine treatment (Sonnenberg and Veress, 1984). *In vivo* studies in the rat report increases in ANF levels with vasopressin, phenylephrine and angiotensin II (Katsube et al., 1985). However, the doses used in these studies were enough to result in marked hemodynamic actions and, therefore, increases in atrial pressures. Thus, in interpreting these studies, the doses applied must be taken into consideration to determine whether the effect observed is truly the result of the agent, or is the consequence of increased hemodynamic actions due to the agent.

Endothelin I, a vasoconstrictor peptide produced by vascular endothelial cells, has been reported to produce a marked enhancement of ANF release in cultured rat atrial myocytes (Fukuda et al., 1988; 1989; Sei and Glembotski, 1990) and in the isolated rat atria (Stasch et al., 1989; Winquist et al., 1989; Schiebinger and Gomez-Sanchez, 1990; de Bold et al., 1991). Endothelin I has been shown to stimulate not only the basal secretion of ANF,

but also to enhance the stretch-stimulated secretion from the isolated perfused heart (Mantymaa et al., 1990) and atria (Schiebinger and Greening, 1992). Endothelin I appears to be, on a molar basis, the most potent and consistent ANF secretagogue identified to date. This *in vitro* effect is independent of the hemodynamic actions that endothelin I causes and, therefore, this implies that it may directly affect ANF secretion. Recently, it has been demonstrated that ANF inhibits endothelin I production and secretion, effects which are believed to be mediated by the C-receptor (Hu et al., 1992). This suggests that these two peptides may interact with one another to regulate blood pressure and salt and water balance.

Other Factors Implicated in the Release ANF

Hypoxia has been reported to stimulate ANF release both *in vivo* (McKenzie et al., 1986; Baertschi et al., 1990) and *in vitro* (Lew and Baertschi, 1988). It has been suggested that this effect may be mediated by stretch, increased heart rate, α- and β- adrenergic mechanisms, metabolic factors and neural reflexes (Baertschi and Teague, 1989; Baertschi et al., 1990). As hypoxia causes an elevated pulmonary artery pressure, ANF, by decreasing pulmonary vasoconstriction, may limit the increased pressure load developed on the right ventricle and thus alleviate pulmonary edema and right heart failure.

Since ANF may be involved in the regulation of electrolyte and water balance it was suggested that changes in osmolality could affect ANF release, however, the data obtained to date is rather conflicting. Some studies demonstrate that increasing plasma osmolarity *in vivo* results in increased plasma ANF levels (Kimura et al., 1986). However, such experiments are also accompanied by intravascular volume expansion, which may explain

the ANF increases observed (Salazar et al., 1986). *In vitro* studies have also yielded varied results as some report no increase in ANF release with increasing osmolarity (Dietz, 1987; Agnolletti et al., 1990) and some report an increase (Arjamaa and Voulteenaho, 1985). Therefore, more work needs to be conducted in this area before any conclusions can be reached regarding the importance of osmolarity in ANF release.

Studies have also shown that increasing temperature results in increased release of ANF (Agnolletti et al., 1990; Bilder et al., 1986). In addition, one group of investigators believe that ANF release is stimulated by a reduction, and not an increase, in atrial distension (Cho et al., 1988). They also report that release is dependent on both the atrial reduction volume and reduction frequency, the latter being the result of an increase in frequency of the length of shortening of atrial myocytes (Cho et al., 1991). However, these findings have not been confirmed by any other investigators.

ANF RECEPTORS

Two major biochemically and functionally distinct classes of ANF-specific receptors have been identified in target tissues (Maack, 1992). One type are the biologically active receptors, named type B, type I (R_1) or guanylate cyclase (GC) receptors and have a molecular weight of 120-140 kDa (Kuno et al., 1986). Following binding to ANF, MgGTP is converted to cGMP (Leitman and Murad, 1987) which is their main second messenger, and the functional effects of the hormone are subsequently mediated. Chinkers and Garbers (1989) have demonstrated by molecular cloning that it is the particulate guanylate cyclase itself which is the biologically active receptor, and possesses an extracellular binding domain and an intracellular guanylate cyclase domain. There are two subtypes of the B-

ANF receptors, namely the B_A or guanylate cyclase-A (GC_A) receptor and the B_B or guanylate cyclase-B (GC_B) receptor (Schulz et al., 1989).

The second type of receptor forms a disulphide-linked homodimer composed of subunits with a molecular weight of 60-70 kDa (Schenk et al., 1985) and is not coupled to guanylate cyclase. Maack and co-workers (1987) have proposed that this is a biologically silent receptor, that is, it does not mediate any of the known vascular or renal effects of ANF, and it serves as a specific clearance binding site for natriuretic peptides. This receptor has been named the clearance receptor (C-receptor) or type II (R_2) receptor and high levels have been found to be expressed on vascular endothelial cells and smooth muscle cells (Leitman et al., 1986; Maack et al., 1987), thereby suggesting it clears ANF from the circulation. C-ANF receptors are the most abundant class of ANF receptors and comprise greater than 95% of the total ANF receptor population in kidney cortex and vascular tissues (Maack et al., 1987). As with the other two receptors, it contains an extracellular domain for ANF binding, but, as is characteristic of clearance receptors, it has a short intracellular domain. Recently, Suga and co-workers (1992) reported a marked species difference in the receptor selectivity of the natriuretic peptide family, especially the BNPs. They observed the rank order of binding affinity for the C-receptor to be ANF > CNP > BNP in rats and humans. The rank order of potency for cGMP production via the B_A receptor was ANP ≥ BNP >> CNP, but that via the B_B receptor was CNP > ANP ≥ BNP.

Many studies have reported that ANF markedly increases guanosine 3',5'-cyclic monophosphate (cGMP) as a result of activation of particulate guanylate cyclase in target tissues and plasma in a dose-dependent fashion (Maack, 1992). In addition, cGMP

analogues mimic the vasorelaxant (Murad, 1986) and renal (Rocha and Kufo, 1990) actions of ANF. The B_A receptor contain a single transmembrane domain that divides each protein in half. The intracellular region has been reported to contain a protein kinase like domain and a carboxyl region which is the cyclase domain (Chinkers and Garbers, 1989). The protein kinase-like domain most probably plays a regulatory role as a repressor for the guanylate cyclase activity. Thus, the binding of ANF to the receptor portion induces conformational changes in the adjacent cyclase domain, and thus increases the rate of cGMP formation (Chinkers and Garbers, 1989). Increases in intracellular cGMP levels in vascular smooth muscle result in the activation of cGMP-dependent protein kinases and phosphorylation of intracellular proteins and the dephosphorylation of myosin light chains required for muscle relaxation (Leitman and Murad, 1987; Murad,1986). In other cell types in which ANF causes elevated cGMP levels, mechanisms of action have not yet been established.

CEARANCE OF ANF

ANF is cleared from the circulation by two main pathways: (1) metabolism by neutral endopeptidase EC24.11 and (2) internalization by clearance receptors. Following incubation with tissue homogenates, Tang and co-workers (1984) observed the rank order of degradative potency to be kidney > liver > lung > plasma > heart. The proximal tubule brush border contains many degradative enzymes including the zinc-dependent cell-surface enzyme neutral endopeptidase EC24.11 (enkephalinase). This enzyme cleaves the ^{106}Cys-^{107}Phe bond which results in disruption of the ANF ring structure (Stephenson and Kenny, 1987; Olins et al., 1987) and therefore, its activity. Inhibition of endopeptidase EC24.11 by agents such as phosphoramidon or thiorphan is reported to result in increased plasma ANF

levels, urinary Na^+ excretion rate and urinary guanosine 3',5'-cyclic monophosphate (cGMP) (Lafferty et al., 1989; Ura et al., 1987). Administration of two synthetic endopeptidase inhibitors (atriopeptidase inhibitors UK79300 and UK69578) have also resulted in similar effects (Samuels et al., 1989).

In addition, ANF has been reported to be removed from the circulation by binding to a "clearance" receptor (the C-ANF or C-receptor) (Maack et al., 1987; Maack, 1992). Administration of C-receptor ligands has been shown to produce a dose-dependent increase in plasma concentration of administered ^{125}I-ANF and a decrease in its metabolic clearance rate and the rate at which the hydrolytic products appear in plasma (Almeida et al., 1989). It has been shown that of the three pathways, the C-receptors have the greatest capacity for clearing circulating ANF (Wilkins and Needleman, 1992).

THE PHYSIOLOGIC EFFECTS OF ANF

The acute administration of exogenous ANF results in natriuresis and diuresis, events that are mediated by cGMP (Appel et al., 1986). Although ANF has been shown to increase glomerular filtration rate (GFR) (de Bold et al., 1981; Burnett et al., 1984), it does not always appear to correlate with the natriuretic and diuretic effects (Mendez et al., 1986), because ANF has been shown to increase sodium and water excretion without concomitant changes in GFR (Murray et al., 1985; Sonnenberg et al., 1982). This suggests that a tubular effect may be involved. Solute transport has been shown to be decreased in the proximal tubule but the presence of ANF causes increased sodium delivery from this segment (Harris et al., 1987). ANF appears to exert little or no effect on the thick or thin limbs of the loop of Henle or the distal convoluted tubule (Peterson et al., 1987; Kondo et al., 1986). There

have been many reports which show that ANF acts on the collecting duct segment by inhibiting sodium reabsorption (Sonnenberg et al., 1982; Van de Stolpe et al., 1988).ANF induces the excretion of phosphate, chloride, magnesium, calcium and cGMP in the urine (Camargo et al., 1984; Wong et al., 1988; Mizelle et al., 1989). Administration of agents which block ANF degradation, such as enkephalinase inhibitors, have been shown to cause a modest increase in circulating ANF which in turn results in increased GFR, natriuresis and diuresis (Lafferty et al., 1989). ANF can dilate renal afferent arterioles and constrict efferent arterioles, thereby causing the hydrostatic pressure within the glomeruli to increase (Dunn et al., 1986; Freid et al., 1986). It has also been shown to relax mesangial cells, resulting in an increased filtration surface area (Appel, 1990; Appel et al., 1987). ANF inhibits vasopressin action on the inner-medullary collecting duct (IMCD) by preventing the production of vasopressin induced cAMP (Dillingham and Anderson, 1986). There are probably a number of interacting factors which are involved in this homeostasis, and ANF may be one of them and may be necessary but not sufficient to induce diuresis or natriuresis (Blaine, 1990).

ANF has been shown to decrease systemic blood pressure by decreasing cardiac output, reducing peripheral vascular resistance and decreasing intravascular volume. The decrease in cardiac output may be in part due to the peptides effect on the sympathetic and parasympathetic nervous systems, and stimulation of the vagal afferent (Ackermann et al., 1984; Thoren et al., 1986). The reduced cardiac output may also be due to a decrease in preload caused by a reduction in central venous and right atrial pressures Pegram et al., 1986; Shapiro et al., 1986) as a result of decreased circulating volume.

ANF has been shown to interact with the renin-angiotensin-aldosterone system. ANF decreases renin secretion and plasma concentrations (Burnett et al., 1984; Maack et al., 1984), an effect which appears to be mediated by cGMP (Henrich et al., 1988). This renin inhibition may also occur indirectly through the inhibition of neural stimuli (Thoren et al., 1986). ANF causes the reduction of circulating angiotensin levels, which in turn result in decreased aldosterone release. ANF also directly inhibits aldosterone production and secretion. ANF has been shown to inhibit the basal and angiotensin-, ACTH-, and potassium-stimulated release of aldosterone (Delean et al., 1984; Schiebinger et al., 1988; Atarshi et al., 1985). This effect was independent of cGMP (Matsuoka et al., 1987). It has been suggested that ANF's inhibition of aldosterone may be as a result of changes in calcium influx seen during the stimulated release of aldosterone, perhaps by direct blockage of calcium channels (Chartier and Schiffrin, 1987; Takagi et al., 1988).

ANF has also been reported to inhibit arginine vasopressin secretion in response to hemorrhage and prolonged dehydration (Samson, 1985). It has been reported that ANF inhibits the firing of vasopressin neurons in the paraventricular nuclei of anaesthetized rats (Standeart et al., 1987). Intracerebroventricular infusion of ANF also results in decreased plasma vasopressin levels and increased urine volume and water excretion, which are effects due to the central suppression of vasopressin secretion (Lee et al., 1987). In lower doses, vasopressin augments ANF secretion (Manning et al., 1985), which suggests a negative feedback system for endocrine antagonism of water homeostasis.

Thus, the combined actions of ANF on the kidneys, adrenals and vasculature serve to decrease systemic blood pressure and intravascular volume by opposing many of the actions of the renin-angiotensin-aldosterone system..

THE ROLE OF ANF IN PATHOPHYSIOLOGICAL STATES

Congestive heart failure (CHF) is a condition characterized by a fluid overload state with a concurrent increase in cardiofilling pressures. The latter induce atrial distension and, as a consequence, the circulating levels of ANF are increased (Fried, 1989). Plasma ANF levels correlate closely with the severity of the CHF, varying directly with right atrial and pulmonary capillary wedge pressures (Brenner et al., 1990). In animal models of CHF, high plasma ANF levels have also been reported to correlate inversely with atrial tissue concentration, implying that there is prompt secretion and decreased tissue storage despite high ANF mRNA levels (Mendez et al., 1987). In addition, ventricular ANF gene expression has been reported in humans and animals with CHF (Day et al., 1987; Ding et al., 1987; Edwards et al., 1988). However, despite often markedly elevated ANF levels in CHF, patients continue to exhibit evidence of volume overload, increased preload and increased systemic vascular resistance, suggesting an acquired insensitivity to the effects of endogenous ANF (Cody et al., 1986; Burnett et al., 1986). However, it has also been demonstrated that despite reduced sensitivity, ANF does contribute towards modulation of the renal, hemodynamic and endocrine effects of CHF, as infusion of an anti-ANF antibody to rats with CHF resulted in increased fluid retention in these animals (Awazu et al., 1989).

Circulating ANF levels have also been reported to be elevated in hypertension. In hypertension mediated primarily by volume overload, such as in the DOCA-salt model, the

increased atrial pressures induced by the volume expansion results in elevated ANF levels (Garcia et al., 1987). In DOCA-salt rats, ANF mRNA and plasma ANF levels were reported to rise sequentially, preceding a natriuresis that reduced the volume overload in these animals (Ballerman et al., 1986). From this, it is suggested that ANF may play a role in an important mechanism in which the body "escapes" from the salt-retaining effects of mineralocorticoid excess (Ballerman et al., 1986; Gonzalez-Campoy et al., 1989). In clinical hypertension, plasma ANF levels have been found to vary considerably and the use of ANF as a hypertensive agent has been limited by the lack of an orally effective analogue. Inhibitors of the ANF-degrading enzyme, neutral endopeptidase (NEP), and of the ANF "clearance" receptor have been reported recently to enhance the antihypertensive actions of endogenous or exogenously administered ANF and human studies are currently in progress (Hollister and Inagami, 1991).

In pathophysiological conditions such as congestive heart failure, ANF likely contributes to modulate the cardiovascular and endocrine responses called upon to compensate for loss of pump function, such as the activation of the renin-angiotensin-aldosterone system (RAAS). Obviously, in situations such as CHF, the RAAS actions predominate over those of ANF. Therapeutically, attempts to increase circulating ANF levels by the development of ANF analogues and agents which inhibit NEP offer new avenues for the treatment of congestive heart failure and hypertension.

Other Natriuretic Peptides

Other peptides have been isolated which are closely related to, but clearly distinct from, ANF. The first, brain natriuretic peptide, or BNP, was so named as it was initially

identified in porcine brain based on its muscle relaxant activity (Sudoh et al., 1989). However, BNP was subsequently found to be far more abundant in cardiac atria than in the central nervous system (Saito et al., 1989). BNP, like ANF, contains a 17-member ring formed by a disulphide bond (Sudoh et al., 1989). The amino acid sequence of BNF differs from ANF at only seven residues within this ring structure. There are greater differences in the amino and carboxyl extensions of the molecules. BNP has also been identified in humans (Sudoh et al., 1989), rats (Kojima et al., 1989) and dogs (Seilhamer et al., 1989). It was reported that the amino acid sequences of these BNPs were less conserved between species than the ANFs. The biosynthetic pathway of BNP is very similar to ANF as transcription yields a precursor molecule which is cleaved to produce a prohormone and then the active hormone, which is species dependent. The circulating form is a 32 amino acid peptide in the human and pig (Kojima et al., 1989) and 45 amino acids (iso-ANP) in rhodents (Flynn et al., 1989). BNP has been shown to be released from the heart (Saito et al., 1989) and rat BNP levels in the atrium have been reported to be about 4% of that of ANF (Nakao et al., 1990). Rat BNP has also been detected in the ventricle where the ratio of BNP to ANF was found to be 30%, however, no mRNA for rat BNP has been detected in the brain (Nakao et al., 1990). Like ANF, BNP has potent natriuretic and hypotensive properties and levels have been reported to be elevated in DOCA-salt hypertensive rats (Yokota et al., 1990). It still remains to be determined how these peptides (ANF and BNP), which have similar origins and sites of action, interact to control body fluid and cardiovascular function.

The newest member of the natriuretic hormone family is a 22-amino acid peptide referred to as type C natriuretic peptide (CNP) (Sudoh et al., 1990). Like ANF and BNP,

CNP has a ring structure formed by an intramolecular disulphide bond, however, it lacks the C-terminal extension from the ring structure. In rats and human, CNP has been detected throughout the brain with the highest concentrations in the hypothalamus and cerebellum where there were no significant amounts of ANF or BNP (Komatsu et al., 1991). In addition, CNP has been shown to selectively bind to the B-type receptor which was reported to be mainly expressed in the brain (Koller et al., 1991), thereby suggesting a central site of action for this peptide. However, recently, the B-type receptor has been detected in peripheral tissues by polymerase chain reaction (PCR) techniques (Canaan-Kuhl et al., 1992). A recent PCR study by Vollmar et al. (1993) reported the detection of CNP mRNA in rat atria and ventricles of the heart as well as in organs of the immune system (thymus, spleen and lymph nodes), although to a lesser extent (1-9%) than that found in the brain. These results suggest that CNP may have a peripheral as well as a central site of action.

Rationale For Study

ANF appears to play an important role in cardiovascular homeostasis, therefore, an understanding of the factors that control the release of ANF may provide an insight into the physiology and pathophysiology of the cardiovascular system. In order to understand and identify the mechanisms that control the release of ANF, it is important to study the kinetics of its secretion, however, to date, there have been few studies which have addressed these aspects.

It has been well established that acute atrial muscle stretch is a stimulus for ANF release through a mechanism referred to as stretch-secretion coupling. However, the effects of chronic stretch on ANF release are more controversial, thereby pointing to factors other than atrial stretch which may regulate release under these conditions. The objective of this study was to characterize the stretch-secretion response in an isolated rat atria preparation. Using a double-label, pulse-chase protocol, the contribution of the newly synthesized and stored hormone pools to basal and stretch stimulated ANF release was determined. Using this approach, it was possible to separate the newly synthesized and stored pools by temporal relationship and also by the use of different tracers (Stachura et al., 1972; Walker and Farquhar, 1980; Maas et al., 1991).

An isolated rat atria preparation previously established by this laboratory (de Bold et al., 1986) was used for the experiments. ANF release was examined after the imposition of acute, continuous and repetitive stretch. The double-label, pulse-chase protocol entails the addition of a labelled amino acid (^{14}C-leucine) to the tissue for 3 hours to allow it to be

incorporated into ANF, followed by a 1 h chase (incubation with unlabelled medium). Release and tissue storage of the labelled peptide is determined by radioimmunoassay and immunoprecipitation. The tissue is then incubated with the second label, ^{3}H-leucine for 1 h and a final 1 h chase period conducted with the tissue under basal (0.2 g load) or stretched (5 g) conditions. During this final chase period, the ^{14}C-ANF represents the older, stored ANF and the ^{3}H-ANF the newly synthesized peptide. ANF specific activities are calculated and compared in order to determine whether there is preferential release of newly synthesized or stored peptide, and if there is any difference between the basal and stimulated states. In this manner, the stretch-secretion coupling response in the atrial myocardium was further characterized.

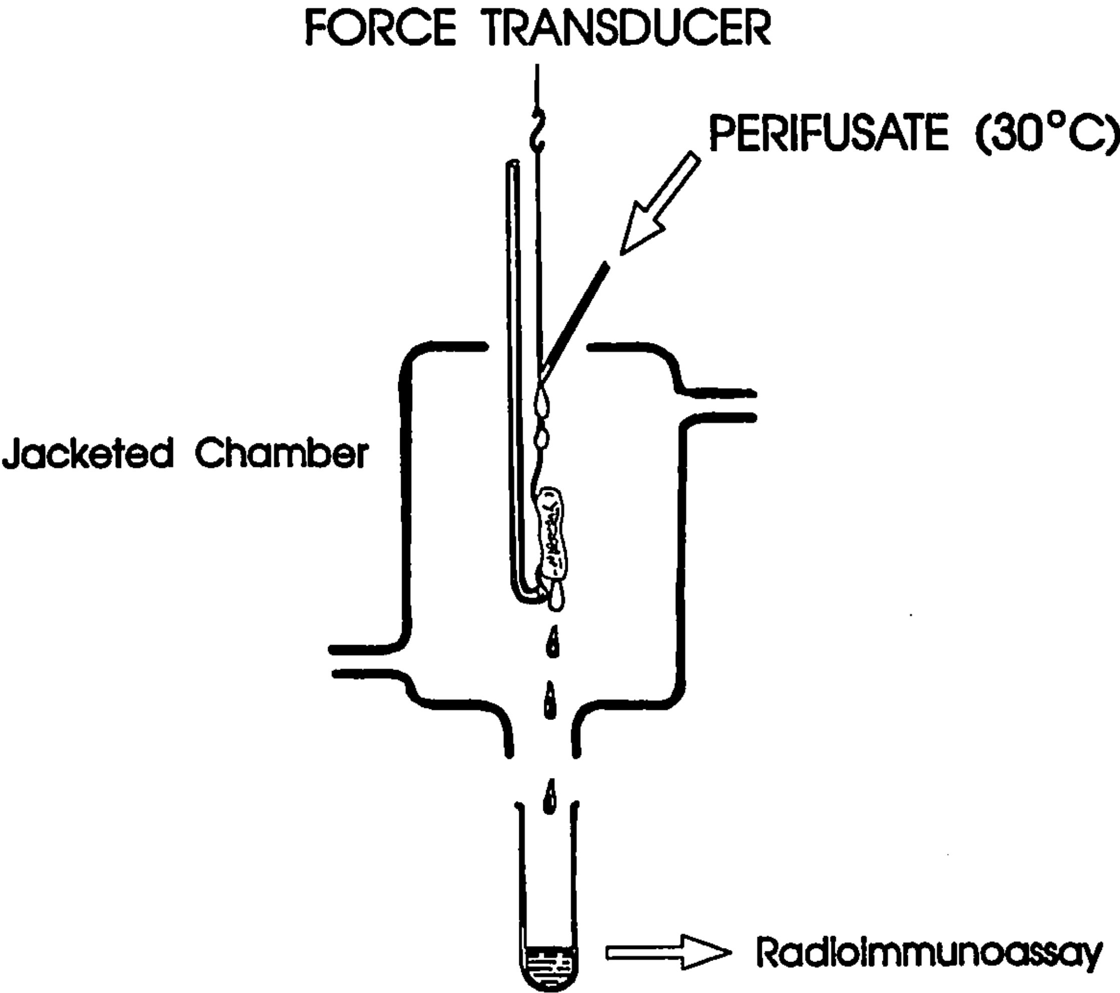
Materials and Methods

Isolated perifused rat atria preparation

The atrial preparation used was as that described by de Bold et al., (1986) with some modifications. Hearts were obtained from male Sprague-Dawley rats (300-350 g). The animals were allowed free access to food until decapitated. The heart was excised rapidly and the atria dissected in supplemented Krebs-Ringer bicarbonate buffer (KRBB), pH 7.2-7.4, oxygenated with 95% O_2-5% CO_2. The composition of the KRBB was (mmol/L): NaCl, 118; KCl, 4.7; NaH_2PO_4, 1.18; $MgSO_4$, 1.18; $NaHCO_3$, 24.9; sodium glutamate, 5; sodium fumarate, 5; sodium lactate, 700; glucose, 11; amino acids and vitamins (Gibco BRL, Ont., Canada) as in Eagle's medium; zinc insulin at a concentration of 25 mU/ml; 0.01% gelatin and 0.001% bovine serum albumin (BSA).

The atria were suspended between two hooks which were inserted into the auricles, and placed in a water-jacketed container (Figure 1). The lower hook was connected to a fixed glass rod and the upper hook to a force transducer (Grass Instruments, Quincy, Mass.) via a silk thread, which in turn was connected to a Narco Physiograph. The tissue was perifused with KRBB by the use of a peristaltic pump at a flow rate of 3 ml/min (for whole atria experiments, i.e. right and left atria) or 1.5 ml/min (for single atrium experiments). The solution was prewarmed to 30ºC by passage through a water jacket and dripped onto the silk thread and the tissue through a 21 1/2 gauge needle. The perifusate was collected on ice at 1 min intervals and frozen at -20ºC until analyzed for ANF by radioimmunoassay (RIA).

Figure 1: Diagram of the Isolated Perifused Atria Preparation (modified with permission, from de Bold *et al.* 1986).



Acute Stretch Experiments

The right atrium was used in these experiments in order to investigate the effect of increasing load on ANF release. After the atrium was placed in the jacketed container, it was immersed for 15 min in oxygenated supplemented KRBB. The bath was then drained, the perifusion started, and the tension adjusted to 0.2 g by a micrometric screw (Narishigi, Japan). The tissue was perifused with supplemented KRBB for a 1 hour equilibration period after which the perifusate was collected at 1 min intervals throughout the course of the experiment for subsequent ANF analysis by radioimmunoassay. For the first 15 min following the equilibration period, the tissue was left at 0.2 g resting tension, after which the load was increased by 2 g every 15 min until the tissue had been stretched to 10 g.

Continuous Stretch Experiments

The left atrium was used to demonstrate the effect of prolonged stretch on ANF release. The tissue was allowed to equilibrate for 1 hour in an organ bath containing 10 ml oxygenated supplemented KRBB. The atrium was paced at a frequency of 3 Hz, 10 ms duration and at a current 150% of threshold. A load of either 0.2 g or 6 g was applied to the tissue for a period of 3 hours following equilibration. The bath was drained every 10 min and refilled with fresh supplemented KRBB and the contents frozen at -20°C until assayed for ANF.

Repetitive Stretch Experiments

The experimental set-up is similar to that described for the continuous stretch experiments. Following a 1 hour equilibration period, the tissue was stretched repetitively to 3 g tension with the following time intervals: every 6 s, 10 min and 40 min. Once again,

the bath was drained every 10 min and the contents frozen at -20°C until ANF RIA was conducted.

Continuous Incorporation Experiments

The whole atria preparation was used for these experiments using the perifusion set-up as previously described. During the 60 min equilibration period, the tissue was perifused with cysteine-free supplemented KRBB after which it was perifused with a "pulse" supplemented KRBB containing ^{35}S-cysteine (NEN Dupont) at a concentration of 0.2 µCi/ml for the following time periods: 10 min, 30 min, 60 min, 120 min and 240 min. At the termination of the experiment, the tissue was placed in 3 ml extractant containing 1.0M acetic acid, 0.1N HCl and 1% NaCl, and frozen at -20°C. The tissue was then either TCA precipitated, or extracted for ANF and immunoprecipitated.

Continuous Incorporation Experiments in the presence of Puromycin

The experimental design was as that described above except that the tissue was perifused for 60 min in cysteine-free supplemented KRBB containing either 10^{-3}M or 10^{-2}M Puromycin. The tissue was then perifused for a further 60 min in ^{35}S-cysteine (0.2 µCi/ml) containing KRBB in the presence of the Puromycin. The tissue was frozen at the end of the experiment until TCA precipitation was conducted.

TCA Precipitation

Atria were placed in 3 ml extractant following the continuous incorporation experiments and homogenized and centrifuged as described in "Extraction of ANF". Trichloroacetic acid (TCA) was added to the supernatant to give a 10% TCA concentration

and left at 4°C overnight. The tubes were then centrifuged for 10 min at 2,000 rpm and the following washes were conducted: 10% TCA (2 times); 95% Ethanol containing 10% Potassium acetate; 10% TCA. The tubes were then placed in a water bath for 15 min at 90°C after which the following washes were carried out: 95% Ethanol / 10% Potassium acetate; 95% Ethanol; Ethanol:Diethyl ether, 3:1. After 15 min in a 70°C water bath, the final washes were conducted: Ethanol:Diethyl ether, 3:1 and Diethyl ether (2 washes). The pellet was then left to dry at room temperature overnight, dissolved in 100 µL H_2O and 1 ml NCS tissue solubilizer (Amersham) was added to solubilize the pellet. Ten ml Cytoscint (Fisher) was added after 24 h at room temperature and the radioactivity counted on a ß-counter.

Experimental Design for Double-label Pulse-Chase Experiments

The whole atria preparation used was as that described by de Bold et al., (1986) and above, with some modifications. The atria were suspended between two hooks which were inserted into the auricles, and placed in a water-jacketed container maintained at 34.5°C. The atria were immersed in 10 ml oxygenated supplemented KRBB containing 10 µCi/ml ^{14}C-leucine (New England Nuclear Corp., Boston, MA; 315 mCi/mmol) for 3 hours. Following this, a 60 min chase period was begun by draining the bath and adding 10 ml unlabelled supplemented KRBB. The supplemented KRBB was collected on ice and replaced every 10 min. The tissue was then incubated for 1 hour in supplemented KRBB containing the second label, 50 µCi/ml ^{3}H-leucine (New England Nuclear; 145 Ci/mmol). The bath was then drained and refilled with unlabelled supplemented KRBB to begin another 60 min chase period with the tissue at either 0.2 g resting tension (basal) or stretched to 5 g by a micrometric device. Once again, the bath was drained every 10 min and the

solution collected. The bath solutions and tissue were frozen at -20°C until analyzed by radioimmunoassay and immunoprecipitation.

As a result of the temporal relationship during the incubation phase, ^{3}H-labelled ANF represents the "newly synthesized" ANF pool and the ^{14}C-labelled ANF the older, "stored" pool.

ANF Extraction

ANF was extracted from atrial tissue by a method described by de Bold and Flynn (1983). At the end of each experiment, the tissue was placed in 3 ml cold extractant containing 1.0 M acetic acid, 0.1 N HCl and 1 % NaCl, and homogenized for 10 sec using a Polytron (with a PEA 10s probe, at 50% of maximum power). The homogenate was left on ice for 1 hour and then centrifuged for 30 min at 10,000 rpm and 4°C. The supernatant was decanted and saved and the pellet homogenized in 3 ml extractant for 10 sec, left on ice for 30 min and centrifuged for 30 min at 10,000 rpm, 4°C. The supernatants were pooled and kept on ice. Sep-Pak cartridges (Millipore Waters, Ont., Canada) were pre-wetted by passing 5 ml 80% HPLC grade acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) through the cartridges, followed by 8 ml 0.1% TFA to rinse. The pooled supernatants were then passed 3 times through the pre-wetted Sep-Pak cartridges, washed with 20 ml 0.1% TFA, and eluted with 3 ml 80% ACN in 0.1% TFA in siliconized Vacutainer glass tubes (100 x 16 mm; Beckton Dickinson) and lyophilized.

Radioimmunoassay for ANF

The radioimmunoassay (RIA) is a sensitive technique for assessing the concentration of biological molecules. The main concept behind RIA is that the binding of a fixed concentration of radioactive tracer antigen to low concentrations of a high-affinity specific antibody is very sensitive to competition by unlabelled antigen and is also very specific for that antigen. In this manner the concentration of antigen in unknown samples can be determined by their ability to compete with tracer for binding to antibody. Thus, as the amount of unknown antigen increases relative to the fixed amount of label present, an increasing fraction of the label will be free.

In order to measure total (immunoreactive) ANF release and tissue content a double antibody separation technique was used to separate the bound from the free ligand. This approach was used because the low concentrations of antibodies (Ab) and antigens (Ag) during the primary reaction do not allow for the formation of a precipitate. Therefore, a second Ag-Ab reaction at a higher concentration of Ab (from a different species) is included so that the Ab in the primary reaction act as the Ag. Since the Ab making up the primary Ab may have been diluted, more Ab from the same species is required to achieve optimal precipitation with the second Ab. Normal rabbit serum (NRS) with its content of Ab is used for this purpose. Following is a description of the protocol used.

The composition of the RIA buffer used was as follows: 0.05 M NaCl, 0.01% sodium azide, 0.1% triton X-100 and 0.1% heat treated bovine serum albumin (HT-BSA). All reagents were prepared in the RIA buffer. Polystyrene tubes (12 x 75 mm; Sarsted Canada Inc.) were used for the reaction. A standard curve was prepared using ANF(99-126)

(Peninsula Laboratories, Belmont, CA) at the following concentrations: 3.125, 6.250, 12.50, 25.00, 50.00 and 100.00 pg/tube.

One hundred µL of the ANF standard, quality control or sample was added to 100 µL of 1:1200 dilution of the antiserum (Peninsula Laboratories) and incubated for 4 h at 4°C. One hundred µL (10,000 cpm) of iodinated ANF(99-126) (New England Nuclear, Mississauga, On) was then added and the tubes incubated at 4°C for 24 h. After this period, free and bound fractions were separated by adding 100 µL goat anti-rabbit gamma globulin (Bio-Rad Laboratories, Mississauga, On) and 100 µL 5% NRS. These tubes were incubated for 2 h at room temperature, after which 1.5 ml polyethylene glycol (PEG) was added. The tubes were centrifuged at 2700 rpm at 4°C for 45 min, the supernatants discarded and the pellets counted for radioactivity on a LKB τ-counter (counting efficiency of 76%). Quality control samples (obtained from pooled samples of perifusate with a known concentration) were run at intervals throughout the experiment. The values obtained from these were used to determine the coefficient of variation for within and between assay and were found to be an average of 8% and 7% respectively.

ANF Immunoprecipitation

Immunoprecipitation was conducted on medium and tissue samples in order to separate the ^{3}H- and ^{14}C-labelled ANF from the total (labelled and unlabelled ANF). The protocol used is as follows. Ten µL of the saline-reconstituted, freeze dried tissue extract or 150 µL of a 1:10 dilution of the bath solution was added to 100 µL of 1:120 dilution of the antiserum (Peninsula Laboratories) and was incubated at 4°C overnight. One hundred µL of goat anti-rabbit gamma globulin and 100 µL of NRS were then added and the tubes left

at room temperature for 2 h. After the addition of 2 ml PEG, the tubes were centrifuged at 2700 rpm for 45 min at 4°C. The supernatants were discarded and the pellets solubilized by the addition of 10 ml NCS (Amersham, Ont., Canada). Ten ml Cytoscint (Fisher Scientific, Ont., Canada) was added and the radioactivity counted using a beta-counter. Tritium and ^{14}C disintegrations per minute (dpm) were computed with the use of external standard-based quench corrections. A standard series method was used to obtain a quench curve calculation, whereby, for each isotope, 10 quenched samples were made up and measured using the dual label standardization program of the Beta counter. In this manner, the two efficiency correction curves (quench curves) and spillover correction curves necessary for the dual labelled samples were calculated.

Data Analysis for the Double-Label Pulse-Chase Experiments

The results are expressed as the mean of 5 experiments ± SEM. The specific activity (S.A.) of either isotope was calculated as immunoprecipitable (IP) disintegrations per min / immunoreactive ANF (dpm/ng) for the tissues and for the labelled ANF released into the medium. Results were expressed as specific activities (dpm/ng) instead of IP (dpm) ^3H- or ^{14}C-ANF so as to account for any differences in total irANF released and stored in individual experiments. The ^3H:^{14}C ratios for the medium were calculated as: IP ^3H-ANF (dpm) / IP ^{14}C-ANF (dpm) released into the medium in the second 1 h chase period. The total IP-ANF (^3H or ^{14}C) represented the sum of the IP ^3H- or ^{14}C-ANF released into the medium and that which was in the tissue at the beginning of the second chase period.

Data were examined by pooled analysis of variance followed by a Tukey HSD test to examine the differences between groups. Differences with a P value < 0.05 were considered significant.

Results

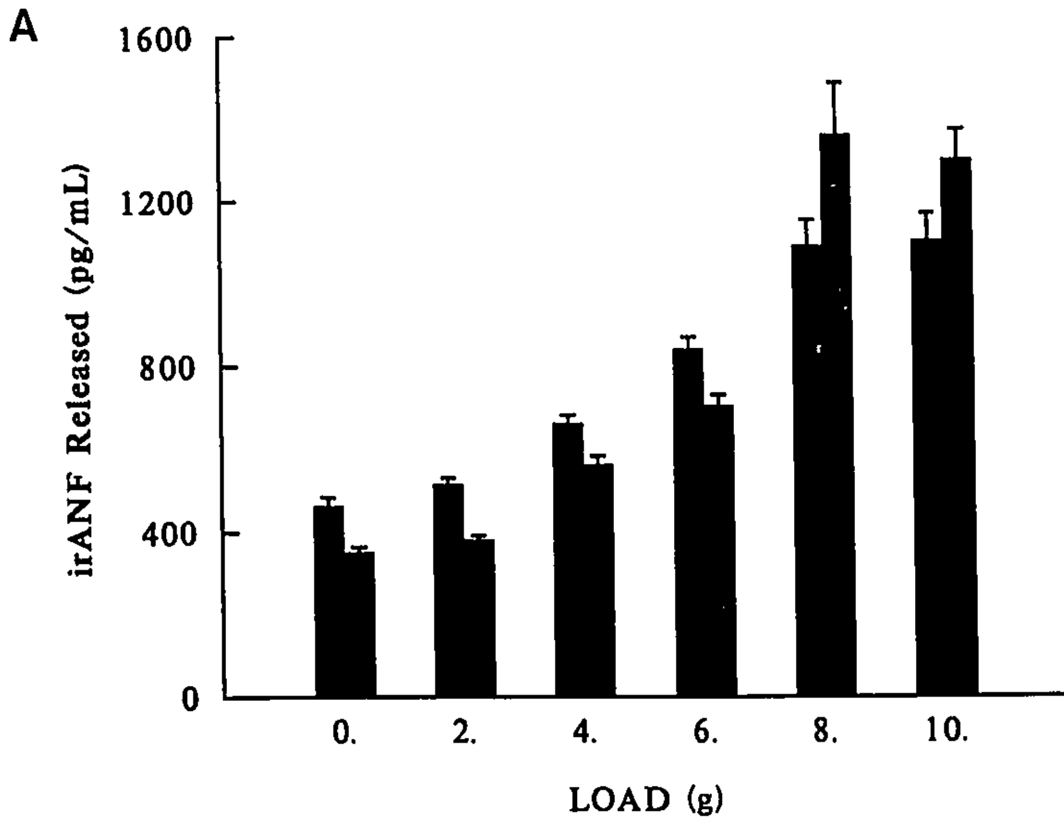
Acute Stretch

With the application of increasing load (2-10 g) on the atrial tissue for 15 min periods, a concomitant increase in ANF release was observed (Figure 2A). However, this increased ANF output reached a maximum with an 8 g load, after which it plateaued and did not increase any further with increasing load. The developed tension also increased with increasing load up to 4 g, after which it plateaued and then decreased at 10 g (Figure 2B).

Continuous Stretch

The tissue was stretched for a period of 3 h with a 0.2 g or 6 g load. The resulting ANF release is shown in Figure 3A and Figure 4A respectively. There was a decrease in ANF release with time in the basal state (0.2 g, Figure 3A), from 208 ± 72.3 pg/ml (at 10 min) to 61 ± 7.9 pg/ml (at 180 min). Upon application of a 6 g load, there was an immediate elevated output of ANF (to 527 ± 93.6 pg/ml at 10 min) after which levels decreased with time (to 82 ± 4 pg/ml) (Figure 4A) even though the increased load (resting tension) was maintained (Figure 4B). Upon application of the 6 g load, developed tension was also increased as compared to basal (0.484 ± 0.003 g versus 0.084 ± 0.007 g) (Figures 3C and 4C) and remained so through the course of the experiment.

Figure 2: Effect of acute increases in resting tension (stretch) from 0.2g to 10 g on (A) ANF release and (B) developed tension. Each bar represents the mean ± SEM of values obtained from a 15 min period (of 1 min collection intervals) for an individual experiment (n=2).


A
1600
1200
800
400
0
irANF Released (pg/mL)
0.
2.
4.
6.
8.
10.
LOAD (g)

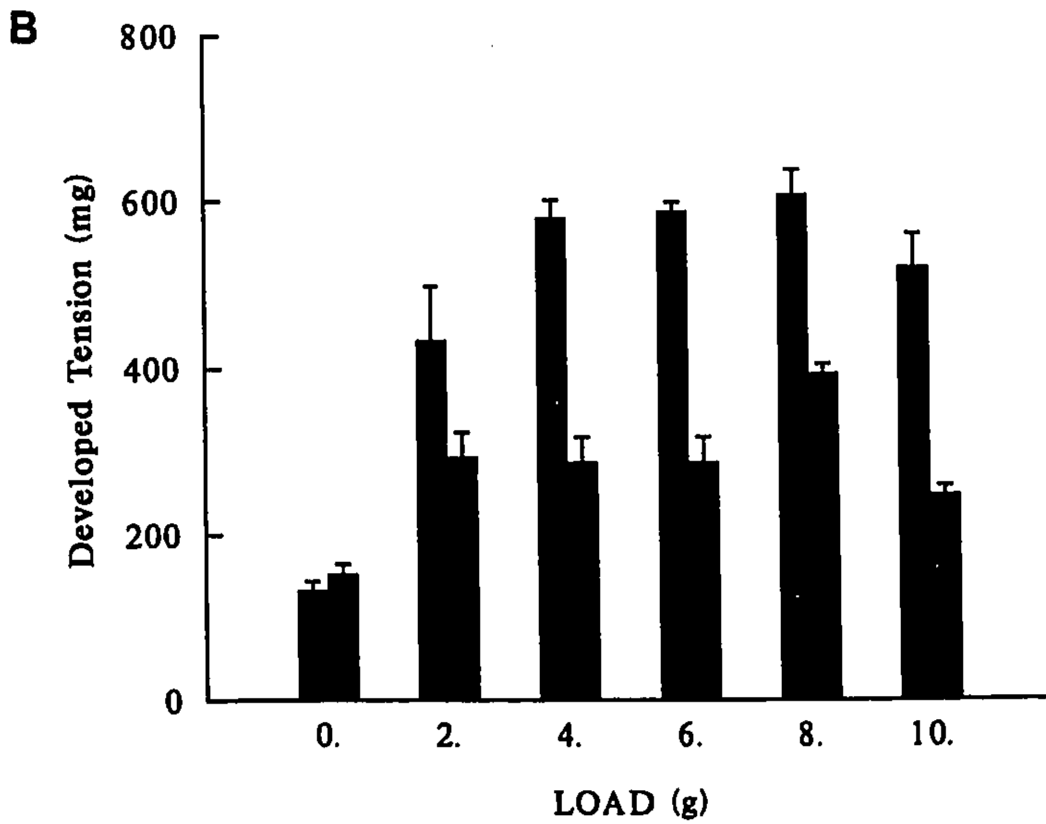
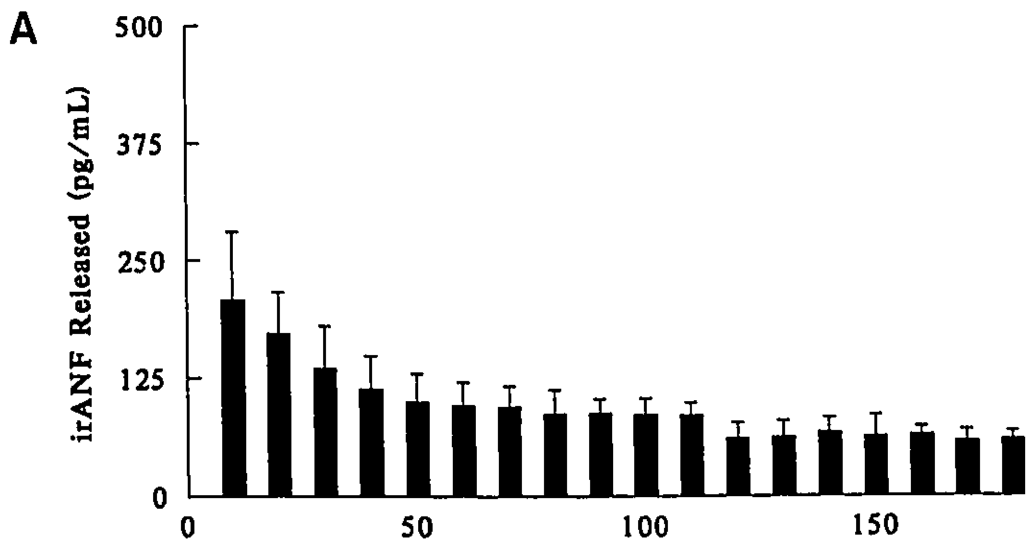
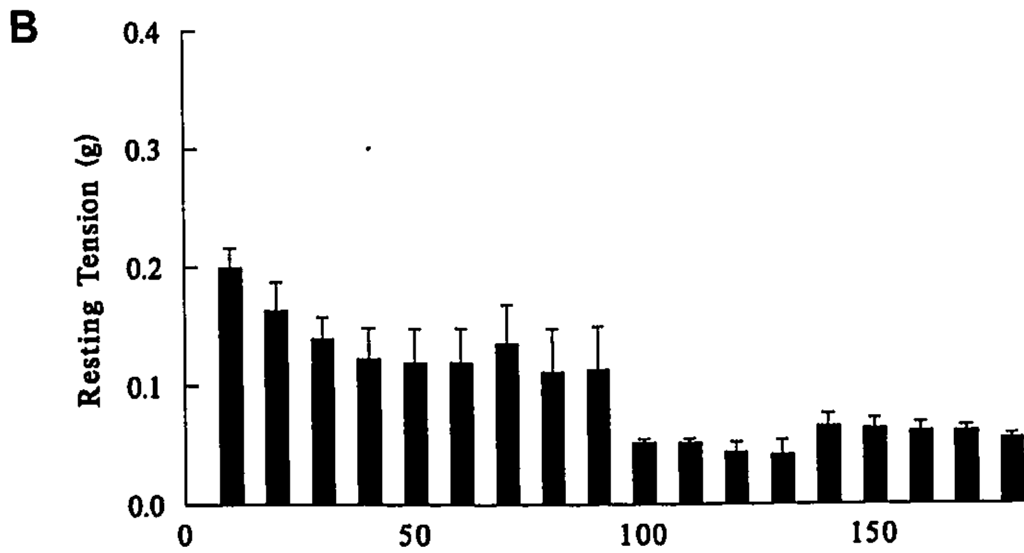

B
800
600
400
200
0
Developed Tension (mg)
0.
2.
4.
6.
8.
10.
LOAD (g)

Figure 3: Effect of basal resting conditions (0.2 g) over a 3 h period on (A) ANF release (B) resting tension and (C) developed tension. Results are the mean ± SEM of 4 experiments.


A
500
375
250
125
0
irANF Released (pg/mL)
0
50
100
150


B
0.4
0.3
0.2
0.1
0.0
Resting Tension (g)
0
50
100
150

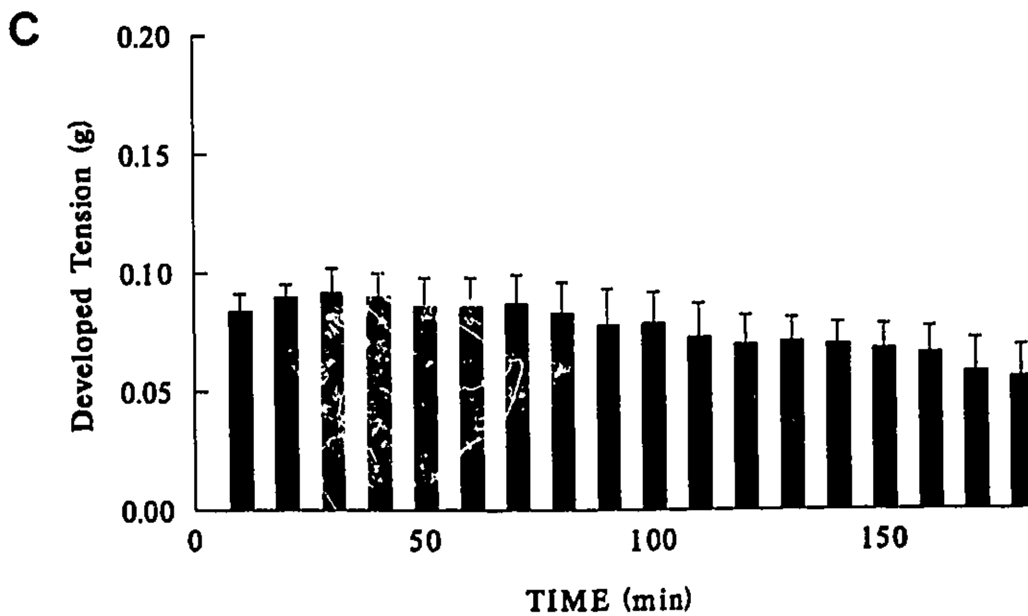
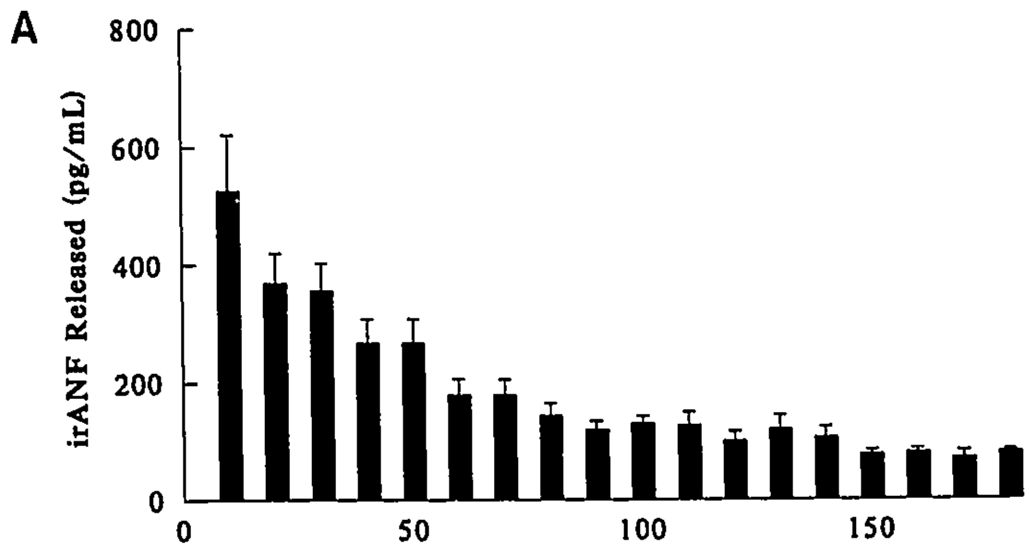
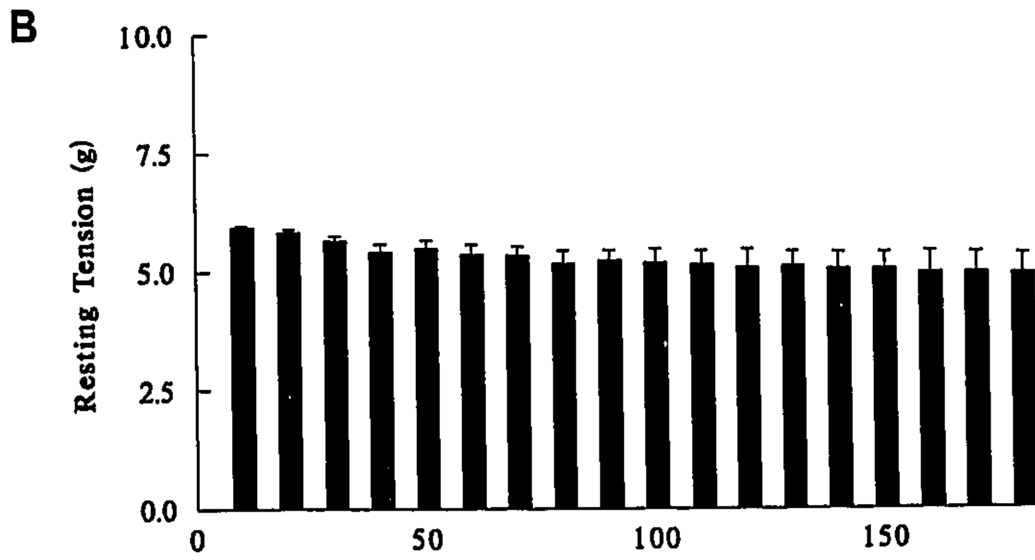
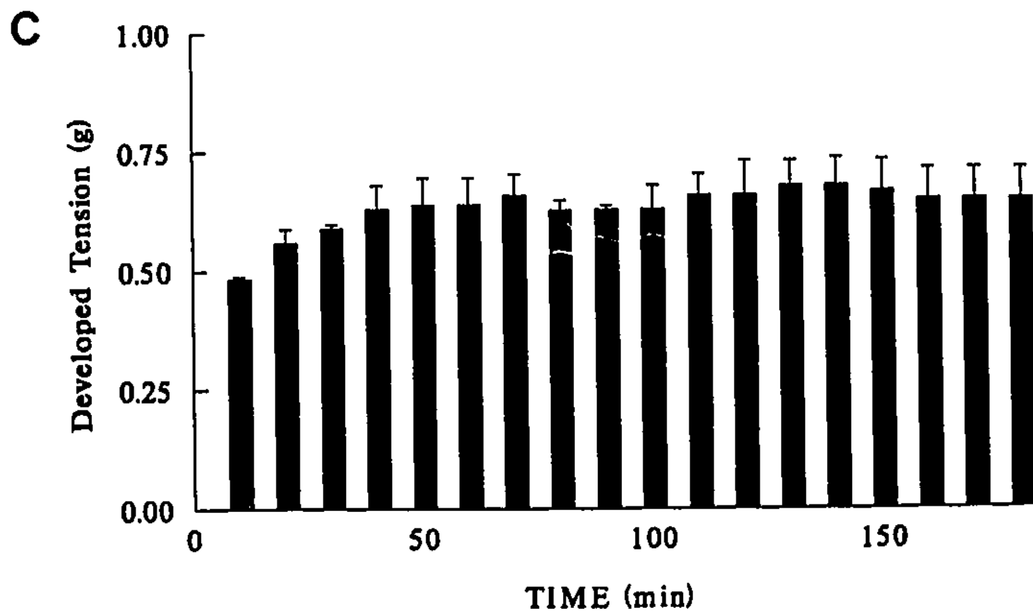

C
0.20
0.15
0.10
0.05
0.00
Developed Tension (g)
0
50
100
150
TIME (min)

Figure 4: Effect of continuous stretch (6 g) for 3 h on (A) ANF release (B) resting tension and (C) developed tension. Results are the mean ± SEM of 4 experiments.


A
irANF Released (pg/mL)
800
600
400
200
0
0
50
100
150


B
Resting Tension (g)
10.0
7.5
5.0
2.5
0.0
0
50
100
150


C
Developed Tension (g)
1.00
0.75
0.50
0.25
0.00
0
50
100
150
TIME (min)

Repetitive Stretch

The effect of repetitive stretch on ANF release is shown in Figure 5. Once again, upon application of the load, there was an immediate increased release of ANF. However, this elevated release was not maintained when the tissue was stretched either every 6 sec (Figure 5A) or every 10 min (Figure 5B). When stretch was imposed on the tissue every 40 min, there was an increase in ANF release with each stretch, but the increase was never as great as the first stretch, and progressively decreased with time and number of stretches (Figure 5C).

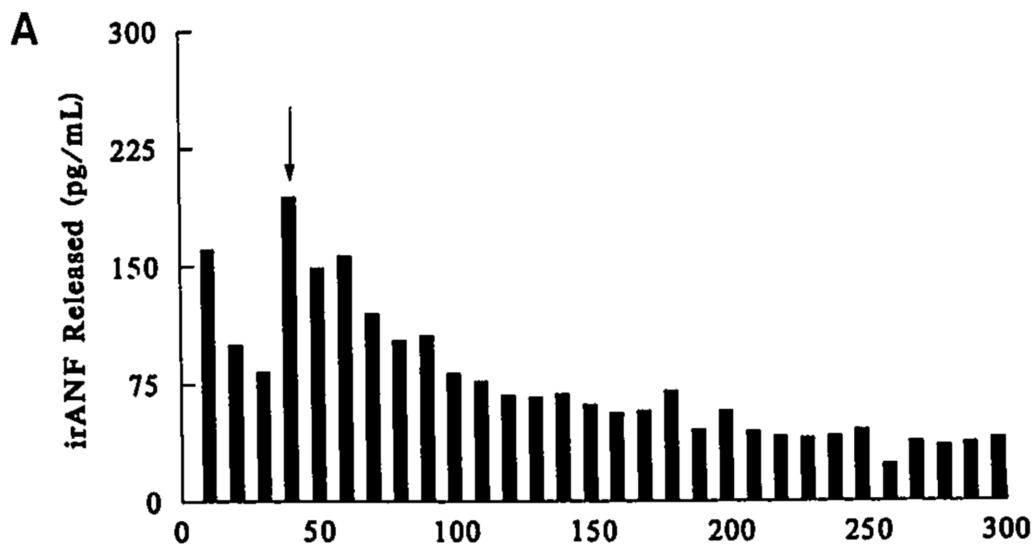
Incorporation of label (^{35}S-cysteine) into TCA Precipitable Protein

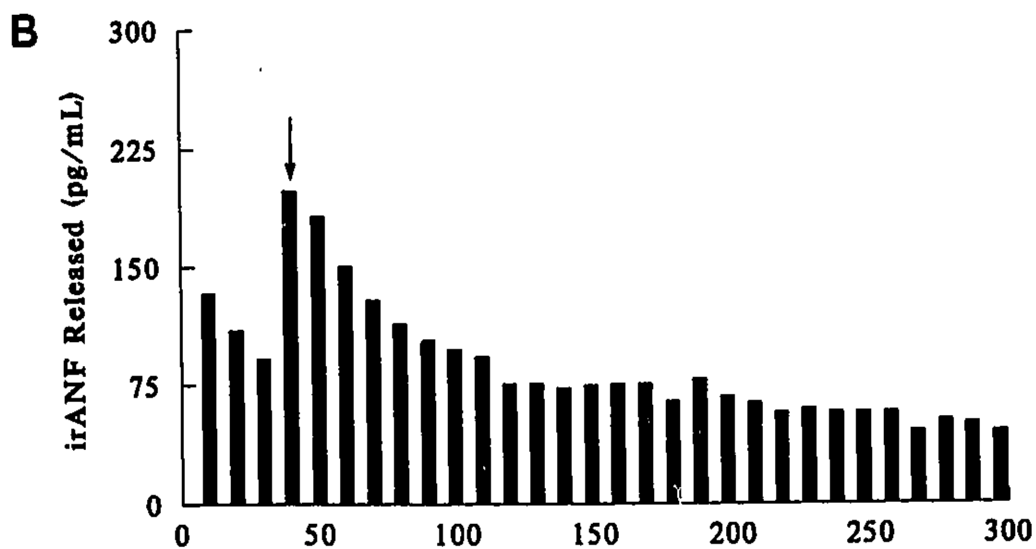
As pulse duration (labelling time) was increased, a concurrent, almost linear, increase in levels of TCA insoluble protein in the atria was observed (Figure 6A). This increase was apparent for labelling times up to 4 h. The TCA soluble fraction also increased with increasing pulse duration (Figure 6B) up to 120 min, after which it plateaued.

Incorporation of ^{35}S-cysteine into protein in the presence of Puromycin

Following perifusion with 10^{-3} M puromycin, there was a decrease of 43% (compared to control) in the amount of label incorporated into TCA insoluble protein which was extracted from the tissue. Exposure of the tissue to 10^{-2} M puromycin resulted in a 65% inhibition of label incorporated into TCA insoluble protein (Table 1).

Figure 5: Effect of repetitive stretch on ANF release Tension was applied for 1-2 sec to the tissue every 6 seconds (A), every 10 min (B) and every 40 min (C). Results are expressed as an average of 2 experiments. Arrows indicate the application of the 3 g load.


A
300
225
150
75
0
irANF Released (pg/mL)
0
50
100
150
200
250
300


B
300
225
150
75
0
irANF Released (pg/mL)
0
50
100
150
200
250
300

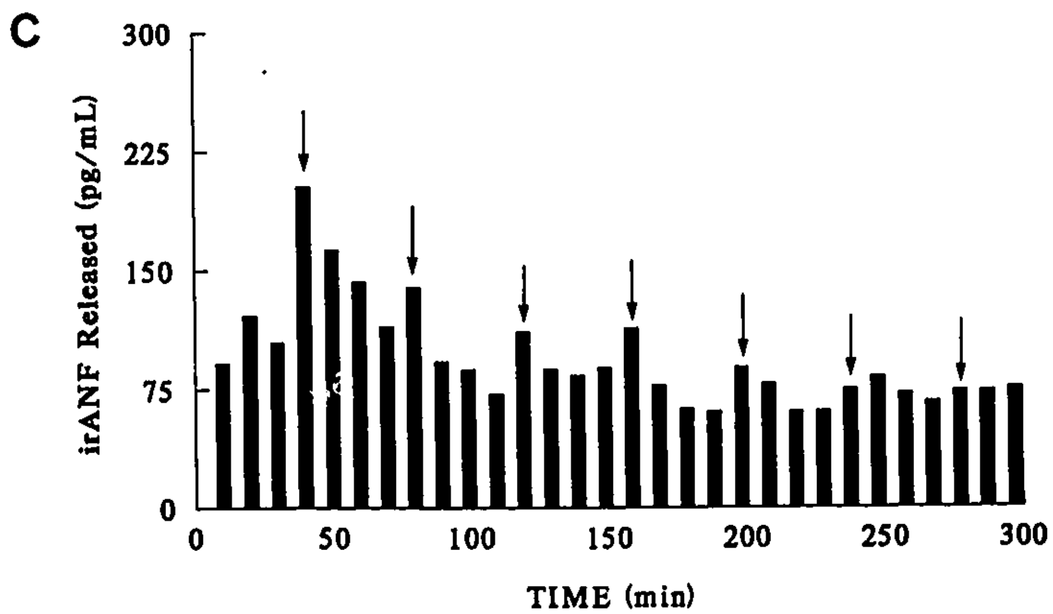
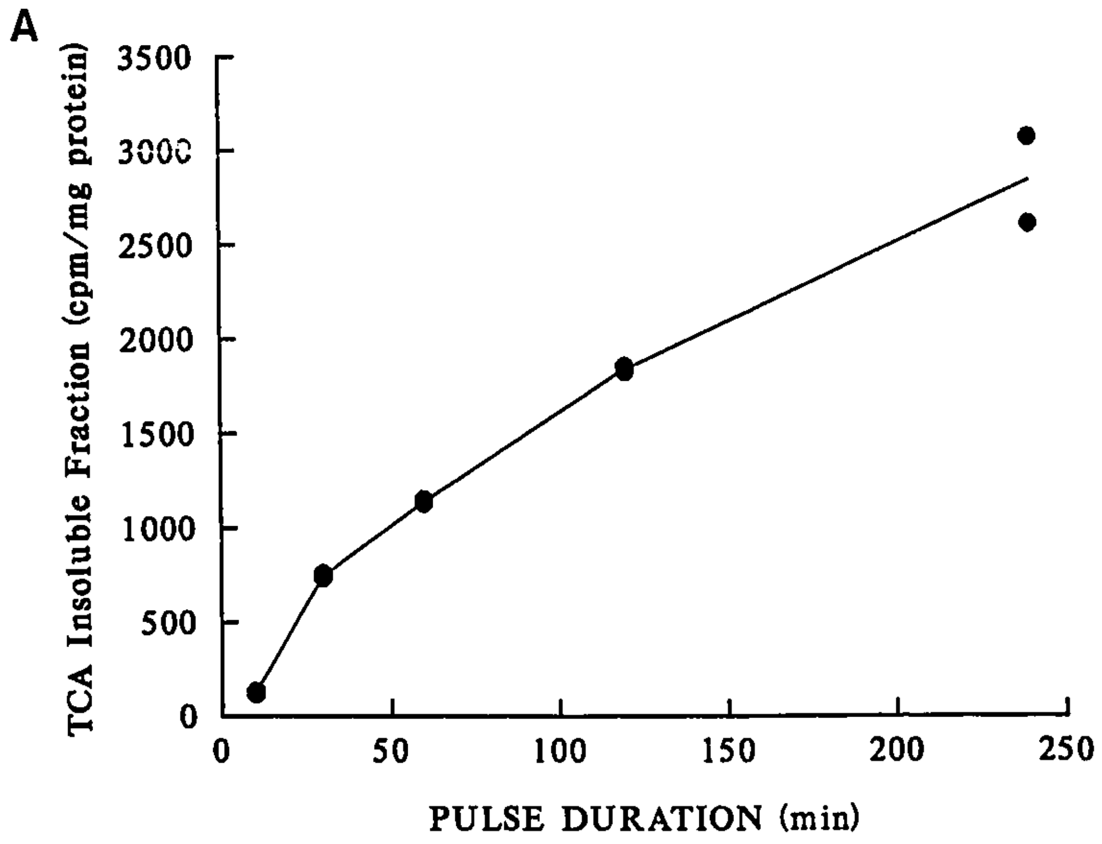

C
300
225
150
75
0
irANF Released (pg/mL)
0
50
100
150
200
250
300
TIME (min)

Figure 6: Effect of increasing pulse duration on tissue TCA insoluble (A) and soluble (B) fractions . Each point represents the results fom an individual experiment (n=2).


A
3500
3000
2500
2000
1500
1000
500
0
TCA Insoluble Fraction (cpm/mg protein)
0
50
100
150
200
250
PULSE DURATION (min)

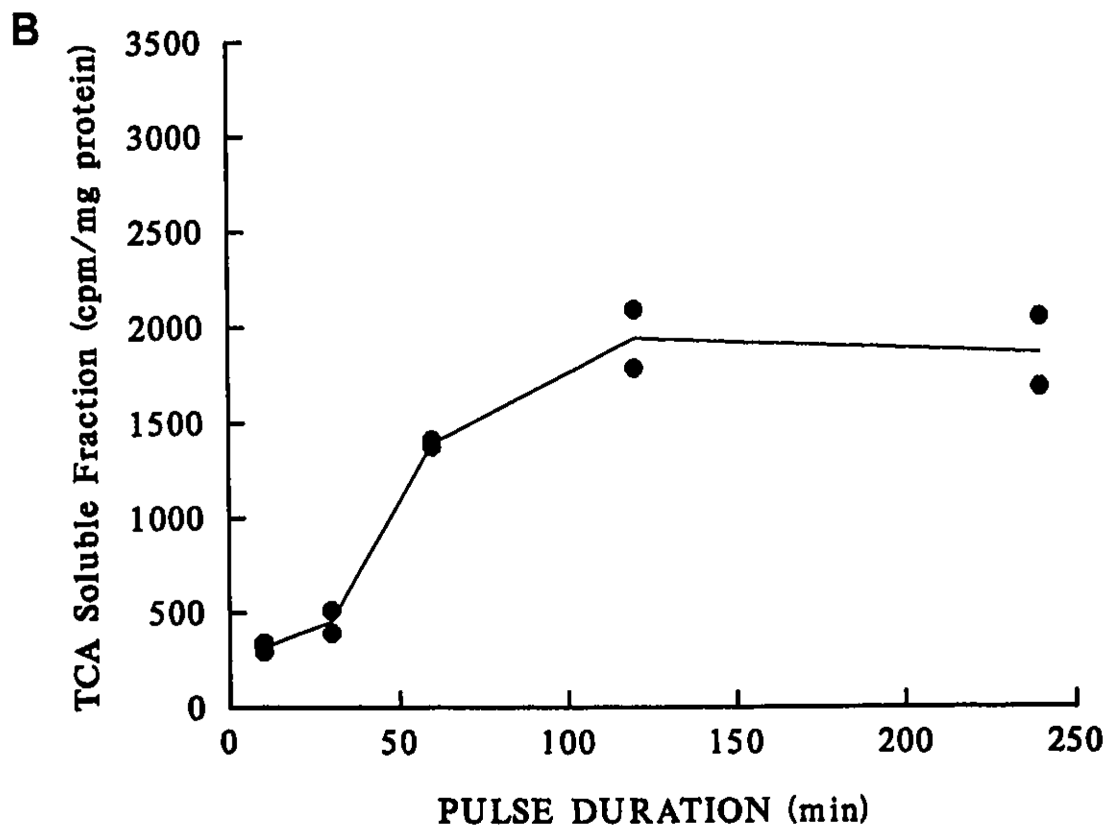

B
3500
3000
2500
2000
1500
1000
500
0
TCA Soluble Fraction (cpm/mg protein)
0
50
100
150
200
250
PULSE DURATION (min)

Table 1 : Effect of Puromycin on levels of TCA Insoluble Protein

EXPERIMENT	CPM / ATRIUM	AVERAGE % INHIBITION
TCA INSOLUBLE PROTEIN	23047, 22646	
10^{-3} M PUROMYCIN	16455, 15817, 15118	43 %
10^{-2} M PUROMYCIN	15797	65 %

Values are those of individual experiments.

Incorporation of label (^{35}S-cysteine) into ANF

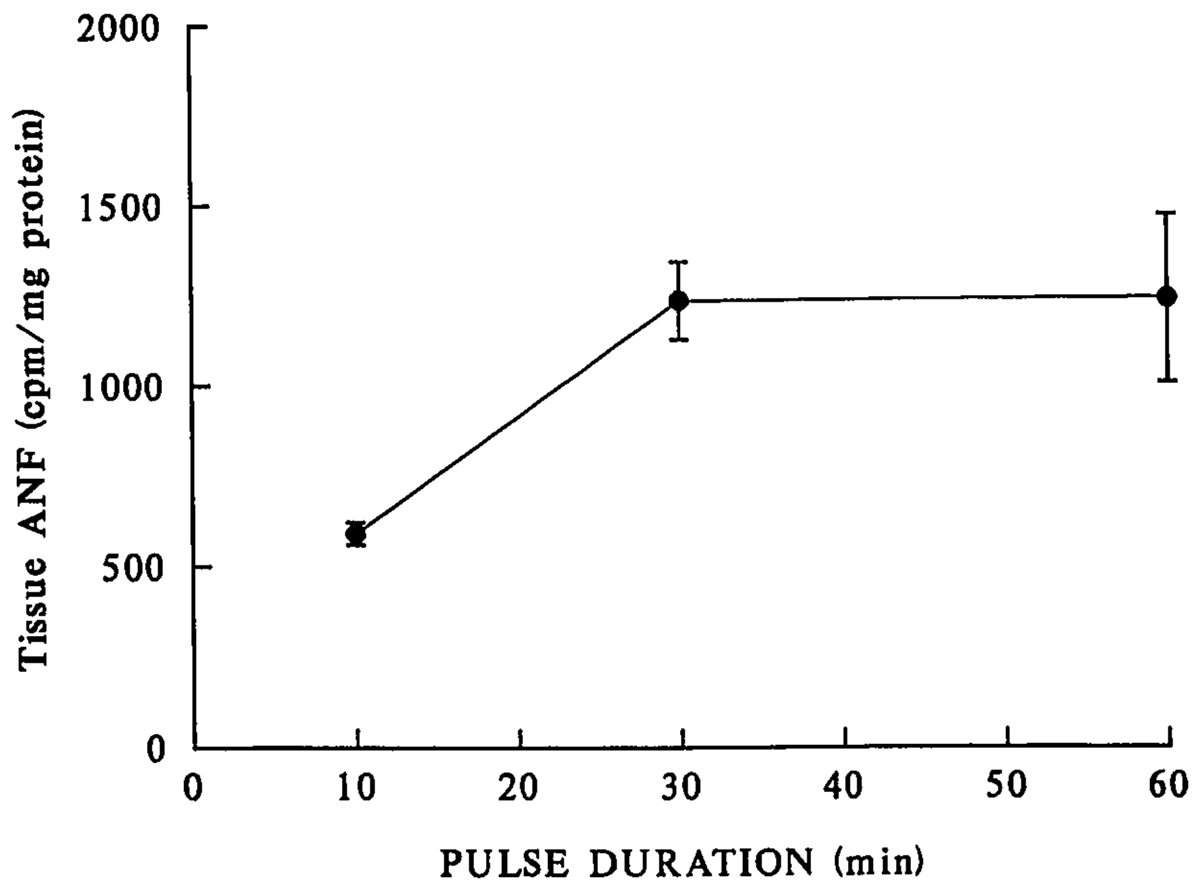
Tissues that were labelled for periods of up to 60 min exhibited increased incorporation of the labelled amino acid (^{35}S-cysteine) into ANF up to 30 min, after which the incorporation reached a plateau (Figure 7).

Double label pulse-chase studies

Basal Conditions

Figure 8A shows that immunoreactive ANF (irANF) release remains stable, during incubation under basal conditions, decreasing only slightly with time, though not significantly. Following pre-incubation of the tissue with ^{14}C-leu KRBB for 3 h, a 1 h chase period was begun. An immediate, yet brief, period of release of immunoprecipitable (IP) ^{14}C-ANF was observed in the first 10 min collection period of the chase that resulted in a significantly ($P < 0.001$) elevated ^{14}C-ANF specific activity (S.A.) levels in the medium (Figure 8B) as compared to the 20 to 180 min collection periods. Therefore, ^{14}C-ANF S.A. levels in the medium decreased after the first 10 min collection period to become stable by 20 min into the chase, and this steady release rate was maintained for the remainder of the experiment, and like the irANF, decreased slightly with time but not significantly. A similar trend was observed following the ^{3}H-leu pulse: initially high ($P < 0.001$) ^{3}H-ANF S.A. levels that decreased to lower, steady levels thereafter (Figure 8C). The S.A.s of the ^{14}C-ANF and ^{3}H-ANF released into the medium (Figures 8B,C) at any of the time points were significantly greater ($P < 0.001$) than the corresponding S.A.s in the tissue at the end of the experiment (Table 2, basal).

Figure 7: Effect of increasing pulse duration on tissue ANF immunoprecipitable counts. Each point represents the mean ± SEM of 5 experiments.



2000
1500
1000
500
0
Tissue ANF (cpm/mg protein)
0
10
20
30
40
50
60
PULSE DURATION (min)

Figure 8: Basal release of ANF over the two chase periods (0-60 min and 120-180 min). A. Release of immunoreactive (ir) ANF. B. Specific activity (S.A.; disintegrations per min per ng ANF per 10 min) of released ^{14}C-ANF. C. Specific activity (S.A.) of released ^{3}H-ANF. Each point is the mean ± SEM of individual specific activities calculated in each of five experiments.

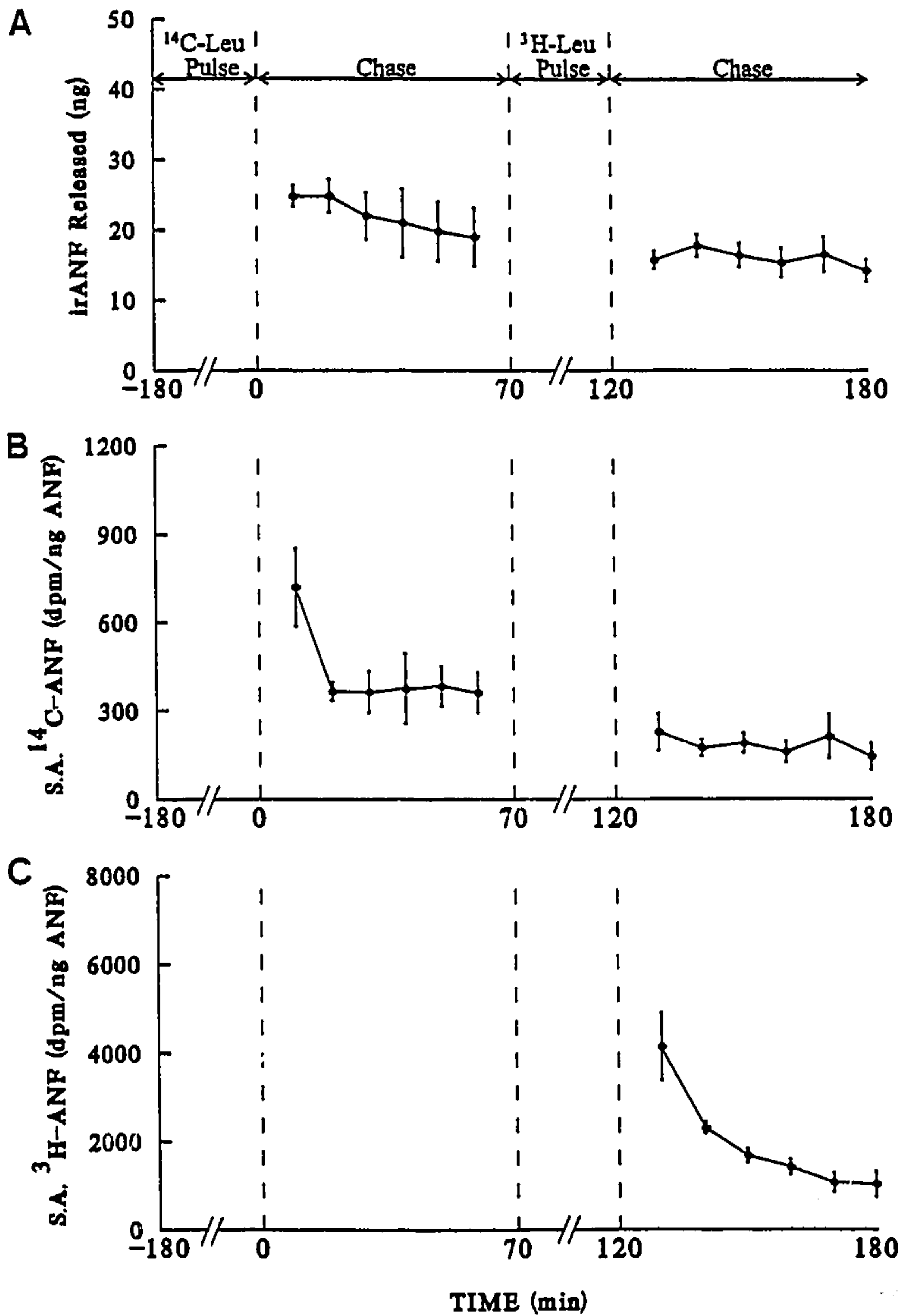

A
50
40
30
20
10
0
¹⁴C-Leu Pulse
Chase
³H-Leu Pulse
Chase
irANF Released (ng)
-180
0
70
120
180
B
1200
900
600
300
0
S.A. ¹⁴C-ANF (dpm/ng ANF)
-180
0
70
120
180
C
8000
6000
4000
2000
0
S.A. ³H-ANF (dpm/ng ANF)
-180
0
70
120
180
TIME (min)

Table 2. Specific Activities of ^{14}C-ANF and ^{3}H-ANF in the Tissue

ANF ISOTOPE	BASAL (dpm/ng ANF)	STRETCH (dpm/ng ANF)
^{14}C-ANF	13.74 ± 1.8	10.8 ± 1.02
^{3}H-ANF	30.70 ± 4.82	17.8 ± 4.6

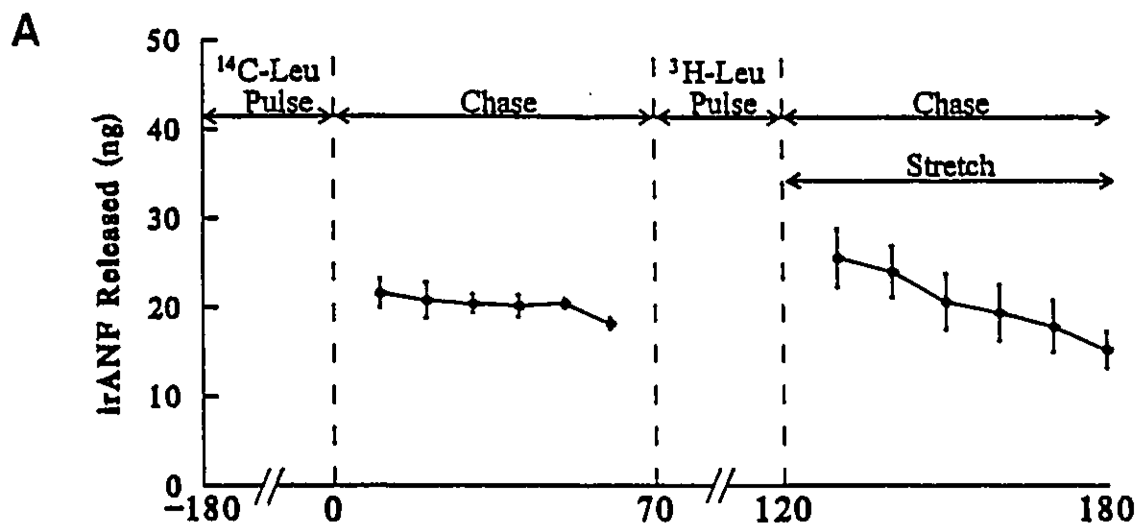
Values are mean ± SEM of five experiments

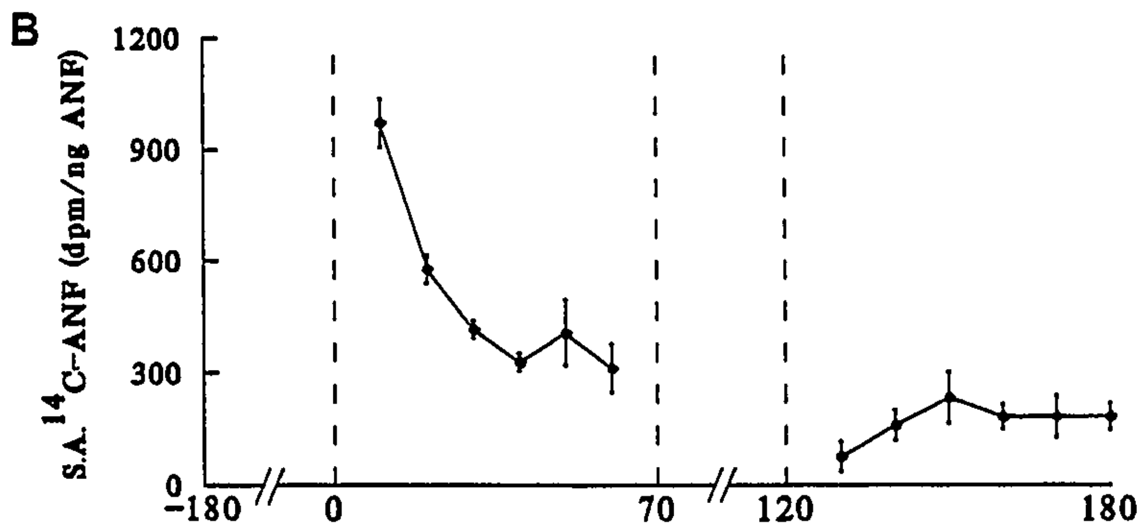
Effect of Stretch

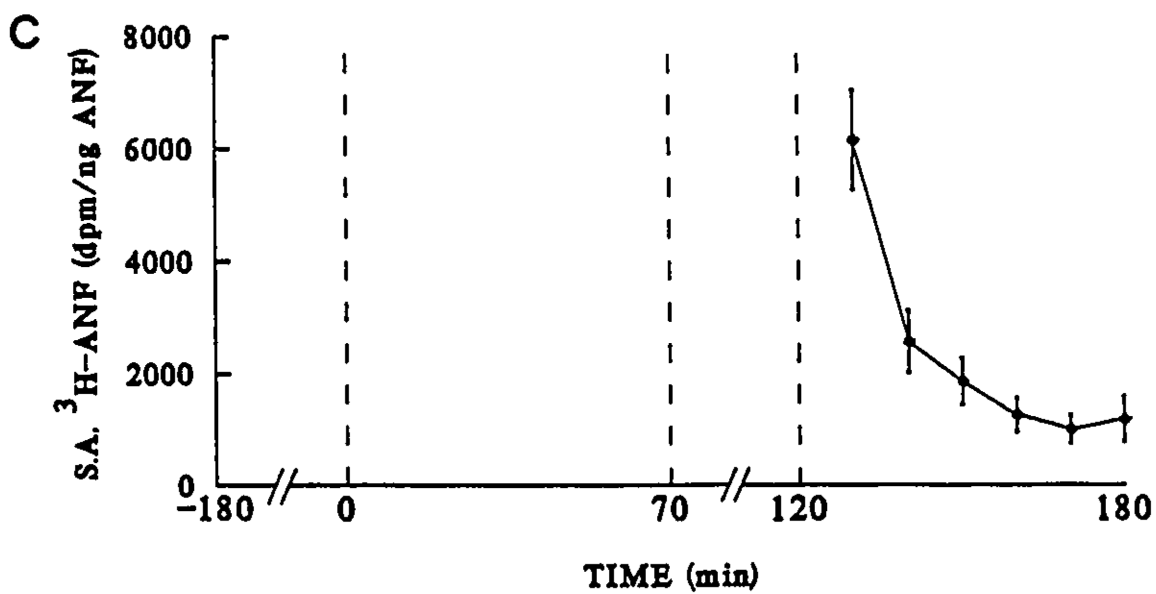
Figure 9A shows the pattern of irANF release during stretch experiments. ANF levels were stable during the 1 h chase period between the first and second pulse; however, upon stretch (at 130 min), there was an immediate significant ($P < 0.05$) increase in irANF release as compared to basal values. The irANF released gradually returned towards basal levels during the chase period, even though there was no change in resting tension (5 g). The levels of released ^{14}C-ANF S.A. followed the same trend as in the basal experiments, prior to the ^{3}H-leu pulse (Figure 9B). However, during the second chase period, while the tissue was being stretched and irANF release into the medium was significantly elevated (Figure 9A), the S.A. of the released ^{14}C-ANF decreased significantly ($P < 0.05$) during the first 10 min of the collection period (130 min), as compared to the 10 to 60 min collection periods prior to stretch, after which it returned to levels comparable to those observed in the basal state.

Upon application of a 5 g load, levels of ^{3}H-ANF S.A. (Figure 9C) in the medium were not significantly different from those observed in the basal state when the tissue was maintained at 0.2 g resting tension (Figure 8C). Again, the S.A.s of the labelled ANF (both ^{14}C and ^{3}H) released into the medium at any of the time points were significantly greater ($P < 0.001$) than those found in the tissue at the end of the experiment (Table 2).

Figure 9: The effect of stretch on ANF release. A 5 g load was applied to the tissue at the onset of the second chase period (i.e. at 120 min). A. Release of irANF. B. Specific activity (S.A.) of released ^{14}C-ANF. C. Specific activity (S.A.) of released ^{3}H-ANF. Each point is the mean ± SEM of individual specific activities calculated in each of five experiments.


A
50
40
30
20
10
0
IrANF Released (ng)
¹⁴C-Leu
Pulse
Chase
³H-Leu
Pulse
Chase
Stretch
-180
0
70
120
180


B
1200
900
600
300
0
S.A. ¹⁴C-ANF (dpm/ng ANF)
-180
0
70
120
180


C
8000
6000
4000
2000
0
S.A. ³H-ANF (dpm/ng ANF)
-180
0
70
120
180
TIME (min)

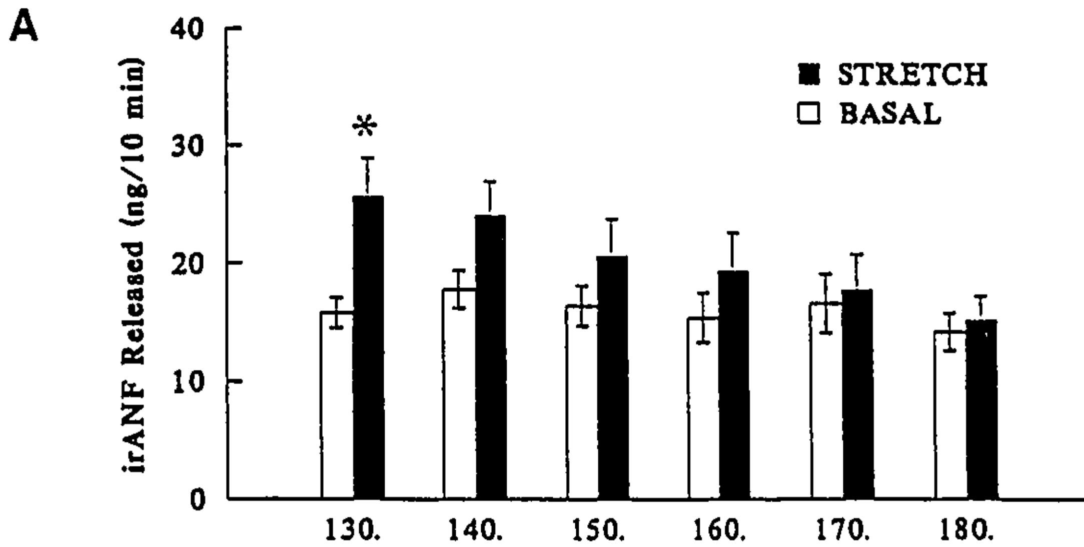
Comparison of Basal vs Stretch Conditions on ANF Release

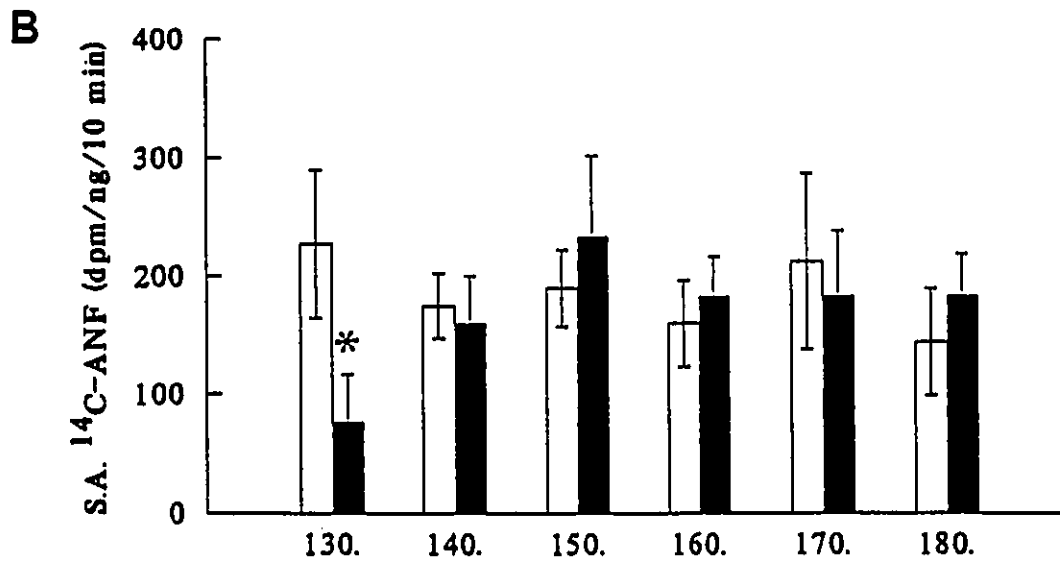
The irANF released following stretch (5 g) was significantly greater ($P < 0.05$) than that observed for the corresponding basal experiments (0.2 g) (Figure 10A) for the first 10 min collection interval (130 min), after which it returned towards basal levels. The level of ^{14}C-ANF S.A. in the medium was lower ($P < 0.05$) than the corresponding basal levels at the 130 min collection period, after which there was no significant difference for the remainder of the experiment (Figure 10B). There was no significant difference between levels of ^{3}H-ANF S.A. released in the basal and stretched conditions (Figure 10C). Comparison of the S.A.s of ^{14}C-ANF and ^{3}H-ANF in the tissue in the basal and stretched states (Table 2) revealed no significant differences.

Analysis of the ^{3}H to ^{14}C ratio (Table 3) of the ANF released into the medium (10.72 ± 0.94) was found to be approximately twice that in the tissue at the start of the second chase period (5.29 ± 0.40) for the basal experiments. In contrast, in the tissues in which ANF release was stimulated by stretch, the ^{3}H- to ^{14}C-labelled ANF ratio in the medium (16.01 ± 1.00) had increased to be approximately 2.5 times that found in the tissue (6.77 ± 0.98). Consequently, there was a significantly greater ($P < 0.05$) ^{3}H to ^{14}C ratio in the medium following stretch when compared to basal. However, there was no significant difference between the total (released plus stored) amounts of labelled ANF in the basal and stretched tissues.

Figure 10 Comparison of ANF release in the basal and stretched states during the second chase period. Each point represents the mean ± SEM of five experiments. A. Release of irANF. B. Specific activity (S.A.) of released ^{14}C-ANF. C. Specific activity (S.A.) of released ^{3}H-ANF.

*: $P < 0.05$, basal versus stretch.


A
irANF Released (ng/10 min)
STRETCH
BASAL
*
0
10
20
30
40
130.
140.
150.
160.
170.
180.


B
S.A. 14C-ANF (dpm/ng/10 min)
*
0
100
200
300
400
130.
140.
150.
160.
170.
180.

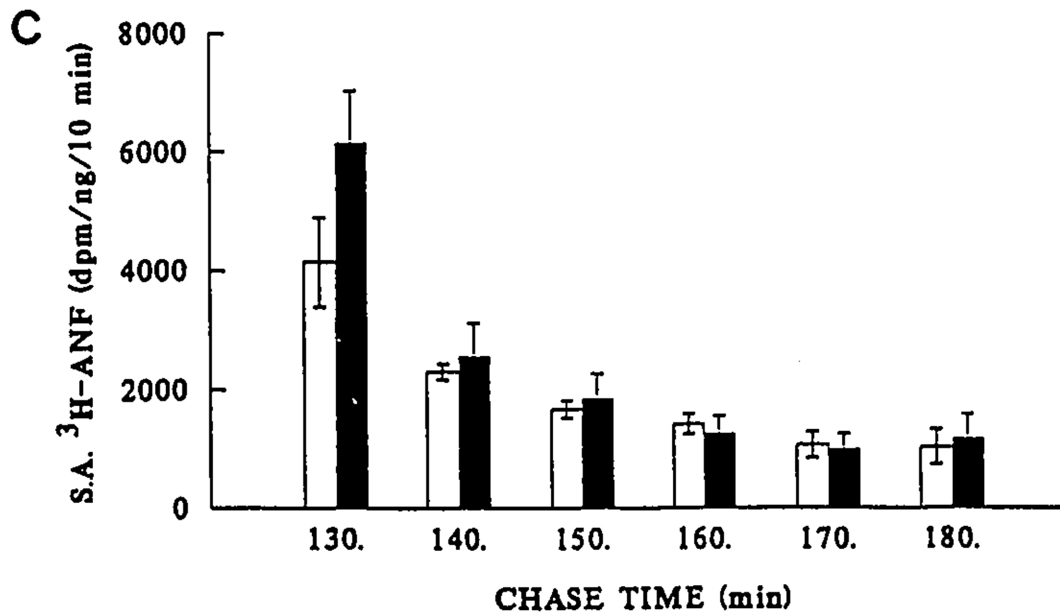

C
S.A. 3H-ANF (dpm/ng/10 min)
0
2000
4000
6000
8000
130.
140.
150.
160.
170.
180.
CHASE TIME (min)

Table 3. ^{3}H to ^{14}C Ratios of Immunoprecipitable ANF released into the Medium and Total Tissue content

	^{3}H:^{14}C RATIO
BASAL	
MEDIUM	10.72 ± 0.94
TOTAL	5.29 ± 0.40
STRETCH	
MEDIUM	16.01 ± 1.00 *
TOTAL	6.77 ± 0.98

Computation of the ^{3}H to ^{14}C ratio is discussed in the text.

Values are mean ± SEM of five experiments.

*: $P < 0.05$, basal vs stretch

Discussion

ANF basal rate of release from the cardiac atria may be increased by a number of different stimuli (Ruskoaho, 1993). Acute increases in atrial muscle stretch brought about by changes in haemodynamics or, *in vitro*, by increasing muscle resting tension, result in a prompt increase in ANF release that is evident within seconds after stimulation (de Bold et al., 1986; de Bold and de Bold, 1991). These observations have led to the establishment of the concept of stretch-secretion coupling (de Bold et al., 1986) which is thought to be the major mediator of physiological and pathophysiological increases of ANF circulating levels. The mechanism underlying transduction of the mechanical stretch stimulus into a secretory response by atrial cardiocytes is unknown.

In the present study, the temporal changes in ANF release with acute, continuous and repetitive stimulation by atrial stretch (by increasing resting tension) were demonstrated in an *in vitro* rat atria preparation. In addition, a double label, pulse-chase protocol was developed in this preparation in order to assess the contribution of the newly synthesized and stored hormone pools to basal and stretch stimulated ANF release from atrial cardiocytes.

Acute atrial distension resulted in a progressive increase in both developed tension and ANF release with increasing resting tension (stretch) of up to 8 g after which both parameters are decreased. These results are in accordance with the Frank-Starling relationship, i.e. the heart responds to increased tension by increasing its ability to perform work, that is, stretching of the heart's sarcomeres results in enhanced contractile

performance. The increase in ANF release to acute atrial muscle stretch demonstrated that the atrial tissue in this preparation is capable of exhibiting the stretch-ANF secretion response, and thus confirms that acute atrial muscle stretch is a stimulus for ANF release. Physiological situations in which acute atrial muscle stretch is exhibited are events leading to an acute increase in central venous pressure such as in head-out water immersion and acute volume load (Miki et al., 1986; Pendergast et al., 1987; Lang et al., 1985). Acute myocardial infarction and rhythm disturbances are pathophysiological situations in which stretch-induced release would occur (Crozier et al., 1987; Mendez et al., 1987; Moe et al., 1991).

Stretch-secretion coupling has been extended to explain chronically elevated ANF circulating levels found in some experimental and clinical conditions (Ding et al., 1987; Edwards et al., 1988). As demonstrated in this work, however, continuous stretch results in an immediate release of ANF after which ANF output gradually returns to basal levels even with constant increased loading. Our results are in agreement with other studies conducted both *in vivo* and *in vitro* (Shin et al., 1991; Walsh et al., 1988; Agnoletti et al., 1987). Agnoletti et al. (1987; 1989) used an *in vitro* rat atria preparation to also show that the release of the peptide was transient, and became readily attenuated. This attenuated response could not be due to depletion of ANF stores as the total ANF tissue content was not significantly reduced. The investigators suggest that the granules whose contents are released from the cell upon stretch are in close contact with the sarcolemma, and provide a small pool of immediately releasable ANF, whereas the majority of specific granules lie in the Golgi complex area from where they originate. This could explain why upon further stimulation, the response is attenuated, as the pool of immediately available ANF becomes

exhausted. In addition, Walsh and coworkers (1988) reported that, following 240 min of rapid cardiac pacing in dogs, heart rate, mean pulmonary wedge pressure and mean right arterial pressure all increased and remained so through the course of the experiment. However, it was observed that arterial ANF concentration increased to a maximum at 30 min, fell after 60 min and went on to decrease further after 240 min of rapid continuous pacing. These investigators also concluded that the atria cannot maintain the peak concentrations of ANF reached after 30 min of rapid pacing despite persistently elevated atrial pressures. In our setting, the results suggested that the tissue may have adapted to the effect of a single stretch, and that repetitive stimulation may be required to maintain a high rate of release. This hypothesis was tested by stretching the tissue repetitively every 6 s, 10 min and 40 min. However, following the initial acute release of ANF upon application of the first stretch, levels returned once again to basal values. Even when the tissue was allowed to "rest" for 40 min between stretches, each successive stretch resulted in a lower elevation of ANF release, and the response was never as great as the initial stretch. There have been reports of other studies examining the effect of repetitive stretch on ANF release. Bilder et al (1986) distended isolated rat atria by the inflation of miniature balloon catheters for periods of 10 min and reported increases in ANF release with increasing distension and also with a moderate amount of distension but with an increasing rate of inflation. At the highest rate tested, immunoreactive ANF was six times greater than that observed with continuous maximal expansion. Thus, it was concluded that the extent of ANF release is influenced by both the rate and magnitude of stretch. Although these results do provide support for the existence of the stretch-secretion coupling mechanism, the periods of stretch (10 min) appear to be rather short, and may not reflect the true response which may be observed had these experiments been conducted for longer periods of time, as was done in

the present work. More evidence for the reduced response to repetitive stimulation was provided by Cuneo et al (1989) who studied the effects of chronic and acute volume expansion on atrial diameter and ANF release in man. These investigators reported increased ANF release and atrial diameter during chronic sodium loading. However, further acute atrial distension failed to increase hormone levels, suggesting that factors in addition to atrial stretch are involved in regulating ANF release in man.

The rapid decline in ANF output seen after acute, prolonged and repetitive atrial muscle stretch suggests the existence within atrial cardiocytes of an acutely releasable hormone pool that is exhausted shortly after the initial stretch stimulus. Situations involving chronic atrial distension and the subsequent effects on ANF release still remains rather unclear. There have been several reports which demonstrate that ANF plasma levels remain elevated despite the termination of the stretch stimulus (Dannanberg et al., 1989; Kaufman et al., 1990) and some which observe increased ANF levels without elevations in atrial pressure (Moe et al., 1991; Fournier et al., 1991), suggesting that atrial distension is not responsible for chronically elevated levels of circulating ANF. It has also been shown that in the chronic situation, ANF mRNA and synthesis is increased, either in the presence or absence of stretch. Shin and co-workers (1991) conducted an *in vivo* study to demonstrate that when atria are stimulated by distension to produce plasma ANF levels as seen in heart failure, the response was greatly attenuated within a few hours. It required several days of maintained stimulation for ANF levels to return to those that had been observed under acute conditions. These investigators conclude that chronically elevated release was the result of increased synthesis and release of ANF. From this, it can be concluded that in the chronic situation, persistent stimulation from sources other than, or in addition to, atrial distension

are required. In addition, chronically elevated ANF release is accompanied by increased ANF synthesis.

Thus, stimulated ANF release seems best viewed as a process that may be brought about by two different mechanisms: one mechanism is based on an immediately releasable, stretch-sensitive, depletable peptide pool. A second mechanism is based on increased gene expression and peptide synthesis which is stimulated by persistent haemodynamic or neuroendocrine stimulation.

From the above discussion, it is evident that following acute stimulation, endocrine tissues producing polypeptide hormones show a behaviour that is compatible with the existence of an acutely releasable pool, followed by a decrease in hormone output and partial depletion of stores under continuous stimulating conditions. There are two well documented pathways responsible for the release of secretory products, namely, the regulated and constitutive pathways (Burgess and Kelly, 1987). The regulated pathway involves concentration of the product into secretory granules and the subsequent release of the product in response to a stimulus. If the newly synthesized product is sorted into the constitutive pathway, it is not concentrated and stored into granules, but is packaged into vesicles which are destined for rapid release in a non-regulated fashion. The *trans*-Golgi network (TGN) appears to be involved in the sorting of the product to either the constitutive or regulated pathways. The mechanisms of protein sorting between these two secretory pathways is unknown.

A number of studies on different hormone systems have reported the preferential release of newly synthesized secretory product over older, stored products from cells of endocrine (Arvan et al., 1991; Macgregor et al., 1975; Stachura et al., 1985; Walker and Farquhar, 1980) and exocrine glands (Rothman, 1976). However, there have also been instances where preferential release of older, stored product has been reported (Sharoni et al., 1976) and others where there is random release. Stachura and collaborators (1986) have proposed a model of intracellular hormone pathways whereby, following synthesis, a small amount of the hormone is released immediately. In this model, the majority of the newly synthesized hormone follows an intracellular pathway through which it is either stored or passed along a path which bypasses storage on its way to release sometime after synthesis.

To test the applicability of the above concepts to ANF, we used a double label, specific immunoprecipitation approach as it allowed for the simultaneous assessment of the effect of stretch on newly synthesized and stored ANF release. The irANF released into the medium is ANF that could have been synthesized at any time in the past. ANF that was released during the second chase period but labelled with isotope from the first pulse incubation (i.e. ^{14}C-labelled ANF) had to have been synthesized during the first incubation and stored during the second pulse period. ANF labelled with ^{3}H-leucine during the second pulse incubation and released during the second chase period represents the most recently synthesized ANF. According to the results obtained in the present work, ANF could also follow the secretory pathway described by Stachura et al. (1986). We have shown that, following both the ^{14}C-and ^{3}H- leu pulses, isotope incorporated into ANF appeared in the chase medium within the first 10 min collection interval. This observation alone implies rapid and direct release of newly synthesized hormone. The S.A. of the released ^{14}C- and

^{3}H- ANF stabilized to a lower level after 20 min into the chase, suggesting that there is also a steady release of newly synthesized ANF some time after the termination of the pulse. The presence of radioactive ANF in the tissue at the end of the experiment suggests that newly synthesized ANF is also stored in the tissue. This finding agrees with the autoradiographic studies reported by de Bold and colleagues (1975) who found that newly synthesized protein was incorporated into specific atrial granules within an hour after the injection of ^{3}H-leu into rats. The fact that the S.A. of ANF in the medium for either isotope was much higher than that in the tissue leads us to conclude that a major fraction of the newly synthesized ANF was preferentially secreted from the tissue either without entering, or equilibrating with, the total granule pool. This could imply that some ANF is released before being packaged into granules, or is released from a group of newly formed secretory granules.

Therefore, there appear to be two pathways of unstimulated release of newly synthesized products. One which is released immediately, and would be analogous to "constitutive" secretion, and the other which is released later and which may come after sorting into the "regulated" pathway. Matsuuchi and Kelly (1991) refer to the latter as "basal" release and they believe it to account for a large fraction of the total secreted protein. From the results presented above, it would appear that "basal" ANF release is the sum of newly synthesized peptide released immediately after synthesis and that which is released some time after synthesis i.e. a combination of the characteristics of both constitutive and regulated secretion.

Stimulation of ANF release by application of a 5 g load on the tissue resulted in an immediate increased irANF release. The ^{14}C-ANF S.A. in the medium was found to decrease

when ANF release was increased implying that there was no change in the immunoprecipitable (IP) ^{14}C-ANF released with stretch. As ANF levels gradually returned towards baseline, so did the ^{14}C-ANF S.A. The observed decrease in S.A. implies the dilution of isotopically labelled hormone with unlabelled hormone. This shows that the increased ANF output with stretch is not due to ^{14}C-ANF that was synthesized, *in vitro,* 2 to 5 hours before stretch. This is further supported by the fact that there was no change in the S.A. of the ^{14}C-labelled ANF in the tissue upon stretch. However, the ^{3}H-ANF S.A. in the medium with the application of a 5 g load was not significantly different from basal values when the tissue was maintained at 0.2 g resting tension. As the ^{3}H-ANF S.A. did not change and the irANF increased upon stretch, then this suggests that there was an increase in immunoprecipitable ^{3}H-ANF released. This observation indicates that there was an increase in newly synthesized ANF release that occurred in parallel to the increase in irANF release following stretch. The tissue S.A. of the ^{3}H-labelled ANF was decreased as compared to the unstretched tissue, thereby supporting the notion that stretch resulted in an increased release and subsequent slightly decreased storage of the newly synthesized ^{3}H-ANF. Further evidence for this was provided by examination of the ^{3}H:^{14}C ratio. Upon stretch, the ^{3}H:^{14}C ratio in the medium was higher than the corresponding ratio in the unstretched tissue, indicating an increase in the amount of immunoprecipitable ^{3}H-ANF released upon stretch.

As there is no change in the immunoprecipitable ^{14}C-ANF released or stored upon stretch, then the accelerated release of the newly synthesized ^{3}H-ANF appears to have occurred via a different path from the one being used to release ^{14}C-ANF from storage. Stretch may increase ANF release by shunting a larger portion of the newly synthesized ANF into an immediately releasable pool at the expense of storing the hormone.

The ^{14}C- and ^{3}H- ANF released into the medium does not appear to be the result of passive cell leakage from damaged cardiocytes. We have previously reported that the isolated atria preparation, as evaluated by several criteria, maintains its integrity over long periods of incubation, as shown by measurement of creatine kinase released into the medium, and by light and electron microscopy (de Bold et al., 1986). Furthermore, the most abundant form of the peptide released into the incubation medium is the processed molecular form, $ANF_{99\text{-}126}$, and not the unprocessed form, $ANF_{1\text{-}126}$, which is the main tissue storage molecular form that would be expected to be released upon tissue disruption. The stable mechanical activity exhibited by the atria and the steady rate of ANF release throughout the course of the experiments demonstrated that the tissue was not disrupted, which would cause ANF release to be increased or erratic. The TCA and ANF incorporation studies showed that both TCA precipitable protein and ANF was being actively synthesized in this preparation and that this protein synthesis was inhibited by Puromycin, thereby confirming that the results were not artifactual. From this, it can be concluded that the tissue in this model is capable of remaining viable over a long period, that the tissue is translationally active and that the release of labelled ANF is not artifactual. The reliability of the preparation used in the present work is further supported by the fact that there was no need to transform results (e.g. to percent of basal values) but, rather, it was possible to average specific activity results between experiments.

The above results were not designed to provide insight into the mechanisms of stretch-secretion coupling. However, some discussion on the subject is relevant. It has been shown that the rate of peptide-chain initiation and elongation / termination increases with an increase in stretch of the myocardium (Kira et al., 1984; Morgan et al., 1987;

Xenophontos et al., 1986). Stretch has been reported to increase the rate of protein synthesis in a variety of muscles including isolated papillary muscle (Cooper et al., 1989; Peterson and Lesch, 1972) and skeletal muscle (Palmer et al., 1985) thereby demonstrating that the ability to sense mechanical load is intrinsic to muscle cells and does not require exogenous neurohormonal factors. The intracellular mediators linking stretch to faster protein synthesis have yet to be identified. Studies have shown that inhibition of contractile activity had no effect on the rate of protein synthesis (Kira et al., 1984; Vandenburgh et al., 1989). In addition, Sadoshima and co-workers (1992) reported that Gd^{3+}-sensitive stretch-activated ion channels, microfilaments, microtubules or contractile activity were not necessary for the transduction of stretch into increased protein synthesis. In skeletal muscle, it has been suggested that increased intracellular Ca^{2+} availability and higher rates of prostaglandin synthesis in response to changes in muscle tension could be involved (Palmer et al., 1983; Vandenburgh et al., 1990). However, these factors do not appear to play a role in the stretched myocardium (Gordon et al., 1985; Morgan et al., 1986). Sodium uptake has been reported to be increased in quiescent or contracting ferret papillary muscle with increasing load (Kent et al., 1989). Addition of streptomycin (a cationic blocker of mechanotransducer ion channels) resulted in the inhibition of the faster rate of protein synthesis, and led to the proposal that deformation-dependent sodium influx is an early signal in the transduction of load into growth. However, there has also been evidence presented in isolated perfused hearts which demonstrates that streptomycin did not block the accelerated protein synthesis which accompanies elevated aortic pressure, and thus does not provide support for the proposed role of sodium influx as a transducer of growth (Morgan et al., 1989). It has also been proposed that intracellular content of cyclic AMP may serve as the intracellular transducer of stretch into cardiac growth (Watson et al., 1989; Xenophontos et al., 1989).

Inositol phosphates have also been reported to be increased with cardiac stretch (Komuro et al., 1991; von Harsdorf et al., 1989) and may be due to activation of phospholipase C. Therefore, it is evident that the molecular mechanisms by which these intracellular signals control cardiac cell growth still remains to be determined.

It cannot be directly determined from these experiments if there was an increase in ANF synthesis with stretch, although this aspect should be considered. Stretch was carried out with the tissue incubated in medium containing unlabelled amino acids, which would decrease the specific activity of the labelled ANF, thereby masking any possible increased synthesis effect caused by stretch.

In conclusion, these studies demonstrate for the first time that, in the basal condition, a portion of ANF is immediately and preferentially released following synthesis, and the rest is presumably taken into tissue stores and released from them at a lower rate. In addition, although atrial stretch has been reported to be the main stimulus for ANF release, these studies have demonstrated that stretch does result in an acute, transient elevated ANF output, however, it is not capable of maintaining these high levels upon either continuous or repetitive stretch. This suggests that factors other than atrial muscle stretch are involved in the long term regulation of ANF release. Furthermore, when ANF release is stimulated by stretch, newly synthesized ANF release is increased but there is no change in older, stored ANF release. These data suggest that stretch may play a role in increasing the translocation of newly synthesized ANF into a stretch-sensitive pool. The fact that this peptide pool is rapidly depletable may be due to its size, or could be related to a depletion or inactivation

of second messenger systems thereby rendering the mechanisms coupling atrial stretch to ANF secretion refractory to further stimulation for a period of time.

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Appendix

Table 1: **Effect of acute increases in resting tension on ANF release (Figure 1)**

LOAD (g)	ANF_1 (pg/ml)	$ANFSE_1$	DT_1 (mg)	$DTSE_1$	ANF_2 (pg/ml)	$ANFSE_2$	DT_2 (mg)	$DTSE_2$
0.2	463	22	133	11	351	12	153	11
2.0	516	15	433	64	381	9	293	30
4.0	662	20	580	20	563	21	287	30
6.0	842	28	587	11	705	25	287	30
8.0	1091	63	607	30	1365	126	393	11
10.0	1.105	66	520	40	1303	73	247	11

Legend:

$ANF_{1,2}$ - ANF release in two (1,2) experiments
$ANFSE_{1,2}$ - Standard erreor of the means for ANF release
$DT_{1,2}$ - Developed tension
$DTSE_{1,2}$ - Standard error of the mean for developed tension

Table 2: **Effect of basal resting conditions (0.2 g) on ANF release (Figure 2)**

Time (min)	MNANF (pg/ml)	SEANF	MNRT (g)	SERT	MNDT (g)	SEDT
10	208	72.3	0.200	0.016	0.084	0.007
20	172	43.5	0.164	0.023	0.090	0.005
30	136	44.3	0.140	0.018	0.092	0.010
40	114	34.7	0.123	0.026	0.090	0.010
50	101	28.7	0.120	0.028	0.086	0.012
60	96	24.3	0.120	0.028	0.086	0.012
70	95	21.6	0.136	0.032	0.087	0.012
80	88	24.6	0.112	0.036	0.083	0.013
90	88	14.3	0.114	0.036	0.078	0.015
100	87	16.9	0.052	0.003	0.079	0.013
110	86	12.7	0.052	0.003	0.073	0.014
120	62	16.3	0.044	0.008	0.070	0.012
130	63	16.3	0.042	0.012	0.071	0.010
140	68	14.7	0.066	0.010	0.070	0.009
150	63	22.4	0.064	0.080	0.068	0.010
160	66	7.8	0.062	0.007	0.066	0.011
170	59	11.4	0.062	0.004	0.058	0.014
180	61	7.9	0.056	0.003	0.056	0.013

Legend:

MNANF - Mean ANF release
SEANF - Standard error of the means for ANF release
MNRT - Mean resting tension
SERT - Standard error of the means for resting tension
MNDT - Mean developed tension
SEDT - Standard error of the means for developed tension

Table 3: Effect of continuous stretch (6 g) on ANF release (Figure 3)

Time (min)	MNANF (pg/ml)	SEANF	MNRT (g)	SERT	MNDT (g)	SEDT
10	527	93.6	5.940	0.028	0.484	0.003
20	369	50.2	5.840	0.057	0.560	0.028
30	357	45.4	5.660	0.085	0.590	0.007
40	268	38.8	5.430	0.156	0.630	0.050
50	268	40.0	5.510	0.156	0.640	0.056
60	179	27.2	5.380	0.198	0.640	0.056
70	179	25.6	5.360	0.170	0.660	0.043
80	144	20.2	5.180	0.254	0.628	0.020
90	121	13.1	5.260	0.198	0.630	0.007
100	132	10.0	5.200	0.283	0.630	0.050
110	128	21.4	5.160	0.283	0.660	0.043
120	102	14.8	5.100	0.368	0.660	0.071
130	122	21.8	5.130	0.297	0.680	0.051
140	107	16.8	5.070	0.325	0.680	0.057
150	78	6.9	5.070	0.325	0.668	0.065
160	81	5.2	4.990	0.438	0.650	0.064
170	71	12.3	4.980	0.424	0.650	0.064
180	82	4.0	4.970	0.410	0.650	0.064

Legend:

- MNANF - Mean ANF release
- SEANF - Standard error of the means for ANF release
- MNRT - Mean resting tension
- SERT - Standard error of the means for resting tension
- MNDT - Mean developed tension
- SEDT - Standard error of the means for developed tension

Table 4: Effect of repetitive stretch on ANF release (Figure 4)

TIME (min)	ANF (pg/ml) 6 Sec	ANF (pg/ml) 10 min	ANF (pg/ml) 40 min	TIME (min)	ANF (pg/ml) 6 sec	ANF (pg/ml) 10 min	ANF (pg/ml) 40 min
10	161	133	92	160	57	76	114
20	100	110	122	170	58	76	78
30	83	92	105	180	71	65	63
40	194	199	203	190	46	79	61
50	149	183	163	200	58	68	89
60	157	151	143	210	45	64	79
70	120	129	115	220	42	58	61
80	103	114	140	230	41	60	61
90	106	104	93	240	42	58	75
100	82	98	88	250	46	58	83
110	77	93	72	260	24	58	72
120	68	76	112	270	39	46	67
130	67	76	88	280	36	53	74
140	69	73	84	290	38	51	74
150	62	75	89	300	41	46	76

Table 5: **Effect of increasing pulse duration on TCA insoluble and soluble fractions (Figure 5)**

Pulse (min)	TCA INSOL.$_1$ (cpm/mg protein)	TCA INSOL.$_2$ (cpm/mg protein)	TCA SOL.$_1$ (cpm/mg protein)	TCA SOL.$_2$ (cpm/mg protein)
10	131	120	339	295
30	737	759	509	391
60	1152	1132	1411	1374
120	1856	1827	1788	2094
240	3077	2616	2052	1686

Table 6: **Effect of increasing pulse duration on immunoprecipitable ANF (IPANF) (Figure 6)**

Pulse (min)	IPANF (cpm/mg protein)	IPANF SE
10	590	30
30	1238	108
60	1246	234

Table 7: **Immunoreactive ANF release in the double-label, pulse-chase studies - basal (0.2 g) conditions (Figure 7A)**

CHASE TIME (min)	H288	H289	H295	H298	H299	MEAN	SEM
10	23	25	28	28	20	24.8	1.53
20	21	21	29	32	21	24.8	2.375
30	16	16	30	30	18	22	3.286
40	14	9	32	33	17	21	4.868
50	13	12	29	31	14	19.8	4.188
60	10	12	32	25	16	19	4.147
130	16	18	14	19	12	15.8	1.281
140	15	18	23	19	14	17.8	1.594
150	13	16	22	18	13	16.4	1.691
160	10	13	22	18	14	15.4	2.088
170	11	13	21	24	14	16.6	2.502
180	12	11	17	19	12	14.2	1.594

Legend:

H288-H299	-	Experiment number
Mean	-	Mean ANF release
SEM	-	Standard error of the means

Table 8: **Release of ^{14}C-ANF (Specific Activities; SA; dpm/ng ANF) - Basal (0.2 g) conditions (Figure 7B)**

CHASE TIME (min)	H288	H289	H295	H298	H299	MEAN	SEM
10	551.000	506.920	1175.00	500.250	867.100	720.054	132.461
20	381.143	444.667	299.000	291.813	412.905	365.906	30.505
30	625.313	375.188	266.800	222.333	333.500	364.627	70.305
40	381.143	815.222	166.750	161.697	353.118	375.586	118.991
50	359.154	555.833	0.000	193.645	428.786	384.355	75.490
60	533.600	444.667	229.281	240.120	0.000	361.917	75.691
130	383.375	370.556	95.286	175.526	111.167	227.182	62.641
140	222.333	185.278	87.000	140.421	238.214	174.649	27.642
150	307.846	166.750	121.273	148.222	205.231	189.864	32.503
160	133.400	256.538	181.909	37.056	190.571	159.895	36.440
170	242.545	461.769	0.000	166.750	190.571	212.327	74.420
180	277.917	60.636	0.000	70.211	166.75	143.879	50.700

Legend:

H288-H299	-	Experiment number
Mean	-	Mean ^{14}C-ANF S.A.
SEM	-	Standard error of the means

Table 9: **Release of ^{3}H-ANF (Specific Activities; SA; dpm/ng ANF) - Basal (0.2 g) conditions (Figure 7C)**

CHASE TIME (min)	H288	H289	H295	H298	H299	MEAN	SEM
130	3084.88	2630.94	3182.85	4458.37	6392.08	4141.57	755.59
140	2490.13	1889.83	2668.00	2246.74	2239.21	2306.78	131.55
150	2001.00	2042.69	1334.00	1371.06	1641.85	1678.12	150.20
160	1734.20	1744.46	1091.46	926.39	1619.88	1423.27	172.55
170	1879.73	1026.15	1016.38	611.42	809.93	1068.72	216.61
180	722.58	2182.91	902.41	631.90	722.58	1032.48	290.94

Legend:
H288-H299 - Experiment number
Mean - Mean ^{3}H-ANF S.A.
SEM - Standard error of the means

Table 10: **Immunoreactive ANF release in the double-label, pulse-chase studies - stretch (5 g) conditions (Figure 8A)**

CHASE TIME (min)	H311	H312	H315	H316	H322	MEAN	SEM
10	21	20	19	20	28	21.6	1.631
20	19	20	16	21	28	20.8	1.985
30	19	20	18	21	24	20.4	1.030
40	17	19	19	23	23	20.2	1.268
50	19	20	21	20	22	20.4	0.510
60	16	20	19	17	19	18.2	0.735
130	20	22	29	20	37	25.6	3.295
140	17	24	30	18	31	24.0	2.915
150	14	18	26	15	30	20.6	3.156
160	12	16	27	15	27	19.4	3.172
170	12	14	26	13	24	17.8	2.973
180	11	15	22	11	17	15.2	2.059

Legend:

H311-H322	-	Experiment number
Mean	-	Mean ANF release
SEM	-	Standard error of the means

Table 11: **Release of ^{14}C-ANF (Specific Activities; SA; dpm/ng ANF) - Stretch (5 g) conditions (Figure 8B)**

CHASE TIME (min)	H311	H312	H315	H316	H322	MEAN	SEM
10	1175.19	967.150	982.947	967.150	762.286	970.945	65.356
20	596.789	533.600	667.000	635.238	452.607	577.047	38.236
30	421.263	366.850	481.722	444.667	361.292	415.159	22.994
40	313.882	280.842	351.053	406.000	290.000	328.355	22.893
50	737.211	400.200	285.857	366.850	242.545	406.533	87.298
60	125.063	333.500	210.632	470.824	421.263	312.256	64.361
130	33.350	181.909	0.000	166.750	0.000	76.402	40.511
140	274.647	83.375	155.633	222.333	64.548	160.107	40.019
150	476.429	148.222	76.962	266.800	200.100	233.703	68.196
160	277.917	166.750	172.926	222.333	74.111	182.807	33.748
170	333.500	285.857	76.962	51.308	166.750	182.875	55.714
180	121.273	222.333	151.591	303.182	117.706	183.217	35.391

Legend:

H311-H322 - Experiment number
Mean - Mean ^{14}C-ANF S.A.
SEM - Standard error of the means

Table 12: Release of ^{3}H-ANF (Specific Activities; SA; dpm/ng ANF) - Stretch (5 g) conditions (Figure 8C)

CHASE TIME (min)	H311	H312	H315	H316	H322	MEAN	SEM
130	8637.65	6427.46	5635.00	6870.10	3190.78	6152.20	888.99
140	3491.94	2084.36	1889.83	4187.28	1183.39	2567.36	551.67
150	2668.00	1334.00	1180.08	3068.20	1044.97	1859.05	419.28
160	1723.08	750.38	667.00	2223.33	938.74	1260.51	304.72
170	833.75	762.29	769.62	2001.00	611.42	995.61	253.98
180	1212.73	578.07	485.09	2728.64	902.41	1181.39	407.53

Legend:

H311-H322 - Experiment number
Mean - Mean ^{3}H-ANF S.A.
SEM - Standard error of the means