

Natriuretic Peptide Secretion and Gene Expression in Isolated Rat Atria  
Following Mechanical or Neuroendocrine Stimulation

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...Il n'est pas aisé de découvrir la Pierre Philosophale. Les alchimistes restaient plusieurs années dans leurs laboratoires, à observer ce feu qui purifiait les métaux. Et tant ils regardaient le feu que, dans leur for intérieur, ils en venaient peu à peu à abandonner toutes les vanités du monde. Alors, un beau jour, ils s'apercevaient que la purification des métaux, en fin de compte, les avaient purifiés eux-mêmes.

Paulo Coelho, "L'Alchimiste"

## ABSTRACT

The natriuretic, diuretic, and vasorelaxant hormones atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) are synthesized and secreted mainly from the mammalian atria of the heart, and are crucial for the maintenance of cardiovascular homeostasis. Direct stimulation of isolated adult rat atrial tissue by stretch, endothelin-1, and phenylephrine was conducted to attempt to define the mechanisms involved in controlling the secretion and gene expression of ANF and BNP in adult atrial cardiocytes. These stimuli represent models for mechanical, autocrine/paracrine, and neuroendocrine stimulation of the endocrine heart. The expression of the early response genes *c-fos*, *c-jun*, *Egr-1*, and *c-myc* were also studied, since they may regulate ANF or BNP gene expression. Isolated rat atria were stimulated by stretch, endothelin-1, or phenylephrine. Radioimmunoassay was used to measure levels of ANF and BNP, and Northern blotting was employed to measure changes in mRNA levels. Stretch resulted in a rapid and short-lived increase in the secretion of ANF and BNP. Calculation of the ratio of ANF to BNP suggested that this may be due to exocytosis of granules that contain both peptides, as well as granules that contain ANF only. Stretch selectively stimulated the expression of the BNP, *c-fos*, *Egr-1*, and *c-myc* genes. Endothelin-1 stimulated the secretion of ANF and BNP, following a time course that is distinct from that elicited by stretch: the increase in secretion was gradual, reached a plateau, and after a few hours returns towards basal levels. The pattern was similar for ANF and BNP, which suggests that they are co-secreted in response to this stimulus. Phenylephrine stimulated ANF and BNP secretion, but their stimulated secretion was not co-regulated. Endothelin-1 and phenylephrine stimulated BNP, *Egr-1*, and *c-myc* gene expression. Phenylephrine also modestly stimulated ANF gene expression. Changes in ANF and BNP mRNA levels were not

coordinated with increased BNP secretion. The results show that ANF and BNP secretion and gene expression are distinctly regulated; this may be due to the relative abundance of each hormone and to partially different mechanisms of secretion for each. Therefore, mechanical and neuroendocrine stimuli contribute in different and specific manners to the modulation of the endocrine heart and hence to the maintenance of cardiovascular homeostasis.

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## ABBREVIATIONS

|             |                                         |
|-------------|-----------------------------------------|
| ADH:        | antidiuretic hormone                    |
| ANF:        | atrial natriuretic factor               |
| AP-1:       | activator protein 1                     |
| ATP:        | adenosine triphosphate                  |
| BNP:        | brain natriuretic peptide               |
| cAMP:       | cyclic adenosine monophosphate          |
| CAT:        | chloramphenicol acetyl transferase      |
| cDNA:       | complementary deoxyribonucleic acid     |
| cGMP:       | cyclic guanosine monophosphate          |
| CNP:        | C-type natriuretic peptide              |
| DOCA:       | deoxycorticosterone acetate             |
| ET-1:       | endothelin-1                            |
| ir:         | immunoreactive                          |
| MAP kinase: | mitogen activated protein kinase        |
| MEK:        | mitogen activated protein kinase kinase |
| MHC:        | myosin heavy chain                      |
| mRNA:       | messenger ribonucleic acid              |
| PCR:        | polymerase chain reaction               |
| PKC:        | protein kinase C                        |
| RIA:        | radioimmunoassay                        |
| rRNA:       | ribosomal ribonucleic acid              |
| SHR:        | spontaneously hypertensive rat          |
| SRE:        | serum response element                  |
| SRF:        | serum response factor                   |

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Bruneau B.G., Piazza L.A., & de Bold A.J.;  $\alpha_1$ -Adrenergic stimulation of isolated rat atria results in discoordinate increases in natriuretic peptide secretion and gene expression and enhances *Egr-1* and *c-myc* expression. *Endocrinology* 1996;137(1):137-143; © The Endocrine Society

## INTRODUCTION

Atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) are diuretic, natriuretic, and vasorelaxant peptides produced and secreted mostly by the atrial cardiocytes of the heart in mammals, and by both atrial and ventricular cardiocytes in non-mammals (de Bold, 1985; Sudoh *et al.*, 1988; Nakao *et al.*, 1992). A third related peptide, named C-type natriuretic peptide (Sudoh *et al.*, 1990), does not have natriuretic activities, and is found mostly in the central nervous system and in vascular endothelial cells (Sudoh *et al.*, 1990; Suga *et al.*, 1992b; Stingo *et al.*, 1992b). Together, these hormones are referred to as the natriuretic peptides. Chronic hemodynamic overload of the atria results in an increase in natriuretic peptide circulating levels and atrial mRNA levels (de Bold *et al.*, 1996; Lattion *et al.*, 1986; Arai *et al.*, 1987; Drexler *et al.*, 1989; Haass *et al.*, 1990; Mukoyama *et al.*, 1991; Fischer *et al.*, 1991; Ogawa *et al.*, 1991; Yokota *et al.*, 1994; Yokota *et al.*, 1995). The importance of the natriuretic peptides in the maintenance of cardiovascular homeostasis in pathophysiological situations has been recently exemplified by experiments involving natriuretic peptide receptor antagonists, or "knockouts" of ANF or ANF receptors (Yokota *et al.*, 1994; Hirata *et al.*, 1994; Nishikimi *et al.*, 1994; Wada *et al.*, 1994; John *et al.*, 1995; Stevens *et al.*, 1995; Lopez *et al.*, 1995). The factor(s) involved in the transduction of the hemodynamic load into enhanced release and gene expression are unknown, as is the time course of these events.

## Historical Perspective

The first mention of a cardiac hormone was made in 1929 by Noël and Morin, in their discussion of a 1922 paper by Tang, in which he proposed the existence in Purkinje cells of birds of a cytological cycle leading to the vesiculation of the chondriome, which he thought was comparable to the mitochondrial cycle of a secretory cell (Noël and Morin, 1929). Noël and Morin stated that the reason for emphasizing the observations of Tang was that "...les récents travaux des physiologistes leur donnent un renouveau d'actualité en soulevant la question des 'hormones cardiaques'". This possibility was substantiated by Kisch, who proposed in 1927 a difference between the atrial cardiocytes and those of the ventricles, based on physiological, toxicological, and pharmacological differences (cited in Kisch, 1963). After the advent of the electron microscope, Kisch extended these early observations by describing that atrial cardiocytes in the guinea pig, unlike ventricular cardiocytes, contained secretory granules similar to those seen in polypeptide hormone-producing endocrine cells (Kisch, 1956). These observations were subsequently confirmed in other vertebrate species, and the suggestion of the secretory nature of the atrium was further emphasized (Bompiani *et al.*, 1959; Palade, 1961; Jamieson and Palade, 1964; Trillo and Bencosme, 1965; Trillo *et al.*, 1966; Hibbs and Ferrans, 1969). Additional studies hinted at the possibility that the granules contained catecholamines (Ostlund *et al.*, 1960; Bloom *et al.*, 1961; Sosa-Lucero *et al.*, 1969), but this was discounted by analysis of purified granules (de Bold and Bencosme, 1973a; de Bold and Bencosme, 1973b). Later autoradiographic studies showed that the granules turned over newly synthesized protein at a high rate (de Bold and Bencosme, 1975; Yunge *et al.*, 1980), and light microscopic investigations indicated that the granule contents were mostly a random-coiled polypeptide that contained sulphur-containing amino acids and tryptophan (de Bold *et al.*, 1978).

Morphological studies revealed that water deprivation and salt restriction, as well as adrenal regeneration hypertension caused a significant increase in the number of granules in the atria of rats (Martinez-Palomo and Bencosme, 1966; Marie *et al.*, 1976; de Bold, 1979), and that mineralocorticoid and salt excess decreased granular content (Marie *et al.*, 1976; de Bold, 1979), suggesting that the granule contents were somehow involved in or affected by changes in water and electrolyte homeostasis. It had long been postulated that the atria were somehow involved in regulation of water and electrolyte balance. In 1952 it was theorized that an extrarenal natriuretic mechanism, disordered in cardiac edema, monitors the volume filling of the arterial tree, and subsequently modulates changes in sodium excretion (Peters, 1952). Some investigators proposed that the sensor might be located in the most compliant part of the cardiovascular system: the great veins and the atria. These researchers showed that distension of the right atrium induced a water diuresis associated with sodium excretion; mediation by vagal afferents was proposed (Gauer *et al.*, 1961). Based on this research, and on observations that saline infusions in essentially hypertensive patients accelerated natriuresis, Homer Smith offered the atria as a likely site for volume regulation (Smith, 1957), but it was not understood what the link between the atria and the kidneys was. A few years later, de Wardener and colleagues (1961) proposed the existence of a circulating "third factor" involved in the renal regulation of sodium balance, the other two factors being aldosterone and glomerular filtration rate. Various attempts at isolating such a circulating substance were unsuccessful (Sealey *et al.*, 1969; Bricker *et al.*, 1968).

Purification of the granules and the characterization of their contents by histochemical and biochemical analysis was achieved in the period of 1969 to 1979 (de Bold and Bencosme, 1973a; de Bold and Bencosme, 1973b; de Bold and Bencosme, 1975; de

Bold *et al.*, 1978). During these years it was determined that the atrial granules did not contain catecholamines as previously thought, but instead contained a cysteine-rich random-coiled polypeptide. To identify the potential role of the granular contents on renal function, crude atrial extracts, using ventricular extracts as controls, were injected into rats (de Bold *et al.*, 1981). This resulted in a pronounced diuretic and natriuretic response, presumably demonstrating that a peptide contained in the granules was responsible for peripheral responses, and was the elusive factor responsible for the link between the atria and the kidneys. The putative peptide was named atrial natriuretic factor (de Bold *et al.*, 1981). Injection of granule fractions from atria confirmed that it was the granular contents that contained ANF (de Bold, 1982a; Garcia *et al.*, 1982). Purification of the content of the atrial granules confirmed that it was in fact a peptide found in the atrial granules that was ANF (de Bold, 1982b; de Bold, 1982a; de Bold and Flynn, 1983; Thibault *et al.*, 1983a; Thibault *et al.*, 1983b).

### ANF Synthesis and Processing

Purification and sequencing of the peptides isolated from atrial tissue revealed a number of different peptides, related in sequence, but differing in length (de Bold, 1982b; de Bold and Flynn, 1983; Flynn *et al.*, 1983; Thibault *et al.*, 1984; Thibault *et al.*, 1983a; Thibault *et al.*, 1983b; Atlas *et al.*, 1984; Currie *et al.*, 1984b; Kangawa *et al.*, 1984; Flynn *et al.*, 1985a; Kangawa *et al.*, 1985; Flynn *et al.*, 1985b). Various names were given to these peptides that were then thought to be distinct members of the atrial natriuretic factor "family" of peptides. The various names assigned to ANF are cardionatrin (de Bold and Flynn, 1983), cardiodilatin (Forssmann *et al.*, 1983), atrial natriuretic peptide (Garcia *et al.*, 1984), atrial

natriuretic polypeptide (Kangawa *et al.*, 1984), auriculin (Atlas *et al.*, 1984; Maack *et al.*, 1984), natriodilatin (Nemer *et al.*, 1984), and atriopeptin (Currie *et al.*, 1984b). Some of these peptides (the atriopeptins) were later found to be artefactual, due to the extraction procedures used to isolate them. The international nomenclature is atrial natriuretic factor (Dzau *et al.*, 1987).

Cloning and sequencing of the ANF cDNA showed that all these different peptides were derived from a single gene, and further analysis of the precursor and circulating forms led to the conclusion that some of the variants isolated were processed fragments of a single polypeptide hormone (Kennedy *et al.*, 1984; Maki *et al.*, 1984; Nakayama *et al.*, 1984; Kangawa *et al.*, 1984; Seidman *et al.*, 1984a; Seidman *et al.*, 1984b; Yamanaka *et al.*, 1984; Zivin *et al.*, 1984; Flynn *et al.*, 1985a; Mägert *et al.*, 1990). The stored proANF is derived from a 152-amino acid-long preproANF; the prepropeptide has a leader sequence which is rapidly cleaved after synthesis. The nomenclature for ANF amino acid sequences begins at the first amino acid of the 126-amino acid propeptide (Dzau *et al.*, 1987). ProANF is then cleaved, probably upon release of the peptide, into an inactive amino-terminal fragment (ANF<sub>1-98</sub>), and the bioactive ANF<sub>99-126</sub> (Flynn *et al.*, 1983; Thibault *et al.*, 1985). Although it was initially shown that ANF<sub>1-98</sub> has no demonstrable biological activities (Flynn *et al.*, 1983; Thibault *et al.*, 1985), some researchers claim that smaller amino-terminal fragments of ANF have various biological activities (Vesely *et al.*, 1987; Vesely, 1992; Vesely *et al.*, 1994); this has not been confirmed by others (Weir *et al.*, 1994). ANF<sub>99-126</sub> has a high degree of homology from one mammalian species to another: all amino acids are conserved except residue 110, which is isoleucine in rats, mice, and rabbits (Flynn *et al.*, 1983; Maki *et al.*, 1984; Seidman *et al.*, 1984a; Seidman *et al.*, 1984b; Yamanaka *et al.*, 1984; Kangawa *et al.*, 1984; Thibault *et al.*, 1985; Oikawa *et al.*, 1985), but methionine in humans, dogs, and

cows (Oikawa *et al.*, 1984; Seidman *et al.*, 1984a; Greenberg *et al.*, 1984; Nemer *et al.*, 1984; Oikawa *et al.*, 1985; Vlasuk *et al.*, 1986). Also, as deduced from the cDNA sequence, a carboxyl-terminal Arg-Arg sequence is present in rat, murine, bovine, and rabbit ANF (Kennedy *et al.*, 1984; Maki *et al.*, 1984; Nakayama *et al.*, 1984; Kangawa *et al.*, 1984; Seidman *et al.*, 1984a; Seidman *et al.*, 1984b; Yamanaka *et al.*, 1984; Zivin *et al.*, 1984; Flynn

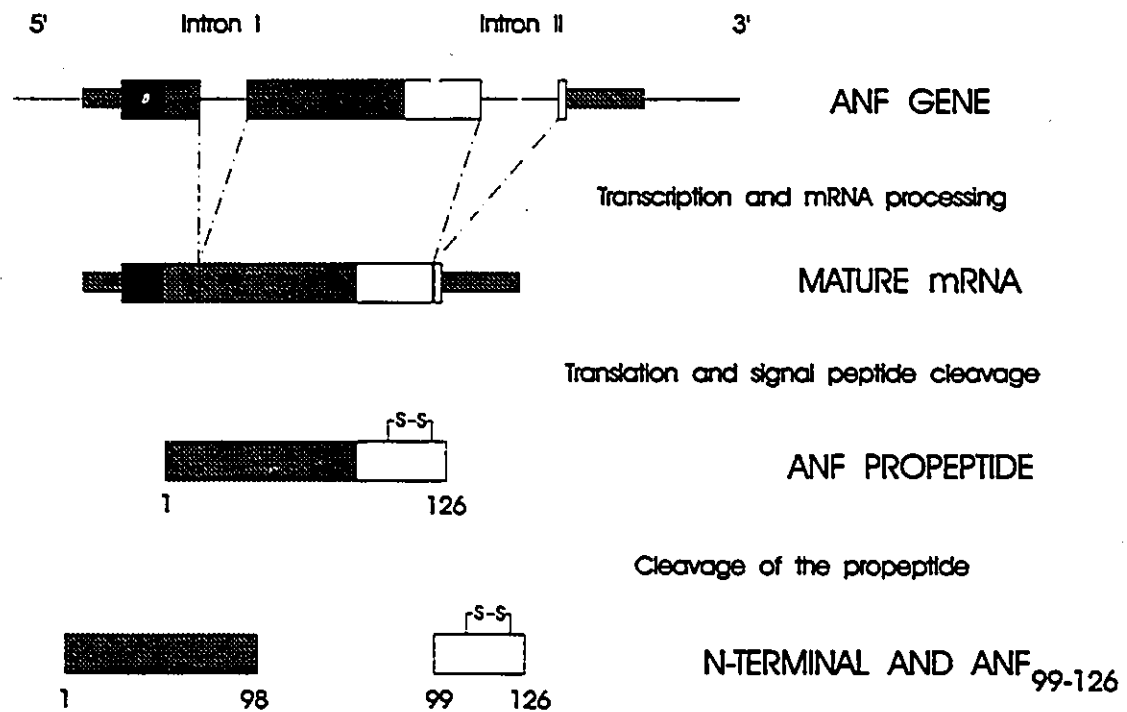


Figure 1: Synthesis and processing of ANF. Thin shaded regions indicate untranslated sequences. Black regions indicate the signal peptide. The wide shaded and clear regions indicate the N-terminal and ANF<sub>99-126</sub>, respectively. The disulphide bridge is shown by the linked "S".

*et al.*, 1985a; Vlasuk *et al.*, 1986; Oikawa *et al.*, 1985), but is not found in ANF from humans, pigs, or dogs (Oikawa *et al.*, 1984; Seidman *et al.*, 1984a; Greenberg *et al.*, 1984; Nemer *et al.*, 1984; Oikawa *et al.*, 1985; Mägert *et al.*, 1990). There are three types of processing events in ANF biosynthesis: 1) formation of a disulphide bond between the cysteines at positions 105 and 121, resulting in the ring form that is essential for ANF activity (Misono *et al.*, 1984), 2) Phosphorylation of the peptide (Bloch *et al.*, 1987; Wildey *et al.*, 1990), and 3) cleavage events. The first cleavage event is the removal of the leader segment of preproANF to yield proANF (Flynn *et al.*, 1985a). The next processing step appears to be the rapid removal of the two C-terminal arginines (Thibault *et al.*, 1989), possibly by carboxypeptidase E (Lynch *et al.*, 1988). This is followed by cleavage of the prohormone at the monobasic site Arg98-Ser99 to yield the bioactive ANF<sub>99-126</sub> (Thibault *et al.*, 1985). Several candidates exist for this final processing step: atrioactivase, a serine protease found in bovine atrial microsomal fractions (Imada *et al.*, 1988), IRCMSP1, a serine protease from rat heart (Seidah *et al.*, 1986) and another serine protease found in bovine granules (Wypij and Harris, 1988; Wypij and Harris, 1992) are potential candidates; kallikrein (Currie *et al.*, 1984a) and thrombin (Michener *et al.*, 1986) have been found to catalyze the reaction *in vitro*. To date, however, no specific enzyme has been unequivocally identified in the processing of ANF<sub>1-126</sub> to ANF<sub>99-126</sub>. Unlike most polypeptide hormones, ANF is not processed during the maturation of the granules; the site of ANF processing is not known, although it is thought to occur by the cardiocytes themselves at the time of secretion (Sei *et al.*, 1992; Dubé *et al.*, 1993). Figure 1 shows the synthesis and maturation of ANF.

## ANF Gene Structure and Expression

The structure of the ANF gene is similar in all mammals (Seidman *et al.*, 1984a; Argentin *et al.*, 1985): three exons, two introns, with the first two exons containing most of the protein-coding sequences (Figure 1); the second intron of the human gene has two repeated sequences of the *Alu* family, as well as sequences with homology to glucocorticoid responsive elements (GRE) (Seidman *et al.*, 1984a). In the atrium of mammals, ANF is expressed at a very high level (1-3% of total mRNA) (Seidman *et al.*, 1985). ANF gene transcripts have also been detected in lower amounts in the ventricles (1% of atrial levels) (Lattion *et al.*, 1986; Day *et al.*, 1987), aortic arch (Gardner *et al.*, 1987a), pituitary (Gardner *et al.*, 1986a), lung (Gardner *et al.*, 1986a), hypothalamus (Gardner *et al.*, 1987c), and kidney (Dagnino *et al.*, 1991; Greenwald *et al.*, 1992; Golomb *et al.*, 1993); however, the atria remain the major site of ANF synthesis. Expression of the ANF gene is an early marker of commitment to the cardiac phenotype of differentiating cardiac myoblasts (Kohtz *et al.*, 1989) and embryonic stem cells (Miller-Hance *et al.*, 1993). Atrial expression of the gene rises continuously during fetal and postnatal development in mammals, whereas ventricular expression, quite high during fetal development, quickly falls to barely detectable levels following birth (at basal levels 20 days postnatal) (Zeller *et al.*, 1987; Wei *et al.*, 1987; Wu *et al.*, 1988). Also, although expression of the gene is found in several extra-cardiac sites, its expression is highest in the heart, and its promoter sequences carry elements that confer it cardiac-specific expression. Finally, its expression can be altered both *in vivo* and *in vitro* by various humoral, neural, and mechanical stimuli.

### *Cis-acting regulatory sequences*

The upstream regulatory sequences of the ANF gene have been well studied. The characteristic TATAA box is found approximately 30 base pairs upstream of the transcription initiation site (Seidman *et al.*, 1984a; Argentin *et al.*, 1985; Seidman *et al.*, 1988). The TATAA box is essential for transcription of most eukaryotic genes, and binds the ubiquitous transcription factor TFIID and its coactivators (Conaway and Conaway, 1993). The other sequences that are important for ANF gene expression are thought to be found in the 2.4 kb preceding the protein-coding sequences (Seidman *et al.*, 1988); This was initially determined by creating gene constructs with various portions of the ANF upstream sequences driving the protein-coding sequences of the chloramphenicol acetyl transferase (CAT) gene, and transfecting these constructs into primary cultures of rat atrial or ventricular cardiocytes. Constructs that carried only the first 1000 bp of the upstream sequences did not confer nearly as much activity to the reporter gene as those that carried the full 2400 bp region, suggesting the presence of important *cis*-acting elements between positions -2400 and -1000.

Further delineation of the upstream regulatory sequences driving the rat ANF gene has been performed. Studies involving ANF enhancer sequences driving a CAT reporter gene in neonatal ventricular cardiocytes have identified an enhancer-like element at position -137 to -693 of the rat gene that decreases ANF gene expression 20- to 30-fold when removed (Rosenzweig *et al.*, 1991); this element is not a classical enhancer since its enhancer activity is orientation- but not position-dependent. In a separate set of experiments, using a luciferase reporter gene, it has been shown that the sequences between -3003 and -638 are responsible for the tissue-specific expression of the gene, while the region between

positions -638 and -323 may be responsible for  $\alpha_1$ -adrenergic inducibility (Knowlton *et al.*, 1991). In this 300 bp region are found elements with homology to serum-responsive element (SRE)/CArG (at position -406), Egr-1 (-459), AP-1 (-493), AP-2 (-525), and cAMP-responsive element (CRE, at nt -601) motifs; some of these elements may be involved in the control of ANF gene expression (Knowlton *et al.*, 1991).

Also by transient transfection assays of ANF promoter sequences driving a reporter gene, two elements important for constitutive ANF gene expression have been defined at positions -1600 to -1000 and -700 to -136 (Argentin *et al.*, 1991). A more recent study has shown that an element located between positions -640 and -136 of the rat ANF gene is responsible for the cardiac-specific expression of ANF, as well as the repression of its expression in other cell types (Argentin *et al.*, 1994). Further delineation of this element pinpointed the region necessary for cardiac-specific expression as being located between positions -480 and -390. Footprint analysis revealed a protected CArG element (also called a serum response element [SRE]), which, when attached to a minimal ANF promoter-reporter construct, restored 90% of cardiac-specific promoter activity in atrial and ventricular cardiocytes (Argentin *et al.*, 1994). A well-conserved SP-1-like  $\alpha_1$ -adrenergic regulatory sequence located at position -80 to -50 has also been described, and it appears to interact with as yet unidentified zinc-dependent proteins (Ardati and Nemer, 1993). It has been recently shown that the SP-1-like site is responsible for basal expression of the ANF promoter in ventricular cardiocytes, and confers part of the responsiveness to  $\alpha_1$ -adrenergic stimulation, in concert with the SRE described at position -406 and an SRE located at position -114 (Sprenkle *et al.*, 1995). The SREs are thought to bind serum response factors (SRFs) (Sprenkle *et al.*, 1995), but the -406 SRE was shown previously not to interact with an SRF (Argentin *et al.*, 1994).

In transgenic mice containing the full-length (2.4 kb) rat construct, atrial-specific and proper developmental expression has been observed, although discrepancies exist between expression of the transgene and that of the endogenous murine ANF gene (Seidman *et al.*, 1991): in these experiments, postnatal ventricular expression of the endogenous murine gene was higher than in the rat, whereas the rat transgene followed the normal pattern of expression (decreased ventricular expression following gestation day 15). A recent report has shown that in transgenic mice harbouring only the 638 bp upstream of the initiation start site, both the transgene and the endogenous ANF gene display appropriate atrial and ventricular developmental expression, consistent with the transfection experiments described above (Knowlton *et al.*, 1995). However, in adult ventricles, either in a transgenic setting or following direct injection of promoter-reporter constructs, the promoter sequences up to position -3003 are not sufficient to confer transcriptional inducibility following pressure overload (Knowlton *et al.*, 1995). This is a significant finding that puts into question the relevance of a great portion of studies on the transcriptional response of the ANF gene to various stimuli, because these experiments have been conducted using transient transfection in neonatal cardiocytes of ANF promoter-reporter gene constructs.

Similar experiments were performed using human gene sequences in rat cardiocytes (LaPointe *et al.*, 1988); these studies showed that the 409 bp upstream of the ANF gene are sufficient to drive a CAT reporter gene in cultured rat neonatal cardiocytes, and following deletion analysis it was found that the element responsible for the observed expression is located at positions -389 to -191. This was confirmed *in vivo* by the transgenic mouse experiments of Field (1988); the 423 bp upstream of the human ANF gene were fused to the SV40 T-antigen coding sequences, and appropriate expression of the transgene was observed, although only the right atria of these animals exhibited oncogene-induced

hyperplasia. As with the rat gene, the human transgene was not inducible in overload-induced hypertrophied ventricles, suggesting that these sequences are sufficient for developmental expression, but not for inducible expression (Rockman *et al.*, 1991). A 40 bp region essential for proper expression of the gene in cultured neonatal rat cardiocytes was identified by deletion analysis at position -381 to -342 of the human upstream sequences (Wu *et al.*, 1989; Wu *et al.*, 1991). DNaseI footprinting and methylation interference studies have identified within the essential 40bp region an 18bp binding segment homologous to that present in the rat at positions -1220 to -1203, but the rat sequences which correspond to the functional human promoter do not seem to confer the same level of expression (Wu *et al.*, 1989). This raises the possibility that although the ANF coding sequences have been conserved over evolution, the promoter sequences may have considerably diverged. As well, silencer elements have been identified at positions -2593 to -1150 and -222 to the CAP site (Wu *et al.*, 1991).

An interesting detail in the rat promoter elements described above is the presence at positions -496 to -489 of a region with considerable homology to the AP-1 binding site (Knowlton *et al.*, 1991; Rosenzweig *et al.*, 1991). The AP-1 transcription factor is a heterodimer of the gene products of the proto-oncogenes *c-fos* and *c-jun*, or a Jun homodimer (Ransone and Verma, 1990), both of which are expressed in ventricular cardiocytes that are exposed to stimuli that lead to a hypertrophic response (Izumo *et al.*, 1988; Komuro *et al.*, 1988; Rockman *et al.*, 1991), before reexpression of ANF. Fos/jun heterodimers have been found, by gel retardation assays, to bind this region (Rosenzweig *et al.*, 1991). This AP-1 site is located within the putative  $\alpha_1$ -adrenergic-responsive element existing in the 638 bp upstream of the rat ANF gene, as described by Knowlton *et al.* (1991), but is not a part of the recently delineated  $\alpha_1$ -adrenergic-responsive element (Ardati and

Nemer, 1993; Sprenkle *et al.*, 1995). Experiments involving *c-fos* and *c-jun* expression in transfected neonatal cardiocytes seem to reveal a paradoxical control of the human ANF gene by *c-fos* and *c-jun* (Kovacic-Milivojevic and Gardner, 1992; Kovacic-Milivojevic and Gardner, 1993): *c-jun* increased ANF gene expression, high levels of *c-fos* inhibited ANF transcription, whereas low levels of *c-fos*, in concert with *c-jun*, activated the ANF gene. The region between -332 and -217 was found to be essential for *c-jun* sensitivity. Another member of the fos family, *fra-1*, also represses ANF promoter activity in atrial cardiocytes (Kovacic-Milivojevic and Gardner, 1995). Another report has shown, using similar techniques to study the rat promoter, that fos and jun repress basal and phenylephrine-stimulated ANF gene expression (McBride *et al.*, 1993). Furthermore, these authors determined that this action does not involve the consensus AP-1 site, but instead that fos and jun act at a cardiac-specific element located between positions -135 and -640 of the rat ANF promoter. This interaction does not require DNA-binding activity, and may be mediated by other cardiac-specific transcription factors. This implies that the regulation of the ANF gene is determined at the level of tissue specificity and agonist response by the same factor, and that this factor is modulated by protein interactions with other putative transcription factors.

Other putative regulatory elements have been detected in the sequences upstream from the ANF gene. Two discrete elements necessary for response to glucocorticoids have been found at positions -431 to -979 and -877 to -904 (Argentin *et al.*, 1991). In addition, an element has been identified at position -2921 that acts as a negative calcium responsive element (nCaRE); this element is composed of a 7 bp inverted repeat preceded by a thymidine-rich region (Okazaki *et al.*, 1992). The inverted repeat of the nCaRE was initially identified in the human parathyroid gene, and is functional in both *in vivo* and *in vitro* assays. The factors that interact with the *cis*-regulatory elements are still unknown. ANF

also has conserved GATA sites in its proximal regulatory regions (Seidman *et al.*, 1984a; Argentin *et al.*, 1985; Grépin *et al.*, 1994). The cloning of the zinc-finger transcription factor GATA-4 in cardiocytes (Arceci *et al.*, 1993; Heikinheimo *et al.*, 1994; Grépin *et al.*, 1994), and its demonstrated control of ANF expression (Grépin *et al.*, 1994), indicate the possibility of the involvement of this factor in the cardiac-specific expression of the ANF gene. GATA-4 is expressed in the developing mouse heart (Heikinheimo *et al.*, 1994), where it first appears during the bending of the heart tube (day 8 post-coitum), and continues to be expressed throughout adulthood. Disruption of GATA-4 expression in P19 embryonal carcinoma cells blocks the potential for these cells to differentiate into cardiac cells (Grépin *et al.*, 1995).

## Factors Affecting ANF Gene Expression

### *Mechanical Overload*

Under overload conditions in the heart, ANF synthesis is increased in both atrial and ventricular cardiocytes (Lattion *et al.*, 1986; Arai *et al.*, 1987; Drexler *et al.*, 1989; Haass *et al.*, 1990; Mukoyama *et al.*, 1991; Fischer *et al.*, 1991; Yokota *et al.*, 1994; Yokota *et al.*, 1995; Ogawa *et al.*, 1996). It has been observed that spontaneously hypertensive rats (SHR) demonstrate increased ANF mRNA levels in the right atrium (Arai *et al.*, 1987), and studies involving infarct-induced volume overload have shown that ANF mRNA levels in the atria increased by 38%, although this was not greater than the general increase in protein synthesis that accompanied the response of the tissue to the infarct (Drexler *et al.*, 1989). In volume overload models such as aortocaval fistula (Lattion *et al.*, 1986; Brown *et al.*, 1993) or mineralocorticoid excess (Lattion *et al.*, 1986; Dananberg and Grekin, 1992; Yokota *et al.*,

1994; Yokota *et al.*, 1995), atrial ANF mRNA levels are elevated as early as 8 to 72 hours after the initiation of treatment. In humans with various types of cardiac disease in which overload of the atria was observed (aortic valve disease, mitral valve disease, or coronary artery disease), increases in ANF mRNA levels in the right atrium closely followed mean pulmonary pressure (Haass *et al.*, 1990; Fischer *et al.*, 1991). As well, dogs with congestive heart failure (CHF) induced by rapid ventricular pacing exhibited a significant increase in atrial, but not ventricular, ANF mRNA levels after 15 days (left atria) or 30 days (right atria) of rapid pacing (Perrella *et al.*, 1992). A study on humans with congestive heart failure showed a significant increase in atrial ANF mRNA levels, versus age-matched normal patients (Mukoyama *et al.*, 1991). In cultured neonatal atrial cardiocytes, two consecutive 30-second stretches result in the upregulation of the ANF gene 24 hours later (Gardner *et al.*, 1992), suggesting that a brief mechanical stimulus is sufficient to trigger a chain of events that leads to increased ANF expression. These data indicate that atrial overload is a significant determinant of atrial ANF gene expression.

The increase in ANF transcripts in the ventricle usually correlates with the degree of hypertrophy (Day *et al.*, 1987; Lee *et al.*, 1988; Izumo *et al.*, 1988; Mercadier *et al.*, 1989; Urbain *et al.*, 1989; Yokota *et al.*, 1995; Ogawa *et al.*, 1996), and varies for different methods of induction of hypertrophy. For example, left ventricular hypertrophy caused by aortic coarctation or experimental infarct raise ventricular ANF mRNA levels to 12-15% of atrial levels, whereas aortocaval fistula and aortic insufficiency result in an increase in ANF transcript levels in the left ventricle to only 6-7% of atrial levels (Urbain *et al.*, 1989). The time course of changes in ANF mRNA levels in the left ventricle following aortic coarctation has been outlined as follows (Izumo *et al.*, 1988; Mercadier *et al.*, 1989): the levels of ANF mRNA begin to rise 2 days after the initial stimulus (aortic coarctation) is applied. The levels rise to

their highest (20-fold basal level) four days post-operation, when the pressure and left ventricular mass are not yet highly increased. The levels drop over the next five days, and rise again to stabilize at a moderate level (ten times basal level). The rise in ANF mRNA levels coincides with the rise in left ventricular end diastolic pressure (LVEDP), which itself partly results from an increase in plasma volume which follows activation of the renin-angiotensin system after aortic coarctation. However, it has become clear recently that increased ANF gene expression in the ventricles is mostly dependent on the increased load on the ventricle, but also depends on a non-hemodynamic stimulus that is related to the process of ventricular hypertrophy (Ogawa *et al.*, 1996). In SHR and SHR stroke-prone rats, ventricular ANF mRNA levels are also augmented (Arai *et al.*, 1988; Dagnino *et al.*, 1992; Roy *et al.*, 1992), and are closely related to mean arterial pressure (Arai *et al.*, 1988). In an *in vitro* model, ANF mRNA was rapidly increased following stretch of rat papillary muscle (Jarygin *et al.*, 1994). The ANF gene is also upregulated by stretch of cultured neonatal ventricular cardiocytes (Sadoshima *et al.*, 1992a), which suggests that it is mechanical stretch of the cardiocytes that is responsible for increased ventricular expression of ANF. Therefore, increased atrial ANF gene expression may also be caused by direct stretching of the atrial cardiocytes.

Besides ANF, another group of genes to be activated by conditions of ventricular overload is that of the proto-oncogenes *c-myc*, *c-fos*, *c-jun*, *junB*, *nur77* (Izumo *et al.*, 1988; Komuro *et al.*, 1988; Schunkert *et al.*, 1991; Rockman *et al.*, 1991; Kolbeck-Ruhmkorff *et al.*, 1993), and the growth response gene *Egr-1* (Rockman *et al.*, 1991). Collectively, these genes are referred to here as early response genes. Many of these genes are also activated by stretch of neonatal ventricular cardiocytes cultured on stretchable silicone membranes (Komuro *et al.*, 1990; Komuro *et al.*, 1991; Sadoshima *et al.*, 1992a). The early response

genes *c-myc*, *c-fos*, *c-jun*, *junB*, *nur77* and *Egr-1* code for DNA-binding proteins (Ransone and Verma, 1990; Sukhatme *et al.*, 1988). Once they are "turned on", the DNA-binding protooncogene protein products could be involved in activating other genes such as contractile protein-coding genes, other messenger proteins, or the gene for ANF. As previously discussed, it has not yet been resolved whether Fos and Jun activate or repress cardiac-specific gene expression (Kovacic-Milivojevic and Gardner, 1992; Kovacic-Milivojevic and Gardner, 1993; McBride *et al.*, 1993).

### ***Endothelin-1***

ANF gene expression is also significantly increased in cultured neonatal rat cardiocytes by the potent vasoconstrictor endothelin-1 (ET-1) (Fukuda *et al.*, 1989; Shubeita *et al.*, 1990; Gardner *et al.*, 1991; Suzuki *et al.*, 1992). Levels of ANF transcript were elevated in neonatal rat cardiocytes of atrial or ventricular origin following 3 to 32 hours of exposure to ET-1 (Fukuda *et al.*, 1989; Shubeita *et al.*, 1990; Gardner *et al.*, 1991; Suzuki *et al.*, 1992). Besides stimulating ANF gene expression, ET-1 induces in cultured neonatal ventricular cardiocytes *c-fos*, *Egr-1*, and the muscle-specific  $\alpha$ -actin, myosin light chain 2 and troponin I genes (Shubeita *et al.*, 1990; Ito *et al.*, 1991; Jones *et al.*, 1992), as well as the  $\alpha$ - and  $\beta$ -myosin heavy chain genes (Wang *et al.*, 1992). This response resembles that of the onset of cardiac hypertrophy, both in the genes involved, and the time course of their expression: the early response genes are induced within 30 minutes, and the contractile protein genes within 3 to 6 hours. In fact, ET-1 has been invoked as a possible autocrine or paracrine factor in the development of cardiac hypertrophy following hemodynamic overload (Ito *et al.*, 1994).

### *Adrenergic agonists*

Early studies using explants of fetal mouse atrial tissue have shown that addition of norepinephrine or epinephrine causes a rapid increase in the number of granules, and a redistribution of these granules, suggesting an increase in ANF secretion and synthesis (Nanot and Le Douarin, 1970). More recently,  $\alpha$ -adrenergic stimulation of cultured neonatal ventricular cardiocytes has been shown to lead to increases in ANF mRNA levels (Knowlton *et al.*, 1991; Sei *et al.*, 1991; McBride *et al.*, 1993; Ardati and Nemer, 1993). Stimulation by the  $\alpha_1$ -adrenergic agonist phenylephrine has been shown to increase ANF mRNA levels after as early as 6 hours of treatment, reaching maximal stimulation after 24-48 hours (Knowlton *et al.*, 1991). Transient transfection assays using ANF promoter-Luciferase constructs determined that the induction of the ANF gene by phenylephrine was due in large part to transcriptional activation of the ANF promoter (Knowlton *et al.*, 1991; McBride *et al.*, 1993). As mentioned above, specific regions of the ANF promoter responsible for phenylephrine responsiveness have been identified; one report states that they are found between positions -638 and -323 (Knowlton *et al.*, 1991) and another reports them between positions -80 and -50 (Ardati and Nemer, 1993).

### *Glucocorticoids*

Glucocorticoids positively regulate expression of the ANF gene both *in vivo* (Gardner *et al.*, 1986b; Day *et al.*, 1987; Dananberg and Grekin, 1992) and *in vitro* (Matsubara *et al.*, 1987a; Matsubara *et al.*, 1987b; Gardner *et al.*, 1988). In neonatal rat atrial cardiocyte cultures (Matsubara *et al.*, 1987b; Gardner *et al.*, 1988), as well as in neonatal ventricular cardiocyte cultures (Matsubara *et al.*, 1987b), the glucocorticoid Dexamethasone

increases ANF mRNA levels, in part by increasing the transcription rate of the ANF gene, as assessed by nuclear run-on transcription assays (Gardner *et al.*, 1988). This observation is supported by the definition of sequences upstream of the rat gene that bind purified glucocorticoid receptors (Argentin *et al.*, 1991). Dexamethasone also increases the levels of ANF mRNA *in vivo* (Gardner *et al.*, 1986b; Day *et al.*, 1987; Dananberg and Grekin, 1992), suggesting that natriuretic glucocorticoids and ANF may interact in some fashion to regulate each other's actions.

### *Other factors*

Thyroid hormone increases ANF mRNA levels in primary cardiocyte cultures (Gardner *et al.*, 1987b; Matsubara *et al.*, 1987a; Mori *et al.*, 1990; Mori *et al.*, 1991) as well as *in vivo* (Gardner *et al.*, 1987b; Ladenson *et al.*, 1988). Addition of thyroxine to dehydrated thyroidectomized animals or triiodothyronine to neonatal primary cardiocyte cultures results in an increase in ANF mRNA levels (Gardner *et al.*, 1987b). The prostaglandin PGF<sub>2 $\alpha$</sub>  (but not PGE<sub>2</sub> or prostacyclin) increases ANF mRNA levels in cultures of neonatal rat ventricular cardiocytes (Gardner and Schultz, 1990). In atrial strips stimulated with the Na-K-ATPase inhibitors ouabain and digoxin, ANF mRNA levels were rapidly increased after only one hour of stimulation (Morise *et al.*, 1991). Stimulation of cultured neonatal ventricular cardiocytes by transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ) (Parker *et al.*, 1991; Takahashi *et al.*, 1994), acidic or basic fibroblast growth factor (Parker *et al.*, 1990),  $\alpha$ -thrombin (Glembotski *et al.*, 1993), myotrophin (Mukherjee *et al.*, 1993), or angiotensin II (Sadoshima and Izumo, 1993b) also causes increases in ANF mRNA levels, while hydroxy vitamin D<sub>3</sub> (Li and Gardner, 1994) and retinoids (Zhou *et al.*, 1995) negatively regulate the transcription of the ANF gene.

## ANF Release

### *Mechanisms of ANF secretion*

From a morphological point of view, the atrial cardiocyte is an endocrine cell that has characteristics of a regulated secretory cell: its dense-core granules are a hallmark of regulated secretion (Burgess and Kelly, 1987). However, ANF storage differs from most regulated secretory pathways, in that it is not processed during the maturation of the granule (Flynn *et al.*, 1985a; Flynn *et al.*, 1985b; Thibault *et al.*, 1985; Thibault *et al.*, 1987). ANF secretion nonetheless retains the classical defining characteristics of regulated secretion. Regulated secretion in endocrine cells involves the concentrated packaging of secretory proteins into secretory granules as they exit the *trans*-Golgi apparatus; the concentration of proteins in secretory granules can reach 200 times the concentration found in the endoplasmic reticulum (Burgess and Kelly, 1987). In the secretory granules, the contents condense, forming an electron-dense core that is sometimes separated from the membrane by a "space". ANF secretion from atrial cardiocytes is independent from protein synthesis, is an energy-dependent process, and is not reduced by the disruption of forward vesicular traffic from the endoplasmic reticulum to the Golgi apparatus (Page *et al.*, 1991; De Young *et al.*, 1994). This is similar to the secretory processes of other endocrine cells such as the pancreas (Rhodes and Halban, 1987) or growth hormone-secreting cells (Chen *et al.*, 1989). Therefore, the main process by which basal ANF secretion from atrial cardiocytes is accomplished is most likely granule exocytosis. This is supported by experiments using immunoelectron microscopy, in which exocytosis of ANF contained in granules was observed (Page *et al.*, 1986; Sugawara, 1987; Nomura *et al.*, 1988).

There is also the possibility that some ANF is released via a constitutive pathway. The constitutive secretory pathway involves passive diffusion of secretory products (Burgess and Kelly, 1987). Sorting to one pathway or the other usually occurs at the *trans*-Golgi complex, but many secretory cells exhibit both pathways of secretion for a single peptide hormone (Rhodes and Halban, 1987; Arvan and Castle, 1987); the constitutive pathway is usually a minor component of secretion for a given peptide compared to a concomitant regulated pathway. This is probably the case in atrial cardiocytes, in which a minor constitutive pathway of ANF secretion may co-exist with a major regulated pathway (Page *et al.*, 1991). The existence of clathrin-coated vesicles in atrial cardiocytes (Page *et al.*, 1986; Iida and Shibata, 1994), which are characteristic organelles of the constitutive pathway (Burgess and Kelly, 1987), support this possibility. However, stimulated release of ANF cannot occur via the constitutive pathway, since stimulated ANF secretion is not dependent on protein synthesis (Page *et al.*, 1991; Doubell and Thibault, 1994).

In addition to the commonly described constitutive and regulated pathways of secretion, there is the possibility that ANF may be secreted by an alternate pathway that does not conform to either of these well established processes. Phasic secretion of newly synthesized ANF has been observed in unstimulated cultured adult atrial cardiocytes (Iida and Shibata, 1994). The phasic secretion of ANF has characteristics of both the constitutive and regulated pathways: it occurs without stimulation, but can be increased by stimulation with phorbol esters. This explains the constant levels of ANF secreted under unstimulated conditions that is consistently observed with *in vitro* preparations. It was proposed that the rapid exocytosis of a portion of granules that contain newly synthesized ANF is responsible for the observed pattern of secretion (Iida and Shibata, 1994). This was based partly on the observation that 60% of newly synthesized ANF remains in the cell, while the rest is secreted,

and partly on the composition of secreted ANF, which retains the same degree of processing as that of ANF released by the regulated pathway. However, although the degree of processing in most secretory cells is directly related to the stage of maturation of the granules (Burgess and Kelly, 1987), ANF is processed co-secretionally (Sei *et al.*, 1992; Dubé *et al.*, 1993), which precludes this type of comparison. This pattern of unstimulated secretion has been observed in the exocrine and endocrine pancreas, in which it has been ascribed to the exocytosis of vesicles budding from immature granules (Arvan and Castle, 1987; Arvan *et al.*, 1991; Kuliawat and Arvan, 1992). Arvan *et al.* refer to this type of secretion as "constitutive-like", and suggest that it continues under conditions in which regulated secretion is absent (Arvan *et al.*, 1991), and is independent from both constitutive and regulated secretion (Kuliawat and Arvan, 1992). In pancreatic islets, constitutive-like secretion occurs by the secretion of newly synthesized hormone *via* exocytosis of vesicles that bud off immature granules, and, as for basal ANF secretion from atrial cardiocytes, is independent from protein synthesis (Kuliawat and Arvan, 1992). Furthermore, it has been shown in the endocrine pancreas that secretion *via* immature granules can be regulated (Arvan *et al.*, 1991).

There is also evidence for a fourth type of mechanism for ANF secretion. ANF has been localized by immunoelectron microscopy in caveolae adjacent to specific atrial granules (Page *et al.*, 1994). Caveolae are flask-shaped invaginations of the plasma membrane, and their permeability is reversibly increased by stretch of atrial cardiocytes (Page *et al.*, 1992). These specialized vesicles appear to be contiguous with specific atrial granules at the plasma membrane (Page *et al.*, 1986; Page *et al.*, 1994), and as such may be involved in regulating part of the exocytosis of granular content (Page *et al.*, 1994). The alternate pathway of ANF secretion proposed by Page *et al.* is from atrial granules, into atrial

caveolae, and then into the interstitial space; this pathway may not necessarily be synchronous with the regulated pathway, and may operate in parallel with it.

#### *Release by atrial stretch*

ANF is acutely released from atria in response to a volume overload, as first demonstrated in heart-lung preparations by Dietz (1984) and *in vivo* by Lang *et al.* (1985). Following *in vitro* studies on perfused rat atrial appendages, it was found that ANF release is a direct consequence of mechanical stretch of the atrial muscle (de Bold *et al.*, 1986; Schiebinger and Linden, 1986a; Agnoletti *et al.*, 1987; Page *et al.*, 1990). Initial speculation about the *in vivo* mechanism of release centred on the theory that atrial pressures were important in determining release of the peptide, but experiments in which atrial transmural pressure was kept constant pointed out that it was volume load-induced stretch of the muscle that was the major determinant of ANF release *in vivo* (Edwards *et al.*, 1988). Nonetheless, contradicting information exists regarding the exact nature of the determinant of ANF release: in a series of experiments involving volume overload and tachycardia in dogs, atrial systolic wall stress was proposed as the major mechanism of ANF release (Christensen *et al.*, 1988), whereas volume expansion experiments in conscious dogs pointed to atrial diastolic wall stress as being more closely related to release of the peptide (Hintze *et al.*, 1985). In humans with cardiac tamponade, the high pressure and low volume in the atria resulting from pericardial blood or effusion is associated with low circulating ANF levels, and relief of this situation results in greater plasma ANF levels (Koller *et al.*, 1987; Northridge *et al.*, 1989). Experiments involving rapid pacing in dogs also show by echocardiogram that it is V-wave (diastolic) stress and not A-wave (systolic) stress that is more significantly related to increased ANF secretion (Stewart *et al.*, 1993). In aortic banded dogs that have developed

atrial and ventricular hypertrophy, volume overload causes a greater proportional increase in plasma ANF levels compared to controls; this is due to increased compliance of the atrial tissue (Hasebe *et al.*, 1995). This clearly shows that it is an increase in atrial dimensions (and therefore stretch) which is the main stimulus for increased ANF release, and not changes in atrial pressure. Experiments involving *in vitro* perfused rat atria subjected to acute mechanical stretch support this notion (de Bold *et al.*, 1986; Schiebinger and Linden, 1986a; Agnoletti *et al.*, 1987; Page *et al.*, 1990).

Each atrium, left or right, can be independently stimulated to release ANF in the rat (Lang *et al.*, 1985; Ledsome *et al.*, 1985; Schiebinger and Linden, 1986a). Although ANF concentrations are twice as high in rat right atrial tissue than in the left (Tanaka *et al.*, 1984; Gulkowska *et al.*, 1984), the quantities of peptide released by each atrium are quite similar, as determined from *in vitro* studies performed on isolated rat atria (Bilder *et al.*, 1986). Also, in human pulmonary hypertension (Adnot *et al.*, 1987), release of ANF is proportional to the level of pulmonary arterial pressure. Pure left heart failure patients with normal right atrial pressure (Raine *et al.*, 1986) have greatly elevated plasma ANF concentrations. It has been suggested that increased ANF release due to volume overload or atrial stretch is partially enhanced by pressor hormones, such as norepinephrine, epinephrine, angiotensin II, ET-1, and vasopressin (Ruskoaho *et al.*, 1989b; Schiebinger and Greening, 1992; Jiao and Baertschi, 1995; Skvorak *et al.*, 1995; Leskinen *et al.*, 1995), although the effects of vasopressin have not been confirmed by others (Schiebinger and Greening, 1992).

### *Release by ET-1*

Experimental evidence indicates that ANF release is stimulated by ET-1. ET-1 rapidly stimulates ANF release from atrial appendages (Hu *et al.*, 1988; Stasch *et al.*, 1989; Winkvist *et al.*, 1989; Schiebinger and Gomez-Sanchez, 1990; de Bold *et al.*, 1991), perfused hearts (Mäntymaa *et al.*, 1990), and cultured atrial cells (Fukuda *et al.*, 1988; Gardner *et al.*, 1991; Suzuki *et al.*, 1992; Horio *et al.*, 1992; Horio *et al.*, 1993; Leite *et al.*, 1994). ET-1 also appears to further increase ANF secretion following volume overload of canine hearts (Donckier *et al.*, 1992). Recent evidence suggests that ET-1 may be a mediator of volume-induced ANF secretion: blocking the effects of ET-1 using ET-1 antibodies (Fyhrquist *et al.*, 1993) and ET-1 receptor antagonists (Leskinen *et al.*, 1995) results in a partial reduction of volume-induced increases in plasma ANF. ET-1 receptor antagonists have also been reported to decrease stretch-induced ANF release from isolated atria (Skvorak *et al.*, 1995).

### *Release by adrenergic agonists*

Secretion of ANF from atrial cardiocytes is also affected by adrenergic stimulation. Conflicting evidence has arisen from experiments attempting to define the secretory effects of adrenergic agonist stimulation of atrial cardiocytes. The initial observation of the adrenergic stimulation by norepinephrine of ANF secretion from isolated atria showed that this effect is predominantly due to  $\alpha$ -adrenergic and not  $\beta$ -adrenergic receptors (Sonnenberg and Veress, 1984). In paced and pre-stretched left atria, it was concluded that norepinephrine stimulation of ANF secretion is mediated by  $\alpha$  and  $\beta$  receptors, with a predominance of the  $\beta$  receptors (Schiebinger *et al.*, 1987). Norepinephrine has varied effects on ANF secretion from cultured atrial cardiocytes (Gibbs, 1987b), and

decreases ANF secretion from perfused atrial strips (Inoue *et al.*, 1988). Norepinephrine in the presence of the  $\beta$ -adrenergic receptor antagonist propranolol increases ANF secretion from atrial tissue and cultured adult atrial cardiocytes, suggesting that  $\alpha_1$ -receptor activation is mainly responsible for norepinephrine-stimulated ANF release (Wong *et al.*, 1988; Ambler and Leite, 1994). Epinephrine increases ANF secretion from freshly isolated adult atrial cardiocytes (Gibbs, 1987b) and perfused right atria (Wong *et al.*, 1988), but decreases ANF release from perfused atrial strips (Inoue *et al.*, 1988). Minced atria show no significant secretory response to the  $\alpha_1$ -adrenergic agonist phenylephrine (Lachance *et al.*, 1986), while ANF secretory rates from perfused paced left atria rapidly increase with equivalent doses of phenylephrine (Schiebinger *et al.*, 1987); similar results were obtained using a heart-lung preparation (Onwochei and Rapp, 1988) and cultured neonatal atrial cardiocytes (Matsubara *et al.*, 1988; Shields and Glembotski, 1989; Sei and Glembotski, 1990). Others have shown stimulated ANF release using the  $\alpha_1$ -adrenergic agonist methoxamine on isolated atria (Wong *et al.*, 1988) and freshly isolated adult atrial cardiocytes (Hayashi *et al.*, 1988). The  $\beta$ -adrenergic agonist isoproterenol has been shown to increase ANF secretion rates from freshly isolated adult atrial cardiocytes (Gibbs, 1987b) and perfused atria (Schiebinger *et al.*, 1987; Wong *et al.*, 1988; Agnoletti *et al.*, 1992), but has no effect on ANF release from a heart-lung preparation (Onwochei and Rapp, 1988) or cultured neonatal atrial cardiocytes (Matsubara *et al.*, 1988), and has no effect on or decreases ANF release from perfused atrial strips (Lachance *et al.*, 1986; Inoue *et al.*, 1988). Stimulation of  $\beta$ -adrenergic receptors or of its main signaling pathway (increased cAMP accumulation) reduces phenylephrine-stimulated ANF secretion from cultured neonatal and adult atrial cardiocytes (Shields and Glembotski, 1989; Ambler and Leite, 1994). Also, some of the effect of both norepinephrine and epinephrine on freshly isolated adult atrial cardiocytes (Gibbs, 1987b) or isolated right

atria (Wong *et al.*, 1988) can be at least partially blocked by the  $\beta$ -adrenergic receptor antagonist propranolol, suggesting the involvement of both  $\alpha$  and  $\beta$  adrenergic receptors.

The discrepancies from one report to another may be due to marked differences in the experimental conditions. In some reports of  $\beta$ -adrenergic stimulation of ANF secretion, researchers have used paced left atria (Schiebinger *et al.*, 1987; Schiebinger, 1989). These same researchers reported that  $\beta$ -adrenergic stimulation of unpaced atria decreases ANF secretion slightly (Schiebinger, 1989), and that in spontaneously beating atria, the response is much smaller than in paced left atria (Schiebinger *et al.*, 1987). Since  $\beta$ -adrenergic stimulation of ANF secretion is dependent on  $\text{Ca}^{2+}$  fluxes from external and internal  $\text{Ca}^{2+}$  sources (Schiebinger, 1989), it is possible that the exaggerated  $\text{Ca}^{2+}$  transients caused by pacing may influence the effects of  $\beta$ -adrenergic agonists. Another report, using spontaneously beating whole atria, showed that isoproterenol, a  $\beta$ -adrenergic agonist, caused a temporary increase in ANF secretion (Agnoletti *et al.*, 1992). These researchers ascribed this observation to a possible dual effect of isoproterenol: an initial stimulus of ANF secretion, followed by an inhibitory effect. Most evidence is in favour of a predominant effect of the  $\alpha_1$ -adrenergic receptor subtype. Precise pharmacological experiments further determined that it is the  $\alpha_{1A}$ -adrenergic receptor subtype, the same that is responsible for adrenergic stimulation of ANF secretion and gene expression in neonatal ventricular cardiocytes (Knowlton *et al.*, 1993), that is involved in the secretory response of cultured adult atrial cardiocytes to norepinephrine (Ambler and Leite, 1994).

### *Release by other factors*

ANF release is believed to be effected by glucocorticoids (Shields *et al.*, 1988), acetylcholine (Hayashi *et al.*, 1988), and Na<sup>+</sup>-K<sup>+</sup>-ATPase inhibitors (Bloch *et al.*, 1988; Morise *et al.*, 1991; Schiebinger and Cragoe, Jr. 1993). The glucocorticoid dexamethasone has been shown to effect ANF release *in vitro* in both cell cultures (Shields *et al.*, 1988; Muir *et al.*, 1993) and isolated atrial appendages (Dananberg and Grekin, 1992). Both dexamethasone and the mineralocorticoid deoxycorticosterone acetate (DOCA), administered *in vivo*, result in increased plasma ANF levels (Dananberg and Grekin, 1992; Yokota *et al.*, 1994), but the effect of DOCA is probably due to a secondary volume overload. Arginine vasopressin (Zongazo *et al.*, 1991), prostaglandins (Gardner and Schultz, 1990), and angiotensin II (Focaccio *et al.*, 1993) have also been suggested as direct stimulators of ANF release.

### *Neural reflexes and ANF release*

Several researchers have implied a link between ANF release in response to volume expansion and neural reflexes. In pithed rats, in which the humoral influences of the nervous system and the direct neural control of the heart through vagal and sympathetic innervation are removed, the increase in ANF release in response to an acute volume expansion is blunted (Eskay *et al.*, 1986; Ruskoaho *et al.*, 1989a). It has also been shown that lesions in the hypothalamus or pituitary reduce volume expansion-induced ANF secretion (Antunes-Rodrigues *et al.*, 1991). It was suggested that muscarinic and  $\alpha_1$ -adrenergic receptors in the hypothalamus are involved in this process (Antunes-Rodrigues *et al.*, 1993). The involvement of the ascending serotonergic system in controlling basal and

stimulated ANF secretion has also been proposed (Reis *et al.*, 1994). These authors put forth the theory that distension of baroreceptors of the atria, carotid sinus, aortic sinus, and kidney, results in alterations in the afferent output to the brain stem noradrenergic neurons, which by the means of axons projecting into the AV3V region of the brain, activated cholinergic neurons *via* an  $\alpha_1$ -adrenergic synapse. It is proposed that the loop makes its way back to the heart to effect release of ANF from the atria. This has gained support from the observations that stimulation of cardiac nerves in perfused heart stimulates ANF secretion, and that chemical sympathectomy abolished this response (Jiao and Baertschi, 1993). Conversely, other researchers have found that denervation in dogs or rats does not affect the release of ANF in response to volume expansion (Ledsome *et al.*, 1985; Goetz *et al.*, 1986; Kabayama *et al.*, 1987). Furthermore, it has been shown that stretch-induced ANF release from isolated atria is not dependent on the release of endogenous neurotransmitters (Schiebinger and Linden, 1986a; Page *et al.*, 1990).

### Physiological Actions of ANF

When taken together, the actions of ANF on the kidneys, vasculature, and adrenal glands serve to regulate body fluid homeostasis, often counteracting or modulating the renin-angiotensin-aldosterone system. ANF affects mainly renal function, acting to increase the glomerular filtration rate (Burnett, Jr. *et al.*, 1984; Bourgoignie *et al.*, 1986), and to alter ion permeabilities at or near the distal segment of the nephron (Sonnenberg *et al.*, 1982; Baum and Toto, 1986). Recent support for important renal actions of ANF has come from studies involving receptor antagonists that are specific for the guanylate cyclase-coupled ANF receptors, in which it has been shown that ANF is an essential component of the

mineralocorticoid escape phenomenon (Yokota *et al.*, 1994). ANF also has pronounced effects on the vasculature (Currie *et al.*, 1983), and on the adrenal glands (Atarashi *et al.*, 1984; Atarashi *et al.*, 1985; Vari *et al.*, 1986).

### *ANF receptors*

ANF interacts with three receptors: two are guanylate cyclase-linked active receptors, and the third is a non-guanylate cyclase coupled clearance receptor. Following the initial discovery of the receptors (Hirose *et al.*, 1985; Vandlen *et al.*, 1985; Leitman *et al.*, 1986; Meloche *et al.*, 1986), support for their existence was obtained by functional studies in bovine aortic smooth muscle cells (Scarborough *et al.*, 1986) as well as *in vivo* (Maack *et al.*, 1987). Purification (Kuno *et al.*, 1986; Paul *et al.*, 1987; Schenk *et al.*, 1987; Shimonaka *et al.*, 1987; Takayanagi *et al.*, 1987) and cloning (Fuller *et al.*, 1988; Chinkers *et al.*, 1989; Schulz *et al.*, 1989; Chang *et al.*, 1989) of the three receptor types gave data on their nature: the two guanylate cyclase-linked receptors were found to be single-chain polypeptides of 120-140 kDa, while the clearance receptor was identified as a 60-70 kDa peptide that forms homodimers. The deduced amino acid sequence of the guanylate cyclase receptors suggested the presence of an intracellular catalytic domain, and an extracellular ligand-binding domain (Chinkers *et al.*, 1989). This was confirmed by expression of receptor cDNAs in cultured mammalian cells, which showed ANF-binding and guanylate cyclase activity (Chinkers *et al.*, 1989). A protein kinase-like domain has also been identified in the intracellular portion of the receptors, and appears to function as a repressor of guanylate cyclase activity until ANF binding to the extracellular domain occurs (Chinkers and Garbers, 1989).

In the rat, the guanylate cyclase receptors have been termed GC-A and GC-B (Schulz *et al.*, 1989). The comparable receptors in man are called ANP-A and ANP-B, with ANP-C being the clearance receptor (Chang *et al.*, 1989). Homology between receptor types, both in humans and rat, is high in the intracellular regions of the receptors (73-74% amino acid homology), but quite low in the ligand-binding domain (43-44% homology), indicating that the two functional receptors may have different affinities for natriuretic peptides. This is indeed the case; the type A receptor has greater affinity than the type B receptor for ANF (Chang *et al.*, 1989; Schulz *et al.*, 1989; Koller *et al.*, 1991).

#### *Effects on the cardiovascular system*

The major effect of ANF on the cardiovascular system is that of a potent vasorelaxant in large arteries (Currie *et al.*, 1983), mediated by cGMP (Winquist *et al.*, 1984). Not all vascular beds respond to ANF; for example, ANF has been found to increase blood flow in lung, heart, spleen mesenteric, renal, and testes vascular beds, but not in brain, skin, or muscle (Garcia *et al.*, 1985), although some studies show little or no effect on blood flow (Fujioka *et al.*, 1985; Lappe *et al.*, 1985a; Lappe *et al.*, 1985b; Pegram *et al.*, 1985). Peripheral resistance has been reported to be slightly decreased by ANF in rats, dogs, and humans (Fujioka *et al.*, 1985; Hirata *et al.*, 1985; Bolli *et al.*, 1987; Dunn *et al.*, 1986; Hintze, 1988; Edwards *et al.*, 1986); however, some report a preferential effect on the renal vasculature (Dunn *et al.*, 1986; Hintze, 1988; Edwards *et al.*, 1986) or no effect at all (Zimmerman *et al.*, 1987). Experiments using the ANF receptor antagonist HS-142-1 show that increases in plasma ANF may not be involved in regulating systemic resistance (Nishikimi *et al.*, 1994). It remains that the result of the vasorelaxant actions of ANF on the vasculature is a decrease in blood pressure (Garcia *et al.*, 1985; Granger *et al.*, 1986;

Fenoy *et al.*, 1989) and a concomitant decrease in cardiac output due to a decreased preload (Breuhaus *et al.*, 1985; Cody *et al.*, 1986; Holtz *et al.*, 1986; Shapiro *et al.*, 1986). ANF also decreases cardiac output through suppression of the sympathetic baroreceptor response to decreased blood volume, presumably by activating vagal afferents (Thören *et al.*, 1986). The observation that ANF infusions lead to increased haematocrit (de Bold *et al.*, 1981; Almeida *et al.*, 1986; Flückiger *et al.*, 1986; Weidmann *et al.*, 1986; Trippodo and Barbee, 1987) suggests that ANF acts to reduce intravascular volume, by either increasing capillary permeability or capillary pressure gradients. Both increases in capillary permeability (Huxley *et al.*, 1987) and capillary filtration pressure (in the renal vasculature) (Dunn *et al.*, 1986) have been observed, but it remains that ANF may reduce preload primarily via these mechanisms, and not directly by dilation of capacitance veins. Debate also exists concerning the role of ANF in the regulation of coronary blood flow: in isolated perfused hearts from guinea pigs, rats, and dogs, ANF seems to reduce coronary blood flow (Wangler *et al.*, 1985; Laxson *et al.*, 1988), but *in vivo* studies in dogs fail to show a strong correlation between ANF levels and changes in regional blood flow (Winqvist *et al.*, 1984; Hintze *et al.*, 1985; Osol *et al.*, 1986; Pegram *et al.*, 1986).

ANF also has anti-growth properties in the vasculature. Hypertrophy of cultured vascular smooth muscle cells is inhibited by ANF (Itoh *et al.*, 1990; Morishita *et al.*, 1994), and this could be a result of a functional antagonism between ANF and angiotensin II (Itoh *et al.*, 1991). Antigrowth properties of ANF on cultured endothelial cells (Itoh *et al.*, 1992; Morishita *et al.*, 1994), and on cardiac fibroblasts (Cao and Gardner, 1995) has also been demonstrated.

### *Renal effects*

The renal effects of ANF include an increase in glomerular filtration rate and filtration fraction, inhibition of renin synthesis in the macula densa, inhibition of Na<sup>+</sup> reabsorption from the collecting duct, and possible inhibition of Na<sup>+</sup> reabsorption in the proximal tubule.

ANF increases GFR and filtration fraction (Cogan, 1985; Camargo *et al.*, 1984; Yukimura *et al.*, 1984; Huang *et al.*, 1985), probably as the result of dilation of afferent arterioles and constriction of efferent arterioles (Dunn *et al.*, 1986), and by counteracting the effects of angiotensin II. ANF may also relax glomerular mesangial cells, thus increasing filtration surface area (Appel *et al.*, 1986; Gunning *et al.*, 1988; Singhal *et al.*, 1989). Effects of ANF have been observed in proximal tubules in several experimental systems (Huang *et al.*, 1985; Harris *et al.*, 1987; van der Stolpe and Jamison, 1988), but little evidence supports a physiological role in fluid and electrolyte balance regulation at this site. In microperfusion studies involving the loop of Henle (Kondo *et al.*, 1986; Peterson *et al.*, 1987) and distal tubule segments (Sonnenberg *et al.*, 1982; Briggs *et al.*, 1982), no effects of ANF were observed, so it is unlikely that ANF has any role at these locations. These observations are supported by the fact that no ANF receptors have been detected in the proximal tubule, loop of Henle, or distal tubule segments of the nephron (Murphy *et al.*, 1985; Healy and Fanestil, 1986; Koseki *et al.*, 1986; Butlen *et al.*, 1987).

In the collecting duct, ANF inhibits reabsorption by the inner medullary collecting duct (IMCD) epithelial cells (Sonnenberg *et al.*, 1982; Sonnenberg *et al.*, 1986), and may even facilitate Na<sup>+</sup> secretion by these cells (Sonnenberg *et al.*, 1986; Rocha and Kudo,

1990b; Rocha and Kudo, 1990a). Also, ANF-induced increases in *vasa recta* hydraulic pressures may contribute to an unfavourable gradient for Na<sup>+</sup> and fluid reabsorption (Mendez *et al.*, 1986).

### *Effects on endocrine functions*

ANF acts to directly inhibit aldosterone release and synthesis from adrenal cortical glomerulosa cells (Kudo and Baird, 1984; Atarashi *et al.*, 1984; De Lean *et al.*, 1984; Atarashi *et al.*, 1985), and endogenous plasma ANF levels reduce *in vivo* aldosterone secretion (Maack *et al.*, 1984; Weidmann *et al.*, 1986; Zimmerman *et al.*, 1987; Metzler and Ramsay, 1989). It is interesting to note that although the guanylate cyclase-associated receptors were isolated from the adrenal cortex (Meloche *et al.*, 1986; Takayanagi *et al.*, 1987), inhibition of aldosterone secretion by ANF does not use cGMP as second messenger (Vari *et al.*, 1986). ANF may instead directly interact with ion channels, as assessed by patch-clamp experiments (Barrett *et al.*, 1991). ANF also reduces renin secretion (Maack *et al.*, 1984; Burnett, Jr. *et al.*, 1984; Kurtz *et al.*, 1986; Opgenorth *et al.*, 1986; Henrich *et al.*, 1987), which results in lower circulating levels of angiotensin II and aldosterone. Actions of ANF on other endocrine tissues include a possible feedback mechanism involving ADH: ANF inhibits ADH secretion in response to haemorrhage and chronic dehydration (Samson, 1985), counters the effects of ADH (Dillingham and Anderson, 1986), and inhibits vasopressin-neuron firing (Standaert *et al.*, 1987), while ADH augments ANF secretion (Manning *et al.*, 1985).

Recent evidence indicates that ANF inhibits the secretion of ET-1 from aortic endothelial cells, and decreases ET-1 synthesis at the translational level, possibly by

interaction with the C-receptor (Hu *et al.*, 1992), which until now was thought to be only a clearance receptor (Maack *et al.*, 1987; Almeida *et al.*, 1989; Maack, 1992). Other studies have shown that ANF inhibits thrombin-induced ET-1 synthesis and secretion in cultured endothelial cells *via* a cGMP-dependent process (Kohno *et al.*, 1992a). A similar effect is observed in mesangial cells in which ET-1 synthesis is stimulated by vasopressin (Kohno *et al.*, 1993). Also, angiotensin II-stimulated ET-1 secretion in porcine and human aorta is reduced by ANF treatment, also presumably through a cGMP-dependent process (Kohno *et al.*, 1992b; Kohno *et al.*, 1991). The synthesis and secretion of C-type natriuretic peptide (CNP, see below) is also stimulated by ANF in bovine aortic endothelial cells (Nazario *et al.*, 1995), implying that CNP may be a mediator between ANF and its actions on adjoining vascular smooth muscle cells. ANF acts to down-regulate the synthesis of the GC-A receptor in vascular smooth muscle cells (Cao *et al.*, 1995).

### *Neural effects*

ANF has effects on the central nervous system, where it is involved in the control of thirst and appetite. ANF binding sites have been detected in areas of the brain that are involved in regulation of cardiovascular homeostasis, including the subfornical organ (Saavedra *et al.*, 1986; Steardo and Nathanson, 1987). Neurons immunoreactive for ANF have also been identified in these regions (Saper *et al.*, 1985). Intracerebroventricular infusion of ANF leads to marked decreases in thirst in rats, sheep, or rabbits (Antunes-Rodrigues *et al.*, 1985; Fioretto *et al.*, 1992; Tarjan *et al.*, 1988; Blackburn *et al.*, 1995). ANF also decreases AVP-induced thirst in man (Burrell *et al.*, 1991). Salt appetite is also inhibited centrally by ANF (Itoh *et al.*, 1986; Weisinger *et al.*, 1992; Tarjan *et al.*, 1988).

Therefore, ANF maintains cardiovascular homeostasis by the central control of thirst and salt appetite in addition to its peripheral actions.

### Other Natriuretic Peptides

There exist two other peptides related to ANF. The first one to be discovered was found in porcine brain, and was thus named brain natriuretic peptide (BNP) (Sudoh *et al.*, 1988). It was later discovered that it is synthesized mostly by the atria and ventricles of the heart (Saito *et al.*, 1989b; Ogawa *et al.*, 1991; Hosoda *et al.*, 1991; Mukoyama *et al.*, 1991). The second related peptide discovered was named C-type natriuretic peptide (CNP) (Sudoh *et al.*, 1990), following the A- B- C-type nomenclature proposed by Sudoh *et al.* (1990). CNP is found mostly in the central nervous system (Kojima *et al.*, 1990) and in vascular endothelial cells (Suga *et al.*, 1992b; Stingo *et al.*, 1992b).

#### *Brain natriuretic peptide*

Following the discovery of BNP in porcine brain, similar peptides in human (Sudoh *et al.*, 1989), rat (Kojima *et al.*, 1989; Flynn *et al.*, 1989), dog (Seilhamer *et al.*, 1989), and mouse (Steinhilber, 1993; Ogawa *et al.*, 1994) were subsequently identified. Like ANF, BNP has vasorelaxant and natriuretic activities, albeit at only a fraction of the potency of the activity of ANF (Sudoh *et al.*, 1988; Ogawa *et al.*, 1991). Unlike ANF, BNP shows little cross-species homology, with only 59% of the amino acids retaining their identity from one species to another (Rosenzweig and Seidman, 1991). BNP is found as a 32-amino acid circulating peptide in pigs and humans (Sudoh *et al.*, 1988; Sudoh *et al.*, 1989), and a non-circulating

26-amino acid functional processing variant is found in porcine brain (Sudoh *et al.*, 1988). In the rat, BNP circulates as a 45-amino acid peptide, and because of this striking difference with other BNPs, it was originally called iso-ANF (Flynn *et al.*, 1989); cloning of the cDNA (Kojima *et al.*, 1989; Flynn *et al.*, 1989) and gene (Roy and Flynn, 1990; Steinhilper, 1993; Ogawa *et al.*, 1994) revealed that iso-ANF is in fact the rodent form of BNP, and that the difference in length between the circulating peptides is due to alternate processing of the prohormone. The alternate processing from one species to another is probably due to the fact that the major part of inter-species divergence occurs in the second exon of the gene, which contains the processing sites (Kojima *et al.*, 1989; Flynn *et al.*, 1989; Roy and Flynn, 1990; Steinhilper, 1993). The discovery of bovine aldosterone secretion inhibitory factor (Nguyen *et al.*, 1989), which is very similar to BNP, points to yet another processing variant of BNP.

Promoter analysis of the rat BNP gene indicates that the elements required for cardiac-specific expression are limited to a 114 bp region upstream of the transcription start site (Grépin *et al.*, 1994; Thuerlauf *et al.*, 1994). This promoter region contains a conserved AP-1-like site that confers it much of its activity (Grépin *et al.*, 1994), and a GATA motif, which also appears to be important for basal expression of BNP in cardiocytes (Grépin *et al.*, 1994; Thuerlauf *et al.*, 1994), and is conserved in other species (Seilhamer *et al.*, 1989; Steinhilper, 1993; Ogawa *et al.*, 1994). The GATA motifs are usually found in hematopoietic cell-specific genes, and are bound by the GATA family of zinc-finger transcription factors (Engel *et al.*, 1992); the rat homolog of the GATA-4 protein has been cloned from cardiocytes (Grépin *et al.*, 1994), and both rat and mouse GATA-4 can activate the BNP and ANF promoters in cardiac and non-cardiac cells (Grépin *et al.*, 1994; Thuerlauf *et al.*, 1994). Blocking GATA-4 expression in P19 embryonal carcinoma cells inhibits the differentiation of these cells into

cardiocytes and prevents the expression of BNP (Grépin *et al.*, 1995). Therefore it appears that the GATA elements of the BNP gene, and perhaps those of the ANF gene, are involved in the cardiac-specific developmental regulation of ANF and BNP expression.

BNP has natriuretic, hypotensive, and vasorelaxant actions that are similar to those of ANF, and also counteracts the renin-angiotensin system (Sudoh *et al.*, 1988; Holmes *et al.*, 1993; Richards *et al.*, 1993). Transgenic mice made to overexpress BNP exhibit a slight level of hypotension (Ogawa *et al.*, 1994), indicating that this peptide may be important in modulating blood pressure. Like ANF, BNP also inhibits stimulated ET-1 synthesis and secretion (Kohno *et al.*, 1991; Kohno *et al.*, 1992b; Kohno *et al.*, 1993), and stimulates CNP production and secretion (Nazario *et al.*, 1995).

BNP is also involved in the response of the heart to overload, although unlike ANF, BNP seems to be recruited more strongly from the ventricles, and would thus appear to be more of a ventricular hormone, at least in conditions of disease (Ogawa *et al.*, 1991; Mukoyama *et al.*, 1991; Yokota *et al.*, 1995; Ogawa *et al.*, 1996). In SHR (Dagnino *et al.*, 1992; Roy *et al.*, 1992) and SHR-stroke prone rats (Ogawa *et al.*, 1991), ventricular BNP mRNA levels are increased (relative to WKY controls) to a much greater degree than ANF mRNA: BNP expression is increased in the atria, but much more so in the ventricle, such that when the heart is taken as a whole, BNP mRNA content in the ventricle is three times that of the atria. This is in sharp contrast with ventricular ANF mRNA, which reach only 7% of atrial levels (Ogawa *et al.*, 1991). In human subjects with congestive heart failure, ventricular BNP levels are at almost double the atrial levels, contributing to 88% of the BNP mRNA levels in the whole heart (Mukoyama *et al.*, 1991). In rats rendered hypertensive by DOCA-salt treatment, BNP mRNA and peptide levels are increased in left atria and in both ventricles only

in animals that had significant atrial and left ventricular hypertrophy, although the increased production of BNP in the right ventricle is not accompanied by significant hypertrophy of the tissue (Yokota *et al.*, 1990; Yokota *et al.*, 1995). These observations have led investigators to believe that BNP may be almost as important as ANF in these pathological states, in part because BNP is cleared from the circulation more slowly than ANF (Mukoyama *et al.*, 1991; Kita *et al.*, 1991). Synthesis and secretion of BNP in cultured neonatal atrial and ventricular cardiocytes can be directly stimulated by ET-1 (Suzuki *et al.*, 1992; Nakagawa *et al.*, 1995) and activators of protein kinase C (PKC) (Suzuki *et al.*, 1992; LaPointe and Sitkins, 1993). In cultured neonatal ventricular cardiocytes, BNP secretion and mRNA levels can be stimulated by phenylephrine (Hanford *et al.*, 1994).

#### *C-type natriuretic peptide*

CNP is strikingly different from ANF and BNP in that it does not have the C-terminal tail which is necessary for most of the biological activity of ANF and BNP (Sudoh *et al.*, 1990). CNP is the natriuretic peptide which retains the most cross-species amino acid homology: 97.1% amino acid identity between rat and pig (Kojima *et al.*, 1990). Following cloning of the cDNA and gene in pig (Tawaragi *et al.*, 1990), rat (Kojima *et al.*, 1990), and human (Tawaragi *et al.*, 1991), it was found that the 3' non-coding sequences of the porcine and human genes do not contain the consensus polyadenylation signal found at the 3' end of most eukaryotic genes (Proudfoot and Brownlee, 1974), and that the rat gene has its poly-A+ tail far downstream of the polyadenylation signal, possibly indicating the presence of a long second intron.

This member of the natriuretic peptide family is found mostly in brain (Kojima *et al.*, 1990), but has recently been identified in vascular endothelial cells, indicating a possible autocrine role in the peripheral vasculature (Suga *et al.*, 1992b; Stingo *et al.*, 1992b). CNP has also been detected by the polymerase chain reaction in the heart, immune organs, kidney, stomach, testis, adrenal gland, and intestine (Vollmar *et al.*, 1993; Suzuki *et al.*, 1993; Minamino *et al.*, 1993), but this might reflect identification in vascular endothelial cells in these tissues. Circulating CNP has been detected at low picomolar concentrations in canine (Clavell *et al.*, 1993) and human plasma (Stingo *et al.*, 1992b; Hama *et al.*, 1994). Notable is the inducible synthesis and secretion of CNP from endothelial cells by stimulation with TGF- $\beta$  (Suga *et al.*, 1992b) and cytokines (Suga *et al.*, 1993). Plasma CNP levels have been shown to increase in patients with septic shock (Hama *et al.*, 1994).

CNP's natriuretic activities are only 1% those of ANF, while its rectum-relaxant activity is 3-4 times more potent than that of ANF (Sudoh *et al.*, 1990). In dogs, CNP has been shown to have potent hypotensive activities, with some natriuretic actions (Stingo *et al.*, 1992a). As with ANF, CNP increases cGMP levels in vascular smooth muscle cells, although the levels of cGMP induced are 3-4 times higher than those induced by ANF (Furuya *et al.*, 1990), implying that it acts via a different mechanism than that of ANF. CNP also may inhibit agonist-induced ET-1 secretion in cultured porcine endothelial cells (Kohno *et al.*, 1992a), but this action is not observed in cultured endothelial cells from the rat (Emori *et al.*, 1993), indicating possible species differences in the type of receptor expressed by these cells (see below). Functional antagonism between CNP and ET-1 in the vasculature is further supported by a report showing that CNP upregulates the ET<sub>B</sub> receptor in vascular smooth muscle cells (Eguchi *et al.*, 1994); the ET<sub>B</sub> receptor is responsible for the vasodilatory actions of ET-1 and ET-2. Infusion of CNP in rats in which vascular injury was induced results in

marked inhibition of neointima formation (Furuya *et al.*, 1993; Furuya *et al.*, 1995), hinting at the possibility that CNP may act in an autocrine or paracrine fashion to inhibit or reduce neointimal proliferation in the development of atherosclerosis or following injury. Supporting this is the observation that CNP reduces the rate of growth of vascular smooth muscle cells (Furuya *et al.*, 1991).

## AIM OF STUDY

Chronic atrial hemodynamic overload *in vivo* results in increased synthesis and release of ANF and BNP under a variety of circumstances (Lattion *et al.*, 1986; Arai *et al.*, 1987; Drexler *et al.*, 1989; Haass *et al.*, 1990; Mukoyama *et al.*, 1991; Fischer *et al.*, 1991; Ogawa *et al.*, 1991; Yokota *et al.*, 1994; Yokota *et al.*, 1995). However, in decompensated heart failure or chronic hypertension, the actions of the natriuretic peptides cannot overcome the detrimental effects of the renin-angiotensin-aldosterone system. This occurs despite the fact that natriuretic peptide receptors can still respond to exogenously administered ANF or BNP (Cody *et al.*, 1986; Yoshimura *et al.*, 1991). It appears that the increased secretion of ANF and BNP is blunted, and cannot maintain the levels of secretion required for compensation of impaired heart function. Therefore, elucidating the mechanism of control of ANF and BNP synthesis and secretion from the endocrine heart is crucial for our understanding of the pathophysiology of heart failure.

The view has been established that the major stimulus for increased natriuretic peptide synthesis and release is stretch of the atrial muscle (Edwards *et al.*, 1988; de Bold *et al.*, 1996). However, it has been observed that although both *in vivo* and *in vitro*, acute atrial muscle stretch results in an immediate increase in the rate of release of ANF, elevated secretory rates cannot be maintained for extended periods of time (Dietz, 1984; Lang *et al.*, 1985; de Bold *et al.*, 1986; Schiebinger and Linden, 1986a). In addition, no direct relationship has been established between acute mechanical stretch of adult atrial tissue and increased ANF gene expression. A recent study has shown a significant increase in BNP secretion and atrial mRNA levels after one hour of atrial stretch in perfused rat hearts (Mäntymaa *et al.*,

1993), but this was only studied for 2 hours; therefore it is not known if this is sustained further. It is not known if acute atrial stretch can modulate the expression of the ANF gene.

The temporary nature of stretch-induced ANF secretion suggests that a stimulus other than stretch might be responsible for the sustained increase in natriuretic peptide gene expression and secretion in the *in vivo* pathophysiological states described above. Several candidates for a neural or endocrine modulation of natriuretic peptide synthesis have been proposed, the most potent of which is ET-1. ET-1 has been shown to stimulate ANF release from perfused atria and perfused hearts (Hu *et al.*, 1988; Stasch *et al.*, 1989; Winquist *et al.*, 1989; Stasch *et al.*, 1989; Schiebinger and Gomez-Sanchez, 1990; de Bold *et al.*, 1991; Mäntymaa *et al.*, 1990), and BNP release from cultured neonatal atrial cardiocytes (Suzuki *et al.*, 1992). ET-1-stimulated natriuretic peptide gene expression has been studied in atrial and ventricular neonatal cardiocytes (Fukuda *et al.*, 1989; Gardner *et al.*, 1991; Shubeita *et al.*, 1990; Suzuki *et al.*, 1992; Nakagawa *et al.*, 1995). The phenotypic differences between neonatal and adult cardiocytes are marked (Wei *et al.*, 1987; Wu *et al.*, 1988; Chien *et al.*, 1993; Knowlton *et al.*, 1995), therefore it is not known what the effects of ET-1 on the expression of natriuretic peptide genes in adult atrial tissue might be, nor is it known if BNP secretion from adult atria can be stimulated by ET-1.

Stimulation of adrenergic receptors has been proposed as a modulator of ANF secretion from atrial tissue and cultured neonatal atrial cardiocytes (Schiebinger *et al.*, 1987; Matsubara *et al.*, 1988; Shields and Glembotski, 1989; Ambler and Leite, 1994), as well as a stimulus for ANF and BNP synthesis and secretion in cultured neonatal ventricular cardiocytes (Knowlton *et al.*, 1991; Hanford *et al.*, 1994). The receptor responsible for the effects of adrenergic stimulation on ANF secretion and gene expression is the  $\alpha_{1A}$ -subtype

(Ambler and Leite, 1994; Knowlton *et al.*, 1993; Hanford *et al.*, 1994), which shares intracellular signalling pathways with those activated by ET-1 (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Ambler and Leite, 1994; De Jonge *et al.*, 1995). The signalling events initiated by ET-1 are known to be rapidly down-regulated in cultured ventricular cardiocytes, while those that follow activation of  $\alpha_1$ -adrenergic receptors are not (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Ambler and Leite, 1994). The atria have extensive adrenergic innervation, and in clinical situations such as congestive heart failure, there is a marked activation of sympathetic activity (Riegger, 1991; Svanegaard *et al.*, 1993; Dzau, 1988; Riegger, 1994). Therefore, since activation of  $\alpha_1$ -adrenergic receptors may be a primary modulator of natriuretic peptide synthesis, the stimulation of isolated atrial tissues by the  $\alpha_1$ -adrenergic agonist phenylephrine was also studied.

The transcriptional regulation of the natriuretic peptide genes has been proposed to be controlled by the products of the early-response genes *c-fos*, *c-jun*, and *Egr-1* (Knowlton *et al.*, 1991; Kovacic-Milivojevic and Gardner, 1992; Kovacic-Milivojevic and Gardner, 1993; McBride *et al.*, 1993). These genes are expressed in response to stretch, ET-1, and phenylephrine in ventricular cardiocytes (Shubeita *et al.*, 1990; Komuro *et al.*, 1990; Iwaki *et al.*, 1990; Ito *et al.*, 1991; Komuro *et al.*, 1991; Rockman *et al.*, 1991; Sadoshima *et al.*, 1992a; Jones *et al.*, 1992; Kolbeck-Ruhmkorff *et al.*, 1993; Neyses *et al.*, 1993). The *c-myc* proto-oncogene is also induced in ventricular cardiocytes in response to hemodynamic overload and  $\alpha_1$ -adrenergic stimulation (Starksen *et al.*, 1986; Izumo *et al.*, 1988; Komuro *et al.*, 1988; Ikeda *et al.*, 1991; Kolbeck-Ruhmkorff *et al.*, 1993), and its expression has been directly implicated in the hypertrophic response of cardiac tissue to mechanical and neuroendocrine stimuli (Neyses *et al.*, 1991; Robbins and Swain, 1992; Komuro and Yazaki, 1993). Whether the early response genes respond in atrial cardiocytes as they do in their

ventricular counterparts is not known. It is expected that they do respond to the three types of stimuli as in ventricular cardiocytes, and they may thus serve as markers for the transcriptional response of the atrial tissue.

The aim of this study was to delineate the determinants of increased natriuretic peptide secretion and gene expression in adult atrial tissue, using stretch, ET-1, or phenylephrine as models of mechanical, autocrine/paracrine, and neuroendocrine stimuli. To this end, *in vitro* isolated atria were stimulated by stretch, ET-1, or phenylephrine for periods of time extending up to 8 hours. The expression of the early response genes *c-fos*, *c-jun*, *Egr-1*, and *c-myc* were also characterized. Secretion of ANF and BNP from isolated atria was measured by radioimmunoassay. Natriuretic peptide and early-response gene expression were examined by analyzing stable transcript levels using Northern blots.

## MATERIALS AND METHODS

### Chemicals and Drugs

Chemicals were obtained from BDH chemicals (Toronto, ON.) or Sigma Chemical (St.Louis, MO.), unless otherwise specified. ET-1 was purchased from Peninsula Laboratories (Belmont, CA.). DNA oligonucleotides were obtained from the University of Ottawa Biotechnology Research Institute or from Bio/Can (Mississauga, ON.).

### Isolated Atrial Preparations

#### *Isolated whole atria*

An *in vitro* perfused rat atrial preparation was used, as described by de Bold et al. (1986). Male Sprague-Dawley rats (300-350 g) were given free access to food and water until sacrificed by decapitation. The hearts were removed, and the atria were dissected away from the ventricles and placed in supplemented Krebs-Ringer bicarbonate buffer (KRBB), pH 7.3-7.4. The composition of the KRBB

is as follows (mmol/L): NaCl (118), KCl (4.7),  $\text{CaCl}_2$  (2.5)  $\text{NaH}_2\text{PO}_4$  (1.18),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

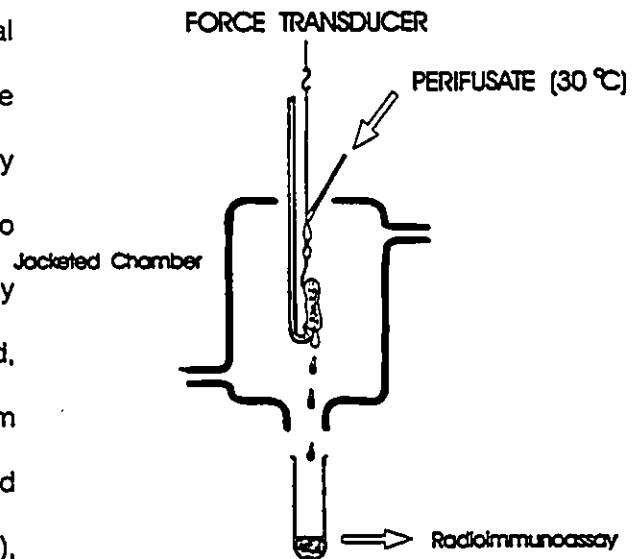


Figure 2: Isolated whole atria. See text for details.

(1.18),  $\text{NaHCO}_3$  (24.9), sodium glutamate (5), sodium fumarate (5), sodium lactate (50), glucose (11). The solution also contained Zinc Insulin (Humulin R, Eli Lilly & Co., Mississauga, ON) at 25 mU/mL, amino acids and vitamins as in Eagle's Medium (Life Technologies, Burlington, ON), 0.001% bovine serum albumin (BSA), and 0.01% gelatin. The atria were suspended between two hooks, one attached to a fixed glass rod, and the other suspended from a silk thread attached to a force transducer (Grass Instruments, Quincy, MA) which was connected to a physiograph (Narco Bio-systems, Houston, TX); a micrometric screw (Nashirigi, Tokyo, JAPAN) allowed precise tension adjustments. The atria thus suspended were kept inside a jacketed container kept at 30°C, and KRBB oxygenated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  and pre-heated at 30°C was dripped onto the cotton thread at a rate of 3 mL/min (see figure 2). The perfusate was collected using a fraction collector, and stored at -20°C until used for radioimmunoassay.

The experimental protocol was as follows. After dissection from the ventricles, the atria were suspended as described above, and were allowed to remain in 50 mL of gassed supplemented KRBB for 15 minutes. Following the bath period, the atria were perfused, given a basal tension of 200 mg, and were left to equilibrate for one hour. The perfusate was collected for 15 minutes following the equilibration period, after which the tissue was either left at the basal tension, or given a 5 g load. The times for the "stretch" period were 30 minutes, 2 hours, or 4 hours. Perfusate was collected every minute for the 30 minutes experiments, and every 10 minutes for the 4 hour experiments.

For stimulation with ET-1, atria were placed in a setup identical to that used for the stretch experiments, except that a 10 mL bath maintained at 30°C was used instead of perfusion. In this manner, atria were bathed in the modified KRBB described above, and

were oxygenated with 95%O<sub>2</sub>/5% CO<sub>2</sub> throughout the duration of the experiment. After dissection, the atria were suspended as described in the perfusion protocol. The tissue was left to equilibrate for two 30 minute periods. Basal secretion was measured from one 15 minute period, designated "0 min". At time 0, the perfusate was replaced with KRBB or KRBB containing 10<sup>-7</sup> mol/L ET-1. The dose of ET-1 used was based on dose-response experiments previously performed (de Bold *et al.*, 1991). The buffer was collected and replaced every 30 minutes for 30 minutes, 2 hours, or 4 hours. Buffer was kept at -20°C until used for radioimmunoassay.

### *Isolated right atria*

To allow study of natriuretic peptide responses over a longer period of time, the latter part of this work involved use of a novel isolated right atrium model that permits experiments that last up to 10 hours (Piazza, Bruneau, & de Bold, unpublished results). Hearts were removed from male Sprague-Dawley rats (300-350 g, Charles River Laboratories, Montréal, PQ), and placed in a Krebs' ringer bicarbonate buffer (KRBB) solution. The KRBB contained (mmol/L): NaCl (78), KCl (4.7), CaCl<sub>2</sub>·2H<sub>2</sub>O (2.54), NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (1.36), MgCl<sub>2</sub>·6H<sub>2</sub>O (1.16), NaHCO<sub>3</sub> (25.0), HEPES (20), sodium glutamate (2.0), sodium fumarate (4.0), sodium lactate (2.0), glucose (11.6). The solution was supplemented with Zinc Insulin (Humulin R, Eli Lilly & Co., Minneapolis, MN) at 2.46 mU/mL, amino acids and vitamins as in Eagle's Medium (Life Technologies, Burlington, ON), 0.001% bovine serum albumin (BSA), 0.01% gelatin, penicillin G (100

U/mL, Sigma Chemicals, St. Louis, MO), and fungizone (0.25  $\mu$ g/mL, Life Technologies, Burlington, ON). The pH was adjusted with 1 N NaOH to 7.40 at 30°C. The osmolarity of the buffer, as measured by an osmometer, was 300 mOsm/kg. The KRBB was filtered through a 0.45  $\mu$ m filter prior to use. The dissection procedure was as follows. A PE-160 cannula (I.D. 1.14 mm), approximately 30 mm in length, was inserted in the inferior vena cava, and out the superior vena cava. The cannula had an opening midway between the inferior and superior cavas, and was positioned such that the opening faced the inside of the right atrial chamber. The cannula was fixed to each vena cava by way of a silk suture. The coronary sinus was ligated, the left auricle was removed, and the aorta and pulmonary vein were severed above the ventricle and removed. The atrium was cleaned of connective tissue and fat. The lower five sixths of the ventricles were removed, and a rigid heat-curved cannula (PE-10), the end of which was heated to form a lip, was inserted in the right atrium via the tricuspid valve; this cannula was held in place by tying a silk suture around the

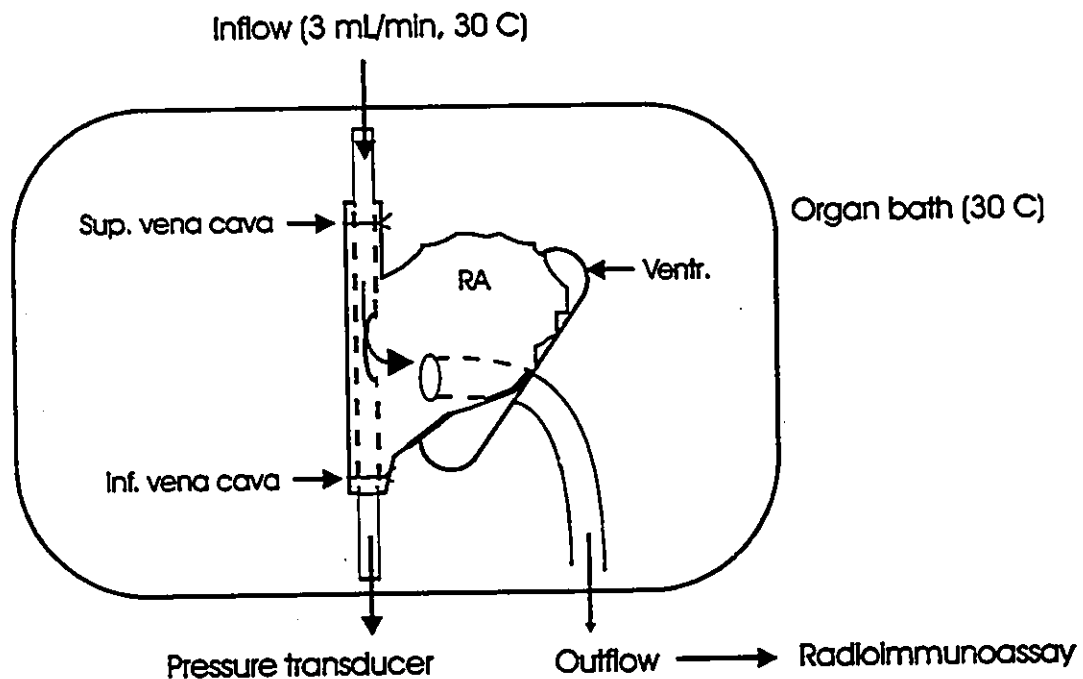


Figure 3: Isolated right atria. See text for details.

atrioventricular junction. The dissection procedure took less than 15 minutes to perform, and the atrium was perfused with fresh KRBB regularly throughout the dissection procedure.

As diagrammed in Fig. 3, the right atrium dissected in this manner was fixed to a plastic disc by inserting the inferior vena cava and outflow cannulas onto tubing-covered syringe needle ends that passed through the disc. The disc was then placed in the opening of an inverted heated (30°C) organ chamber. The superior vena cava was attached to a cannula from which KRBB (pre-heated to 30°C and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>) was infused by a programmable solvent delivery module (iModel 560, Waters, Milford, MA) at a rate of 3 mL/minute. The outflow from the inferior vena cava was connected to a pressure transducer (Narco Bio-Systems, Houston, TX) that was connected to a physiograph (Narco Bio-Systems, Houston, TX). Thus, the flow of KRBB was from the superior vena cava, into the right atrium, and out the tricuspid valve. The organ chamber contained 50 mL of KRBB, which was replenished at a rate of 2.5 mL/minute, and which was gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> throughout the duration of the experiment. The level of the bath medium was adjusted to be 2 cm above the atrium. To accurately measure intra-atrial pressure, the tips of the pressure transducer and outflow cannulas were positioned at the same height. To adjust the intra-atrial pressure, two methods were used. The first was employed to adjust the basal pressure to approximately 0.5 mmHg; this was accomplished by slightly raising or lowering the atrium in relation to the tips of the outflow and pressure transducer cannulas, and observing the tracings on the physiograph until the desired intra-atrial pressure was attained. The second was for the imposition of a rhythmic volume overload on the atrium; to this end, a solenoid driving a piston was placed midway along the outflow cannula, acting as a valve. The solenoid was under the control of a function generator (Wavetek), with which solenoid closing rate and duration were altered. The solenoid rate was set at 84 cycles per minute,

which was the fastest rate at which the intra-atrial pressure could be precisely controlled. Closing duration was adjusted to increase intra-atrial pressure in a pulsatile manner. Secretion medium was collected for 5 min periods in siliconized glass tubes using a fraction collector (ISCO Retriever II, Lincoln, NE.). A 50  $\mu$ L aliquot was quickly transferred to another tube for use in the ANF RIA; this and the remainder of the perfusate were immediately placed at -20°C, and were thawed only once for use in RIA or for extraction of BNP. Atria were then left at the basal intra-atrial pressure, or stimulated by stretch, ET-1, or L-phenylephrine for 1.5, 4, 6, or 8 hours. The degree of stretch (8 mmHg) and the dose of ET-1 ( $10^{-8}$  mol/L) were derived from dose-response curves previously performed (L.A. Piazza and A.J. de Bold, unpublished observations). Phenylephrine dose-response experiments were performed using phenylephrine at 10, 50, and 100  $\mu$ mol/L; these concentrations were chosen based on previous experiments that involved ANF secretion from atrial tissue (Schiebinger *et al.*, 1987; de Bold *et al.*, 1991) and ANF gene expression in cultured cardiocytes (Sei *et al.*, 1991). Drugs at 9 times their final concentration or their vehicle were infused via the inflow cannula, using a syringe pump (Sage Instruments, Cambridge, MA) set at 0.34 mL/minute; the flow from the delivery module was set such that flow into the atrium was maintained at 3 mL/minute. Phenylephrine was dissolved in KRBB containing 100  $\mu$ mol/L ascorbic acid as an antioxidant; ET-1 was dissolved in KRBB. There was no difference in natriuretic peptide secretion or atrial mechanical function between atria perfused with KRBB alone or KRBB containing ascorbic acid. At the end of the perfusion period, the tissue was rapidly removed, and placed in a dissecting dish containing KRBB. The cannulas were removed, and remaining ventricular tissue and connective tissue were swiftly cut away. The right atrium was flash-frozen in liquid nitrogen.

## Extraction of BNP

For BNP secretion analysis, secretion medium was extracted using C<sub>18</sub> Sep-Pak cartridges (Millipore-Waters, Milford, MA): Sep-Pak cartridges were pre-wetted with 80% acetonitrile in 0.1% trifluoroacetic acid (TFA), rinsed with 0.1% TFA. Secretion medium was passed three times through the cartridge at a rate of approximately 1 mL/min; adsorbed samples were eluted with 80% acetonitrile in 0.1% TFA (de Bold and Flynn, 1983). Eluates were freeze-dried and reconstituted first in 25  $\mu$ L 10 mM HCl for 20 min, to which 225  $\mu$ L RIA buffer (see below) was subsequently added; 100  $\mu$ L were used for BNP RIA. Extraction efficiency of BNP was  $77 \pm 2\%$ .

## Radioimmunoassay

Radioimmunoassay was performed using the double-antibody technique as described by Sarda et al. (1989). The RIA buffer used consisted of 0.1 mol/L sodium phosphate, 0.05 mol/L NaCl, 0.01% sodium azide, 0.1% Triton X-100, and 0.1% heat-treated BSA. This buffer was used for the preparation of all reagents. Standard curves were calculated using ANF<sub>99-126</sub> (Peninsula Laboratories, Belmont, CA) at the following concentrations (pg/mL): 31.25, 62.50, 125.0, 250.0, 500.0, and 1000. The protocol is as follows: 100  $\mu$ L of ANF standard or samples was added to 100  $\mu$ L of 1:30,000 dilution of antiserum raised against ANF<sub>99-126</sub> (Peninsula Laboratories, Belmont, CA) and left at 4°C for 4 hours. Following this incubation period, 100  $\mu$ L (10,000 cpm) of iodinated ANF<sub>99-126</sub> (NEN/DuPont, Boston, MA) was added to the tubes, and the samples were left at 4°C for 20 hours. Goat anti-rabbit gamma globulin (Peninsula Laboratories, Belmont, CA) and 100  $\mu$ L of normal rabbit serum were added to the tubes to separate the free and bound fractions.

Following a 2 hour incubation at room temperature, 1.5 mL polyethylene glycol was added to the tubes. The tubes were centrifuged for 45 minutes at 2000Xg; the supernatants were discarded and the radioactive pellets counted in a  $\gamma$ -counter. For BNP RIA, the same procedure was carried out, except that iodinated BNP<sub>64-95</sub> (Peninsula Laboratories, Belmont, CA) and anti-rat antiserum raised against BNP<sub>64-95</sub> (Peninsula Laboratories, Belmont, CA) were used. Cross-reactivity of ANF and BNP antibodies with BNP and ANF peptides, respectively, was less than 0.01%. Intra-assay and inter-assay coefficients of variation for the BNP RIA were 9.8% and 6.5%, respectively. The sensitivity of the BNP RIA was 1.5 pg/tube.

#### RNA Extraction and Northern Analysis

Atria were kept at -80°C until needed. Total RNA was isolated by modifications of the acid guanidium isothiocyanate method of Chomczynski & Sacchi (1987), using 1 mL/100 mg tissue Ultraspec® RNA (Biotecx, Houston, TX) or Trizol® (Life Technologies, Burlington, ON). The final RNA pellet was resuspended in sterile diethylpyrocarbonate (DEPC)-treated water, and the RNA concentrations were measured by spectrophotometry at 260 and 280 nm. A 10 µg fraction of each RNA sample was denatured in 50% formamide, 7% formaldehyde, 1X MOPS buffer (10X MOPS buffer is 0.2 mol/L 3-[N-Morpholino]propane-sulphonic acid (MOPS), 100 mmol/L Na acetate pH 7.0, 20 mmol/L ethylenediaminetetraacetic acid (EDTA)), and 0.1% bromophenol blue at 65°C for 10 minutes, and was electrophoresed through a 1.5% agarose gel containing 0.22 mol/L formaldehyde and 1X MOPS buffer. Following electrophoresis, the RNA was transferred overnight by capillary action, using 10X standard saline citrate (SSC; 20X SSC is 3 mol/L NaCl, 0.3 mol/L Na<sub>3</sub>citrate) as transfer medium, to a positively charged nylon membrane (Hybond-N+.

Amersham, Oakville, ON). The RNA was cross-linked to the membrane by UV irradiation. The blot was pre-hybridized for 1 hour at 65°C in a hybridization solution containing 6X SSC, 5X Denhardt's solution (100X Denhardt's solution is 2% (w/v) BSA, 2% (w/v) Ficoll™, 2% (w/v) polyvinylpyrrolidone), 0.5% sodium dodecyl sulphate (SDS), 0.5 mol/L NaPO<sub>4</sub>, 200 µg/mL sheared salmon sperm DNA (ssDNA). Alternatively, this solution was supplemented with 50% formamide, in which case pre-hybridization and hybridization were carried out at 42°C. Fresh solution was used for overnight hybridization at 65°C or 42°C with the following probes: a 900 bp EcoRI/HindIII fragment containing the full-length rat ANF cDNA (Flynn *et al.*, 1985a), a 595 bp Sall fragment of the rat BNP cDNA (see "Cloning of the BNP and CNP cDNAs" below), a 1.1 kb PstI fragment of the mouse *v-fos* gene (Curran *et al.*, 1982), a 1.3 kb PstI fragment of the human *c-jun* cDNA (de Groot *et al.*, 1990), a 1.1 kb PstI fragment of the human *c-myc* gene (Marcu *et al.*, 1983), a 2 kb EcoRI fragment of mouse *Egr-1* (clone OC68) (Sukhatme *et al.*, 1988), a 2 kb BamHI/BglII fragment of the mouse phosphoglycerate kinase (PGK) cDNA (*pgk-1*) (Mori *et al.*, 1986), and a 5 kb EcoRI/Sall fragment of the mouse 28S rRNA cDNA probe. The *Egr-1* and 28S rRNA probes were a generous gift from Dr. Kenneth R. Chien (University of California at San Diego); the *pgk-1*, *v-fos*, *c-jun*, and *c-myc* probes were provided by Dr. P.H. Boer (University of Ottawa Heart Institute). The DNA probes were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP using a nonanucleotide random-priming kit (Megaprime, Amersham, Oakville, ON). Following hybridization, the blots were washed twice for 20 min in 2X SSC/1% SDS at 65°C and twice in 0.2X SSC/0.1% SDS at 65°C. Membranes were exposed to X-Ray film (X-AR, Eastman Kodak, Rochester, NY). Autoradiographs were quantitated by scanning laser densitometry, using an LKB Ultrosan XL (Pharmacia LKB, Uppsala, Sweden), and analyzed using the Gelscan XL2000 software package (Pharmacia LKB, Uppsala, Sweden). For each blot, probes were hybridized simultaneously with control probes; the 28S rRNA probe was used as control for the ANF cDNA, and the *pgk-1* probe as

control for all others. To eliminate variation due to reprobing, blots were probed simultaneously with the cDNAs for the genes of interest and the housekeeping controls. ANF mRNA signals were divided by the 28 rRNA signals, and the values of the signals of the other probes were divided by the *pgk-1* values. The different housekeeping probes were used because of their signal intensities relative to the genes of interest: the 28S rRNA signal is of comparative strength to that of ANF mRNA, while it would mask the signals of the BNP, *Egr-1*, and *c-myc* mRNAs following the longer exposure times required for these genes; similarly, the high signal strength of the ANF mRNA masks the *pgk-1* values.

### Cloning of the BNP and CNP cDNAs

Oligonucleotide primers corresponding to the -53 to -35 position (downstream primer) and 542 to 523 position (complementary strand upstream primer) nucleotides of the BNP cDNA sequence (Kojima *et al.*, 1989) were synthesized. The sequences were chosen to obtain matched oligonucleotide GC content for the pair. An additional 3 nucleotides were added to the sequence to complete *Sall* restriction sites at the ends of the primers, and an extra four nucleotides added to the ends of the primer for complete extension of the desired sequence during the PCR reactions.

The sequence of the primers is as follows (Italics indicate the *Sall* restriction site, underlined sequences indicate the nucleotides which correspond to the published BNP cDNA sequence):

- 1) Downstream primer (-53 to -35): GAGAGTCGACAAGAGAGAGCAGGACAC
- 2) Upstream primer (542 to 523): GAGAGTCGACACTGTGGCAAGTTTGTGC

Rat atria mRNA was isolated using the Fasttrack® Poly-A+ isolation kit (Invitrogen, San Diego, CA), and was used in a two-step Polymerase chain reaction using the above primers (at 1 µmol/L) in a PCR reaction mix (10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 0.01% (w/v) gelatin, 200 µmol/L dNTPs); the first step was a 15 minute, 50°C reverse transcription reaction (AMV reverse transcriptase, Bio/Can, Mississauga, ON) using the upstream primer to produce a single-stranded cDNA; the second step was 30 cycles of amplification (1.5 minutes at 94°C, 2 minutes at 50°C, 3 minutes at 72°C) using a thermostable Taq DNA polymerase (Bio/Can, Mississauga, ON). After the PCR reaction, 10% of the reaction mix was electrophoresed through a 1% agarose gel to determine if the proper size fragment was obtained. The expected fragment size of 595 bp was obtained, and 10% of the remaining PCR product was cloned without purification in a pUC-based vector using the TA Cloning kit (Invitrogen, San Diego, CA), and propagated in E.Coli. The cDNA thus obtained was submitted to analytical restriction digests with BamH1, HaeIII, HpaII, and to confirm its identity. This BNP cDNA hybridized to a single band of the appropriate size and intensity when used to probe a Northern blot (see "Results"), and hybridized specifically to a PCR fragment amplified using different BNP-specific primers.

To clone the CNP cDNA, the following primers were designed using Oligo™ software (National Biosciences, Plymouth, MN) (Rychlik and Rhoads, 1989; Rychlik *et al.*, 1990) (underlined sequence corresponds to the CNP cDNA sequence (Kojima *et al.*, 1990)):

- 1) Downstream primer (109-129): CUACUACUACUATTCGGCACCATGCACCTCTCC
- 2) Upstream primer (649-664): CAUCAUCAUCAUTCCCCTCCCCTCCCCAAATA

Brain total RNA (50 ng) was reverse transcribed using the Superscript II reverse transcriptase (RT) kit (Life Technologies, Burlington, ON), under the conditions

recommended by the manufacturer. The primers were used in a PCR reaction consisting of 35 cycles of amplification of brain cDNA; cycling parameters were set for 60 seconds denaturation (95°C), 45 seconds annealing (62°C), 90 seconds extension (72°C), using Amplitaq® DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The reaction conditions were as follows: 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3 at 24°C), 1.5 mmol/L MgSO<sub>4</sub>, 0.1% gelatin, 0.2 µmol/L dNTPs, 0.2 µmol/L each primer, and 1.25 units of enzyme, in a total volume of 50 µL. The amplification of a single 575 bp fragment (nucleotides 109 to 684 of the CNP cDNA (Kojima *et al.*, 1990)) was confirmed by electrophoresing 5 µL of the reaction through a 2% agarose gel. The amplified fragment was then cloned using the ligase-independent CloneAmp system (Life Technologies, Burlington, ON) (Rashtchian *et al.*, 1992). Briefly, 2 µL of the PCR reaction was incubated for 30 min at 37°C in annealing buffer (20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>) with the pUC-derived pAMP1 vector (50 ng), and 1 unit of uracil DNA glycosylase. Two microliters of this reaction were used to transform DH5α competent cells (Life Technologies, Burlington, ON). The identity of the clone was confirmed by digestion with EcoRI, Aval, PvuII, and EcoRI/BamHI. A 400 bp PvuII fragment was isolated from an agarose gel using the Quiaex DNA isolation procedure (Quiagen, Chatsworth, CA), labelled with [ $\alpha$ -<sup>32</sup>P]dCTP as described in "RNA Extraction and Northern Analysis", and used to probe a northern blot of brain, liver, and atria total RNA; a single band of the expected size (1.1 kb) was obtained only in the lanes containing brain RNA, thus further confirming the identity of the CNP clone.

### Statistical Analysis

All data are reported as mean±SEM. Unpaired Student's *t*-test was used to determine statistical significance between pairs. Analysis of variance (ANOVA) was

performed to determine statistical differences among multiple groups. When significance was obtained by ANOVA, Fisher's least squares difference post-hoc analysis was used to determine pairwise differences. Significance was accepted at  $P < 0.05$ .

## RESULTS

### Isolated whole atria

Isolated atria showed steady beating rates and developed tension under basal load, throughout the 4 hour perfusion period (Fig. 4). The initial 5 g stretch stabilized to approximately 2 g after 5 minutes ( $n=6$ ,  $P<0.001$  vs basal and initial 5 g resting tension). Following stretch, the beating rate increased slightly, and the developed tension rose from  $26\pm7$  mg to  $180\pm24$  mg ( $n=6$ ,  $P<0.001$  vs. 0 minutes and vehicle) (Fig. 4). Basal immunoreactive (ir) ANF levels in the perfusate were stable throughout the experimental period (Fig. 5); imposition of the 5 g stretch caused irANF output to increase from  $406\pm11$  pg/mL/min to  $743\pm28$  pg/mL/min ( $n=12$ ,  $P<0.001$  vs. 0 minutes and vehicle) (Fig. 5). irANF levels returned to basal levels after 100 min of continuous stretch (Fig. 5). irBNP levels released from the perfused atria remained constant during the stretch period, but showed a tendency to increase after 15 min of stretch, when compared to non-stretched controls (Fig. 6).

Addition of ET-1 ( $10^{-7}$  mol/L) caused developed tension to increase over one hour from  $43\pm12$  to  $194\pm31$  mg ( $n=6$ ,  $P<0.001$  vs. 0 minutes and vehicle); this decreased after 90 minutes of stimulation, returning to near basal levels (Fig. 7). Beating rate increased from  $117.5\pm4.8$  bpm to  $145.0\pm5.0$  bpm ( $n=4$ ,  $P<0.05$  vs. 0 minutes and vehicle). Resting tension rose from the basal  $200\pm0$  mg load to  $545\pm103$  mg ( $n=4$ ,  $P<0.001$  vs. 0 minutes and vehicle) and remained at this level throughout the stimulation period (Fig. 7). IrANF release from the non-stimulated atria remained steady, and addition of ET-1 caused a gradual increase from  $172\pm25$  pg/mL/min to  $395\pm45$  pg/mL/min ( $n=4$ ,  $P<0.001$  vs. 0 minutes and vehicle) over one

hour of stimulation (Fig. 8). The stimulated ANF levels remained high for another 30 minutes, and gradually returned towards basal levels after 3.5 hours of continuous stimulation. IrBNP levels also increased with ET-1 stimulation (Fig. 8) from a basal level of  $138 \pm 24$  fg/mL min to  $417 \pm 88$  fg/mL min ( $n=4$ ,  $P<0.05$  vs. 0 minutes and vehicle) after 30 minutes of stimulation. These levels remained significantly elevated for 90 minutes, and returned towards basal levels thereafter.

A representative northern blot of the genes under study is shown in Fig. 9. The blots shown are from total RNA isolated from whole atria that were left unstimulated for 2 hours following the equilibration period, or were stimulated for two hours with either stretch or ET-1. Densitometric analysis of the northern blots at time points of 30 minutes, 2 hours, and 4 hours, showed that with stretch, ANF and BNP mRNA levels remained constant (Fig. 10). Two hours of continuous stimulation by ET-1 caused a  $33 \pm 7\%$  increase ( $n=5$ ,  $P<0.05$ ) in BNP transcript levels, compared to non-stimulated time controls; this increase was temporary, returning to basal levels after 4 hours (Fig. 10). ANF mRNA levels remained constant after stimulation by ET-1 ( $10^{-7}$  mol/L), although a tendency to increase was observed after 4 hours of stimulation (Fig. 10). Relative levels of early response gene expression are shown in Figure 11. Two hours of stretch resulted in a  $55 \pm 16\%$  increase in *c-fos* mRNA levels ( $n=4$ ,  $P<0.025$ ), and a  $70 \pm 5\%$  increase in *Egr-1* mRNA levels ( $n=4$ ,  $P<0.01$ ), compared to control levels. *C-myc* mRNA levels were elevated  $69 \pm 28\%$  ( $n=6$ ,  $P<0.05$ ) over control levels after 4 hours of stretch. A  $201 \pm 14\%$  increase ( $n=5$ ,  $P<0.001$ ) in *Egr-1* expression was observed in atria that had been stimulated for 2 hours with ET-1, when compared to their respective non-stimulated controls; at 4 hours of ET-1 stimulation, *Egr-1* mRNA levels had further increased to  $767 \pm 30\%$  of basal levels ( $n=4$ ,  $P<0.001$ ). Stimulation by ET-1 also led to increases in *c-fos* and *c-myc* mRNA levels after 2 hours, and an increase in *c-jun* mRNA

levels after 4 hours; these changes were not statistically significant.

**Figure 4: Mechanical variables of isolated atria, under basal or stretch conditions. Triangles indicate non-stimulated atria (n=6), circles represent stretched tissues (n=6). Stretch was applied at time 0 as indicated by the arrows. \* $P < 0.05$ , \*\* $P < 0.01$  vs 0 minutes (by ANOVA).**

Fig. 4

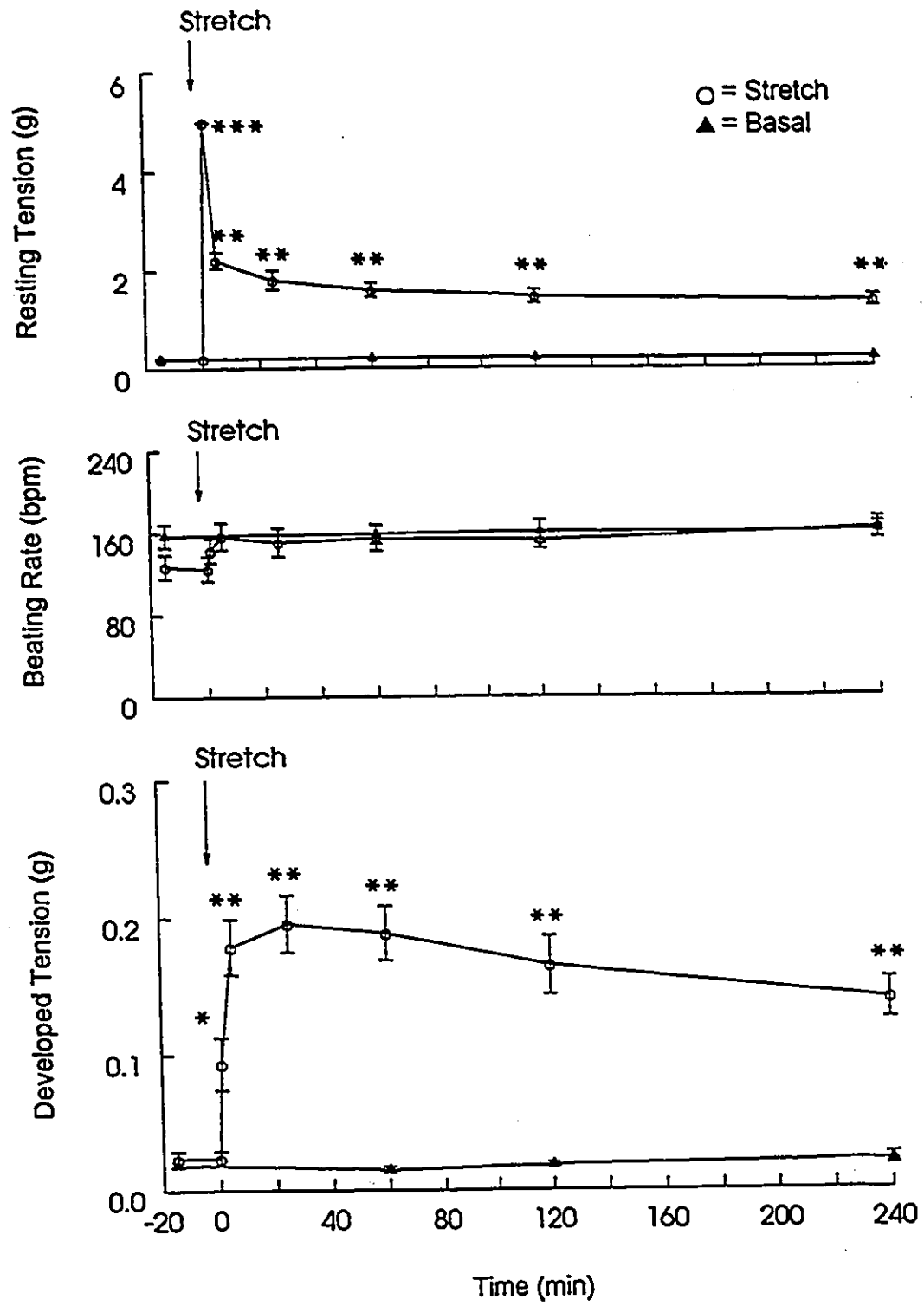


Figure 5: Basal and stretch-induced ANF release from isolated whole atria. Black bars represent ANF release from non-stimulated atria (n=10), while white bars represent stretched tissues (n=12). Stretch (5g) was applied at time 0, as indicated by the arrows. \* $P < 0.05$  vs 0 minutes (by ANOVA) and corresponding basal (by  $t$ -test), \*\* $P < 0.01$  vs. all time points (by ANOVA) and corresponding basal (by  $t$ -test), † $P < 0.01$  vs. corresponding basal (by  $t$ -test).

Fig. 5

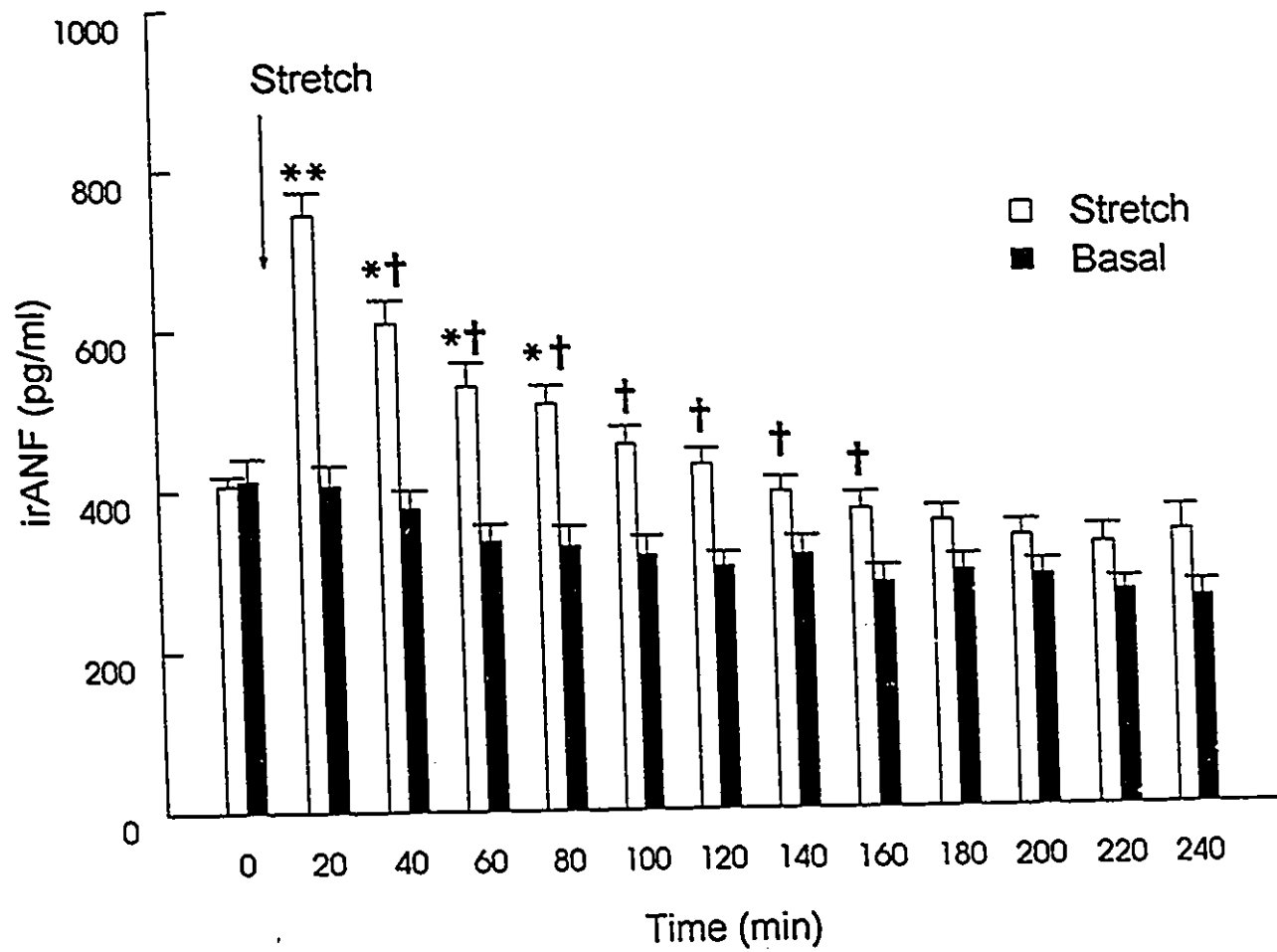


**Figure 6:** Basal and stretch-induced irBNP release from isolated whole atria. Black bars represent irBNP release from non-stimulated atria (n=4), while white bars represent stretched tissues (n=4). Stretch (5g) was applied at time 0, as indicated by the arrows. Periods of 15 minutes were pooled and extracted before determination of irBNP levels by RIA.

Fig. 6

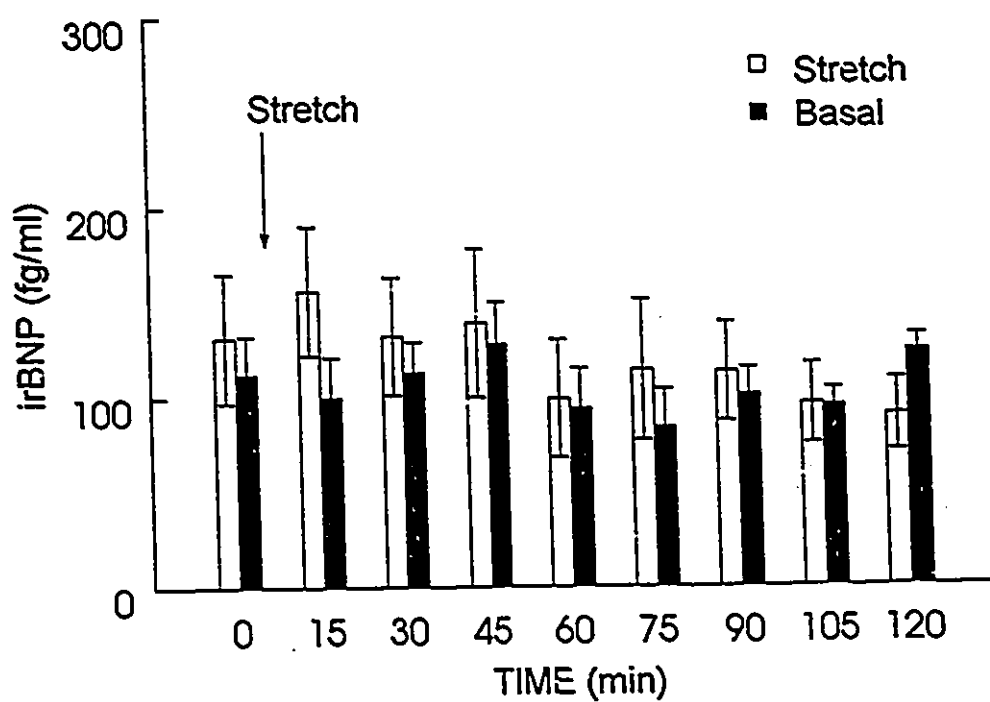


Figure 7: Mechanical variables of isolated atria, under basal or endothelin-1-stimulated conditions. Triangles indicate non-stimulated atria (n=6), circles represent tissues stimulated with  $10^{-7}$  mol/L endothelin-1 (n=6). Endothelin-1 was applied at time 0 as indicated by the arrows. \* $P<0.05$ , \*\* $P<0.01$  vs 0 minutes (by ANOVA).

Fig. 7

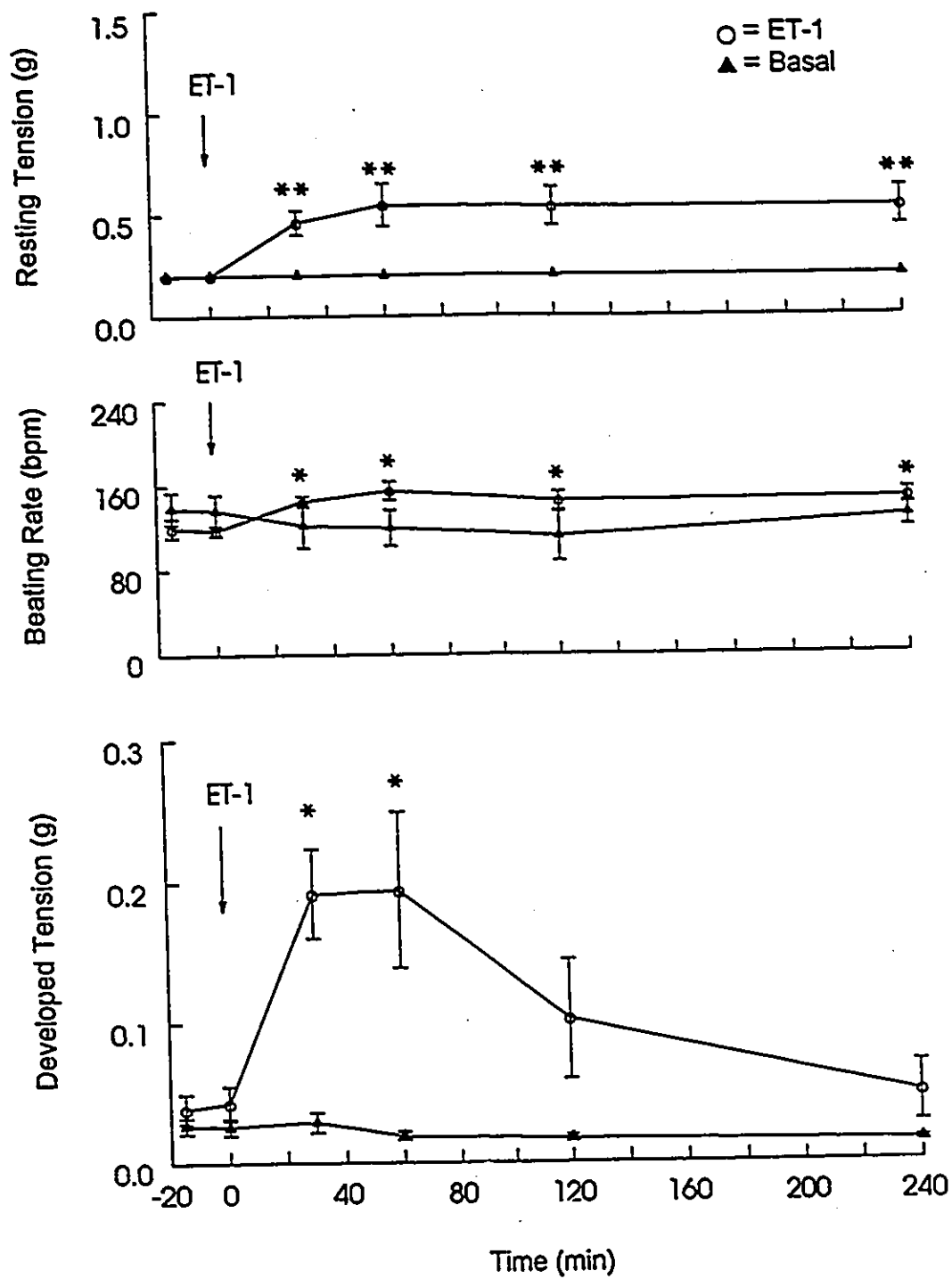


**Figure 8:** Basal and endothelin-1-stimulated irANF and irBNP release from isolated whole atria. Black bars represent ANF release from non-stimulated atria (n=4), while white bars represent stretched tissues (n=4). Endothelin-1 ( $10^{-7}$  mol/L) was applied at time 0, as indicated by the arrows. n.d.= not determined. \* $P<0.05$ , \*\* $P<0.01$  vs 0 minutes (by ANOVA) and corresponding basal (by *t*-test).

Fig. 8

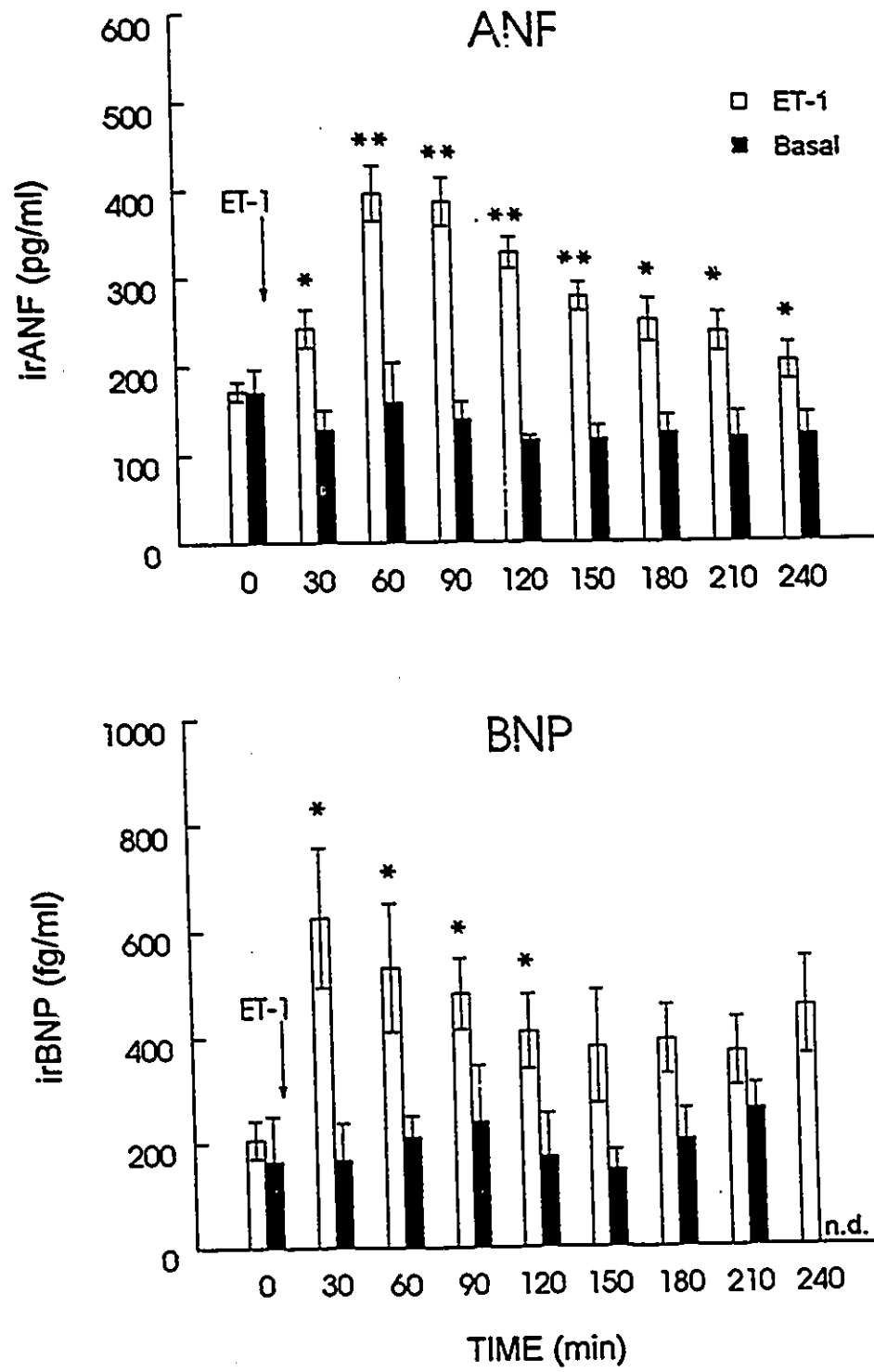


**Figure 9:** Northern blot analysis of RNA extracted from isolated whole atria, probed with 28S rRNA (28S), ANF, BNP, *Egr-1*, *c-myc*, and *pgk-1* probes. The 28S and PGK signals are housekeeping controls. The northern blots presented are a representative of the results obtained from atria perfused for 2 hours under basal conditions (Basal), or stimulated by stretch (Stretch) or endothelin-1 (ET-1). The individual signals in each group are derived from Northern blots performed with RNA obtained from the same tissue sample. Exposure times were 15 minutes at 22°C (ANF and 28S rRNA), 6 h at -80°C (BNP and *Egr-1*), 40 h at -80°C (PGK), and 48 h at -80°C (*c-myc*), and were identical for all four groups.

Fig. 9

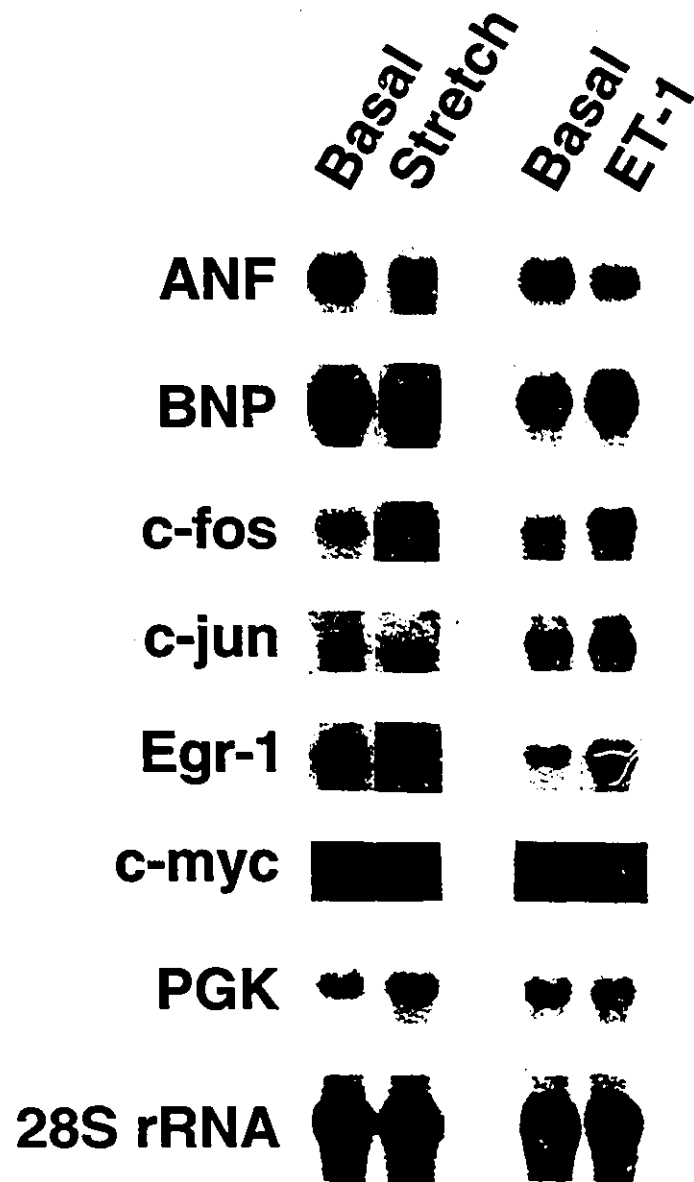


Figure 10: Densitometric quantitation of Northern blots performed with ANF and BNP cDNA probes, using 28S rRNA and *pgk-1* cDNA probes as controls. Data are expressed as the mean $\pm$ SEM of the percentage of change from values obtained from corresponding unstimulated time controls. \* $P<0.05$  vs. non-stimulated basal controls (by *t*-test).

Fig. 10

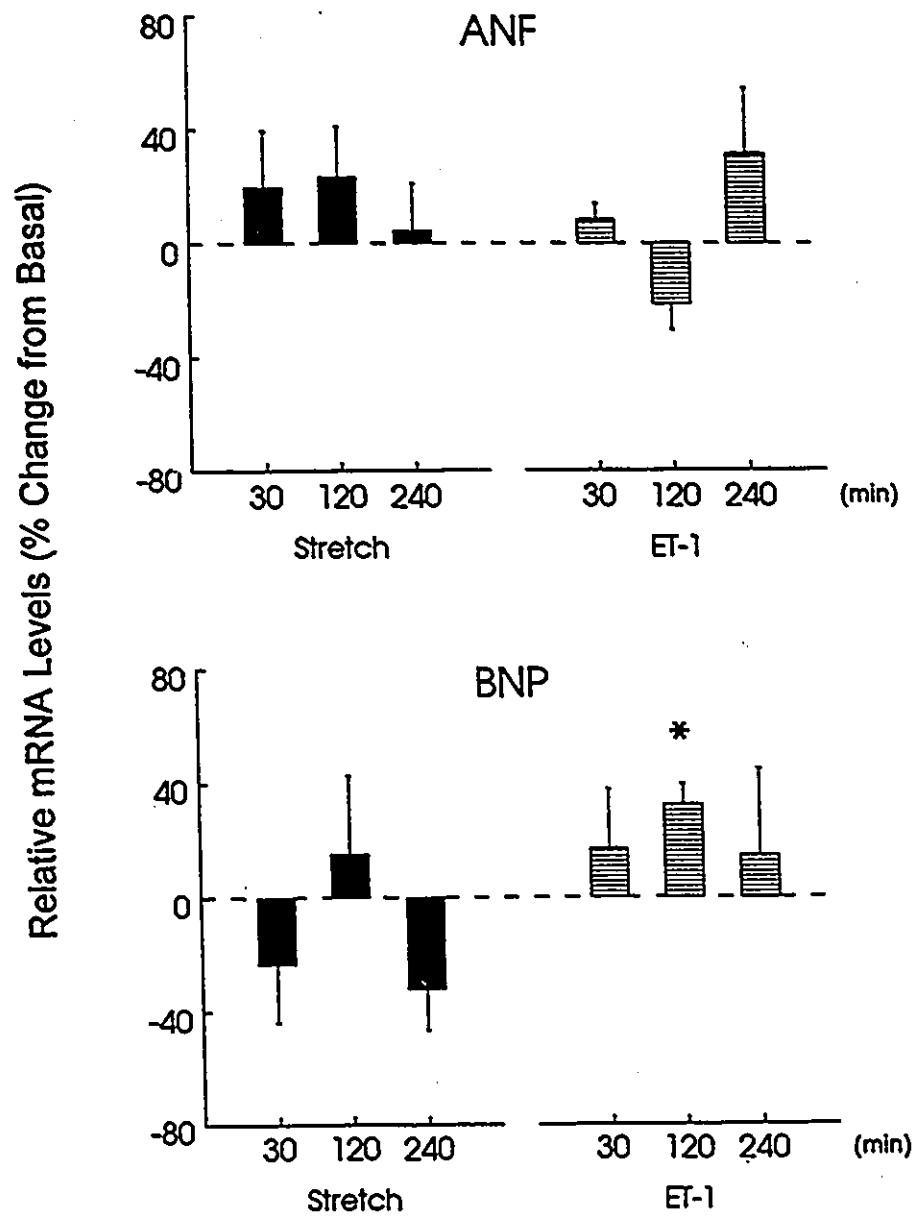
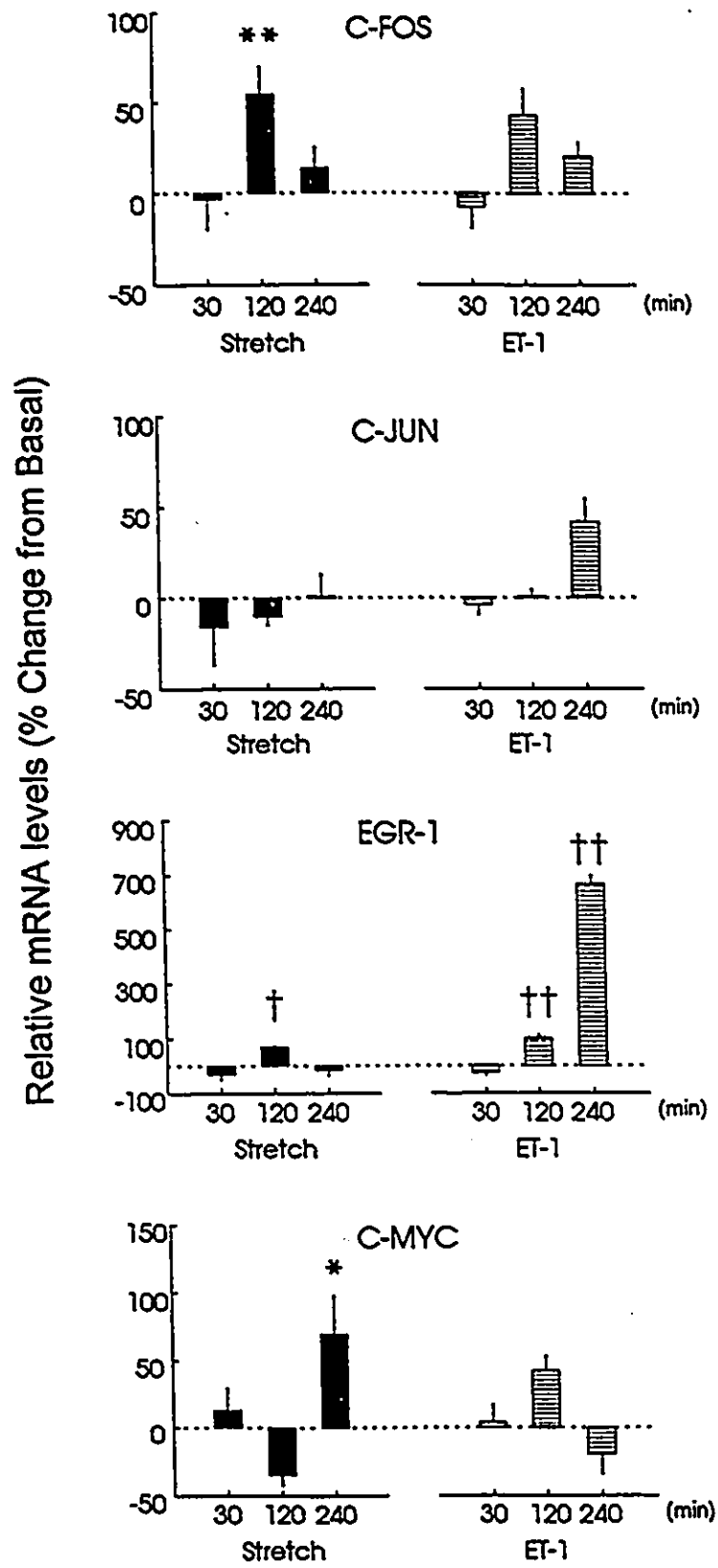


Figure 11: Densitometric quantitation of Northern blots performed with probes for *c-fos*, *c-jun*, *Egr-1*, and *c-myc*, using the *pgk-1* cDNA probe as a control. Data are expressed as the mean $\pm$ SEM of the percentage of change from values obtained from corresponding unstimulated time controls. \* $P$ <0.05, \*\* $P$ <0.025, † $P$ <0.01, †† $P$ <0.001 vs. non-stimulated basal controls (by *t*-test). Note that the scales for the changes in *Egr-1* and *c-myc* mRNA levels are different from those of *c-fos* and *c-jun*.

Fig. 11



## Isolated right atria

The mechanical activity of the isolated atria under basal or stretch-stimulated conditions is shown in figure 12. To stretch isolated right atria, an increase from 0.5 mmHg intraatrial pressure to 8 mmHg was used. The increase in intraatrial pressure decreased rapidly, reaching a constant level of  $4.80 \pm 0.58$  mmHg after 2 hours; the decrease was due to increased compliance of the atria following stretch, as is seen for the whole atria. The increase in intraatrial pressure resulted in an increase in heart rate from  $158 \pm 6$  min<sup>-1</sup> to  $190 \pm 5$  min<sup>-1</sup> ( $n=5$ ,  $P<0.001$ ); beating rates remained significantly elevated during the 8 hour stretch period. Basal levels of irANF release remained steady throughout the 8 hour period of perfusion (Fig. 13). Secretion of irANF was stimulated from a basal level of  $399 \pm 44$  pg/mL to a maximum of  $1135 \pm 85$  pg/mL after 15 minutes (Fig. 13a;  $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle). Stretch-stimulated irANF secretion thereafter declined, reaching basal levels after 3 hours of stretch.

IrBNP secretion was also stimulated by stretch in this model (Fig. 13b). Imposition of the load increased irBNP secretion from a basal level of  $2.00 \pm 0.13$  pg/mL to a peak of  $4.10 \pm 0.04$  pg/mL after 15 minutes ( $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle). This increase was also temporary, returning to basal levels after 2 h of stretch. Calculation of the ratios of irANF to irBNP secreted in response to stretch showed that this ratio was significantly increased in the first 15 minutes of stretch (Fig. 16), indicating that the two peptides are not initially co-secreted in response to stretch.

Addition of ET-1 ( $10^{-8}$  mol/L) caused an increase in intra-atrial pressure from  $0.48 \pm 0.004$  mmHg to  $1.38 \pm 0.17$  mmHg after 15 minutes ( $n=5$ ,  $P<0.001$ ), which gradually

decreased towards basal levels throughout the period of stimulation (Fig. 14). ET-1 increased the beating rate from  $150 \pm 5 \text{ min}^{-1}$  to  $176 \pm 13 \text{ min}^{-1}$ , although this was not statistically significant (Fig. 14). IrANF release was stimulated by ET-1 after 15 minutes of stimulation from a basal level of  $289 \pm 35 \text{ pg/mL}$ , and peaked after 60 minutes at  $976 \pm 120 \text{ pg/mL}$  (Fig 15a;  $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle); the stimulated ANF secretion slowly declined, reaching basal levels after 5 hours of stimulation. IrBNP secretion was also stimulated by ET-1 after 60 minutes from its basal level of  $1.94 \pm 0.27 \text{ pg/mL}$ , peaking at  $3.95 \pm 0.93 \text{ pg/mL}$  after 90 minutes (Fig. 15b;  $n=5$ ,  $P<0.05$  vs. 0 minutes and  $P<0.01$  vs. vehicle). IrBNP secretion thereafter decreased towards basal levels. Calculation of the ratios of ET-1-stimulated irANF to irBNP secretion showed that they were co-secreted (Fig. 16)

Representative Northern blots are shown in Figure 17 for basal, stretch-stimulated, and ET-1-stimulated gene expression at the 6 hour time point. Densitometric quantitation of the blots is shown in Figure 18. ANF mRNA levels were not significantly affected by stretch or ET-1. Stretch caused a  $63 \pm 15\%$  increase in BNP mRNA levels after 1.5 hours of stimulation ( $n=5$ ,  $P<0.01$ ). This increased further, peaking at a  $133 \pm 20\%$  increase after 6 hours ( $n=5$ ,  $P<0.001$ ), and remained significantly elevated for the remainder of the period of stimulation ( $+105 \pm 12\%$  after 8 hours;  $n=5$ ,  $P<0.01$ ). *Egr-1* mRNA levels were increased by  $92 \pm 27\%$  following 4 hours of stretch ( $n=5$ ,  $P<0.05$ ); the increase was no longer significant after 6 or 8 hours of stretch. Stretch also resulted in a  $80 \pm 25\%$  increase in *c-myc* mRNA levels after 1.5 hours ( $n=5$ ,  $P<0.05$ ), which remained significantly elevated after 4 hours ( $+82 \pm 34\%$ ;  $n=5$ ,  $P<0.05$ ). BNP mRNA levels were increased by  $10^{-8} \text{ M}$  ET-1 after 4 hours of stimulation ( $+83 \pm 20\%$ ,  $n=5$ ,  $P<0.001$ ). The increase peaked at  $+120 \pm 56\%$  after 6 hours ( $n=5$ ,  $P<0.05$ ), and decreased thereafter, although it was still significantly higher than controls after 8 hours ( $+49 \pm 11\%$ ,  $n=5$ ,  $P<0.05$ ). ET-1 also stimulated *Egr-1* mRNA levels after

4 hours ( $+134\pm33\%$ ,  $n=5$ ,  $P<0.001$ ); this increase was temporary. *C-myc* mRNA levels were stimulated by ET-1, gradually increasing over the 8 hour period, and reaching a significant increase of  $+93\pm18$  vs controls after 8 hours ( $n=5$ ,  $P<0.05$ ).

Perfused right atria were stimulated with phenylephrine at doses of 10  $\mu\text{mol/L}$ , 50  $\mu\text{mol/L}$ , or 100  $\mu\text{mol/L}$ . Phenylephrine at all doses caused a significant sustained increase in intra-atrial pressure and heart rate (Fig. 19). Beating rate increased from  $145\pm18$  to  $208\pm15 \text{ min}^{-1}$  at 2 hours with 10  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle), from  $134\pm11$  to  $223\pm14 \text{ min}^{-1}$  at 2 hours with 50  $\mu\text{mol/L}$  phenylephrine ( $n=6$ ,  $P<0.001$  vs. 0 minutes and vehicle), and from  $149\pm4$  to  $213\pm15 \text{ min}^{-1}$  at 1.5 hours with 100  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle). Intra-atrial pressure increased from  $0.56\pm0.02$  to  $1.22\pm0.27 \text{ mmHg}$  at 1 hour with 10  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle), from  $0.51\pm0.04$  to  $1.57\pm0.14 \text{ mmHg}$  at 15 minutes with 50  $\mu\text{mol/L}$  phenylephrine ( $n=6$ ,  $P<0.001$  vs. 0 minutes and vehicle), and from  $0.52\pm0.05$  to  $1.28\pm0.25 \text{ mmHg}$  at 1 hour with 100  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.001$  vs. 0 minutes and vehicle). Prazosin (10  $\mu\text{mol/L}$ ) added at the same time as 50  $\mu\text{mol/L}$  phenylephrine for 6 hours inhibited the increase in developed tension (Fig. 19;  $P<0.05$  vs. 50  $\mu\text{mol/L}$  phenylephrine,  $P>0.10$  vs. vehicle).

Immunoreactive (ir) ANF secretion was stimulated by all three doses of phenylephrine tested (Fig. 20a). IrANF secretion was increased from  $316\pm64$  to  $522\pm53 \text{ pg/mL}$  at 1.5 hours with 10  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.01$  vs. vehicle), from  $266\pm25$  to  $702\pm108 \text{ pg/mL}$  at 1.5 hours with 50  $\mu\text{mol/L}$  phenylephrine ( $n=6$ ,  $P<0.001$  vs. 0 minutes and vs. vehicle), and from  $317\pm34$  to  $854\pm134 \text{ pg/mL}$  at 1.5 hours with 100  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.001$  vs. 0 minutes and vs. vehicle). Phenylephrine also stimulated irBNP secretion

(Fig. 20b). This was increased from  $1.97 \pm 0.10$  to  $3.59 \pm 0.52$  pg/mL at 1.5 hours with 50  $\mu\text{mol/L}$  phenylephrine ( $n=6$ ,  $P<0.01$  vs. 0 minutes and  $P<0.001$  vs. vehicle), and from  $2.24 \pm 0.15$  to  $3.86 \pm 0.55$  pg/mL at 1 hour with 100  $\mu\text{mol/L}$  phenylephrine ( $n=5$ ,  $P<0.015$  vs. 0 minutes and  $P<0.001$  vs. vehicle). IrANF secretion remained significantly elevated during the 8 hour period with 100  $\mu\text{mol/L}$  phenylephrine, and reached basal levels after 5 and 6 hours of stimulation with 10 and 50  $\mu\text{mol/L}$  phenylephrine, respectively. Phenylephrine-stimulated irBNP secretion remained above basal levels throughout the period of stimulation. Prazosin added at the same time as 50  $\mu\text{mol/L}$  phenylephrine for 6 h prevented the increase in irANF and irBNP secretion (Fig. 20;  $P<0.05$  vs. 50  $\mu\text{mol/L}$  phenylephrine,  $P>0.10$  vs. vehicle). When total secretion during the period of stimulation is calculated (Table 1), 10  $\mu\text{mol/L}$  phenylephrine significantly stimulated total irANF secretion, and the two higher doses of phenylephrine significantly stimulated total irANF and irBNP secretion.

Determination of the ratios of irANF to irBNP secreted in response to phenylephrine (Fig. 21) indicated that BNP was preferentially released in the first 15 minutes after phenylephrine addition, as exhibited by the tendency of irANF/irBNP ratio to decrease. Thereafter, the irANF/irBNP ratio significantly increased over time, indicating that there was a proportionally greater amount of irANF released compared to irBNP during this time. After 2.5 hours, the ratio of irANF to irBNP significantly decreased, reflecting the maintained increase in BNP secretion and the decay of the ANF response. The ratio of irANF to irBNP under basal conditions remained constant throughout the period examined (not shown).

A representative Northern blot of the genes under study at the 6 hour time point is shown in Fig. 22. Densitometric analysis of the Northern blots is presented in Fig. 23. No significant changes in mRNA levels were observed for any of the genes studied under basal

conditions (data not shown). Stimulation with 50  $\mu\text{mol/L}$  phenylephrine for 6 hours led to a  $49 \pm 13\%$  increase in relative ANF mRNA levels ( $n=5$ ,  $P<0.001$ ) and a  $135 \pm 18\%$  increase in relative BNP mRNA levels ( $n=5$ ,  $P<0.001$ ). After 8 hours a  $77 \pm 19\%$  increase in relative BNP mRNA levels was measured ( $n=7$ ,  $P<0.01$ ). Relative *Egr-1* mRNA levels were increased by  $81 \pm 26\%$  after 4 hours ( $n=5$ ,  $P<0.01$ ),  $167 \pm 26\%$  after 6 hours ( $n=5$ ,  $P<0.001$ ), and  $40 \pm 11\%$  after 8 hours ( $n=7$ ,  $P<0.05$ ). Increases in relative *c-myc* mRNA levels were observed after 4 hours ( $+49 \pm 14\%$ ,  $n=5$ ,  $P<0.01$ ), and after 6 hours ( $+53 \pm 7\%$ ,  $n=5$ ,  $P<0.001$ ). Prazosin added at the same time as 50  $\mu\text{mol/L}$  phenylephrine for 6 hours abolished any changes in ANF, BNP, *Egr-1*, and *c-myc* gene expression (Fig. 22 and data not shown,  $n=5$ , all  $P<0.05$  vs. 50  $\mu\text{mol/L}$  phenylephrine and  $P>0.10$  vs. vehicle).

Figure 12: Effect of stretch on atrial contraction rate (A) and intra-atrial pressure (B) of isolated right atria. HR=heart rate, IAP=intra-atrial pressure. Atria were left at the basal 0.5 mmHg intra-atrial pressure (open circles, n=5), or were distended by increasing the intra-atrial pressure to 8 mmHg (closed circles, n=5) at time 0, as indicated by the arrow.  $\pm P < 0.001$  vs. time 0 (by ANOVA).

Fig. 12

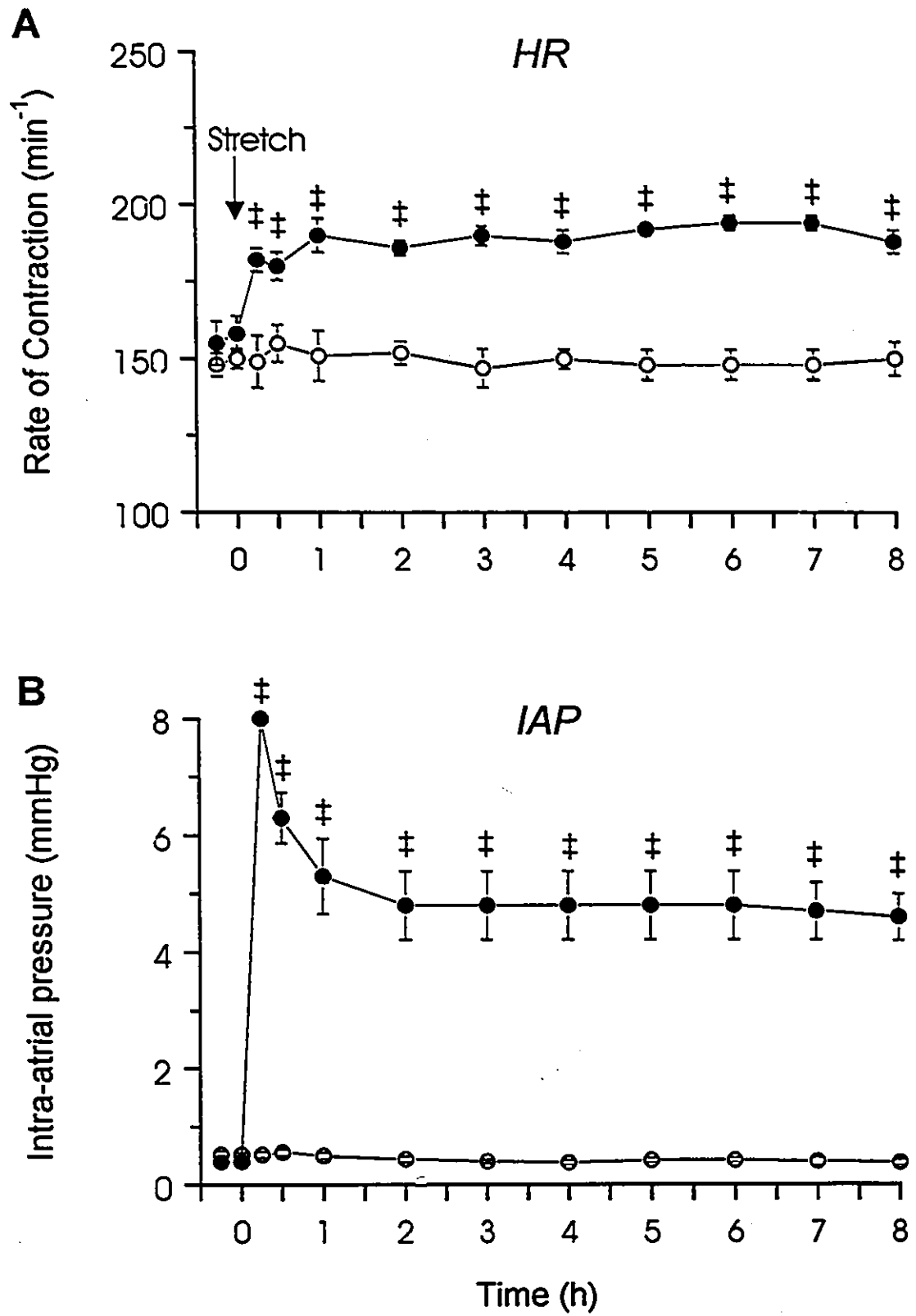


**Figure 13:** Effect of stretch on irANF (A) and irBNP (B) secretion from isolated right atria. Atria were left at the basal 0.5 mmHg intra-atrial pressure (open circles, n=5), or were distended by increasing the intra-atrial pressure to 8 mmHg (closed circles, n=5) at time 0, as indicated by the arrow. \* $p < 0.05$ , † $p < 0.01$ , ‡ $P < 0.001$  vs. time 0 (by ANOVA) and vs. basal (by *t*-test); § $P < 0.05$  vs. basal (by *t*-test).

Fig. 13

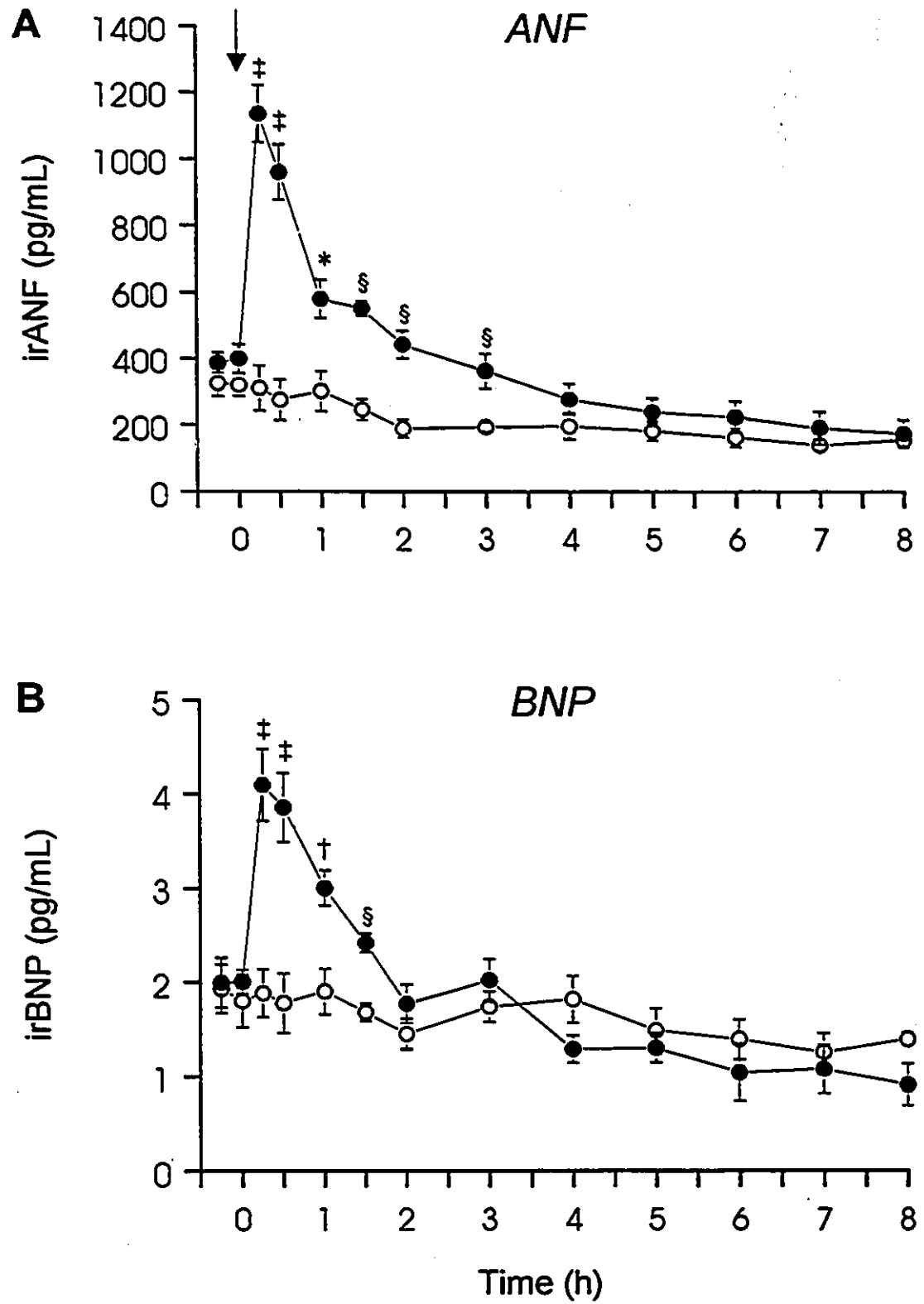


**Figure 14:** Effect of endothelin-1 on atrial contraction rate (A) and intra-atrial pressure (B) of isolated right atria. HR=heart rate; IAP=intra-atrial pressure. Atria were left at the basal 0.5 mmHg intra-atrial pressure and perfused with vehicle (open circles, n=5), or were stimulated by adding  $10^{-8}$  mol/L endothelin-1 (closed circles, n=5) at time 0, as indicated by the arrow. \* $p < 0.05$ , † $p < 0.01$ , ‡ $p < 0.001$  vs. time 0 (by ANOVA); § $p < 0.05$  vs. vehicle (by *t*-test).

Fig. 14

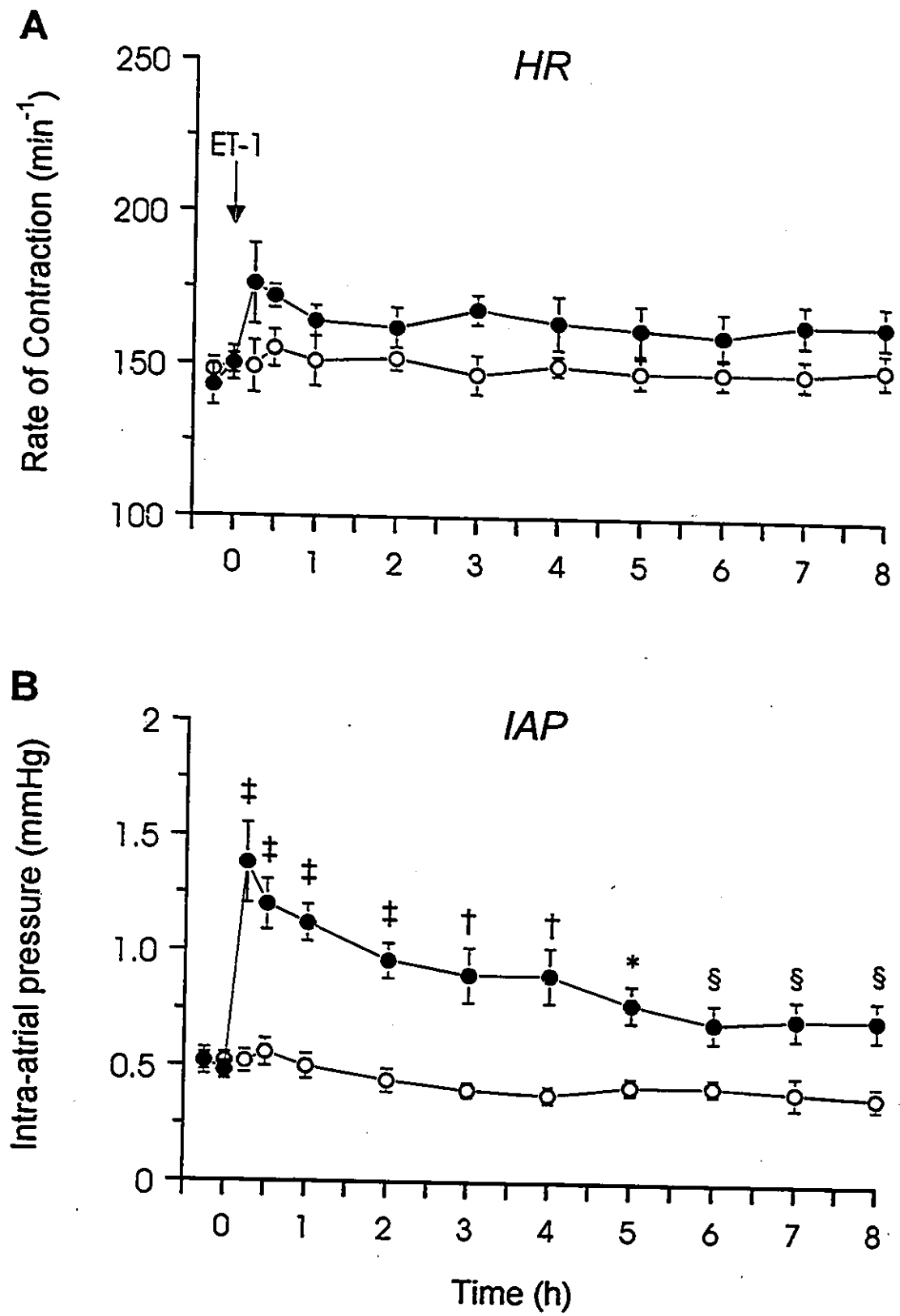


Figure 15: Effect of endothelin-1 on irANF (A) and irBNP (B) secretion from isolated right atria. Atria were left at the basal 0.5 mmHg intra-atrial pressure and perfused with vehicle (open circles, n=5), or were stimulated by adding  $10^{-8}$  mol/L endothelin-1 (closed circles, n=5) at time 0, as indicated by the arrow. \* $p < 0.05$ , † $p < 0.01$ , ‡ $P < 0.001$  vs. time 0 (by ANOVA) and vs vehicle (by *t*-test); § $P < 0.05$  vs. vehicle (by *t*-test).

Fig. 15

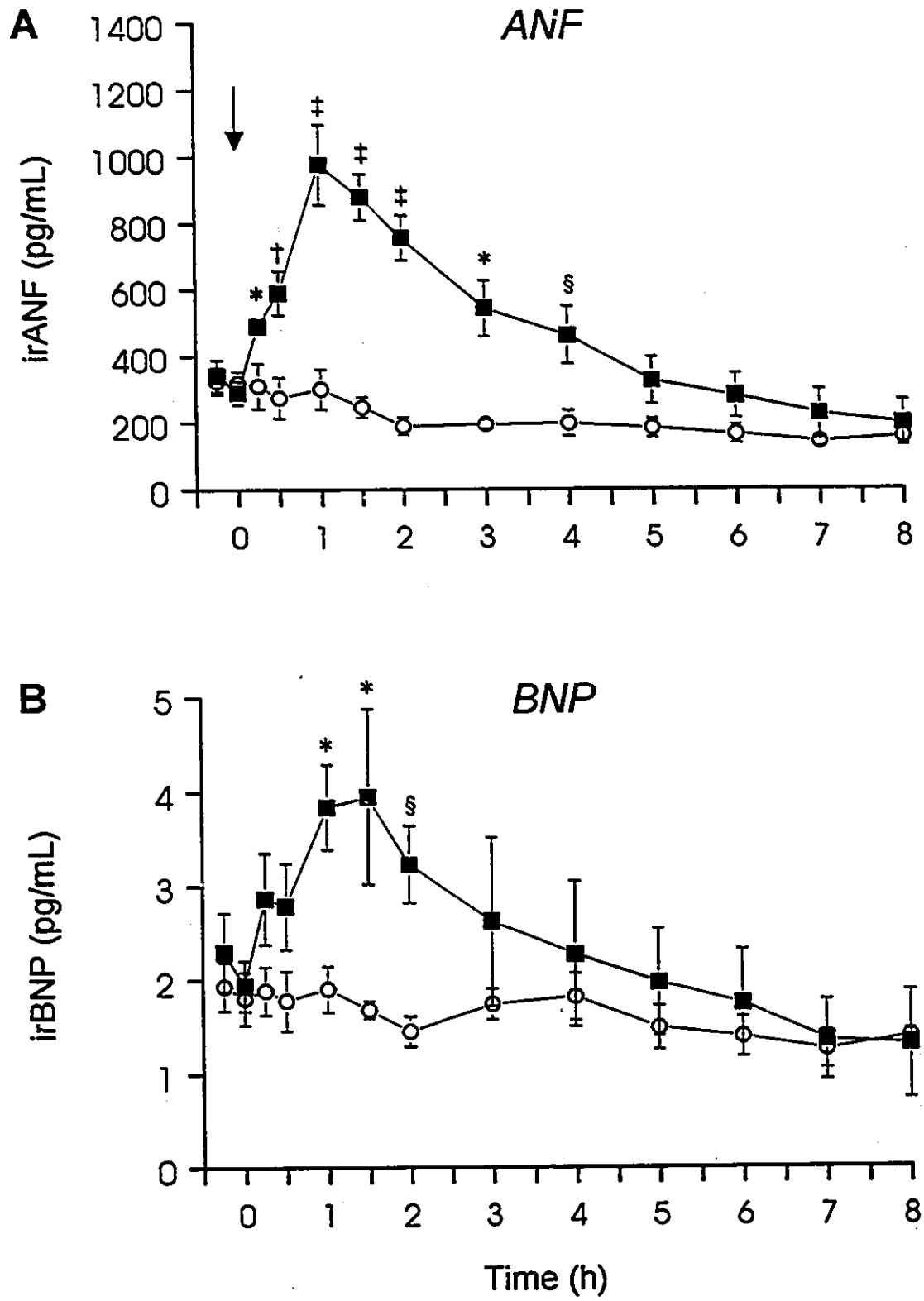


Figure 16: Ratios of irANF to irBNP secreted in response to (A) stretch or (B) endothelin-1 ( $10^{-8}$  mol/L, n=5). Stretch or endothelin-1 were applied at time 0, as indicated by the arrow. \* $P < 0.05$  vs. time 0 (by ANOVA).

Fig. 16

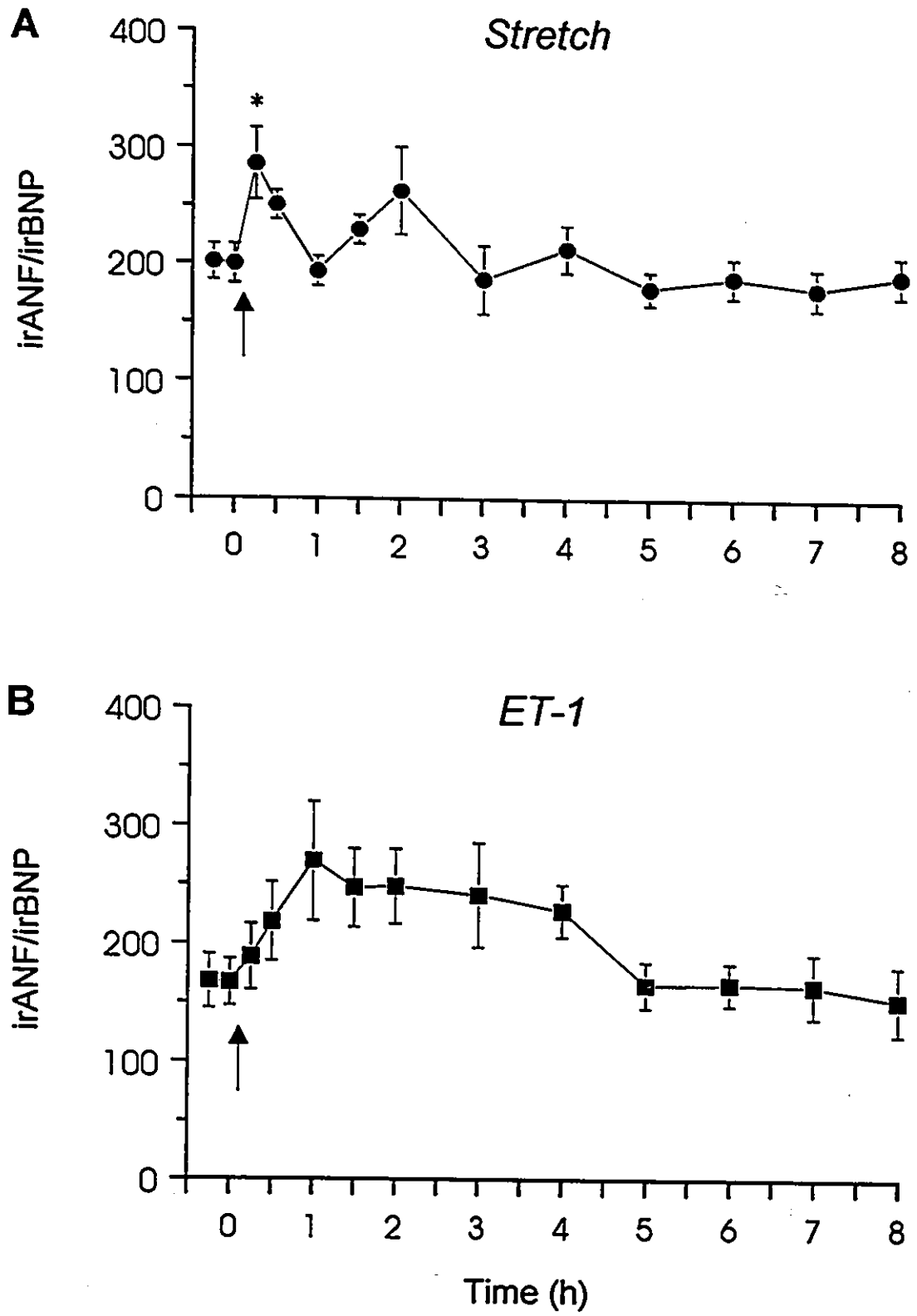


**Figure 17:** Northern blot analysis of RNA extracted from perfused right atria, probed with 28S rRNA (28S), ANF, BNP, *Egr-1*, *c-myc*, and PGK probes. The 28S and PGK signals are housekeeping controls. The northern blots presented are a representative of the results obtained from atria perfused for 6 hours at 0.5 mmHg with vehicle (Basal), or were stimulated by stretch (Stretch, 8 mmHg) or  $10^{-8}$  mol/L endothelin-1 (ET-1). The individual signals in each group are derived from Northern blots performed with RNA obtained from the same tissue sample. Exposure times were 15 minutes at 22°C (ANF and 28S rRNA), 6 hours at -80°C (BNP and *Egr-1*), 40 hours at -80°C (PGK), and 48 hours at -80°C (*c-myc*), and were identical for all three groups.

Fig. 17

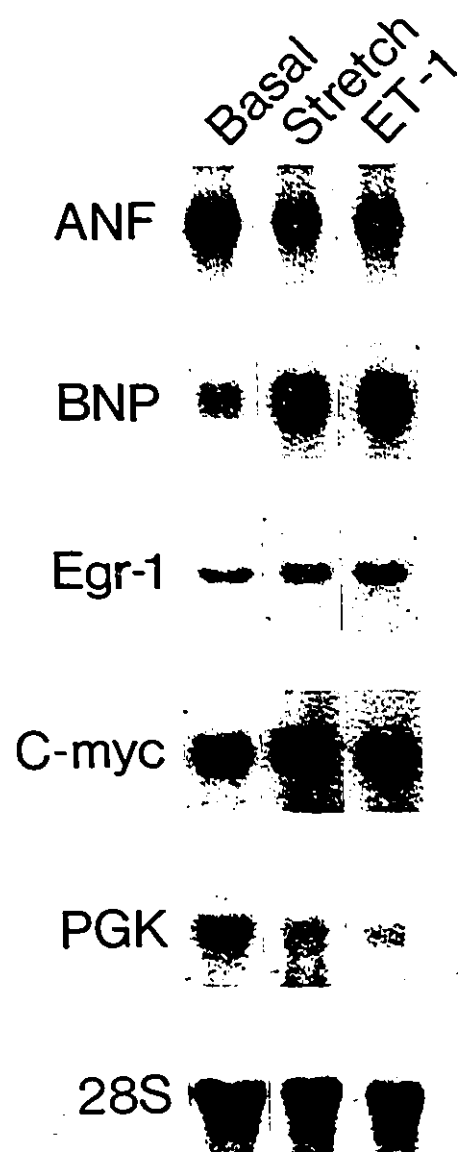


Figure 18: Densitometric analysis of Northern blots performed on atrial RNA extracted from right atria that were perfused at 0.5 mmHg with vehicle alone (n=5) or were stimulated by stretch (closed bars) or endothelin-1 (hatched bars) for 1.5 h (n=5), 4 h (n=5), 6 h (n=5), or 8 h (n=5-7). Relative signals quantitated are (from top to bottom): ANF, BNP, *Egr-1*, and *c-myc*. Signals were normalized to 28S rRNA signals (for ANF mRNA) or to *pgk-1* mRNA signals (for all other mRNAs). Data are expressed as mean $\pm$ SEM of the percent change from unstimulated controls. \* $P$ <0.05, † $P$ <0.01, ‡ $P$ <0.001 vs. vehicle (by ANOVA).

Fig. 18

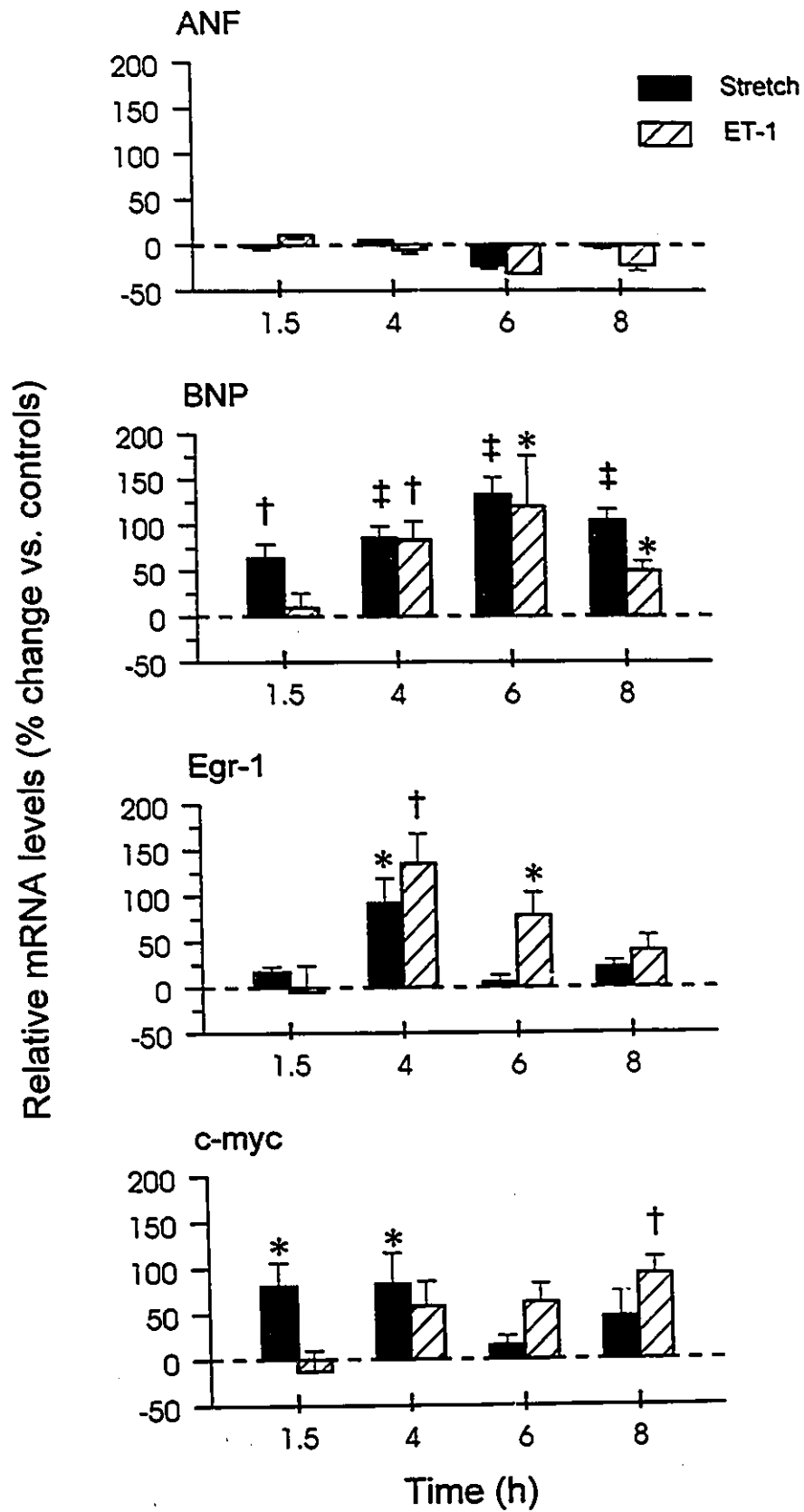


**Figure 19:** Effect of phenylephrine on (A) rate of contraction and (B) intra-atrial pressure of isolated right atria. HR=heart rate; IAP=intra-atrial pressure. Atria were perfused with vehicle (100  $\mu$ M ascorbic acid,  $\circ$ , n=5), 10  $\mu$ M phenylephrine ( $\bullet$ , n=4), 50  $\mu$ M phenylephrine ( $\blacksquare$ , n=6), 100  $\mu$ M phenylephrine ( $\blacktriangle$ , n=5), or 50  $\mu$ M phenylephrine with 10  $\mu$ M prazosin ( $\square$ , n=5) at time 0, as indicated by the arrow. Data are mean $\pm$ SE. \* $P$ <0.05, † $P$ <0.01 vs. time 0 (by ANOVA) and vs. vehicle (by  $t$ -test); significance applies to all doses of phenylephrine.

Fig. 19

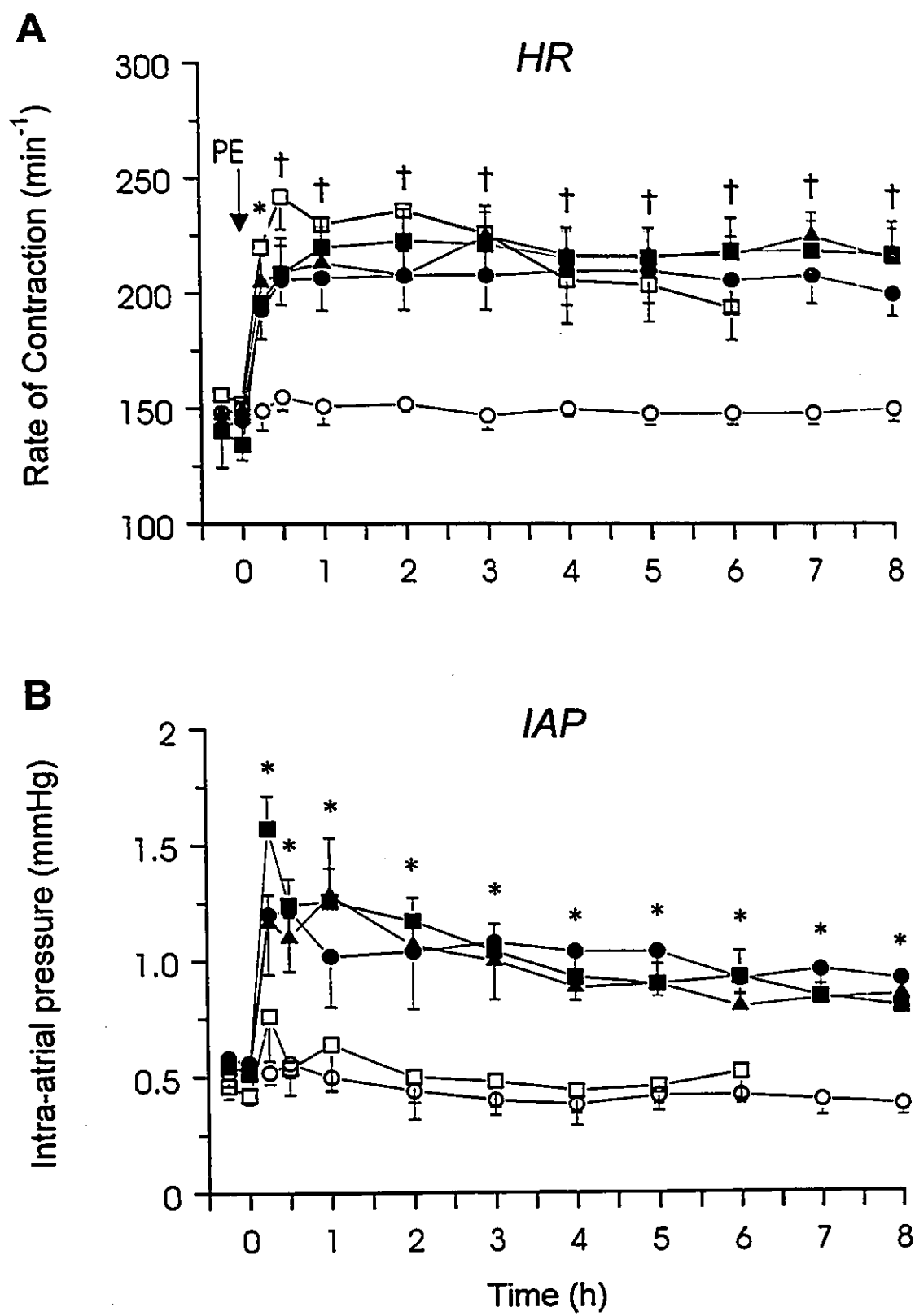


Figure 20: Effect of phenylephrine on (A) irANF and (B) irBNP secretion from isolated right atria. Atria were perfused with vehicle (100  $\mu$ M ascorbic acid,  $\circ$ ,  $n=5$ ), 10  $\mu$ M phenylephrine ( $\bullet$ ,  $n=4$ ), 50  $\mu$ M phenylephrine ( $\blacksquare$ ,  $n=6$ ), 100  $\mu$ M phenylephrine ( $\blacktriangle$ ,  $n=5$ ), or 50  $\mu$ M phenylephrine with 10  $\mu$ M prazosin ( $\square$ ,  $n=5$ ) at time 0, as indicated by the arrow. Data are mean $\pm$ SE. \* $P<0.05$ , † $P<0.01$ , ‡ $P<0.001$  vs time 0 (by ANOVA) and vs. vehicle (by  $t$ -test), § $P<0.05$  vs. vehicle (by  $t$ -test).

Fig. 20

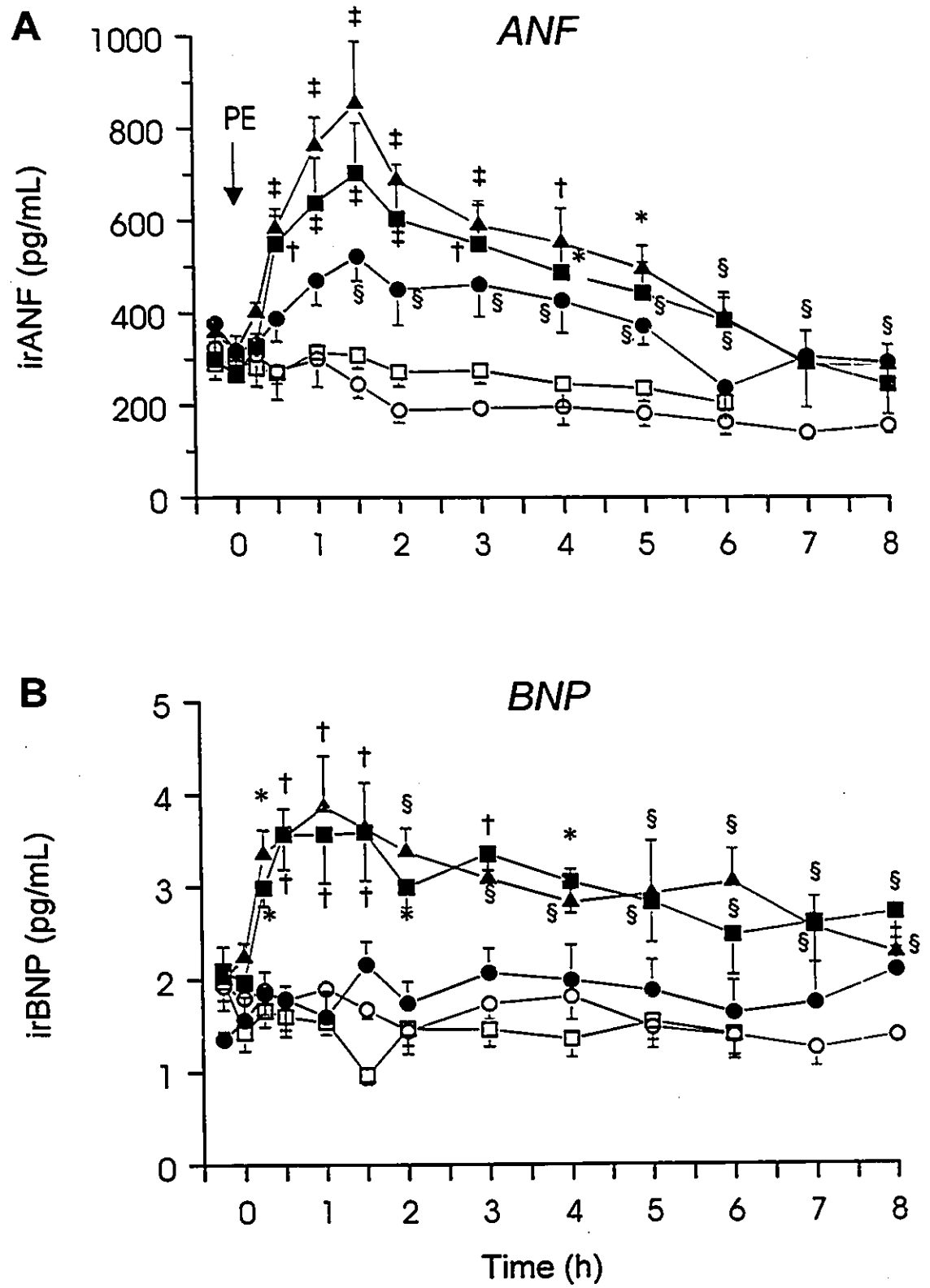


Figure 21: Ratios of irANF to irBNP secreted in response to phenylephrine (50 and 100  $\mu$ M, n=9). Phenylephrine was applied at time 0, as indicated by the arrow. Data are mean $\pm$ SE. \* $P$ <0.05 vs. 0 min and 3-8 h, † $P$ <0.05 vs. 15 min.

Fig. 21

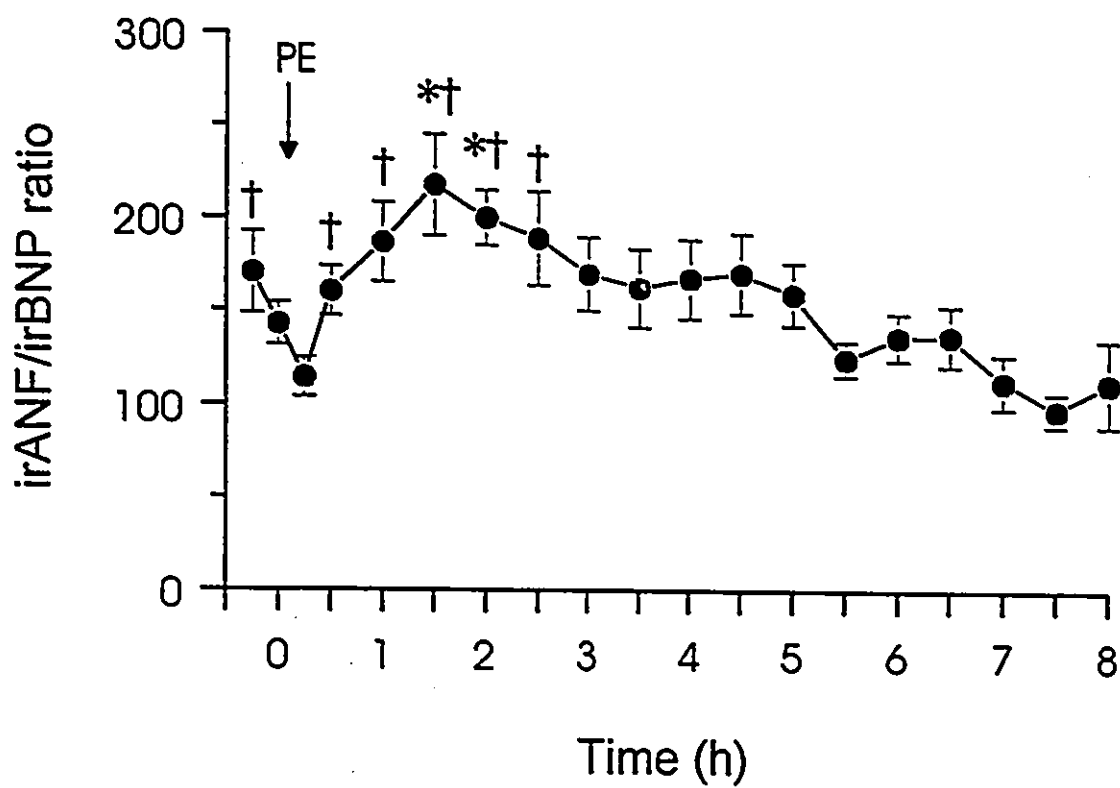




Table 1: Total irANF and irBNP secretion.

|                              | irANF (ng/8 h) | irBNP (ng/8 h) |
|------------------------------|----------------|----------------|
| Vehicle (n=5)                | 312±26         | 5.14±0.22      |
| Phenylephrine (10 µM) (n=5)  | 536±75*        | 5.65±0.42      |
| Phenylephrine (50 µM) (n=5)  | 690±90†        | 6.93±0.21‡     |
| Phenylephrine (100 µM) (n=6) | 767±59†        | 6.88±0.27‡     |

Total irANF or irBNP secreted during the 8 h experimental period, with stimulation by various doses of phenylephrine. \* $P<0.05$ , † $P<0.01$  vs. vehicle; ‡ $P<0.01$  vs. vehicle and  $P<0.05$  vs. 10 µM phenylephrine (by ANOVA).

Figure 22: Northern blot analysis of RNA extracted from perfused right atria, probed with 28S rRNA (28S), ANF, BNP, *Egr-1*, *c-myc*, and PGK probes. The 28S and PGK signals are housekeeping controls. The northern blots presented are a representative of the results obtained from atria perfused for 6 h with vehicle (Veh), 50  $\mu$ M phenylephrine (PE), or 50  $\mu$ M phenylephrine with 10  $\mu$ M prazosin (PE+PZ). The individual signals in each group are derived from Northern blots performed with RNA obtained from the same tissue sample. Exposure times were 15 min at 22°C (ANF and 28S rRNA), 6 h at -80°C (BNP and *Egr-1*), 40 h at -80°C (PGK), and 48 h at -80°C (*c-myc*), and were identical for all three groups.

Fig. 22

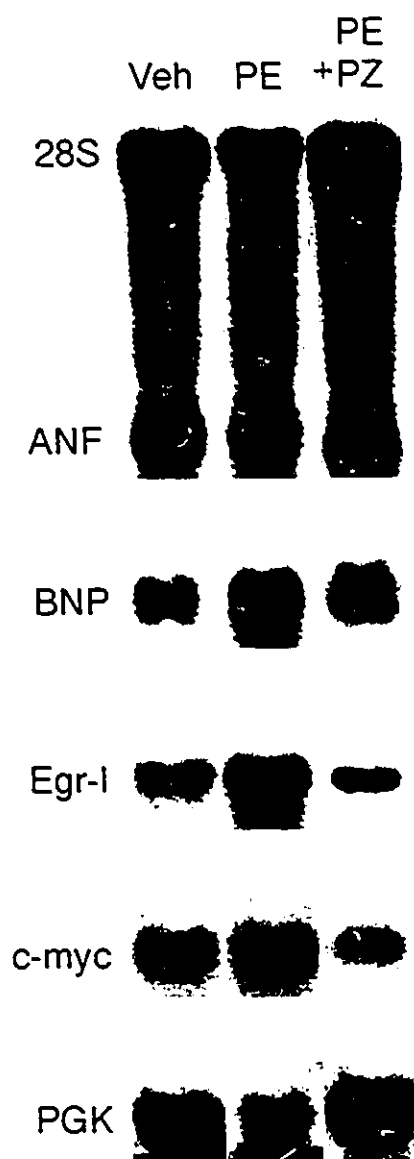
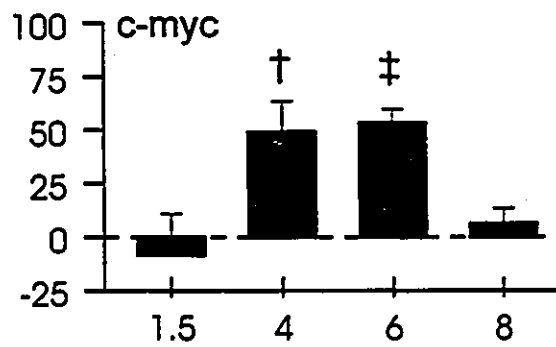
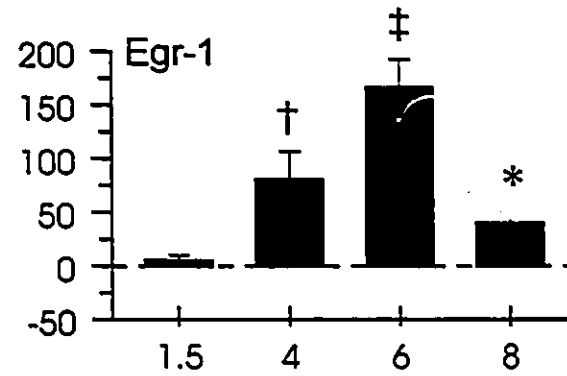
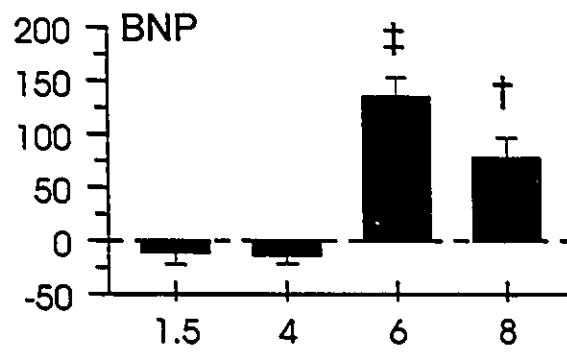
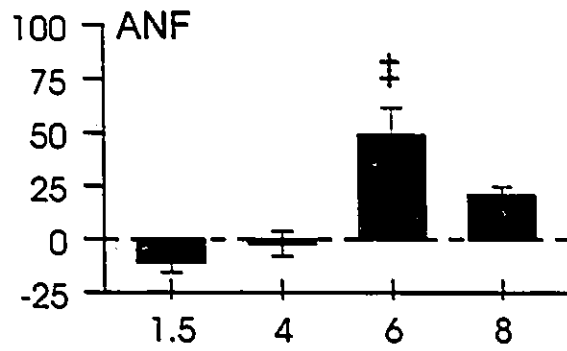


**Figure 23:** Densitometric analysis of Northern blots performed on atrial RNA extracted from right atria that were perfused with vehicle alone (n=5) or with 50  $\mu$ M phenylephrine (PE) for 1.5 h (n=5), 4 h (n=5), 6 h (n=5), or 8 h (n=5-7). Relative signals quantitated are (from top to bottom): ANF, BNP, *Egr-1*, and *c-myc*. Signals were normalized to 28S rRNA signals (for ANF mRNA) or to PGK mRNA signals (for all other mRNAs). Data are expressed as mean $\pm$ SEM of the percent change from unstimulated (vehicle) controls. \* $P$ <0.05, † $P$ <0.01, ‡ $P$ <0.001 vs. vehicle (by ANOVA). Please note that the scales for changes in ANF and *c-myc* mRNA levels are different than those for changes in BNP and *Egr-1* mRNA levels.

Fig. 23

Relative mRNA levels (% change vs. controls)



Time (h)

## DISCUSSION

Atrial ANF and BNP gene expression and secretion are altered in parallel with changes in hemodynamic or neuroendocrine balance in many physiological and pathophysiological situations, but the precise factors involved in the modulation of natriuretic peptide gene expression remain to be determined. In this study, direct stimulation of adult atrial tissue by stretch, ET-1, and phenylephrine *in vitro* was conducted to attempt to define the mechanisms involved in controlling ANF and BNP secretion and gene expression in adult atrial cardiocytes *in situ*. These stimuli represent models for mechanical, autocrine/paracrine, and neuroendocrine stimulation of the endocrine heart. The results of this study reveal that the endocrine heart responds to specific stimuli by distinct modulation of each of its components at the levels of synthesis and secretion.

### Natriuretic peptide secretion following stretch

Atrial stretch induced a rapid increase in ANF secretion, as previously shown in other studies that used isolated atria (de Bold *et al.*, 1986; Schiebinger and Linden, 1986a; Bilder *et al.*, 1986; Agnoletti *et al.*, 1987; de Bold and de Bold, 1989; Page *et al.*, 1990; Kuroski-de Bold and de Bold, 1991). It is unlikely that ANF release from the isolated atrial preparations is due to cell leakage due to damage or death. Previous studies have shown that both models of isolated atrial tissue used in the present study synthesize RNA and protein at a constant rate, and retain normal morphological and cytological features (de Bold *et al.*, 1986 and Piazza, Bruneau, and de Bold, unpublished results). Also, creatine kinase levels in the perfusate remain low, under both basal and stretch conditions (de Bold and de

Bold, 1989). The molecular form of ANF and BNP released from the whole atria and right atrial preparation is the mature, processed form of the peptides, ANF<sub>99-126</sub> and BNP<sub>64-95</sub> (de Bold and de Bold, 1989 and Bruneau and de Bold, unpublished data), clearly indicating that there is no cell leakage, since only the unprocessed variant of ANF (ANF<sub>1-126</sub>) is present in the atrial cardiocytes (Flynn *et al.*, 1965a; Flynn *et al.*, 1985b; Thibault *et al.*, 1989), while BNP is stored in its processed form (Thibault *et al.*, 1992; Yokota *et al.*, 1995). Further support for the intact state of the preparation can be obtained from the mechanical parameters presented here: beating rate and strength of contraction remain steady throughout the basal period, and respond to stimulation as expected.

The observations that acute atrial stretch *in vitro* results in a rapid increase in ANF secretion further confirms that it is stretch of the atrial tissue that is responsible for the release of ANF under conditions of volume load, and not pressure on the atrial wall (Edwards *et al.*, 1988; Hintze *et al.*, 1985; Koller *et al.*, 1987; Northridge *et al.*, 1989; Stewart *et al.*, 1993; Hasebe *et al.*, 1995). Experiments involving *in vitro* perfused rat atria subjected to acute mechanical stretch have previously shown that pure stretch is a sufficient and potent stimulus for ANF release (de Bold *et al.*, 1986; Schiebinger and Linden, 1986a; Agnoletti *et al.*, 1987; Page *et al.*, 1990). The results presented here support this contention, and the use of two different models further emphasizes this. In fact, in the isolated right atria, the stretch stimulus is measured in terms of intraatrial pressure, but it has been shown in this model that increases in intraatrial pressure are directly related to increased atrial volume, therefore supporting the view that it is stretch alone that influences ANF release from the atria.

It has been proposed that basal and volume expansion-induced secretion of ANF is modulated by neural reflexes (Eskay *et al.*, 1986; Ruskoaho *et al.*, 1989a;

Antunes-Rodrigues *et al.*, 1991; Antunes-Rodrigues *et al.*, 1993; Reis *et al.*, 1994), although others find conflicting results (Ledsome *et al.*, 1985; Goetz *et al.*, 1986; Kabayama *et al.*, 1987). The present experiments support the notion that stretch-induced ANF release from the atria is a process that can occur independently from neural reflexes. It remains that there may be a partial contribution, either direct or indirect, of neural reflexes on the increased circulating ANF levels measured following volume expansion. It has been suggested from experiments with *in vitro* perfused hearts that cardiac sympathetic stimulation potentiates the stretch-induced increase in ANF release (Jiao and Baertschi, 1995). Therefore, the most likely effect of neural reflexes is one of potentiation of the stretch effect on ANF secretion, but the primary and direct stimulus for ANF secretion following volume expansion is most likely stretch of the atrial cardiocyte *per se*.

The mechanism underlying the mechanotransduction process responsible for stretch-induced ANF release remains to be elucidated. Basal and stretch-induced ANF secretion is an energy-dependent process that has many of the characteristics of regulated secretion, and is mainly dependent on exocytosis of secretory granules (Page *et al.*, 1991). Recent evidence using immunoelectron microscopy of stretched atrial tissue fixed by tannic acid has shown that stretch-induced ANF secretion also occurs at least in part by this process (Newman and Severs, 1993). Morphological studies *in vitro* and *in vivo* have suggested that stretch causes the translocation of atrial specific granules from a perinuclear to a peripheral distribution (Agnoletti *et al.*, 1989). This appears to be a preparatory step, which is soon followed by fusion of the granules with the plasma membrane, resulting in extrusion of the granule contents (Page *et al.*, 1986; Sugawara, 1987; Nomura *et al.*, 1988; Newman and Severs, 1993). Stretch-induced ANF secretion is due to the rapid release of newly synthesized hormone (Mangat and de Bold, 1993); it is possible that regulated

secretion from immature granules is partly responsible for this (Iida and Shibata, 1994; Arvan *et al.*, 1991), although this requires experimental testing. Stretch-induced ANF secretion may also occur *via* caveolae. It is not known what the contribution of this pathway to total ANF secretion is, nor if it is regulated by stretch; however, the increase in caveola permeability following stretch suggests at least a partial contribution of this pathway to stretch-induced ANF secretion (Page *et al.*, 1992).

Stretch-induced ANF secretion rates returned to basal levels after 3 hours of stretch, although mechanical stimulation was maintained throughout the stimulation period, as observed by the sustained increase in mechanical activity. A consistent feature of stretch-induced ANF secretion *in vitro* and *in vivo* is that it is short-lived (de Bold *et al.*, 1986; Agnoletti *et al.*, 1987; Walsh *et al.*, 1988; de Bold and de Bold, 1989; Kuroski-de Bold and de Bold, 1991; Mangat and de Bold, 1993), and a subsequent stretch does not elicit a response of the same magnitude (Agnoletti *et al.*, 1987; Walsh *et al.*, 1988). There is no discernible change in atrial ANF content following stretch (Agnoletti *et al.*, 1987; Mangat and de Bold, 1993; Mäntymaa *et al.*, 1993), which indicates that total ANF content is not depleted by acute stretch. In fact, during *in vivo* volume overload, it takes up to 72 hours to significantly decrease atrial ANF stores (Yokota *et al.*, 1994). Microscopic examination of the atrial cardiocytes following stretch reveals no apparent change in the amount of granules present (Agnoletti *et al.*, 1989; Page *et al.*, 1991; Newman and Severs, 1993), suggesting that there is not a complete depletion of ANF-containing granules, but instead that there is a relatively small population of granules that is sensitive to stretch, and which is rapidly depletable. This was conclusively determined by experiments that made use of a double-label pulse-chase protocol, in which it was determined that it is a rapidly-depletable pool of newly-synthesized hormone that responds to stretch (Mangat and de Bold, 1993). Therefore, the decrease in

stretch-induced ANF secretion observed here is due to this depletion of a discreet pool of granules.

The co-localization of ANF and BNP in atrial granules of porcine, human, and rat atrial cardiocytes (Hasegawa *et al.*, 1991; Nakamura *et al.*, 1991; Thibault *et al.*, 1992; Kuroski-de Bold *et al.*, 1992) implies that both peptides should be released simultaneously. In rat, porcine and human atrial myocardium, there are distinct populations of granules: some containing ANF alone, and some containing both ANF and BNP (Hasegawa *et al.*, 1991; Nakamura *et al.*, 1991; Kuroski-de Bold *et al.*, 1992). The distribution of these two types of granules in the porcine atrial myocardium is also different; the greater proportion of ANF-only granules are found mostly on the epicardial side of the atrial muscle, while the bi-hormonal granules are found predominantly in the endocardium (Hasegawa *et al.*, 1991). Therefore, the different types of granules may be destined for alternate release pathways. Furthermore, the amount of BNP associated with the granules is only a small portion of total cellular BNP, and is proportionally very small compared to granule-associated ANF, suggesting that there are differences in the post-translational fates of these peptides (Yokota *et al.*, 1995). Indeed, using tissue fractionation, it has recently been shown that while 40% of atrial ANF is found in a granule-associated component, only 8% of BNP is granule-associated. In rats made hypertensive by DOCA-salt treatment, total atrial BNP content does not change, whereas there is a decrease in the granule-associated BNP content (Yokota *et al.*, 1995), thus supporting the notion that there are pathways involved in the secretion of BNP that are both dependent on and independent from specific atrial granules.

In the isolated whole atria, BNP secretion rates remained constant following stretch, although there appeared to be a tendency for BNP levels to increase over control

levels after 15 minutes of stretch. Since BNP levels in the perfusate were predicted to be very low, 15 minute perfusion periods were pooled to measure BNP secretion. Therefore, it cannot be ruled out that an acute response of BNP secretion to stretch may have been masked by a dilution effect. In the isolated right atria, stretch imposed by a pulsatile increase in intra-atrial pressure to 8 mmHg resulted in a marked increase in BNP secretion which paralleled that of ANF. The apparent discrepancies between the two models are most likely related to two major differences in the models used. First, a linear stretch was applied to the whole atria, unlike the isolated right atria, which received a radial stretch; the conformation of the atrial cardiocytes in relation to the stretch may affect the degree of exocytosis of BNP-containing granules. It is more likely that the radial stretch of the isolated right atria results in a more uniform stretch of all cardiocytes than does the linear stretch applied to the whole atria. Second, it has been shown for ANF that rapid cyclical stretching is more efficient at stimulating peptide release than constant load (Bilder *et al.*, 1986). A similar effect may be responsible for the difference in BNP secretion between the whole atria that were given a constant load, and the isolated right atria that received a cyclical stretch at a rate similar to that described as the optimal rate for ANF release (Bilder *et al.*, 1986). In addition, the relative increase in ANF secretion was greater from the isolated right atria than from the whole atria; this translates into a smaller increase in BNP secretion from the whole atria, especially considering that the proportional increase in BNP secretion is smaller than that of ANF in the isolated right atria. Therefore, it appears that the BNP secretion is more sensitive to the degree of stretch, than ANF secretion. The differing sensitivities of ANF and BNP to atrial stretch suggest that the stretch-secretion coupling mechanism regulates the secretion of either hormone independently to precisely address the requirements of an overloaded cardiovascular system.

The increase in BNP release in response to stretch of the isolated right atria is similar to that reported in a recent study by Mäntymaa *et al.* (1993), who used a modified Langendorff preparation, and who also reported a proportionally smaller increase in BNP release compared to that of ANF. The present experiments also support the observation that volume overload due to aorto-caval fistula causes coordinate increases in circulating ANF and BNP (Thibault *et al.*, 1992). However, not all studies report a concomitant increase in ANF and BNP secretion following volume overload. Acute volume loading in humans results in a rapid increase in plasma ANF levels, while BNP concentrations are unchanged (Lang *et al.*, 1993). In DOCA-treated rats, in which the resulting volume overload translates into elevated circulating levels of ANF, plasma BNP levels remain at basal levels (Yokota *et al.*, 1994). It is possible that, since BNP secretion is less sensitive to stretch than ANF secretion is, the atrial stretch found in these models is not sufficient to significantly increase BNP secretion. Otherwise, the apparent discrepancies between the *in vitro* data presented here and some of the *in vivo* studies might be explained by the observation that the response to acute stretch is rapid and transient; combined with the low levels of BNP in the circulation and clearance of the peptide, a rapid and modest increase in BNP secretion may not be readily detectable in the circulation.

Stretch-induced BNP release from the isolated right atria followed a time course that was similar to that of ANF. Therefore it is likely that the population of granules destined for rapid stretch-induced release also contain BNP, and that as for ANF, granule exocytosis is the primary mechanism of stretch-induced BNP secretion. Support for the possibility of granule-mediated BNP secretion has come from recent investigations carried out using the Na<sup>+</sup> ionophore monensin. Monensin specifically blocks the formation of nascent secretory granules at the level of the *trans* Golgi network (Mollenhauer *et al.*, 1990), and has been

shown to decrease basal ANF secretion from cultured adult atrial cardiocytes (Iida *et al.*, 1988). In isolated right atria, pre-incubation with monensin inhibits stretch-stimulated secretion of both ANF and BNP (A.J. de Bold, unpublished results), indicating that the secretion of both peptides in response to stretch is dependent on the exocytosis of newly formed granules. Calculation of the ratios of irANF to irBNP secreted in response to stretch revealed that there was an initial preferential release of ANF compared to BNP, and that thereafter the two peptides were released together. This indicates that the two hormones are not initially co-secreted in response to stretch. This may reflect the proportionally greater amount of ANF stored in the specific atrial granules (Kuroski-de Bold *et al.*, 1992; Yokota *et al.*, 1995), but if bihormonal granules were exclusively involved in stretch-stimulated secretion, the ratio of irANF/irBNP secreted would remain constant throughout the stretch-stimulated period. The most likely explanation is that two granule populations, one containing both ANF and BNP and one containing ANF only, are recruited for exocytosis in the early phase of stretch-induced secretion, and subsequently granules with an equal proportion of both hormones are involved in the secretion of ANF and BNP. It is unclear why the change in ratios of irANF/irBNP occurs during the stretch-stimulated period, but it most likely reflects the more rapid depletion of one pool of peptide compared to the other.

It is not known what the intermediates between stretch of the cardiocyte and increased secretion of ANF or BNP are. One of the possible mechanisms proposed as an initiator of the stretch-induced increase in natriuretic peptide secretion is the action of mechanosensitive ion channels. A recent report has shown that gadolinium ( $Gd^{3+}$ ), a non-specific inhibitor of stretch-activated ion channels in *Xenopus* oocytes and chick ventricular cardiocytes (Yang and Sachs, 1989; Sigurdson *et al.*, 1992) can inhibit stretch-induced ANF release from perfused atria (Laine *et al.*, 1994a). Mechanosensitive  $K^+$ -selective, cation-

selective, and anion-selective channels have been described in rat atrial cardiocytes (Kim, 1992; Kim and Fu, 1993; Kim, 1993; Hagiwara *et al.*, 1992); however, these channels are insensitive to  $Gd^{3+}$ . Therefore, the effects of  $Gd^{3+}$  on ANF secretion might occur either through an undefined  $Gd^{3+}$ -sensitive channel, or are due to effects of  $Gd^{3+}$  that are distinct from their effects on mechanosensitive ion channels. It has been pointed out, however (Laine *et al.*, 1994a), that the mechanosensitive ion channels may be in atrial cells other than the cardiocytes, such as endothelial cells (Hoyer *et al.*, 1994), and their activation might lead to the release of a factor that would influence ANF secretion. It has been suggested that autocrine release of ET-1 in response to stretch is partly responsible for stretch-induced ANF secretion from atrial tissue (Skvorak *et al.*, 1995).

The involvement of plasmalemmal cation flux in the stretch-stimulated release of ANF is controversial. In cultured cardiocytes, the increase in ANF secretion following membrane stretch induced by hypo-osmotic swelling can be further increased by decreasing external calcium ion ( $Ca^{2+}$ ) concentrations or by chelating intracellular  $Ca^{2+}$ , suggesting that  $Ca^{2+}$  negatively modulates ANF secretion (Gibbs, 1987a; Greenwald *et al.*, 1989). Similarly, in isolated rat or rabbit atria it has been shown that eliminating external  $Ca^{2+}$  increases basal ANF secretion, while it does not affect the response to stretch (de Bold and de Bold, 1989; Cho *et al.*, 1994). Others have shown that blocking L-type or T-type  $Ca^{2+}$  channels partly decreases the response of ANF secretion to stretch, thus implicated transplasmalemmal  $Ca^{2+}$  influx as a primary mediator of stretch-induced ANF secretion (Page *et al.*, 1990). However, this observation was contradicted by others using similar atrial preparations and pharmacological treatments (Laine *et al.*, 1994a). It appears, therefore, that external  $Ca^{2+}$  influx is not an important mediator of ANF secretion, under basal or stimulated conditions.

Although plasmalemmal  $\text{Ca}^{2+}$  transport may not be involved in regulating stretch-induced ANF secretion, there exists conclusive evidence for the involvement of  $\text{Ca}^{2+}$  transport from the sarcoplasmic reticulum. Experiments using isolated rat atria have shown that ryanodine, a blocker of sarcoplasmic reticulum  $\text{Ca}^{2+}$  channels, can partly inhibit the release of ANF following stretch (Kuroski-de Bold and de Bold, 1991; Laine *et al.*, 1994b). As well, depletion of intracellular  $\text{Ca}^{2+}$  stores by caffeine decreases both basal and stretch-induced ANF secretion (Page *et al.*, 1990; Kuroski-de Bold and de Bold, 1991). Studies in cultured neonatal atrial cardiocytes have shown that stretch-induced ANF secretion is not dependent on influx of  $\text{Ca}^{2+}$  but can be blocked by calmidazolium, a calmodulin inhibitor (Gardner *et al.*, 1992). Others, using a different calmodulin antagonist (W-7) provide evidence that supports (Kato *et al.*, 1990) or opposes (Ishida *et al.*, 1988) the potential role of calmodulin in the regulation of ANF secretion. The identification of the  $\text{Ca}^{2+}$ -binding peptides Annexin V and VI as granule-associated proteins (Doubell *et al.*, 1991) suggests their involvement and a possible role for  $\text{Ca}^{2+}$  in the regulated exocytosis of specific atrial granules.

The manipulation of cationic environment also reveals an essential feature of ANF secretion: its independence from mechanical activity. A major insight from the studies on the effects of cation replacement (Kuroski-de Bold and de Bold, 1991) was the finding that although the different cations used to replace  $\text{Ca}^{2+}$  exerted different effects on atrial ANF release, they all inhibited atrial beating and developed tension, suggesting that in general, ANF release is independent from atrial contraction. Evidence in support of this view was obtained in studies with rat atria *in vitro* (de Bold and de Bold, 1987; de Bold and de Bold, 1987) in which it was demonstrated that the rates of basal ANF release do not differ in electrically driven or quiescent atrial muscle. Moreover, there are no changes in ANF

release from either the rat left auricle or human atrial trabeculae subjected to different pacing frequencies even though the tissues showed their characteristic positive and negative (staircase) changes in developed tension with increasing pacing frequency (de Bold and de Bold, 1987; de Bold and de Bold, 1987). This is supported by other reports in which changes in pacing frequency had no effect on ANF release from perfused rat hearts (Katoh *et al.*, 1990), rat atria (Agnoletti *et al.*, 1992), or rabbit atria (Cho *et al.*, 1991). However, other investigators have found a direct correlation between frequency of contraction and ANF release from atria *in vitro* (Schiebinger and Linden, 1986b; Naruse *et al.*, 1987; Bilder *et al.*, 1989) or in rapidly paced animals (Rankin *et al.*, 1987; Walsh *et al.*, 1987; Walsh *et al.*, 1988). The increased levels of ANF in plasma observed in chronotropic stimulation *in vivo* are accompanied by an increase in intra-atrial pressures (Rankin *et al.*, 1987; Walsh *et al.*, 1987) and pulmonary wedge pressures (Walsh *et al.*, 1987), which could be responsible for the increased secretion of ANF. In fact, increased ANF plasma levels in rapidly paced dogs is dependent mostly on the increase in passive atrial diastolic stretch (Stewart *et al.*, 1993). In addition, rapid pacing cannot sustain elevated ANF plasma levels (Walsh *et al.*, 1988). It has also been reported that in humans with atrial paralysis and in patients with normal atrial activity paced at 70 beats/minute in VVI mode for complete heart block, atrial contraction is not necessary for ANF release (Vardas *et al.*, 1988). Further, in humans, atrial pacing does not increase atrial pressure nor circulating ANF (Burnett, Jr. *et al.*, 1989). However, ventricular pacing results in a significant increase in intra-atrial pressure, resulting in increased circulating ANF levels. In the *in vitro* studies, paced atria that are given a large non-physiological preload were used to show the effect of pacing on ANF secretion (Schiebinger and Linden, 1986b; Naruse *et al.*, 1987; Bilder *et al.*, 1989). In fact, it was not possible to stimulate ANF secretion using a preload smaller than 1 gram, and very rapid pacing rates were required to effect significant changes in ANF secretion. One of these

investigators suggests that the degree of resting tension applied directly influences the magnitude of the secretory response to increased pacing frequencies (Bilder *et al.*, 1989). These findings suggest that atrial tachycardia *per se* does not stimulate ANF release. Therefore, the increase in contractile rate due to stretch that is observed in the present study is not the primary mediator of increased ANF secretion.

Atrial stretch causes a rapid increase in phosphoinositol hydrolysis (von Harsdorf *et al.*, 1989), which may be involved in regulating the release of specific atrial granules. Right atrial dilatation in isolated perfused rat hearts for 10 minutes results in an increase in inositol monophosphate (IP), inositol biphosphate (IP<sub>2</sub>), and inositol-1,4,5(tris)phosphate (IP<sub>3</sub>) accumulation (von Harsdorf *et al.*, 1989). IP<sub>3</sub> binds to receptors on the sarcoplasmic reticulum membrane, and causes release of Ca<sup>2+</sup> from the sarcoplasmic reticulum (Berridge, 1993). Since release of Ca<sup>2+</sup> from the ryanodine-sensitive sarcoplasmic reticulum stores has been implicated in stretch-induced ANF release (Kuroski-de Bold and de Bold, 1991; Laine *et al.*, 1994b), the increase in IP<sub>3</sub> is probably at least partially involved in mediating this process. The IP<sub>3</sub> receptor on the sarcoplasmic reticulum is ryanodine-insensitive (Berridge, 1993), which suggests that Ca<sup>2+</sup> release from intracellular stores in response to stretch is due in part to a signalling molecule that is distinct from IP<sub>3</sub>, or may be due to Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release. In cultured cardiocytes, stretch results in an increase in the accumulation of inositol-1,3,4(tris)phosphate, inositol-1,4,5(tris)phosphate, and inositol-1,3,4,5-tetrakisphosphate (IP<sub>4</sub>) (Dassouli *et al.*, 1993). Importantly, in the cardiocyte cultures, the stretch-induced increase in IPs is not affected by depolarization by high K<sup>+</sup> (Dassouli *et al.*, 1993); this supports the contention that stretch-induced ANF release is independent from contractile activity (Kuroski-de Bold and de Bold, 1991). An indirect link between IP<sub>3</sub> and ANF release has been established in studies comparing the response of atria from aging normotensive or

genetically hypertensive rats (Brunner *et al.*, 1995); it was found that the decrease in stretch-responsiveness of the secretory response of the hypertensive animals over time was accompanied by a decrease in stretch-stimulated IP<sub>3</sub> accumulation.

The increase in inositol phosphate metabolism in response to stretch is most likely due to activation of phospholipase C (von Harsdorf *et al.*, 1989; Komuro *et al.*, 1991; Sadoshima and Izumo, 1993a), and appears to be mediated by a pertussis toxin-insensitive protein (Dassouli *et al.*, 1993). Activation of phospholipase C results in the accumulation of IP<sub>3</sub> and diacylglycerol; the downstream target of diacylglycerol is activation of the serine/threonine kinase, protein kinase C (PKC) (Nishizuka, 1992), which has been implicated in the regulation of ANF secretion by stretch (Page *et al.*, 1990; Ruskoaho *et al.*, 1990). Activation of PKC by phorbol esters has been shown to cause a potentiation of stretch-induced ANF release (Ruskoaho *et al.*, 1990). PKC also appears to be partly responsible for stretch-induced ANF and BNP secretion from ventricular myocardium (Kinnunen *et al.*, 1993). The potential involvement of PKC in the stimulation of ANF or BNP secretion by stretch is complicated by the observations that various isoforms of PKC exist in the heart, and may be differentially activated temporally by the same agonist (Steinberg *et al.*, 1995). Also, various PKC isoforms are specifically activated by particular stimuli (Gu and Bishop, 1994; Bogoyevitch *et al.*, 1993; Puceat *et al.*, 1994; Clerk *et al.*, 1994). Therefore it is not known which isoforms are activated by stretch in atrial cardiocytes. In addition, the different PKC isoforms may have distinct sites of action in the same cell. Various species of PKC have been shown to migrate to specific compartments in cardiocytes following stimulation. Upon activation and translocation to membrane-associated intracellular compartments, the PKC isoforms may be involved in nuclear, cytoskeletal, or cytosolic phosphorylation events. Such events include activation of other kinases, phosphorylation of

microtubule-associated proteins, or regulation of contractility (Nishizuka, 1992). Phosphorylation of cytoskeletal components has been associated with secretion of insulin from pancreatic  $\beta$  cells (Schubart *et al.*, 1982; Brown *et al.*, 1985; Howell and Tyhurst, 1986; Ashcroft and Hughes, 1990) and secretion of thromboxane A<sub>2</sub> from platelets (Gerrard *et al.*, 1985). PKC may also phosphorylate ANF, which is found in a phosphorylated state in granules (Bloch *et al.*, 1987; Wildey *et al.*, 1990).

Another possible mediator of stretch-induced ANF or BNP release is the prostaglandin PGF<sub>2</sub>. Increases in plasma ANF due to volume loading can be partially reduced by indomethacin, a cyclooxygenase inhibitor (Gardner and Schultz, 1990). PGF<sub>2</sub> itself can induce secretion of ANF from cultured neonatal atrial cardiocytes (Gardner and Schultz, 1990) or perfused hearts (Rayner *et al.*, 1993). Stimulation of ANF secretion from atrial strips by arginine vasopressin (Zongazo *et al.*, 1991) or from isolated hearts by platelet-activating factor (Rayner *et al.*, 1993) is also at least partly dependent on PGF<sub>2</sub>. The right atrium is a rich source of prostaglandins (Evers *et al.*, 1987), which supports the possibility of endogenously produced prostaglandins affecting ANF secretion. Stretch has also been shown to increase prostaglandin release from vascular tissue *in vitro* (Quadt *et al.*, 1982). Also, eicosanoid production is enhanced in right atria of rabbits following left ventricular infarction (Evers *et al.*, 1987). Therefore, autocrine or paracrine release of PGF<sub>2</sub> may be an important mediator of stretch-induced ANF or BNP secretion. The actions of PGF<sub>2</sub> in the cardiocyte appears not to be *via* adenylate cyclase (Gardner and Schultz, 1990; Kovacic-Milivojevic *et al.*, 1991); instead, they may be mediated by calmodulin (Kovacic-Milivojevic *et al.*, 1991), with the initial locus of action at the level of phosphoinositide hydrolysis, as has been shown in other systems. This is consistent with the

possible role of increased phosphoinositide hydrolysis and calmodulin in the regulation of ANF secretion.

The relevance of stretch-induced natriuretic peptide release observed here is reflected in observations made in *in vivo* conditions of volume overload, during which stretch of the atrium results in the rapid release of both ANF and BNP into the circulation (Lang *et al.*, 1985; Ledsome *et al.*, 1985; Lattion *et al.*, 1986; Thibault *et al.*, 1992). The natriuretic, diuretic, and vasorelaxant effects of both hormones then serve to counterbalance the increased load on the cardiovascular and renal systems. It is not immediately obvious why two hormones with similar spectra of activity would be released concomitantly. This becomes more clear when the relative plasma half-lives and receptor affinities of each peptide are taken into consideration. The large increase in ANF secreted in response to stretch will have an immediate effect on the GC-A receptors, for which it has the strongest affinity (Chang *et al.*, 1989; Suga *et al.*, 1992a). BNP has a longer plasma half-life than ANF (Kita *et al.*, 1991; Mukoyama *et al.*, 1991); thus, the proportionally smaller increase in BNP will supplement the response to ANF over a longer period of time. It is conceivable, based on the present observations and on previously published reports (Thibault *et al.*, 1992; Lang *et al.*, 1993; Yokota *et al.*, 1994; Yokota *et al.*, 1995), that increased BNP secretion in response to load accompanies only the most severe overloads. This dual natriuretic peptide system is therefore well adapted to deal with varying degrees of cardiac overload.

In summary, stretch of isolated atrial tissue results in a rapid and short-lived increase in the secretion of ANF and BNP. This may be due to exocytosis of granules that contain both peptides, as well as granules that contain ANF only. It is not clear what the signalling mechanisms responsible for this secretory process are, but it is likely that

phosphoinositide hydrolysis, increased release of  $\text{Ca}^{2+}$  from intracellular stores, and PKC may be involved. The initial trigger that follows mechanical stimulation is not known.

#### Natriuretic peptide mRNA levels following stretch

Stretch-stimulated ANF release is associated with the rapid depletion of an immediately releasable pool of newly synthesized peptide (Mangat and de Bold, 1993) and is independent from protein synthesis (Page *et al.*, 1991), suggesting that acute stretch is not immediately accompanied by increased ANF synthesis. Supporting this hypothesis, the present study shows that ANF mRNA levels remained constant after 8 hours of stretch. It is possible that the high levels of ANF mRNA already present in the atria are sufficient to maintain the synthesis requirements of the tissue for the time course studied. However, during longer periods of atrial stretch, as seen in volume overload *in vivo*, atrial ANF peptide content is partly depleted, while ANF mRNA levels are elevated (Lattion *et al.*, 1986; Dananberg and Grekin, 1992; Perrella *et al.*, 1992; Yokota *et al.*, 1994; Yokota *et al.*, 1995; de Bold *et al.*, 1996).

Most evidence linking stretch to changes in ANF gene expression has come from *in vivo* studies in which chronic atrial overload was observed. In patients with heart failure (Haass *et al.*, 1990; Fischer *et al.*, 1991), increases in atrial ANF mRNA levels significantly correlate with increases in pulmonary artery pressure and left atrial pressure, as well as with plasma ANF levels, suggesting that increases in ANF plasma levels due to increased atrial pressure or volume are related to increased ANF mRNA levels in the atria. In rats, significant increases in atrial ANF mRNA levels have been reported following volume overload induced

by either ureter ligation, bilateral nephrectomy, aortocaval fistula, DOCA-salt treatment, or DOCA treatment (Lattion *et al.*, 1986; Dananberg and Grekin, 1992; Yokota *et al.*, 1994; Yokota *et al.*, 1995). In rats with myocardial infarction, increased ANF plasma levels and increased atrial weight are associated with increased ANF mRNA levels, although this is not specific to the ANF gene (Mendez *et al.*, 1987; Drexler *et al.*, 1989). Following pressure overload, as seen in aortic banded rats, significant increases in atrial ANF mRNA levels were observed after 10 weeks; this is associated with increased right atrial pressure (Matsubara *et al.*, 1990). Spontaneously hypertensive rats also have increased atrial ANF mRNA levels compared to normotensive Wistar-Kyoto controls (Arai *et al.*, 1987), although reduced ANF mRNA levels have been reported in the atria of spontaneously hypertensive-stroke-prone rats (Ogawa *et al.*, 1991). Rapid-pacing-induced heart failure in dogs is associated with increased ANF mRNA levels; this increase is proportional to the increase in right atrial pressure that develops in this model, and is inversely related to mean arterial pressure (Perrella *et al.*, 1992). In this model, ventricular ANF synthesis is not increased, indicating that the increase in ANF plasma levels measured 15 days after the initiation of treatment is due to increased atrial synthesis and secretion alone, without a significant contribution from the ventricles.

In all the animal models described above, the increase in ANF mRNA levels is associated with a decrease in atrial stores of the hormone, indicating an increased demand over the available supply. Therefore, the increase in ANF gene expression can be seen as a response required to increase the levels of ANF peptide available for secretion. Furthermore, in an *in vitro* model, it has been shown that two consecutive 30 second stretches of neonatal atrial cardiocytes in culture resulted in a significant increase in ANF mRNA levels 24 hours later (Gardner *et al.*, 1992). Therefore, the endocrine heart

compensates for increased demand of ANF by increasing its synthesis only after the available stores are significantly depressed. This suggests that after a certain time of constant overload, the atrial cardiocytes cannot maintain increased secretion of ANF by regulated secretion alone, and must increase the synthesis of the peptide to compensate for the lack of available secretable hormone. This is consistent with the proposed regulated mechanism of secretion of ANF in response to stretch (Page *et al.*, 1991; De Young *et al.*, 1994), and further suggests that although a discreet pool of ANF is depleted in response to acute stretch (Mangat and de Bold, 1993), increased synthesis is not an immediate response for its replenishment. The increase in gene expression required for increased ANF synthesis therefore occurs only after a period of time that is longer than the period of time studied here.

BNP mRNA levels were elevated after 1.5 hours of stretch in the isolated right atria, increased further until they peaked after 6 hours, and remained elevated throughout the 8 hour period of study. However, in the whole atria, stretch did not cause significant changes in BNP mRNA levels. As with the stretch-induced secretion observed with this model, the discrepancies with the results obtained with the isolated whole atria are most likely due to differences in the models. Other researchers, using modified Langendorff-perfused hearts, have observed an increase in BNP mRNA levels due to an increase in intraatrial pressure from 0.5 mmHg to 4.1 mmHg, following a similar time course, although stimulation was carried out for only 2 hours (Mäntymaa *et al.*, 1993). The results presented here and those of Mäntymaa *et al.* (1993) are comparable in that the changes in BNP mRNA levels occur after the stretch-induced increase in BNP secretion had subsided. This suggests that the increased BNP gene expression is brought about to effect a sustained increase in hormone release, such as is observed in chronic overload situations (Dagnino *et al.*, 1992; Yokota *et*

*et al.*, 1995), or to replenish stores of BNP that may have been depleted by stretch, as is observed for ANF (Mangat and de Bold, 1993).

Increased atrial BNP mRNA levels have been observed in experimental animals with acute volume overload. In rats treated with DOCA, BNP mRNA levels increase in the atria after 72 hours of treatment (Yokota *et al.*, 1994). In more chronic models of overload, BNP mRNA levels are also elevated in the atria, although not to the same extent as in the ventricles. Several studies have suggested that in these conditions of overload, BNP is recruited more strongly from the ventricles than from the atria, which makes it a predominantly ventricular rather than atrial hormone (Mukoyama *et al.*, 1991; Ogawa *et al.*, 1991). This is perhaps the case in long-term overload situations such as genetic hypertension, congestive heart failure, or cardiomyopathies (Mukoyama *et al.*, 1991; Ogawa *et al.*, 1991; Hasegawa *et al.*, 1993a; Hasegawa *et al.*, 1993b). However, as delineated in models of overload such as mineralocorticoid excess (Yokota *et al.*, 1994) or by infusion of pressor agents such as phenylephrine or arginine vasopressin (Magga *et al.*, 1994), the endocrine heart appears to respond to acute or sub-acute stimuli by increasing the synthesis of BNP in the atria long before the ventricular cardiocytes respond in the same manner, which suggests an immediate response of the atrial cardiocytes at the level of secretion and synthesis, which is later supplemented by a similar response in the ventricular cardiocytes. The results presented here support the possibility of a dual chamber-specific response of BNP gene expression to the progression of cardiac overload from acute to chronic stimulation.

The apparently paradoxical increase in BNP mRNA levels without a detectable concomitant increase in secretion is reminiscent of acute volume overload in rats, in which

BNP mRNA levels are increased in the atria without a concomitant change in plasma or tissue levels (Yokota *et al.*, 1994). If the stretch-induced increase in BNP secretion is due to granule exocytosis, and is followed in chronic overload situations by an increase in constitutive secretion, the increased synthesis of BNP may be directed to the replenishment of the granule stores as well as to increased constitutive secretion. Alternatively, if BNP secretion is exclusively accomplished via a regulated pathway, it is not immediately obvious why two hormones that are co-secreted in response to a stimulus respond differently at the level of transcript abundance. The differences in the response of ANF and BNP to stretch at the levels of gene expression may be related to the relative proportion of peptide stores in the atrial cardiocytes. The proportionally smaller amount of BNP contained in granules compared to ANF (Hasegawa *et al.*, 1991; Thibault *et al.*, 1992; Kuroski-de Bold *et al.*, 1992; Yokota *et al.*, 1995) may result in a more rapid depletion of a discrete pool of BNP that requires a rapid increase in synthesis to replenish, and suggests that the requirement for replenishment of stores by increased synthesis is lesser for ANF than for BNP. Therefore, the increased peptide production that follows the increase in mRNA levels may not be immediately reflected by a detectable increase in either tissue content or secretion. It follows that recruitment of BNP synthesis by acute stretch is a requirement for the maintenance of BNP secretion in conditions of volume overload.

Although the promoters for both the ANF and BNP genes have similarities, it is likely that these are not involved in the response of these genes to stretch. In fact, in a transient transfection assay in cultured neonatal ventricular cardiocytes subjected to static stretch, the entire characterized region of the ANF promoter (>3 kb) was unresponsive to stretch (Sadoshima *et al.*, 1992a). As well, in a transgenic model, the full-length ANF promoter was also found to be unresponsive to pressure overload (Knowlton *et al.*, 1995).

However, the response of the ANF promoter differs between atrial and ventricular cardiocytes (Kovacac-Milivojevic and Gardner, 1992; McBride *et al.*, 1993; Kovacic-Milivojevic and Gardner, 1993); therefore it is difficult to derive definitive conclusions regarding atrial ANF gene expression from observation made only in ventricular cardiocytes. It is unknown, therefore, if the regulation of the ANF or BNP genes in atrial cardiocytes is coordinated at the transcriptional level, nor is it known if either process is responsive to the intracellular cascade of events that is initiated by stretch.

The difference in the response of the ANF and BNP genes to stretch may not be related to differences in the response of their respective promoters. Unlike ANF mRNA, BNP mRNA levels may be regulated by stretch at the post-transcriptional level, by stabilization of the mRNA; the BNP transcript has conserved AUUUA motifs that are involved in this mechanism of gene expression regulation (Kojima *et al.*, 1989; Sudoh *et al.*, 1989; Flynn *et al.*, 1989; Seilhamer *et al.*, 1989; Steinhilper, 1993; Ogawa *et al.*, 1994). BNP mRNA levels are in fact stabilized in cultured neonatal ventricular cardiocytes following the addition of phorbol esters, phenylephrine, or ET-1 (LaPointe and Sitkins, 1993; Hanford *et al.*, 1994; Nakagawa *et al.*, 1995). The ANF mRNA does not have such a destabilizing motif (Kennedy *et al.*, 1984; Maki *et al.*, 1984; Nakayama *et al.*, 1984; Kangawa *et al.*, 1984; Seidman *et al.*, 1984a; Seidman *et al.*, 1984b; Yamanaka *et al.*, 1984; Zivin *et al.*, 1984; Flynn *et al.*, 1985a), and therefore is not under the same type of post-transcriptional control that the BNP mRNA is under.

As for stretch-induced secretion, mechanosensitive ion channels have also been conjectured as mediators of stretch-induced changes in cardiocyte gene expression. Linear stretch of neonatal ventricular cardiocytes plated on silicone membranes results in increased

ANF mRNA levels, as well as increased early-response gene,  $\alpha$  myosin heavy chain, and  $\alpha$  skeletal actin gene expression (Sadoshima *et al.*, 1992a). A  $Gd^{3+}$ -sensitive stretch-activated ion channel has been characterized in neonatal ventricular cardiocytes, but blocking it with  $Gd^{3+}$  is ineffective in inhibiting the stretch-induced increase in early-response gene mRNA levels (Sadoshima *et al.*, 1992b). It has since been shown that the stretch-induced increase in ANF mRNA levels is due to autocrine release of angiotensin II from the ventricular cardiocytes (Sadoshima *et al.*, 1993). Cultured neonatal atrial cardiocytes stretched by hypotonic swelling (Tokola *et al.*, 1991) or by cyclical stretching on flexible membranes (Gardner *et al.*, 1992) have increased ANF mRNA levels compared to unstretched controls; however, atrial cardiocytes do not appear to synthesize angiotensin II (Sadoshima *et al.*, 1993), thus eliminating this as a possible mediator of stretch-induced changes in BNP gene expression in atrial cardiocytes. The mechanosensitive channels described in atrial cardiocytes might be involved in regulating stretch-induced changes in BNP gene expression. However, until specific blockers are available for these channels, this cannot be conclusively demonstrated. Therefore, the involvement of stretch-activated ion channels and the mechanisms involved in modulating stretch-induced BNP gene expression in atrial cardiocytes are still undefined.

Stretch of cultured neonatal ventricular cardiocytes activates PKC (Sadoshima and Izumo, 1993a). Direct activation of PKC in cultured ventricular cardiocytes by phorbol esters or by transfection of constitutively active PKC constructs has been shown to increase ANF and BNP mRNA levels by increasing its rate of transcription (Suzuki *et al.*, 1992; Shubeita *et al.*, 1992; LaPointe and Sitkins, 1993; Decock *et al.*, 1994). Stimulation of ANF and BNP gene expression by phorbol ester, phenylephrine, ET-1, basic fibroblast growth factor, acidic fibroblast growth factor, and thrombin can be blunted by PKC inhibitors (Sei *et*

*et al.*, 1991; LaPointe and Sitkins, 1993; Glembofski *et al.*, 1993; Tokola *et al.*, 1994; Hanford *et al.*, 1994; Nakagawa *et al.*, 1995), thus further emphasizing the importance of this enzyme in the activation of ANF and BNP gene expression in cardiocytes. As mentioned above, the precise role of PKC following stretch of the cardiocyte is unclear, due to the diversity of isoforms and their cellular locations. As well, it is not known if pharmacological blockade of PKC activity can inhibit the stretch-induced increase in natriuretic peptide gene expression. As important in this context is the distinct developmental pattern of expression of the various PKC isoforms (Bogoyevitch *et al.*, 1994; Puceat *et al.*, 1994). Therefore it is difficult to ascertain the precise role of PKC in stretch-induced BNP gene expression in the model of adult atria presented here by comparison with results from neonatal cardiocytes in culture.

Other kinases are also activated by stretch in neonatal ventricular cardiocytes that are essential for increased ANF gene expression following stimulation by stretch. One of these is the mitogen-activated protein kinases (MAP kinase) (Sadoshima and Izumo, 1993a; Yamazaki *et al.*, 1993). MAP kinases are serine/threonine kinases, and have been found to phosphorylate many nuclear proteins such as c-fos, c-myc, and p62<sup>TCF</sup> (Blenis, 1993; Crews and Erikson, 1993). The c-fos protein has been implicated in the regulation of the ANF and BNP promoters (Kovacic-Milivojevic and Gardner, 1992; McBride *et al.*, 1993; Kovacic-Milivojevic and Gardner, 1993; Grépin *et al.*, 1994), and thus its regulation by MAP kinases may be an important element of the response of the ANF gene to stimuli that activate the MAP kinases. C-myc has been implicated in the hypertrophic and hyperplastic response of the cardiocyte (Jackson *et al.*, 1990; Robbins and Swain, 1992), and as shown here, is induced by stretch in atrial tissue. p62<sup>TCF</sup> is part of the serum response factor (SRF), which is the major component responsible for the activation of the c-fos gene following stretch

(Sadoshima and Izumo, 1993a), and therefore may be an intermediate in the modulation of the ANF or BNP promoters *via fos*.

S6 kinase, also known as pp90<sup>RSK</sup>, is also activated by stretch in neonatal ventricular cardiocytes (Sadoshima and Izumo, 1993a; Yamazaki *et al.*, 1993; Sadoshima *et al.*, 1995). Although it has not been directly connected to changes in natriuretic peptide gene expression, S6 kinase might be involved in lamin phosphorylation, and thus may be involved in the mechanotransduction pathway that leads to increased ANF or BNP mRNA levels. Alternatively, S6 kinase may phosphorylate the serum response factor (SRF) that in turn activates the *c-fos* gene (Sadoshima and Izumo, 1993a), which may subsequently modulate the expression of the ANF or BNP genes (Kovacic-Milivojevic and Gardner, 1992; McBride *et al.*, 1993; Kovacic-Milivojevic and Gardner, 1993; Grépin *et al.*, 1994). S6 kinase may even phosphorylate, and thus activate, the c-fos protein (Chen *et al.*, 1993). Alternatively, since the ANF promoter contains SREs that appear to interact with SRF (Sprenkle *et al.*, 1995), these may be involved in the regulation of the ANF gene following stretch. Definite proof of these possibilities remains to be obtained experimentally.

A parallel to the stretch-stimulated ANF and BNP secretion and BNP gene expression described here can be made with parathyroid hormone-related peptide (PTHrP), a vasorelaxant peptide originally described in tumours associated with hypercalcemia (Suva *et al.*, 1987). PTHrP is synthesized in and secreted from smooth muscle cells, and can be regulated by stretch. In uterus and urinary bladder, sustained stretch *in vivo* or *in vitro* leads to increased PTHrP mRNA levels, following a time course that resembles that of stretch-induced increases in BNP mRNA levels (Daifotis *et al.*, 1992; Yamamoto *et al.*, 1992). Vascular smooth muscle cells also respond to stretch: balloon distension of abdominal aorta

increases PTHrP mRNA levels (Pirola *et al.*, 1994), as does flow-motion stress (Pirola *et al.*, 1994) or cyclical stretch of vascular smooth cell cultures (Noda *et al.*, 1994). Cyclical stretch *in vitro*, while causing a modest increase in PTHrP mRNA levels, significantly increases its secretion (Noda *et al.*, 1994); this is analogous to the initial response to atrial stretch, in which ANF and BNP secretion is increased without a concomitant increase in transcript levels. As for cardiocytes, stretch-induced PTHrP secretion or gene expression is not dependent on  $Gd^{3+}$ -sensitive stretch-activated channels or on  $Ca^{2+}$  influx (Noda *et al.*, 1994). Similarly, the flow-stress induction of PTHrP expression is independent of PKC, but can be partially inhibited by disruption of microtubule assembly by colchicine (Pirola *et al.*, 1994), although this treatment does not affect stretch-induced expression of *c-fos* in cardiocytes (Sadoshima *et al.*, 1992b). It may be of importance that PTHrP is also synthesized and secreted by cardiocytes, is found predominantly in specific atrial granules, and exhibits a tissue-specific pattern of expression that resemble that of ANF (Deftos *et al.*, 1993).

In conclusion, stretch selectively stimulates the expression of the BNP gene in atrial cardiocytes. The lack of an increase in ANF mRNA levels following stretch most likely reflects the lack of requirement for increased ANF synthesis by the atrial cardiocyte following acute stretch. BNP is present in only a portion of granules destined for release; it is possible that the increase in BNP mRNA levels that follow stretch reflects an increase in synthesis necessary to replenish rapidly-depletable granule stores of the peptide, or to increase constitutive secretion of the hormone.

## Natriuretic peptide secretion following ET-1 stimulation

The doses of ET-1 used in this study are higher than the concentrations reported for circulating levels of ET-1 in rat plasma, which are in the  $10^{-12}$  mol/L range (Saito *et al.*, 1989c). Recent studies have shown that low doses ( $10^{-10}$ - $10^{-11}$  mol/L) of ET-1 inhibit ANF secretion from perfused hearts, suggesting that physiological levels of ET-1 do not stimulate ANF release (Shirakami *et al.*, 1993). However, the reported effects of low-dose ET-1 were only studied for a short period of time, during which the inhibitory effect was significant only in the first few minutes. On the other hand, pre-infusion of ET-1 antiserum has been shown to reduce both basal and volume expansion-induced plasma ANF levels (Fyhrquist *et al.*, 1993), suggesting that ET-1 is required to maintain ANF secretion, and to increase its secretion following volume overload. The local cardiac production of ET-1 (Yanagisawa *et al.*, 1988; Eid *et al.*, 1994; Ito *et al.*, 1993; Arai *et al.*, 1995; Brown *et al.*, 1995; Larivière *et al.*, 1995) supports the possibility that under certain pathophysiological conditions, locally produced ET-1 could act directly on the ET receptors of cardiocytes at concentrations that would approach those used in this study. This has recently been supported by reports that show that blocking ET-1 receptors can inhibit stretch-induced ANF release from isolated perfused hearts (Skvorak *et al.*, 1995) or increased ANF plasma levels following volume overload *in vivo* (Leskinen *et al.*, 1995).

The ET-1-stimulated increase in ANF secretion reported here coincides with results obtained in other studies that used isolated atria (Hu *et al.*, 1988; Stasch *et al.*, 1989; Winquist *et al.*, 1989; Schiebinger and Gomez-Sanchez, 1990; de Bold *et al.*, 1991; Schiebinger and Greening, 1992). The increase in release ranges in these studies from 1.5 to 4.5 times basal levels, with the increases generally proportional to the initial preload. ET-1

and stretch have been shown to act in a partly additive fashion in the stimulation of ANF release from perfused hearts (Mäntymaa *et al.*, 1990), perfused atria (Hu *et al.*, 1988; Schiebinger and Greening, 1992), and cultured atrial cardiocytes (Gardner *et al.*, 1991). The data presented here may in fact be more indicative of the actual stimulatory effects of ET on ANF secretion, since the atria are given a smaller preload than that in other studies (Stasch *et al.*, 1989; Schiebinger and Gomez-Sanchez, 1990), resulting in steady basal ANF release rates. In all previous studies, however, stimulation was continued for only a maximum of 45 minutes, therefore not allowing further observation of the pattern of ANF release later in the stimulatory period. In the present study, there was a return towards basal levels of ET-1-stimulated ANF secretion after 1.5 hours of continuous stimulation. Thus, although the stimulation by ET-1 of ANF secretion is more sustained than that of stretch, this process is also temporary. This has been previously observed in cultured neonatal atrial cardiocytes stimulated with ET-1, in which there is a tendency for stimulated ANF secretion to decline during the 2 hour period of stimulation (Sei and Glembofski, 1990).

BNP secretory rates from atrial tissue were rapidly and potently stimulated by ET-1. The pattern of ET-1-stimulated BNP secretion is similar to that of ANF, and calculation of the ratios of irANF to irBNP secreted in response to ET-1 indicate that the two hormones are cosecreted. This suggests that, as with stretch, the population of granules destined for stimulated release contains both hormones, or, alternatively, similar signal transduction processes are responsible for the secretion of both peptides. Studies using cultured neonatal atrial cardiocytes have also shown that ANF and BNP are co-secreted in response to ET-1; it was concluded from these studies that ANF and BNP release from atrial granules was responsible for ET-1-stimulated secretion of both peptides (Suzuki *et al.*, 1992; Horio *et al.*, 1993; Thibault *et al.*, 1994). As with the stretch-induced secretion of ANF and BNP,

monensin has also been shown to decrease ET-1-stimulated secretion of both ANF and BNP (A.J. de Bold, unpublished results), which supports the possibility that ET-1 causes the exocytosis of ANF and BNP contained in granules, and further suggests that the same population of granules is responsible for the ET-1-stimulated secretion of both ANF and BNP. There was, however, a tendency for a larger proportional increase in ANF secretion, suggesting that as with stretch-induced release, monohormonal ANF-containing granules are also recruited by ET-1.

The mechanisms involved in the regulation of ANF or BNP secretion from atrial cardiocytes by ET-1 are not well defined. Specifically, it is not known whether the ET-1-stimulated secretion is exclusively dependent on a regulated pathway of granule exocytosis, or if a constitutive pathway is also a contributing factor. ANF and BNP secretion from ventricular cardiocytes can be stimulated by ET-1 (Shubeita *et al.*, 1990; Horio *et al.*, 1992; Horio *et al.*, 1993; Irons *et al.*, 1993b; Nakagawa *et al.*, 1995). Ventricular cardiocytes do not secrete natriuretic peptides via a dense-core granule-dependent pathway (Bloch *et al.*, 1986; Irons *et al.*, 1993b), but can rapidly increase ANF secretion in response to ET-1, in a manner that resembles regulated secretion (Irons *et al.*, 1993b). However, this type of secretion differs between atrial and ventricular cardiocytes: basal ANF secretion from atrial cardiocytes is associated with long peptide transit times, indicating secretion based on secretion from dense-core granules, whereas secretion from ventricular cardiocytes is associated with a much shorter transit time, which indicates a rapid secretion of newly synthesized peptide (Bloch *et al.*, 1986; Irons *et al.*, 1993b). As mentioned above, atrial cardiocytes exhibit phasic secretion of newly synthesized peptide (Iida and Shibata, 1994), indicating that the distinctions between secretion from atrial and ventricular cardiocytes are not clearly defined.

Most evidence, however, is in favour of a purely granule-mediated regulated pathway of stimulated secretion.

In atrial and ventricular cardiocytes, ET-1 binds to the ET<sub>A</sub> ET receptor subtype (Irons *et al.*, 1993a; Thibault *et al.*, 1994). This receptor is a serpentine, or seven transmembrane spanning domain receptor (Arai *et al.*, 1990), which is normally coupled to GTP-binding and hydrolysing proteins (G-proteins) (Hepler and Gilman, 1992). G-proteins are composed of three subunits designated  $\alpha$ ,  $\beta$ , and  $\gamma$ . The  $\alpha$ -subunits are generally considered to define the post-G-protein interaction (Hepler and Gilman, 1992). There are three families of G-protein  $\alpha$ -subunits; those to which the ET<sub>A</sub> receptor is coupled is of the pertussis toxin-insensitive Gq type, which is in turn coupled to phospholipase C $\beta$  (Hepler and Gilman, 1992). In ventricular cardiocytes, binding of ET-1 to its receptor results in a rapid and transient increase in phosphoinositide hydrolysis (Shubeita *et al.*, 1990; Van Heugten *et al.*, 1993; McDonough *et al.*, 1993). This is accompanied by a more sustained increase in diacylglycerol formation (Shubeita *et al.*, 1990; McDonough *et al.*, 1993). ET-1 has been shown to increase phospholipid hydrolysis in rat atria, thus reflecting activation of phospholipase C (Kuraja *et al.*, 1990). The down-regulation of ET-1-stimulated ANF and BNP secretion might be due to partial homologous desensitization of ET-1 receptor binding and ET-1-stimulated phosphoinositide hydrolysis as observed in cultured adult atrial or neonatal ventricular cardiocytes following 30-45 minutes of stimulation by ET-1 (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Leite *et al.*, 1994). The time courses for receptor binding, ANF secretion, and desensitization parallel each other in adult atrial cardiocytes in culture, indicating that these processes are directly related to one another (Leite *et al.*, 1994).

As with stretch,  $\text{Ca}^{2+}$  influx has been involved in ET-1-stimulated ANF and BNP secretion from atrial cardiocytes. In paced left atria or cultured neonatal atrial cardiocytes, reducing external  $\text{Ca}^{2+}$  concentration from 1.8 to 0.2 mmol/L or lower significantly (but not completely) reduces ET-1-stimulated ANF secretion, as does the addition of voltage-dependent  $\text{Ca}^{2+}$  channel antagonists (Schiebinger and Gomez-Sanchez, 1990; Sei and Glembofski, 1990; Irons *et al.*, 1992; Doubell and Thibault, 1994). However, another report has shown that diltiazem, a blocker of L-type voltage-sensitive  $\text{Ca}^{2+}$  channels, does not affect ET-1-stimulated ANF secretion from neonatal atrial cardiocytes cultured on microcarrier beads (Uusimaa *et al.*, 1992). Chelating external or internal  $\text{Ca}^{2+}$  in cultured neonatal cardiocytes eliminates ET-1-stimulated secretion (Doubell and Thibault, 1994). There appears to be no requirement for mobilization of  $\text{Ca}^{2+}$  for ET-1-stimulated ANF release, since blocking  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum with ryanodine does not affect the secretory response to ET-1 in paced left atria or cultured neonatal atrial cardiocytes (Schiebinger and Gomez-Sanchez, 1990; Uusimaa *et al.*, 1992; McDonough *et al.*, 1994; Doubell and Thibault, 1994). It appears, however, that calmodulin and a calmodulin-dependent kinase are partly required for the effects of ET-1 on ANF secretion (Irons *et al.*, 1992; Gardner *et al.*, 1991), although this has not been confirmed by others (Doubell and Thibault, 1994).

This apparent  $\text{Ca}^{2+}$  requirement may extend to the involvement of PKC as an intracellular mediator of the effects of ET-1. In cultured neonatal or adult atrial cardiocytes, PKC inhibition by H-7 or staurosporine, or desensitization of PKC by pretreatment with phorbol esters, reduces the increased ANF secretion that follows stimulation by ET-1 (Irons *et al.*, 1992; Uusimaa *et al.*, 1992; Leite *et al.*, 1994). However, many PKC isoforms present in cardiocytes are  $\text{Ca}^{2+}$ -independent (Steinberg *et al.*, 1995), which suggests that a

$\text{Ca}^{2+}$ -dependent component, probably mediated by calmodulin, and a  $\text{Ca}^{2+}$ -independent component, mediated by PKC, are required for the stimulation of ANF secretion by ET-1. As for stretch, the developmentally regulated distribution of the various PKC isoforms complicates comparison of the responses to ET-1 of neonatal and adult cardiocytes (Bogoyevitch *et al.*, 1993; Puceat *et al.*, 1994; Clerk *et al.*, 1994).

It remains to be determined if the down-regulation of ANF and BNP secretion is due to the down-regulation of receptor signalling as described for cultured atrial and ventricular cardiocytes (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Leite *et al.*, 1994), or if, as with stretch (Mangat and de Bold, 1993), a sub-population of specific atrial granules is depleted. It is also of interest to determine if it is the same population of granules that responds to stretch and ET-1. Stretch and ET-1 interact in a partly additive fashion to enhance ANF release (Hu *et al.*, 1988; Mäntymaa *et al.*, 1990; Schiebinger and Greening, 1992), suggesting either: 1) the intracellular signalling events initiated by both stimuli are similar and can be enhanced by the combination of the two stimuli; or 2) at least a portion of the hormone secreted in response to either stimulus originates from the same population of granules, and that the rate of exocytosis of this population is not maximally stimulated by either stimulus. Additionally, it has been proposed that ANF can down-regulate its own secretion by an autocrine negative feedback mechanism (Nachshon *et al.*, 1995). A combination of these possible mechanisms is also possible.

In heart failure, there is a marked increase in circulating levels of ET-1 (Shichiri *et al.*, 1990; Stewart *et al.*, 1992; McMurray *et al.*, 1992). Although plasma levels are not elevated in these cases to levels that reach those used in the present experiments, increased receptor density on the cardiocytes (Arai *et al.*, 1995; Brown *et al.*, 1995) and the possibility

of pooling of the peptide bring forth the possibility that the increase in circulating ET-1 contributes to the increase in ANF and BNP secretion that is seen in these conditions. Furthermore, the local increase in ET-1 synthesis and ET<sub>A</sub> receptor synthesis in the heart in experimental overload situations (Ito *et al.*, 1993; Arai *et al.*, 1995; Brown *et al.*, 1995) suggests that a local cardiac ET system is also activated to respond to overload. The natriuretic peptides and ET-1 antagonize each others actions on the vasculature and on the kidneys (Zimmerman *et al.*, 1990; Munger *et al.*, 1991; Ota *et al.*, 1992). Furthermore, the natriuretic peptides directly antagonize ET-1 synthesis and secretion in the vasculature (Kohno *et al.*, 1991; Hu *et al.*, 1992; Kohno *et al.*, 1992b; Kohno *et al.*, 1993). The increase in ANF and BNP secretion in response to ET-1 indicates that there exists a balance between the two systems; this balance may be deregulated in heart failure.

In conclusion, ET-1 stimulates the secretion of ANF and BNP, following a time course that is distinct from that elicited by stretch: the increase in secretion is gradual, reaches a plateau, and after a few hours returns towards basal levels. The pattern is similar for ANF and BNP, which suggests that they are co-secreted in response to this stimulus. The return towards basal levels probably reflects down-regulation of ET-1 receptor signalling.

#### Natriuretic peptide mRNA levels following ET-1 stimulation

ET-1 stimulation of ANF secretion in cultured neonatal atrial cardiocytes, as for stretch in adult atrial tissue, is independent from protein synthesis (Doubell and Thibault, 1994). Therefore it is not surprising that ANF mRNA levels remained constant after 8 hours of continuous ET-1 stimulation. However, increases in ANF mRNA levels following stimulation

with ET-1 have been shown in cultured neonatal atrial cardiocytes after 3 to 24 hours of continuous stimulation (Fukuda *et al.*, 1989; Gardner *et al.*, 1991; Suzuki *et al.*, 1992). It is possible that neonatal atrial cardiocytes in culture respond to ET-1 stimulation differently than their adult counterparts, and that a longer period of stimulation by ET-1 is required to effect significant changes in ANF mRNA levels in adult atrial tissue. This is similar to the response of atrial tissue to stretch, in which secretion is not coupled to increased ANF gene expression. This supports the possibility that both stimuli induce the release of ANF from a pool of granules destined for immediate release, and therefore do not require *de novo* synthesis of ANF peptide. It follows that only after a longer period of time, when the demand for increased secretion is no longer met by the available stores, is the synthesis of ANF increased. Thus, in atrial cardiocytes, acute secretory events, resulting from both mechanical or endocrine stimuli, appear to be independent from changes in ANF gene expression.

ET-1 enhanced the expression of the BNP gene in isolated atria. In the whole atria model, ET-1 modestly and temporarily stimulated BNP gene expression, whereas in the isolated right atria, the increase in BNP mRNA levels was more pronounced and was sustained for the length of the experimental period. These responses are distinct from those observed in cultured neonatal atrial cardiocytes, in which stimulation with a similar dose of ET-1 resulted in only a modest (+33%) increase in BNP mRNA levels after 16 hours of stimulation (Suzuki *et al.*, 1992). The more rapid and (in the case of the right atria) larger increases in BNP gene expression reported here further emphasize the major differences between adult and neonatal cardiocytes. As with stretch, the specific induction of BNP gene expression may be due to differences in the mechanisms of regulation between the ANF and BNP genes, either at the level of the promoter, or at the level of mRNA stability. Experiments

in cultured neonatal ventricular cardiocytes have shown that the increase in BNP mRNA levels by ET-1 is in large part due to increased transcription, and is also dependent on an increase in mRNA stability (Nakagawa *et al.*, 1995). The transcriptional response of the BNP gene to ET-1 in neonatal ventricular cardiocytes is not dependent on protein synthesis (Nakagawa *et al.*, 1995), indicating that it is responsive to alterations in the activities of transcription factors already present in the cell. However, the stability of the BNP mRNA is dependent on protein synthesis (Nakagawa *et al.*, 1995), which suggests that this component of the ET-1-induced response requires increased synthesis of a stabilizing protein.

The rapid ET-1-induced desensitization may explain the temporary nature of ET-1-stimulated ANF and BNP secretion from atrial cardiocytes observed in the present study, but the changes in BNP gene expression, as well as those reported in other studies (Shubeita *et al.*, 1990; Suzuki *et al.*, 1992; Nakagawa *et al.*, 1995), occur at a much later time point, which points to alternate pathways. In ventricular cardiocytes, PKC has been identified as an important intermediate between ET-1 receptor binding and the increase in BNP mRNA levels (Nakagawa *et al.*, 1995). PKC activation is usually the result of the activation of phospholipase C and the subsequent generation of diacylglycerol and IP<sub>3</sub> (Nishizuka, 1992). Diacylglycerol accumulates in two phases: a rapid transitory phase, and a slower more sustained phase (Nishizuka, 1992). This molecule can originate from both phosphatidylinositol-(4,5)bisphosphate, which results from phospholipase C activation, or from phosphatidylcholine, which results from phospholipase D activation; in fact, the sustained increase in diacylglycerol is considered to be mostly derived from phosphatidylcholine (Nishizuka, 1992). ET-1 has been shown to cause an increase in the accumulation of phosphatidic acid in cultured ventricular cardiocytes, which reflects activation of phospholipase D (Ye *et al.*, 1994). This may be related to activation of PKC by

a diacylglycerol molecule that is derived from phosphatidylcholine, instead of from phosphatidylinositol bisphosphate (Leach *et al.*, 1991).  $IP_3$  may contribute to activation of PKC by causing  $Ca^{2+}$  release from intracellular stores (Berridge, 1993). The increase in active  $IP_3$  metabolites is short-lived, while the increase in intracellular diacylglycerol is not (Shubeita *et al.*, 1990; Van Heugten *et al.*, 1993; McDonough *et al.*, 1993), suggesting that the diacylglycerol branch of phospholipase activation may be more important in affecting changes in natriuretic peptide gene expression in cardiocytes. It is possible that partial contribution from both phospholipases C and D results in the sustained increase in diacylglycerol observed after ET-1 stimulation, and may explain how ET-1 can effect changes in BNP gene expression long after its effects on phosphoinositol metabolism have subsided.

As with stretch, ET-1 binding to its receptor in ventricular cardiocytes also activates tyrosine kinases, raf-1 kinase, MAP kinase, and S6 kinase (Sadoshima *et al.*, 1995; Bogoyevitch *et al.*, 1995; Yamazaki *et al.*, 1996). This activation appears to be dependent on  $Ca^{2+}$  influx, and not on PKC. As well, ET-1 stimulation of glomerular mesangial cells results in the activation of ras, and the subsequent induction of *c-fos* via its serum response element (Herman and Simonson, 1995), as has been shown for stretch induced *c-fos* expression (Sadoshima and Izumo, 1993a). This suggests that multiple pathways are initiated by the activation of the  $ET_A$  receptor, and that these pathways are common to the response to both stretch and ET-1. However, it is not clear what the precise long-term effects of each signalling pathways are. Some, such as the short-lived increase in phosphoinositide hydrolysis, may be involved in the regulation of ANF and BNP secretion, or the initiation of longer-acting cascades of intracellular events. Others, such as the ras-mediated, raf-1-mediated, or MAP kinase pathways, might be involved in regulating BNP and early response gene expression. Activation of these pathways by themselves are sufficient to mimic the

effects of ET-1 on cultured neonatal ventricular cardiocytes (Thorburn *et al.*, 1993; Thorburn *et al.*, 1994a; Thorburn *et al.*, 1994b).

ET-1 may act in a paracrine or autocrine manner following stretch of the atria. Recent reports have suggested that paracrine actions of ET-1 in atria mediate stretch-induced ANF secretion and volume-induced increases in ANF plasma levels (Skvorak *et al.*, 1995; Leskinen *et al.*, 1995). Therefore, it is likely that stretch-induced ET-1 release acts on the atrial cardiocytes to modulate ANF and BNP secretion and synthesis. This is supported by the observations in cultured endothelial cells that fluid shear stress stimulates ET-1 production and secretion (Yoshizumi *et al.*, 1989), and that in perfused hearts, increased perfusion pressure or flow results in increased ET-1 release in the venous effluent of the heart (McClellan *et al.*, 1994). In DOCA-salt hypertensive rats, increased ET-1 gene expression has been observed in coronary arteries and in the atrial and ventricular endocardium (Larivière *et al.*, 1995). However, others have shown that fluid shear stress down-regulates ET-1 synthesis and secretion (Malek and Izumo, 1992). It has not been determined if ET-1 synthesis or secretion is similarly affected in endothelial or mesothelial cells of the atrial myocardium, but it is likely that a similar process occurs in these cells. This possibility is supported by the observation that ET-1 released from epicardial mesothelial cells can be regulated by angiotensin II in a manner similar to that found in vascular endothelial cells (Eid *et al.*, 1994).

Additionally, since cardiocytes themselves synthesize and secrete ET-1 in a regulated manner (Ito *et al.*, 1993), it is possible that stretch causes the autocrine or paracrine activation of ET-1. ET-1 mRNA levels in ventricular cardiocytes are increased in hearts that are hypertrophied following suprarenal aortic banding (Arai *et al.*, 1995),

suggesting that this response in fact exists, and thus can be of importance. In the aorto-caval fistula model, which is characterized mainly by volume overload to the right side of the heart, the increase in ET-1 mRNA levels responds earlier and is more pronounced in the right atrium than in any other chamber (Brown *et al.*, 1995). This and other models of volume overload are also characterized by increased ANF and BNP mRNA levels in the atrial chambers (Lattion *et al.*, 1986; Brown *et al.*, 1993; Yokota *et al.*, 1994; Yokota *et al.*, 1995). Importantly, the density of ET-1-specific ET<sub>A</sub> receptors in all cardiac chambers is also increased in both the pressure overload and volume overload models (Arai *et al.*, 1995; Brown *et al.*, 1995), indicating that both components of the ET-1 system are present in cardiocytes and are positively regulated by increased load. Therefore, it is possible that locally synthesized and secreted ET-1 is a crucial initiating factor for the stimulation of ANF or BNP gene expression in overloaded atria. The increase in BNP mRNA levels by ET-1 that is observed in the isolated atria is in keeping with this possibility. However, the increase in BNP mRNA levels was not sustained, which suggests that additional stimuli are required for the maintenance of natriuretic peptide gene expression. The combination of stretch and ET-1 may be required for a more robust and sustained increase in BNP mRNA levels.

In summary, ET-1 stimulation of isolated atria results in a specific increase of BNP mRNA levels. As with stretch, ANF mRNA levels are not affected, suggesting that the increased natriuretic peptide secretion due to ET-1 stimulation is not associated with a synthetic requirement for ANF, but does require a compensatory increase in BNP gene expression.

## Natriuretic peptide secretion following phenylephrine stimulation

ANF secretion was stimulated by the  $\alpha_1$ -adrenergic agonist phenylephrine. Therefore, the results presented here support an important role for  $\alpha_1$ -adrenergic receptors in the control of ANF secretion. The precise adrenergic receptor responsible for stimulated ANF secretion is controversial (Sonnenberg and Veress, 1984; Schiebinger *et al.*, 1987; Schiebinger, 1989; Wong *et al.*, 1988; Agnoletti *et al.*, 1992; Azizi *et al.*, 1993; Onwochei and Rapp, 1988; Hayashi *et al.*, 1988; Ambler and Leite, 1994; Matsubara *et al.*, 1988; Shields and Glembotski, 1989; Sei and Glembotski, 1990), but appears to be the  $\alpha_{1A}$  subtype (Ambler and Leite, 1994). In the present experiments, it is clear that  $\alpha_1$ -adrenergic receptor stimulation can result in a considerable and sustained increase in the secretion of ANF, which suggests that this adrenergic receptor subtype is important in the stimulation of ANF secretion by adrenergic activation.

The return towards basal levels of phenylephrine-stimulated ANF secretion from atrial tissue after 1.5 hours has not previously been observed, since stimulation was only maintained for 15-80 minutes in other studies that used atrial tissue (Schiebinger *et al.*, 1987; Wong *et al.*, 1988; Onwochei and Rapp, 1988). However, a similar trend was seen in cultured neonatal atrial cardiocytes (Sei and Glembotski, 1990). The gradual rise followed by a slow return towards basal levels is similar to the pattern of ANF secretion that follows stimulation by ET-1. This supports the hypothesis that these agonists stimulate ANF secretion through similar intracellular signalling cascades (De Jonge *et al.*, 1995), and further suggests that the secretory response of atrial cardiocytes to phenylephrine is subject to down-regulation in a manner similar to that observed for ET-1 (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Ambler and Leite, 1994; Leite *et al.*, 1994). The increase in  $IP_3$  in

atrial tissues due to stimulation by norepinephrine follows the same transient pattern as the one stimulated by ET-1 (Kuraja *et al.*, 1990). However, it has been shown that receptor density and the stimulation of intracellular signalling cascades following occupancy of  $\alpha_1$ -adrenergic receptors on atrial or ventricular cardiocytes, unlike that elicited by ET-1, is not subject to down-regulation (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Ambler and Leite, 1994). Therefore, the down-regulation observed may be due to other mechanisms. It is also possible that ANF down-regulates its own secretion, through an autocrine negative feedback mechanism (Nachshon *et al.*, 1995).

The stimulatory effects of  $\alpha_1$ -adrenergic receptor activation on ANF secretion from atrial cardiocytes is thought to be mediated primarily through the activation of phospholipase C, and the subsequent generation of  $IP_3$  and diacylglycerol, and possibly via a PKC-dependent pathway. In all models studied, stimulation of ANF secretion by  $\alpha_1$ -adrenergic agonists depends on influx of extracellular  $Ca^{2+}$  (Sei and Glembofski, 1990; Schiebinger *et al.*, 1992; Ambler and Leite, 1994). In isolated atria, initiation, but not maintenance, of phenylephrine-induced ANF secretion depends on extracellular  $Ca^{2+}$  influx, and is independent from a ryanodine-sensitive intracellular  $Ca^{2+}$  compartment (Schiebinger *et al.*, 1992). In cultured neonatal atrial cardiocytes, blocking either extracellular  $Ca^{2+}$  influx and ryanodine-sensitive channels partially inhibited phenylephrine-induced ANF secretion (Sei and Glembofski, 1990). In cultured adult atrial cardiocytes, a similar dependency on extracellular and intracellular  $Ca^{2+}$  can be observed (Ambler and Leite, 1994); in addition, the  $\alpha_1$ -adrenergic stimulated ANF secretion in this model partially relies on calmodulin-dependent processes. The involvement of a calmodulin-dependent kinase in the modulation of ANF secretion by ET-1 has also been shown for cultured neonatal atrial cardiocytes (Irons *et al.*, 1992). In addition to calmodulin-dependent kinase, it appears that a PKC-dependent

pathway may be involved; in both neonatal and adult cultured atrial cardiocytes, inhibition or down-regulation of PKC inhibits  $\alpha_1$ -adrenergic stimulation of ANF secretion (Sei and Gliembotski, 1990; Ambler and Leite, 1994).

It has not been determined if the increased ANF secretion following stimulation by  $\alpha_1$ -adrenergic stimulation is due to granule exocytosis. However, most evidence suggests that this is the pathway most often used for ANF secretion. Stretch-induced ANF secretion relies on a rapidly-depletable pool of newly synthesized peptide (Mangat and de Bold, 1993); the depletion of a discrete ANF storage pool or the selective down-regulation of its recruitment may contribute to the return to basal levels observed following stimulation with phenylephrine. Therefore, the down-regulation of ANF secretion after its stimulation by phenylephrine may be due to this mechanism, and not to the desensitization of receptor signalling. This raises the intriguing possibility that the down-regulation of ANF and BNP secretion following ET-1 stimulation is also due to the depletion of a pool of peptide destined for immediate release. The nature of the pool of ANF in this potential subset of secretory granules has not been characterized.

BNP secretion was also stimulated by phenylephrine. The increase in BNP secretion was slightly more rapid than that of ANF, and remained significantly higher than that from vehicle-perfused atria throughout the experimental period (8 hours). Calculation of the ratios of irANF to irBNP secreted following addition of phenylephrine shows that the stimulated secretion of these peptides was not coordinately regulated. It is not clear at this point whether differences in the kinetics of stimulated ANF and BNP secretion reflect different secretion pathways or regulation by alternate intracellular signalling cascades. Whereas ANF secretion from atrial cardiocytes is thought to function exclusively through a regulated

pathway (Bloch *et al.*, 1986; Page *et al.*, 1991; De Young *et al.*, 1994), BNP secretion may occur through both a granule-dependent pathway (Thibault *et al.*, 1992) and a constitutive pathway that does not rely on packaging in dense-core granules, as is found for ANF in cultured neonatal ventricular cardiocytes (Bloch *et al.*, 1986). As discussed above, there are profound differences between the proportional amounts of ANF and BNP associated with specific atrial granules (Hasegawa *et al.*, 1991; Kuroski-de Bold *et al.*, 1992; Yokota *et al.*, 1995). It has been shown in cultured adult atrial cardiocytes that basal ANF secretion might be dependent on a secretory mechanism that shares features of both constitutive and regulated secretion (Iida and Shibata, 1994). It is possible that the so-called constitutive-like pathway of secretion (Arvan *et al.*, 1991; Kuliawat and Arvan, 1992), involving immature granules, may be partly responsible for the increase in ANF and BNP secretion. Therefore, since BNP is co-stored with ANF in a subset of secretory granules (Hasegawa *et al.*, 1991; Thibault *et al.*, 1992; Kuroski-de Bold *et al.*, 1992), it is possible that these granules, in their immature stage, can be recruited for stimulated secretion, resulting initially in a greater proportion of BNP released compared to ANF, and subsequently in a more sustained increase in BNP secretion.

Increased sympathetic activity accompanies hemodynamic disturbances. In experimental models in which ANF and BNP are elevated, such as DOCA-salt hypertensive rats, or rapid pacing in dogs, there is a marked increase in circulating norepinephrine (Armstrong *et al.*, 1986; Eid and de Champlain, 1988; Moe *et al.*, 1989). Sustained elevation in sympathetic activity can also be observed in patients with heart failure (Dzau, 1988; Riegger, 1994). Therefore, in addition to the pressor effects of the activation of adrenergic receptors in the heart and in the vasculature, there is a compensation via the  $\alpha_1$ -adrenergic-mediated increase in ANF and BNP secretion. The actions of ANF and BNP generally

antagonize those of the sympathetic nervous system (Zukowska-Grojec *et al.*, 1986; Volpe *et al.*, 1986; Holtz *et al.*, 1987; Kuchel *et al.*, 1987; Genovesi *et al.*, 1990; Awazu *et al.*, 1991; Morita *et al.*, 1989). It follows that in decompensated heart failure, the increase in ANF and BNP secretion can no longer counter the deleterious effects of the sympathetic adrenergic system, and of the actions of angiotensin, aldosterone, and ET-1.

In summary, stimulation of  $\alpha_1$ -adrenergic receptors on atrial cardiocytes by phenylephrine stimulates the secretion of ANF and BNP. Unlike that seen with stretch and ET-1, the stimulated secretion of the two hormones is not co-regulated. This implies that different granule populations or pathways of secretion are recruited in the atrial cardiocyte by stimulation with phenylephrine.

#### Natriuretic peptide mRNA levels following phenylephrine stimulation

ANF and BNP mRNA levels were increased following 6 hours of continuous stimulation by phenylephrine. Such changes in atrial ANF and BNP gene expression, especially the robust and sustained increase in BNP mRNA levels, strongly support the possibility of the involvement of  $\alpha_1$ -adrenergic stimulation in the response of the endocrine heart to hemodynamic challenge. In the low-renin DOCA-salt rat model of hypertension, for example, in which increased atrial expression of ANF and BNP is observed (Lattion *et al.*, 1986; Yokota *et al.*, 1995), the changes may be due to increased circulating catecholamines or increased catecholamine turnover, which are characteristics of this model (Eid and de Champlain, 1988). In this and other models, the secretion of ANF and BNP is sustained, as reflected by the persistent increase in plasma levels, and their actions are sustained, as

reflected by an increase in urinary cGMP and the fact that blockade of natriuretic peptide receptors results in further increases in mean arterial pressure (Hirata *et al.*, 1994; Yokota *et al.*, 1994). Increased atrial BNP mRNA levels have been observed in response to phenylephrine infusion in Wistar-Kyoto and spontaneously hypertensive rats (Magga *et al.*, 1994), and although this was partly attributed to the hemodynamic effects of phenylephrine, direct stimulation of  $\alpha_1$ -adrenergic receptors in atrial cardiocytes might have at least partially mediated the response. The results presented here support this possibility. The increases in ANF and BNP mRNA levels are also consistent with observations made in cultured neonatal ventricular cardiocytes (Knowlton *et al.*, 1991; Hanford *et al.*, 1994), and suggest that sustained increases in ANF and BNP secretion from both cell types may be dependent on increased gene expression.

It has been shown in cultured cardiocytes that  $\alpha_1$ -adrenergic stimulation directly stimulates natriuretic peptide secretion or gene expression independent from contraction (Matsubara *et al.*, 1988; Sei and Glembofski, 1990; Knowlton *et al.*, 1991; Ambler and Leite, 1994; Hanford *et al.*, 1994). Similarly, it is unlikely that the changes in ANF and BNP secretion and gene expression presented here are due to the effects of phenylephrine on atrial mechanical activity because of the following observations: First, at the doses used here, there is maximal inotropic and chronotropic stimulation by phenylephrine, whereas the changes in natriuretic peptide secretion respond maximally to only the higher doses. Second, the response of contractile activity to phenylephrine is sustained throughout the period of stimulation, while that of ANF secretion decays over time. Third, although it abolished changes in the inotropic response, secretion, and gene expression, prazosin did not prevent the phenylephrine-induced chronotropy, which proves that increased rate of contraction is unrelated to increased natriuretic peptide secretion or gene expression. This

is consistent with previous findings that suggest that the effects of phenylephrine on the sino-atrial node are mediated by a receptor subtype that is distinct from the  $\alpha_1$ -subtype responsible for its inotropic actions (Jahnel *et al.*, 1994).

The discoordinate increase in natriuretic peptide secretion and gene expression observed here suggests that, as with stretch and ET-1, these processes are not directly related in an acute setting. Instead, the increase in mRNA levels for the natriuretic peptides may be related to the beginning of a chronic phase of increased natriuretic peptide secretion that is dependent on synthesis, as is observed in cultured neonatal ventricular cardiocytes (Knowlton *et al.*, 1991; Hanford *et al.*, 1994). The effects of phenylephrine on transcriptional responses in ventricular cardiocytes are effected through activation of the  $\alpha_{1A}$ -adrenergic receptor (Knowlton *et al.*, 1993), leading to an intracellular signalling cascade that involves phosphoinositide hydrolysis via phospholipase C activation (Van Heugten *et al.*, 1993; McDonough *et al.*, 1993; Knowlton *et al.*, 1993). This is subsequently followed by activation of PKC, a calmodulin-dependent kinase, and mitogen activated protein kinases (De Jonge *et al.*, 1995). The intracellular mechanisms leading to such changes in atrial cardiocytes have not been fully characterized, but it is known that  $\alpha_1$ -adrenergic-mediated ANF secretion from adult cultured atrial cardiocytes is also effected through the  $\alpha_{1A}$ -adrenergic receptor subtype, and is dependent on PKC- and calmodulin kinase-dependent pathways (Ambler and Leite, 1994), suggesting that the changes in ANF and BNP secretion and mRNA levels observed in the present study are due to related intracellular signalling pathways. Therefore, the divergence in the time course of secretion and gene expression probably reflects the different effects of events downstream of phospholipase C activation, such as the activation of various PKC isoforms (Steinberg *et al.*, 1995), or alternate signalling pathways that are

effective in stimulating ANF gene expression in cultured ventricular cardiocytes, such as ras- or tyrosine kinase-dependent pathways (LaMorte *et al.*, 1994; Thorburn and Thorburn, 1994).

While PKC may be important for the activation of ANF gene expression, it appears to be only partly responsible for the transduction of the pathways leading to this event. The involvement of a  $\text{Ca}^{2+}$ -dependent calmodulin (CaM) kinase in the transduction of phenylephrine stimulation of cardiocytes to increased ANF gene expression has been suggested (Sei *et al.*, 1991). It is suggested that this pathway is essential for increases in ANF gene expression, and that it is enhanced by the PKC-mediated pathway. The apparent requirement for calcium in the phenylephrine- or ET-1-induced increases in ANF and BNP gene expression may reflect this pathway, and not the PKC-mediated pathway, since it appears that mostly  $\text{Ca}^{2+}$ -independent PKC species are involved in this response. Also, stimulation of MAP kinase and S6 kinase in cultured ventricular cardiocytes is independent from PKC activation (Sadoshima *et al.*, 1995), which suggests a possible pathway for CaM kinases. Overexpression of calmodulin in the cardiocytes of transgenic mice results in cardiocyte hypertrophy and hyperplasia, indicating that this pathway by itself can affect pronounced changes in cardiac growth (Gruver *et al.*, 1993). The expression of the ANF gene in the ventricles of these animals was slightly increased, suggesting that calmodulin overexpression in itself can stimulate ANF gene expression. The relative levels of ANF in the atria, which had the highest degree of hypertrophy, were not examined. The results of these experiments suggests that a calmodulin-dependent pathway is important for increased cardiocyte growth and gene expression. It is still unknown how CaM kinase may contribute to increased ANF gene expression.

It has recently been shown that  $\alpha_1$ -adrenergic stimulation of cultured neonatal ventricular cardiocytes increases tyrosine phosphorylation, and that this effect can be blocked by the tyrosine kinase inhibitor genistein (Thorburn and Thorburn, 1994). Genistein can also inhibit phenylephrine-induced increases in ANF promoter activity (Thorburn and Thorburn, 1994). As well, MAP kinase activation by phenylephrine (but not that elicited by stretch) can be inhibited by genistein (Yamazaki *et al.*, 1993; Thorburn and Thorburn, 1994). The activation of a tyrosine kinase by a G-protein-coupled receptor occurs probably *via* cross-talk from one of the pathways initially activated by agonist stimulation. For example, it is known that MEK phosphorylates MAP kinases on tyrosine and threonine residues (Blenis, 1993; Crews and Erikson, 1993), and evidence for this has been obtained in cardiocytes (Sadoshima *et al.*, 1995). Activation of the MAP kinases has also been found to be essential for the increase in ANF mRNA levels observed following stimulation of cultured neonatal ventricular cardiocytes by phenylephrine (Thorburn *et al.*, 1994a). Therefore, a MAP kinase pathway is partly responsible for the transduction of phenylephrine receptor binding to changes in cardiocyte gene expression, and this may be modulated in part by PKC and tyrosine kinases.

Tyrosine kinases are generally coupled to the guanine nucleotide-binding protein p21<sup>ras</sup>, also simply known as ras. Activated ras by itself can induce increases in ANF gene expression in cultured neonatal ventricular cardiocytes (Thorburn *et al.*, 1993). A dependence on ras for MAP kinase activation and ANF transcriptional activation by phenylephrine has been demonstrated in the same model (LaMorte *et al.*, 1994; Thorburn, 1994). Although G<sub>αq</sub> is not normally coupled to ras, the activation of ras may occur through the βγ subunits of the heterotrimeric G-protein instead of *via* the α subunit (Hepler and Gilman, 1992). Alternatively, ras activation may be independent from tyrosine

phosphorylation, and may instead occur as a result of PKC action (Hirai *et al.*, 1994). PKC activation of ras is not sufficient for ras activation of AP-1/Jun, although ras is an essential component of this event (Hirai *et al.*, 1994), suggesting that ras-dependent pathways require additional pathways downstream of PKC to affect nuclear events. Whatever the precise mechanism of its actions may be, it remains that activation of ras is an essential component of changes in ANF gene expression due to phenylephrine and possibly other  $G_q$ -linked agonists such as ET-1.

Ras acts directly on the serine/threonine kinase Raf-1, for which the only known substrate is MEK (Daum *et al.*, 1994). A requirement for raf-1 exists for phenylephrine-stimulated MAP kinase activation and increased ANF gene expression in cultured neonatal ventricular cardiocytes (Thorburn *et al.*, 1994b), indicating that the ras-raf-MEK-MAP kinase pathway is essential for at least a portion of the transcriptional activation effected by G-protein-coupled agonists in cardiocytes. The activation of raf-1 may be directly downstream of ras, but there is evidence that PKC can directly activate raf-1 (Daum *et al.*, 1994). The possible downstream actions of raf-1 are also unclear. It is known that raf-1 is necessary for MAP kinase activation and ANF transcriptional activity by phenylephrine in cardiocytes, but raf-1 cannot effect the morphological changes associated with phenylephrine stimulation (Thorburn *et al.*, 1994b). It is also unknown whether the activation of raf-1 occurs for all  $G_{\alpha q}$ -coupled receptors, although it has also been shown for stimulation by ET-1 (Bogoyevitch *et al.*, 1995). The activation of raf-1 in cultured neonatal ventricular cardiocytes is less marked following stimulation by phenylephrine than following stimulation by ET-1 (Bogoyevitch *et al.*, 1995); this may be partly related to the differences in the response of the BNP gene following these two stimuli.

The signalling pathways responsible for changes in natriuretic peptide gene expression and secretion are similar to those effected by ET-1. The convergence of similar pathways resulting from stimulation of distinct receptors on natriuretic peptide secretion and gene expression is particularly relevant, considering that the effects of ET-1 and phenylephrine on the endocrine heart are distinct; the sustained increase in BNP secretion that is observed only with phenylephrine stimulation suggests that this is a specific response that is not readily down-regulated, and therefore may be physiologically important, especially considering that BNP has a longer plasma half-life than ANF (Kita *et al.*, 1991). This also becomes relevant when comparing the patterns of ET-1 and catecholamine levels in plasma; these do not change in unison in experimental models such as DOCA-salt hypertension (Eid and de Champlain, 1988; Nguyen *et al.*, 1992) or in patients with congestive heart failure (Stewart *et al.*, 1992).

It is less clear whether the phenylephrine-stimulated signalling events converge on the same nuclear factors that result in increased ANF or BNP gene expression. As discussed for stretch and ET-1, the ANF and BNP genes may be under similar transcriptional control by phenylephrine, especially since they have regions of similarity in the promoter regions that respond to  $\alpha_1$ -adrenergic stimulation. Both have functional GATA, Egr-1, and AP-1 motifs that may be involved in this response (Knowlton *et al.*, 1991; McBride *et al.*, 1993; Grépin *et al.*, 1994; Thuerauf *et al.*, 1994), although distinct phenylephrine response elements have been characterized in the ANF promoter (Ardati and Nemer, 1993; Sprenkle *et al.*, 1995), and the AP-1 site of the ANF promoter does not seem to be directly involved in mediating  $\alpha_1$ -adrenergic stimulation (McBride *et al.*, 1993; Sprenkle *et al.*, 1995). In fact, there is evidence that in neonatal ventricular cardiocytes in culture, BNP mRNA levels are partly regulated by phenylephrine at the level of mRNA stability (Hanford *et al.*, 1994) as

well as at the transcriptional level (Thuerlauf *et al.*, 1994; Hanford *et al.*, 1994), whereas ANF mRNA levels appear to be regulated exclusively at the transcriptional level (Knowlton *et al.*, 1991; McBride *et al.*, 1993; Ardati and Nemer, 1993; Hanford *et al.*, 1994). Whether this is the case in adult atrial cardiocytes remains to be determined. The increased expression of *Egr-1* observed in response to phenylephrine suggests that this transcription factor may be at least partially involved in the changes in natriuretic peptide gene expression observed here.

In summary, phenylephrine stimulates the expression of the ANF and BNP genes in isolated atrial tissue. The increase in BNP mRNA levels is more pronounced than that of ANF, and unlike ANF mRNA levels, it is sustained throughout the period of stimulation. The increase in BNP mRNA levels has a slower onset than that elicited by stretch or ET-1; this may be related to the different pattern of BNP secretion, further suggesting that the increase in BNP secretion is not regulated by phenylephrine in the same manner as by stretch or ET-1.

#### Early response gene expression

The regulation of transcription of cardiac genes, including the ANF and BNP genes, is thought to be partly mediated by Fos, Jun, c-myc, and *Egr-1* (Knowlton *et al.*, 1991; Kovacic-Milivojevic and Gardner, 1992; McBride *et al.*, 1993; Kovacic-Milivojevic and Gardner, 1993; Thuerlauf *et al.*, 1994). Therefore, the characterization of the expression of early response genes following stimulation of atrial tissue by stretch, ET-1, and phenylephrine is an important first step in determining the pathways that lead to changes in ANF and BNP gene expression. In the present work, stretch increased mRNA levels of the early response

genes *c-fos*, *Egr-1*, and *c-myc*. ET-1 and phenylephrine enhanced the expression of *Egr-1* and *c-myc*, following stimulus-specific temporal patterns of expression. This response reveals distinct and specific modes of actions for mechanical and neuroendocrine stimuli on atrial gene expression.

The induction of *c-fos*, *Egr-1*, and *c-myc* by mechanical loading has been previously observed in ventricular cardiocytes (Izumo *et al.*, 1988; Komuro *et al.*, 1988; Komuro *et al.*, 1990; Komuro *et al.*, 1991; Schunkert *et al.*, 1991; Rockman *et al.*, 1991; Sadoshima *et al.*, 1992a; Kolbeck-Ruhmkorff *et al.*, 1993); it is evident from the present study that the same is true for atrial tissue subjected to a similar stress. The time course and degree of stimulation of *c-fos* and *c-myc* in atrial is similar to that observed in *in vitro* volume overloaded ventricles (Schunkert *et al.*, 1991; Kolbeck-Ruhmkorff *et al.*, 1993), and in *in vivo* pressure overloaded ventricles (Izumo *et al.*, 1988; Komuro *et al.*, 1988). In cultured ventricular cardiocytes, the peak response of *c-fos*, *c-jun*, and *Egr-1* occurs after 30 minutes of continuous stretch (Komuro *et al.*, 1990; Sadoshima *et al.*, 1992a), while that of *c-myc* peaks at 1 hour, and remains elevated until 2 hours of stretch (Sadoshima *et al.*, 1992a). In perfused ventricles, volume overload-induced *c-fos*, *c-jun*, and *c-myc* expression is maximal after 1 hour, and remains elevated for 1.5 hours (Schunkert *et al.*, 1991; Kolbeck-Ruhmkorff *et al.*, 1993). Pressure overload *in vivo* in rats requires a longer time for peak expression of *c-fos* and *c-myc*, ranging from 2 to 8 hours (Komuro *et al.*, 1988; Izumo *et al.*, 1988); a model of transverse aortic coarctation in mice results in a quicker response, probably due to the severity of the imposed load (Rockman *et al.*, 1991). This indicates that the temporal response of the early response genes is variable from one model to another, and appears to depend on the severity of the load. The nomenclature "early response gene" was originally derived from the observation in cultures cells that these genes responded with a rapid and

transient increase in transcript levels. It is clear from the present experiments and from some *in vivo* studies (Komuro *et al.*, 1988; Izumo *et al.*, 1988) that in fact *Egr-1* and *c-myc* do not respond as early response genes.

The response observed in atrial tissue is similar to that seen in ventricular cardiocytes, except for the lack of response of *c-jun*. This marked difference between atrial and ventricular tissues reflects the pronounced differences in phenotype between the two cell types (Chien *et al.*, 1993), and suggests that differences such as these are responsible for the variation in the responses to a similar stimulus of one tissue compared to the other. This difference extends to the regulation of cardiac gene expression by the protein products of these cellular oncogenes. In particular, the modulation of ANF promoter activity by *fos*, *jun*, and their related transcription factors is markedly different between atrial and ventricular cardiocytes (Kovacic-Milivojevic and Gardner, 1992; Kovacic-Milivojevic and Gardner, 1993; Kovacic-Milivojevic and Gardner, 1995).

The increased expression of the early response genes following stretch can also be observed in other cell types. In cardiac non-myocytes in culture, static stretch induces *c-fos* (Sadoshima *et al.*, 1992a). This is similar to the reported localization of *c-myc* in cardiac non-myocytes following pressure overload of ventricular tissue (Snoeckx *et al.*, 1991; Hannan *et al.*, 1994). The *c-fos* and *Egr-1* genes are induced by cyclical stretch of cultured rat mesangial cells (Akai *et al.*, 1994). As with cardiocytes, the stretch-induced early response gene expression in mesangial cells is related to activation of PKC, which suggests that stretch induces similar intracellular signalling cascades in both cell types. Furthermore, it suggests that mechanical stimuli activate transduction and transcriptional mechanisms that are common to most cell types.

The induction of *c-fos* and *Egr-1* by ET-1 has been shown in cultured ventricular cardiocytes (Shubeita *et al.*, 1990; Jones *et al.*, 1992; Neyses *et al.*, 1993). The time course of induction of these genes by ET-1 ranges from 15 minutes in cultured neonatal or adult rat ventricular cardiocytes (Ito *et al.*, 1991; Neyses *et al.*, 1993) to 2 hours in adult feline ventricular cardiocytes (Jones *et al.*, 1992). The longer response lag observed in atrial tissue may be related to differences in the models used, in a manner similar to that observed with the response to stretch. The responses of *c-jun* or *c-myc* to ET-1 have not been studied in cardiocytes; therefore, it is not known whether the response observed in atrial tissue is similar to that of ventricular cardiocytes. The induction of *c-myc* is unique in its time course, which is characterized by a long onset, reaching statistically significant levels after 8 hours of stimulation. This is in contrast with BNP and *Egr-1*, the expression of which peaks at 4 or 6 hours and thereafter declines. The specific time course of *c-myc* expression may be due to signalling pathways that converge on this gene and not on the others; it is known that MAP kinases, which are activated by ET-1 in cardiocytes, can activate *c-myc* directly (Blenis, 1993; Crews and Erikson, 1993). Alternatively, the signalling cascade activated by ET-1 may activate a transcription factor that autoregulates itself, thus maintaining its activity for a longer period of time than that elicited by ET-1 receptor signalling.

The increased expression of *Egr-1* and *c-myc* observed in response to phenylephrine is similar to observations in ventricular cardiocytes, in which expression of these two early response genes is increased in response to  $\alpha$  (but not  $\beta$ ) adrenergic stimulation. Stimulation of neonatal ventricular cardiocytes in culture by norepinephrine leads to an increase in *c-myc* expression after 30 minutes of stimulation, which peaks at 1 hour, and thereafter decreases to control levels (Starksen *et al.*, 1986). This response can be abolished by inhibitors of the  $\alpha$  subtype of adrenergic receptors, but not those specific

for  $\beta$ -adrenergic receptors. A similar response to norepinephrine is found in adult ventricular cardiocytes (Ikeda *et al.*, 1991) and in the ventricles of perfused hearts (Kolbeck-Ruhmkorff *et al.*, 1993). *C-myc* can also be induced by phenylephrine in arrested isolated rat ventricles (Moalic *et al.*, 1992). In neonatal ventricular cardiocyte cultures, *Egr-1* is also induced by norepinephrine following a time course similar to that of *c-myc*, and is dependent solely on  $\alpha$ -adrenergic receptor activation (Iwaki *et al.*, 1990). *In vivo*, infusion of  $\alpha$ -adrenergic agonists, but not  $\beta$ -adrenergic agonists, results in activation of *Egr-1* (Brand *et al.*, 1993); this could be attributed to effects on the vasculature or on the mechanical properties of the cardiocytes that are characteristic of each type of agonist, but it is more likely that the effects are those of direct stimulation of the adrenergic receptors on the cardiocytes. The specific responses of *c-myc* and *Egr-1* to  $\alpha$ -adrenergic stimulation contrasts with those of *c-fos* and *c-jun* in similar experiments, which respond to both  $\alpha$  and  $\beta$  agonists (Iwaki *et al.*, 1990; Moalic *et al.*, 1992; Brand *et al.*, 1993). Therefore, the transcriptional response of atrial tissue to  $\alpha_1$ -adrenergic stimulation is similar to that observed in ventricular cardiocytes. However, the time course of induction in atria as compared with ventricles is slower, which indicates that the response of the atrial cells is different from that of their ventricular counterparts. The increases in *c-myc* and *Egr-1* mRNA levels are also consistent with their marked induction in atria by ET-1, which shares common signalling pathways with phenylephrine (De Jonge *et al.*, 1995).

The relationship between ANF gene expression and *c-fos* and *c-jun* expression is still unclear. *In vitro* studies involving cultured neonatal atrial or ventricular cardiocytes transfected with *c-fos* and *c-jun* expression constructs suggest that low levels of Fos/Jun activity tend to increase ANF transcription, but that higher levels of this transcription factor inhibit ANF promoter activity (Kovacic-Milivojevic and Gardner, 1992; McBride *et al.*, 1993;

Kovacic-Milivojevic and Gardner, 1993). These reports are consistent with observations that increases in ANF gene expression both *in vivo* and *in vitro* generally occur after early response gene expression has returned to basal levels (Izumo *et al.*, 1988; Rockman *et al.*, 1991; Sadoshima *et al.*, 1992a). In the present study, *c-fos* and *c-jun* mRNA levels were elevated, compared to *in vivo* levels, in all isolated atrial tissues, whether they were stimulated or not. This may be related to the sensitivity of these genes to tissue manipulation and cellular stress: it has been shown that in perfused rat ventricles, *c-fos* and *c-jun* induction occurred with perfusion alone, even without additional myocyte stretch (Schunkert *et al.*, 1991; Kolbeck-Ruhmkorff *et al.*, 1993) suggesting that the stress of perfusion was sufficient to induce the expression of these early response genes. In the isolated whole atria, two hours of stretch stimulated *c-fos* expression further, indicating that the expression of this gene was not maximally stimulated by tissue manipulation or by the basal load. The persistent expression of these genes throughout the duration of these experiments may prevent or reduce increases in ANF mRNA levels by stretch, ET-1, or phenylephrine, in a manner similar to that observed in the transfection studies discussed above (Kovacic-Milivojevic and Gardner, 1992; McBride *et al.*, 1993; Kovacic-Milivojevic and Gardner, 1993). The regulation of the BNP gene by the AP-1 complex is not yet clear (Thuerlauf *et al.*, 1994; Grépin *et al.*, 1994); it is therefore difficult to relate *c-fos* or *c-jun* expression to the response of the BNP gene. However, the increase in BNP mRNA levels in response to stretch, ET-1, and phenylephrine suggests that *fos* or *jun* do not significantly inhibit BNP gene expression.

*Egr-1* appears to have an important role in ET-1-mediated stimulation of cardiocytes. Recent studies have shown that ET-1-stimulated protein synthesis in ventricular cardiocytes can be blocked by the introduction of antisense *Egr-1* oligonucleotides (Neyses

*et al.*, 1991). ET-1 has also been shown to increase myosin heavy chain (MHC) mRNA levels in cultured ventricular cardiocytes (Wang *et al.*, 1992), and *Egr-1* directly modulates  $\alpha$ -MHC transcription (Gupta *et al.*, 1991), thus suggesting that *Egr-1* links ET-1 stimulation to changes in  $\alpha$ -MHC gene expression. The presence of an *Egr-1* binding site in the upstream region of the ANF and BNP genes (Knowlton *et al.*, 1991; Thuerlauf *et al.*, 1994) raises the possibility of *Egr-1*-mediated ANF and BNP gene expression control, although the functionality of these binding site has not yet been assessed. Taken together, these observations indicate that induction of *Egr-1* is an important component of the response of cardiocytes to stimulation by ET-1. Since *Egr-1* expression is also stimulated by stretch and phenylephrine, it is likely that this gene also plays an important role in the response of atrial cardiocytes to these stimuli.

*C-myc* is developmentally regulated in cardiocytes, and is thought to be involved in the regulation of cardiocyte proliferation during development (Jackson *et al.*, 1990). Adult cardiocytes lose the capability to proliferate; it is unclear therefore what the role of the *myc* protein would be in these cells. The DNA-binding site of *c-myc* is similar to that of the myogenic regulatory protein MyoD (Blackwood and Eisenman, 1991; Marcu *et al.*, 1992; Kretzner *et al.*, 1992), which suggests that *c-myc* may be involved in regulating the transcription of muscle-specific genes in the cardiocytes. However, no experimental data exists to support this possibility. *C-myc* overexpression in transgenic mice leads to cardiocyte proliferation and enlargement (Jackson *et al.*, 1990), and has been shown to exaggerate the increase in cardiac mass due to thyroid hormone infusion, suggesting that its expression modulates the hypertrophic response of cardiac myocytes (Robbins and Swain, 1992); *c-myc* may perform similar functions in overloaded atrial tissue.

It is not possible from the present data to determine the cell type that expresses of the early response genes. However, sufficient evidence exists to support the possibility of cardiocyte expression of these genes. In perfused ventricle (Schunkert *et al.*, 1991) and cultured neonatal ventricular cardiocytes (Sadoshima *et al.*, 1992a), stretch-induced Fos expression was localized to the cardiocytes by immunofluorescence. Furthermore, all studies using cultured cardiocytes unequivocally demonstrate that the expression of the early response genes following mechanical or neuroendocrine stimulation can be stimulated directly in the cardiocytes (Starksen *et al.*, 1986; Shubeita *et al.*, 1990; Ikeda *et al.*, 1991; Komuro *et al.*, 1991; Sadoshima *et al.*, 1992a; Jones *et al.*, 1992; Neyses *et al.*, 1993). In hearts of animals infused with isoproterenol, strong immunoreactivity for c-fos was reported in atrial and ventricular cardiocytes, as well as in non-myocytes (Brand *et al.*, 1993). Following pressure overload *in vivo*, it has been reported that the myc protein localizes predominantly to vascular endothelial cells in the ventricles (Snoeckx *et al.*, 1991). A subsequent study using a similar model showed that *c-myc* expression could be localized to the nuclei of cardiocytes (Pollack *et al.*, 1994); the differences between the two reports were ascribed to the previous study's limitation to early time points (Snoeckx *et al.*, 1991; Pollack *et al.*, 1994). In animals treated with norepinephrine for 2 hours, myc immunoreactivity was located predominantly in non-myocytes of ventricular tissue; however, although *c-myc* mRNA levels were increased in atrial tissues, no myc immunoreactivity was detected in this tissue (Hannan *et al.*, 1994). These discrepancies may also be due to the very limited period of time studied. Therefore, it is likely that the atrial cardiocytes express these genes in response to stretch or ET-1; non-myocytes may also contribute to the observed expression.

In conclusion, mechanical and receptor-mediated stimulation of isolated atrial tissue result in the enhanced expression of several early response genes. Stretch-induced expression of *c-fos*, *Egr-1*, and *c-myc* is similar to that observed in ventricular cardiocytes, but the lack of induction of *c-jun* distinguishes this response from the ventricular cells. The specific induction of *Egr-1* and *c-myc* by ET-1 delineates a marked difference in the transcriptional response of atrial tissue to mechanical and neuroendocrine stimuli. The expression of *c-fos*, *Egr-1*, and *c-myc* in response to stretch, ET-1, and phenylephrine suggests that these transcription factors are involved in the transcriptional and growth response of the atrial cardiocytes to these stimuli.

## SUMMARY

This study has demonstrated that the control of ANF and BNP secretion and gene expression in atrial cardiocytes is a complex process that can be specifically and distinctly modulated by mechanical and neuroendocrine factors.

Stretch of atrial tissue results in an immediate increase in ANF and BNP secretion. This increased hormone release is short-lived, and is not related to the mechanical activity of the tissue. From their patterns of secretion, it appears that ANF and BNP are co-secreted in response to stretch, which implies that granules that contain both peptides are involved in the stretch-secretion coupling of the atrial cardiocytes. However, there is initially a preferential increase in the secretion of ANF, indicating that both monohormonal and bihormonal granules may be involved in the secretory response to stretch. Since the decrease in ANF secretion after the initial stretch-induced increase results from the depletion of a pool of newly synthesized hormone, it is likely that the same process occurs for BNP. Increased BNP gene expression was observed following 1.5 hours of stretch. This response is not mirrored by ANF, which shows that each gene is regulated by distinct mechanisms. The difference in regulation of the natriuretic peptide genes may be due to post-transcriptional regulation of the BNP gene, compared to the exclusively transcriptional regulation of the ANF gene. The rapid increase in BNP mRNA levels following stretch may be a required compensatory mechanism for the replenishment of granule stores or for increased secretion *via* a non-granule-dependent pathway.

ET-1 stimulation resulted in the increased secretion of ANF and BNP. The rise in secretory rate was more gradual and sustained than with stretch, but was also transient. The

decrease in ET-1 stimulated natriuretic peptide secretion is most likely due to desensitization of receptor signalling, although the depletion of a pool of ET-1-sensitive granules cannot be discounted. ANF and BNP appear to be co-secreted in response to ET-1; therefore, a population of bi-hormonal granules is most likely the target of ET-1 receptor signalling. Increased BNP mRNA levels followed ET-1 stimulation. This increase was not coordinately regulated with the increase in BNP secretion. This implies that different signalling mechanisms are responsible for BNP secretion and gene expression, or that the mechanisms that regulate BNP mRNA levels respond in a delayed fashion to ET-1 stimulation. Alternatively, the regulation of BNP gene expression may be related to a mechanism that responds to increased demand for BNP synthesis, which is not directly related to ET-1 receptor activation.

Stimulation of natriuretic peptide secretion by phenylephrine reveals distinct regulation of secretion of ANF and BNP. The secretion of both hormones is characterized by a gradual increase; however, the proportional increase in ANF secretion is greater than that of BNP, and the increase in BNP secretion is more sustained than that of ANF. Therefore, unlike that which follows stretch or ET-1 stimulation, increased ANF and BNP secretion following stimulation of  $\alpha_1$ -adrenergic receptors is dependent on distinct secretory pathways. It is possible that different granule populations, containing distinct proportions of ANF and BNP, are independently recruited by phenylephrine stimulation; however, it is more likely that the more sustained increase in BNP secretion is due to secretion *via* a non-granule-dependent pathway. The activation of  $\alpha_1$ -adrenergic receptors on atrial cardiocytes results in a modest and transient increase in ANF mRNA levels, and a more robust and sustained increase in BNP mRNA levels. This shows that, as with stretch and ET-1, BNP gene

expression is more acutely sensitive to the endocrine requirements of the atrial cardiocytes than ANF.

The expression of the early response genes *Egr-1* and *c-myc* was increased by stretch, ET-1, and phenylephrine, albeit following a different time course from one gene or stimulus to the other. Stretch also increased the expression of the *c-fos* gene. Therefore, the expression of these transcription factors is a consistent response of the cardiocytes to mechanical and receptor-mediated stimuli, and may be important for subsequent changes in atrial gene expression, potentially including the regulation of the natriuretic peptide genes.

## PERSPECTIVES

In heart failure, compensatory neurohormonal activation includes sustained increase in ANF and BNP secretion (Sugawara *et al.*, 1986; Burnett, Jr. *et al.*, 1986; Saito *et al.*, 1989a; Mukoyama *et al.*, 1990; Mukoyama *et al.*, 1991; Wei *et al.*, 1993). In this study, the potential for regulation of natriuretic peptide secretion and synthesis by mechanical, endocrine/paracrine, and neurohormonal stimuli has been investigated. It has been shown that increased secretion and gene expression of ANF and BNP in atrial tissue can be brought about by these three types of stimuli, and that each modulates the function of the endocrine heart in a distinct fashion.

Stretch, ET-1, and phenylephrine stimulation all lead to rapid and transient increases in ANF secretion, without greatly affecting ANF mRNA levels. In chronic overload situations, however, there is a persistent increase in both ANF secretion and gene expression in the atria (de Bold *et al.*, 1996; Lattion *et al.*, 1986; Arai *et al.*, 1987; Drexler *et al.*, 1989; Haass *et al.*, 1990; Mukoyama *et al.*, 1991; Fischer *et al.*, 1991; Ogawa *et al.*, 1991; Yokota *et al.*, 1994; Yokota *et al.*, 1995). Therefore, there must be a biphasic response of the endocrine heart to hemodynamic or neuroendocrine stress. It is also possible that a full and sustained response of ANF synthesis and secretion requires a combination of mechanical and receptor-mediated stimuli. In studies involving *in vitro* perfused hearts or isolated atria, it has been suggested that stretch may act in an additive fashion with ET-1 or adrenergic stimulation (Schiebinger and Greening, 1992; Jiao and Baertschi, 1995). This type of combination is likely to occur in heart failure, in which increased load is accompanied by increased sympathetic activity and enhanced presence of pressor agents such as ET-1 and

angiotensin II (Dzau, 1988; Swedberg *et al.*, 1990; Riegger, 1991; Stewart *et al.*, 1992; McMurray *et al.*, 1992; Svanegaard *et al.*, 1993; Riegger, 1994).

Mechanical and neuroendocrine stimulation also led to marked increases in BNP secretion. The differences in the temporal patterns and proportional increases in secretion between ANF and BNP point to a bifunctional hormonal system that permits finely tuned responses to the demands of the cardiovascular system. This is particularly exemplified by the stretch- and phenylephrine-induced responses. Stretch causes a proportionally larger increase in ANF secretion, compared to that of BNP, which indicates that the atria have the capacity to fine-tune their response to stretch, releasing one or both hormones, depending on the severity of the load. In the case of phenylephrine, the more rapid and more sustained, albeit proportionally smaller, increase in BNP secretion demonstrates a second type of control, in which the decreasing effects of ANF may be supplemented by the continuing effects of BNP.

The increased BNP mRNA levels, despite a return to basal levels of stretch- and ET-1-stimulated secretion, signals an increase in synthesis that most likely precedes a second phase increase in secretion. This may also be related to the proportional amounts of natriuretic peptide stores available; if BNP stores are rapidly decreased, an increase in synthesis may be required for the replenishment of these. Following adrenergic stimulation, the sustained increase in BNP secretion is accompanied after a longer period of time by increased mRNA levels. It appears likely that the signalling pathways activated by phenylephrine that increase BNP secretion are distinct and complementary to those activated by stretch or ET-1. Therefore, as suggested for ANF secretion, a combination of stimuli would

lead to a synergistic increase in BNP secretion and synthesis that might be more sustained than with either stimulus in isolation.

The increased secretion and synthesis of natriuretic peptides by mechanical or neuroendocrine stimuli in isolated rat atria observed in this study serves as an ideal model to study the modulation of the endocrine heart by these types of stimuli. The intracellular mechanisms involved in intracellular ANF and BNP peptide trafficking require elucidation, as do the precise means by which their genes are regulated at the transcriptional and post-transcriptional levels. The information acquired from this and future studies will present an important understanding of the mechanisms of regulation of the endocrine heart, and how these mechanisms may be improperly regulated in decompensated heart failure.

## REFERENCES

- Adnot, S., Chabrier, P.E., Andrivet, P., Viossat, I., Piquet, J., Brun-Buisson, C., Gutkowska, Y., and Braquet, P. (1987). Atrial natriuretic peptide concentrations and pulmonary hemodynamics in patients with pulmonary artery hypertension. *Am. Rev. Respir. Dis.* 136, 951-956.
- Agnoletti, G., Rodella, A., Ferrari, R., and Harris, P. (1987). Release of atrial natriuretic peptide-like immunoreactive material during stretching of the rat atrium in vitro. *J. Mol. Cell. Cardiol.* 19, 217-220.
- Agnoletti, G., Ferrari, R., Slade, A.M., Severs, N.J., and Harris, P. (1989). Stretch-induced centrifugal movement of atrial specific granules--a preparatory step in atrial natriuretic peptide secretion. *J. Mol. Cell. Cardiol.* 21, 235-239.
- Agnoletti, G., Rodella, A., Cornacchiari, A., Panzali, A.F., Harris, P., and Ferrari, R. (1992). Isoproterenol induces release of atrial natriuretic peptide from rat atrium in vitro. *Am. J. Physiol.* 262, H285-H292.
- Akai, Y., Homma, T., Burns, K.D., Yasuda, T., Badr, K.F., and Harris, R.C. (1994). Mechanical stretch/relaxation of cultured rat mesangial cells induces protooncogenes and cyclooxygenase. *Am. J. Physiol.* 267, C482-C490.
- Almeida, F.A., Suzuki, M., and Maack, T. (1986). Atrial natriuretic factor increases hematocrit and decreases plasma volume in nephrectomized rats. *Life Sci.* 39, 1193-1199.
- Almeida, F.A., Suzuki, M., Scarborough, R.M., Lewicki, J.A., and Maack, T. (1989). Clearance function of type C receptors of atrial natriuretic factor in rats. *Am. J. Physiol.* 256, R469-R475.
- Ambler, S.K. and Leite, M.F. (1994). Regulation of atrial natriuretic peptide secretion by alpha 1-adrenergic receptors: the role of different second messenger pathways. *J. Mol. Cell. Cardiol.* 26, 391-402.

- Antunes-Rodrigues, J., McCann, S.M., Rogers, L.C., and Samson, W.K. (1985). Atrial natriuretic factor inhibits dehydration- and angiotensin II-induced water intake in the conscious, unrestrained rat. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 8720-8723.
- Antunes-Rodrigues, J., Ramalho, M.J., Reis, L.C., Menani, J.V., Turrin, M.Q., Gutkowska, J., and McCann, S.M. (1991). Lesions of the hypothalamus and pituitary inhibit volume-expansion-induced release of atrial natriuretic peptide. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2956-2960.
- Antunes-Rodrigues, J., Marubayashi, U., Favaretto, A.L., Gutkowska, J., and McCann, S.M. (1993). Essential role of hypothalamic muscarinic and alpha-adrenergic receptors in atrial natriuretic peptide release induced by blood volume expansion. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10240-10244.
- Appel, R.G., Wang, J., Simonson, M.S., and Dunn, M.J. (1986). A mechanism by which atrial natriuretic factor mediates its glomerular actions. *Am. J. Physiol.* **251**, F1036-F1042.
- Arai, H., Nakao, K., Saito, Y., Morii, N., Sugawara, A., Yamada, T., Itoh, H., Shiono, S., Mukoyama, M., Ohkubo, H., and et, a.l. (1987). Simultaneous measurement of atrial natriuretic polypeptide (ANP) messenger RNA and ANP in rat heart—evidence for a preferentially increased synthesis and secretion of ANP in left atrium of spontaneously hypertensive rats (SHR). *Biochem. Biophys. Res. Commun.* **148**, 239-245.
- Arai, H., Nakao, K., Saito, Y., Morii, N., Sugawara, A., Yamada, T., Itoh, H., Shiono, S., Mukoyama, M., Ohkubo, H., and et, a.l. (1988). Augmented expression of atrial natriuretic polypeptide gene in ventricles of spontaneously hypertensive rats (SHR) and SHR-stroke prone. *Circ. Res.* **62**, 926-930.
- Arai, H., Hori, S., Aramori, I., Ohkubo, H., and Nakanishi, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348**, 730-732.
- Arai, M., Yoguchi, A., Iso, T., Takahashi, T., Imai, S., Murata, K., and Suzuki, T. (1995). Endothelin-1 and its binding sites are upregulated in pressure overload cardiac hypertrophy. *Am. J. Physiol.* **268**, H2084-H2091.
- Arceci, R.J., King, A.A., Simon, M.C., Orkin, S.H., and Wilson, D.B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell. Biol.* **13**, 2235-2246.

Ardati, A. and Nemer, M. (1993). A nuclear pathway for alpha 1-adrenergic receptor signaling in cardiac cells. *EMBO J.* 12, 5131-5139.

Argentin, S., Sun, Y., Lihrmann, I., Schmidt, T., Drouin, J., and Nemer, M. (1991). Distal cis-Acting promoter sequences mediate glucocorticoid stimulation of cardiac atrial natriuretic factor gene transcription. *J. Biol. Chem.* 266, 23315-23322.

Argentin, S., Nemer, M., Drouin, J., Scott, G.K., Kennedy, B.P., and Davies, P.L. (1985). The gene for rat atrial natriuretic factor. *J. Biol. Chem.* 260, 4568-4571.

Argentin, S., Ardati, A., Tremblay, S., Lihrmann, I., Robitaille, L., Drouin, J., and Nemer, M. (1994). Developmental stage-specific regulation of atrial natriuretic factor gene transcription in cardiac cells. *Mol. Cell. Biol.* 14, 777-790.

Armstrong, P.W., Stopps, T.P., Ford, S.E., and de Bold, A.J. (1986). Rapid ventricular pacing in the dog: pathophysiologic studies of heart failure. *Circulation* 74, 1075-1084.

Arvan, P., Kuliawat, R., Prabakaran, D., Zavacki, A.M., Elahi, D., Wang, S., and Pilkey, D. (1991). Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* 266, 14171-14174.

Arvan, P. and Castle, J.D. (1987). Phasic release of newly synthesized secretory proteins in the unstimulated exocrine pancreas. *J. Cell Biol.* 104, 243-252.

Ashcroft, S.J. and Hughes, S.J. (1990). Protein phosphorylation in the regulation of insulin secretion and biosynthesis. *Biochem. Soc. Trans.* 18, 116-118.

Atarashi, K., Mulrow, P.J., Franco-Saenz, R., Snajdar, R.M., and Rapp, J. (1984). Inhibition of aldosterone production by an atrial extract. *Science* 224, 992-994.

Atarashi, K., Mulrow, P.J., and Franco-Saenz, R. (1985). Effect of atrial peptides on aldosterone production. *J. Clin. Invest.* 76, 1807-1811.

Atlas, S.A., Kleinert, H.D., Camargo, M.J., Januszewicz, A., Sealey, J.E., Laragh, J.H., Schilling, J.W., Lewicki, J.A., Johnson, L.K., and Maack, T. (1984). Purification, sequencing and synthesis of natriuretic and vasoactive rat atrial peptide. *Nature* 309, 717-719.

Awazu, M., Kon, V., Harris, R.C., Imada, T., Inagami, T., and Ichikawa, I. (1991). Renal sympathetic nerves modulate glomerular ANP receptors and filtration. *Am. J. Physiol.* 261, F29-F35.

Azizi, C., Carayon, A., Masson, F., Noe, E., Barthelemy, C., Eurin, J., Maistre, G., and Legrand, J.C. (1993). Mechanisms of isoproterenol-induced atrial natriuretic peptide release from superfused rabbit atria. *Am. J. Physiol.* 265, H1283-H1288.

Barrett, P.Q., Isales, C.M., Bollag, W.B., and McCarthy, R.T. (1991).  $\text{Ca}^{2+}$  channels and aldosterone secretion: modulation by  $\text{K}^{+}$  and atrial natriuretic peptide. *Am. J. Physiol.* 261, F706-F719.

Baum, M. and Toto, R.D. (1986). Lack of a direct effect of atrial natriuretic factor in the rabbit proximal tubule. *Am. J. Physiol.* 250, F66-F69.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature* 361, 315-325.

Bilder, G.E., Schofield, T.L., and Blaine, E.H. (1986). Release of atrial natriuretic factor. Effects of repetitive stretch and temperature. *Am. J. Physiol.* 251, F817-F821.

Bilder, G.E., Siegl, P.K., Schofield, T.L., and Friedman, P.A. (1989). Chronotropic stimulation: a primary effector for release of atrial natriuretic factor. *Circ. Res.* 64, 799-805.

Blackburn, R.E., Samson, W.K., Fulton, R.J., Stricker, E.M., and Verbalis, J.G. (1995). Central oxytocin and ANP receptors mediate osmotic inhibition of salt appetite in rats. *Am. J. Physiol.* 269, R245-R251.

Blackwood, E.M. and Eisenman, R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* 251, 1211-1217.

Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. U. S. A.* 90, 5889-5892.

- Bloch, K.D., Seidman, J.G., Nattilan, J.D., Fallon, J.T., and Seidman, C.E. (1986). Neonatal atria and ventricles secrete atrial natriuretic factor via tissue-specific secretory pathways. *Cell* 47, 695-702.
- Bloch, K.D., Jones, S.W., Preibisch, G., Seipke, G., Seidman, C.E., and Seidman, J.G. (1987). Proatrial natriuretic factor is phosphorylated by rat cardiocytes in culture. *J. Biol. Chem.* 262, 9956-9961.
- Bloch, K.D., Zamir, N., Lichtstein, D., Seidman, C.E., and Seidman, J.G. (1988). Ouabain induces secretion of proatrial natriuretic factor by rat atrial cardiocytes. *Am. J. Physiol.* 255, E383-E387.
- Bloom, G., Ostlund, E., von Euler, U.S., Lishajko, F., Ritzen, M., and Adams-Ray, J. (1961). Studies on catecholamine-containing granules of specific cells in cyclostoma hearts. *Acta Physiol. Scand. Suppl.* 53, 1-34.
- Bogoyevitch, M.A., Parker, P.J., and Sugden, P.H. (1993). Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. *Circ. Res.* 72, 757-767.
- Bogoyevitch, M.A., Glennon, P.E., Andersson, M.B., Clerk, A., Lazou, A., Marshall, C.J., Parker, P.J., and Sugden, P.H. (1994). Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J. Biol. Chem.* 269, 1110-1119.
- Bogoyevitch, M.A., Marshall, C.J., and Sugden, P.H. (1995). Hypertrophic agonists stimulate the activities of the protein kinases c-raf and A-raf in cultured ventricular myocytes. *J. Biol. Chem.* 270, 26303-26310.
- Bolli, P., Müller, F.B., Linder, L., Raine, A.E., Resink, T.J., Erne, P., Kiowski, W., Ritz, R., and Bühler, F.R. (1987). The vasodilator potency of atrial natriuretic peptide in man. *Circulation* 75, 221-228.
- Bompiani, G.D., Roulher, C., and Hatt, P.Y. (1959). Le tissu de conduction du coeur chez le rat. Etude au microscope electronique. I. Le tronc commun du faisceau de His et les cellules claires de l'oreillette droite. *Arch. Mal. Coeur.* 52, 1257-1274.
- Bourgoignie, J.J., Gavellas, G., and Hwang, K.H. (1986). Renal effects of atrial natriuretic factor in primate. *Am. J. Physiol.* 251, F1049-F1054.

Brand, T., Sharma, H.S., and Schaper, W. (1993). Expression of nuclear proto-oncogenes in isoproterenol-induced cardiac hypertrophy. *J. Mol. Cell. Cardiol.* 25, 1325-1337.

Breuhaus, B.A., Saneii, H.H., Brandt, M.A., and Chimoskey, J.E. (1985). Atriopeptin II lowers cardiac output in conscious sheep. *Am. J. Physiol.* 249, R776-R780.

Ericker, N.S., Klahr, S., Purkerson, M., Schultze, R.G., Avioli, L.V., and Birge, S.J. (1968). In vitro assay for a humoral substance present during volume expansion and uremia. *Nature* 219, 1058-1059.

Briggs, J.P., Steipe, B., Schubert, G., and Schnermann, J. (1982). Micropuncture studies of the renal effects of atrial natriuretic substance. *Pflüegers Arch.* 395, 271-276.

Brown, B.L., Walker, S.W., and Tomlinson, S. (1985). Calcium calmodulin and hormone secretion. *Clin. Endocrinol.* 23, 201-218.

Brown, L.A., Nunez, D.J.R., and Wilkins, M.R. (1993). Differential regulation of natriuretic peptide receptor messenger RNAs during the development of cardiac hypertrophy in the rat. *J. Clin. Invest.* 92, 2702-2712.

Brown, L.A., Nunez, D.J., Brookes, C.I.O., and Wilkins, M.R. (1995). Selective increase in endothelin-1 and endothelin A receptor subtype in the hypertrophied myocardium of the aorto-venacaval fistula rat. *Cardiovasc. Res.* 29, 768-774.

Brunner, F., Mouton, R., Lochner, A., and Opie, L.H. (1995). Defective stretch-induced release of atrial natriuretic peptide from aging hypertensive rat heart: possible role of phosphatidylinositol pathway. *J. Cardiovasc. Pharmacol.* 25, 168-176.

Burgess, T.L. and Kelly, R.B. (1987). Constitutive and regulated secretion. *Annu. Rev. Cell. Biol.* 3, 243-293.

Burnett, J.C., Jr., Granger, J.P., and Opgenorth, T.J. (1984). Effects of synthetic atrial natriuretic factor on renal function and renin release. *Am. J. Physiol.* 247, F863-F866.

- Burnett, J.C., Jr., Kao, P.C., Hu, D.C., Heser, D.W., Heublein, D., Granger, J.P., Opgenorth, T.J., and Reeder, G.S. (1986). Atrial natriuretic peptide elevation in congestive heart failure in the human. *Science* 231, 1145-1147.
- Burnett, J.C., Jr., Osborn, M.J., Hammill, S.C., and Heublein, D.M. (1989). The role of frequency of atrial contraction versus atrial pressure in atrial natriuretic peptide release. *J. Clin. Endocrin. Metab.* 69, 881-884.
- Burrell, L.M., Lambert, H.J., and Baylis, P.H. (1991). Effect of atrial natriuretic peptide on thirst and arginine vasopressin release in humans. *Am. J. Physiol.* 260, R475-R479.
- Butlen, D., Mistaoui, M.H., and Morel, F. (1987). Atrial natriuretic peptide receptors along the rat and rabbit nephrons: [125I] alpha-rat atrial natriuretic peptide binding in microdissected glomeruli and tubules. *Pflüegers Arch.* 408, 356-365.
- Camargo, M.J.F., Kleinert, H.D., Atlas, S.A., Sealey, J.E., Laragh, J.H., and Maack, T. (1984). Ca-dependent hemodynamic and natriuretic effects of atrial extract in isolated rat kidney. *Am. J. Physiol.* 246, F447-F456.
- Cao, L., Wu, J., and Gardner, D.G. (1995). Atrial natriuretic peptide suppresses the transcription of its guanylyl cyclase-linked receptor. *J. Biol. Chem.* 270, 24891-24897.
- Cao, L. and Gardner, D.G. (1995). Natriuretic peptides inhibit DNA synthesis in cardiac fibroblasts. *Hypertension* 25, 227-234.
- Chang, M.S., Lowe, D.G., Lewis, M., Hellmiss, R., Chen, E., and Goeddel, D.V. (1989). Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* 341, 68-72.
- Chen, R.H., Abate, C., and Blenis, J. (1993). Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc. Natl. Acad. Sci. U. S. A.* 90, 10952-10956.

Chen, T.T., Kineman, R.D., Betts, J.G., Hill, J.B., and Frawley, L.S. (1989). Relative importance of newly synthesized and stored hormone to basal secretion by growth hormone and prolactin cells. *Endocrinology* 125, 1904-1909.

Chien, K.R., Zhu, H., Knowlton, K.U., Miller-Hance, W., van-Bilsen, M., O'Brien, T.X., and Evans, S.M. (1993). Transcriptional regulation during cardiac growth and development. *Annu. Rev. Physiol.* 55, 77-95.

Chinkers, M., Garbers, D.L., Chang, M.S., Lowe, D.G., Chin, H.M., Goeddel, D.V., and Schulz, S. (1989). A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* 338, 78-83.

Chinkers, M. and Garbers, D.L. (1989). The protein kinase domain of the ANP receptor is required for signaling. *Science* 245, 1392-1394.

Cho, K.W., Seul, K.H., Kim, S.H., Seul, K.M., and Koh, G.Y. (1991). Atrial pressure, distension, and pacing frequency in ANP secretion in isolated perfused rabbit atria. *Am. J. Physiol.* 260, R39-R46.

Cho, K.W., Kim, S.H., Seul, K.H., Hwang, Y.H., and Kook, Y.J. (1994). Effect of extracellular calcium depletion on the two-step ANP secretion in perfused rabbit atria. *Regul. Pept.* 52, 129-137.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.

Christensen, G., Ilebekk, A., Aakeson, I., and Kiil, F. (1988). The release mechanism for atrial natriuretic factor during blood volume expansion and tachycardia in dogs. *Acta Physiol. Scand.* 134, 263-270.

Clavell, A.L., Stingo, A.J., Wei, C.M., Heublein, D.M., and Burnett, J.C., Jr. (1993). C-type natriuretic peptide: a selective cardiovascular peptide. *Am. J. Physiol.* 264, R290-R295.

Clerk, A., Bogoyevitch, M.A., Anderson, M.B., and Sugden, P.H. (1994). Differential activation of protein kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultured from neonatal rat hearts. *J. Biol. Chem.* 269, 32848-32857.

- Cody, R.J., Atlas, S.A., Laragh, J.H., Kubo, S.H., Covit, A.B., Ryman, K.S., Shakhovich, A., Pondolfino, K., Clark, M., Camargo, M.J., Scarborough, R.M., and Lewicki, J.A. (1986). Atrial natriuretic factor in normal subjects and heart failure patients. Plasma levels and renal, hormonal, and hemodynamic responses to peptide infusion. *J. Clin. Invest.* 78, 1362-1374.
- Cogan, M.G. (1985). Atrial natriuretic factor ameliorates chronic metabolic alkalosis by increasing glomerular filtration. *Science* 229, 1405-1407.
- Conaway, R.C. and Conaway, J.W. (1993). General initiation factors for RNA polymerase II. *Annu. Rev. Biochem.* 62, 161-190.
- Crews, C.M. and Erikson, R.L. (1993). Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell* 74, 215-217.
- Curran, T., Peters, G., Van Beveren, C., Teich, N.M., and Verma, I.M. (1982). FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* 44, 674-682.
- Currie, M.G., Geller, D.M., Cole, B.R., Boylan, J.G., YuSheng, W., Holmberg, S.W., and Needleman, P. (1983). Bioactive cardiac substances: potent vasorelaxant activity in mammalian atria. *Science* 221, 71-73.
- Currie, M.G., Geller, D.M., Chao, J., Margolius, H.S., and Needleman, P. (1984a). Kallikrein activation of a high molecular weight atrial peptide. *Biochem. Biophys. Res. Commun.* 120, 461-466.
- Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Eubanks, S.R., Galluppi, G.R., and Needleman, P. (1984b). Purification and sequence analysis of bioactive atrial peptides (atriopeptins). *Science* 223, 67-69.
- Dagnino, L., Drouin, J., and Nemer, M. (1991). Differential expression of natriuretic peptide genes in cardiac and extracardiac tissues. *Mol. Endocrinol.* 5, 1292-1300.
- Dagnino, L., Lavigne, J.P., and Nemer, M. (1992). Increased transcripts for B-type natriuretic peptide in spontaneously hypertensive rats. Quantitative polymerase chain reaction for atrial and brain natriuretic peptide transcripts. *Hypertension* 20, 690-700.

- Daifotis, A.G., Weir, E.C., Dreyer, B.E., and Broadus, A.E. (1992). Stretch-induced parathyroid hormone-related peptide gene expression in the rat uterus. *J. Biol. Chem.* 267, 23455-23458.
- Dananberg, J. and Grekin, R.J. (1992). Corticoid regulation of atrial natriuretic factor secretion and gene expression. *Am. J. Physiol.* 263, H1377-H1381.
- Dassouli, A., Sulpice, J.C., Roux, S., and Crozatier, B. (1993). Stretch-induced inositol trisphosphate and tetrakisphosphate production in rat cardiomyocytes. *J. Mol. Cell. Cardiol.* 25, 973-982.
- Daum, G., Eisenmann-Tappe, I., Fries, H., Troppmair, J., and Rapp, U.R. (1994). The ins and outs of Raf kinases. *Trends Biochem. Sci.* 19, 474-480.
- Day, M.L., Schwartz, D., Wiegand, R.C., Stockman, P.T., Brunnert, S.R., Tolunay, H.E., Currie, M.G., Standaert, D.G., and Needleman, P. (1987). Ventricular atriopeptin. Unmasking of messenger RNA and peptide synthesis by hypertrophy or dexamethasone. *Hypertension* 9, 485-491.
- de Bold, A.J., Raymond, J.J., and Bencosme, S.A. (1978). Atrial specific granules of the rat heart: light microscopic staining and histochemical reactions. *J. Histochem. Cytochem.* 26, 1094-1102.
- de Bold, A.J. (1979). Heart atria granularity effects of changes in water-electrolyte balance. *Proc. Soc. Exp. Biol. Med.* 161, 508-511.
- de Bold, A.J., Borenstein, H.B., Veress, A.T., and Sonnenberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extracts in rats. *Life Sci.* 28, 89-94.
- de Bold, A.J. (1982a). Tissue fractionation studies on the relationship between an atrial natriuretic factor and specific atrial granules. *Can. J. Physiol. Pharmacol.* 60, 324-330.
- de Bold, A.J. (1982b). Atrial natriuretic factor of the rat heart. Studies on isolation and properties. *Proc. Soc. Exp. Biol. Med.* 170, 133-138.
- de Bold, A.J. (1985). Atrial natriuretic factor: a hormone produced by the heart. *Science* 230, 767-770.
- de Bold, A.J., de Bold, M.L., and Sarda, I.R. (1986). Functional-morphological studies on in vitro cardionatriin release. *J. Hypertens.* 4(Suppl 2), S3-S7.

de Bold, A.J., Kuroski-de Bold, M.L., Boer, P.H., Dubé, G., Mangat, H., and Johnson, F. (1991). A decade of ANF research. *Can. J. Physiol. Pharmacol.* 69, 1480-1485.

de Bold, A.J., Bruneau, B.G., and Kuroski-de Bold, M.L. (1996). Mechanical and neuroendocrine regulation of the endocrine heart. *Cardiovasc. Res.* 31, 7-18.

de Bold, A.J. and Bencosme, S.A. (1973a). Studies on the relationship between the catecholamine distribution in the atrium and the specific granules present in atrial muscle cells: 2. Studies on the sedimentation pattern of atrial noradrenaline and adrenaline. *Cardiovasc. Res.* 7, 364-369.

de Bold, A.J. and Bencosme, S.A. (1973b). Studies on the relationship between the catecholamine distribution in the atrium and the specific granules present in atrial muscle cells: 1. Isolation of a purified specific granule subfraction. *Cardiovasc. Res.* 7, 351-363.

de Bold, A.J. and Bencosme, S.A. (1975). Autoradiographic analysis of label distribution in mammalian atrial and ventricular cardiocytes after exposure to tritiated leucine. In *Recent Advances in Studies on Cardiac Structure and Metabolism. The Cardiac Sarcoplasm*. P. Roy and P. Harris, eds. (Baltimore: University Park Press), pp. 129-138.

de Bold, A.J. and de Bold, M.L. (1987). Factors affecting cardionatrin release. In *Highlights in endocrinology*. C. Christiansen and B.J. Riis, eds. (Denmark: N.Bogtrykkeri), pp. 161-163.

de Bold, A.J. and Flynn, T.G. (1983). Cardionatrin I - a novel heart peptide with potent diuretic and natriuretic properties. *Life Sci.* 33, 297-302.

de Bold, M.L. and de Bold, A.J. (1987). Atrial stretch-secretion coupling and immunoreactive cardionatrin release in vitro: effect of calcium and EGTA. In *Biologically active atrial peptides*. B.M. Brenner and J.H. Laragh, eds. (New York: Raven Press), pp. 173-175.

de Bold, M.L. and de Bold, A.J. (1989). Effects of manipulations of  $Ca^{2+}$  environment on atrial natriuretic factor release. *Am. J. Physiol.* 256, H1588-H1594.

de Groot, R.P., Schoorlemmer, J., van Genesen, S.T., and Kruijer, W. (1990). Differential expression of *jun* and *fos* genes during differentiation of mouse P19 embryonal carcinoma cells. *Nucl. Acids Res.* 18, 3195-3202.

De Jonge, H.W., Van Heugten, H.A.A., and Lamers, J.M.J. (1995). Signal transduction by the phosphatidylinositol cycle in myocardium. *J. Mol. Cell. Cardiol.* 27, 93-106.

De Lean, A., Racz, K., Gutkowska, J., Nguyen, T.T., Cantin, M., and Genest, J. (1984). Specific receptor-mediated inhibition by synthetic atrial natriuretic factor of hormone-stimulated steroidogenesis in cultured bovine adrenal cells. *Endocrinology* 115, 1636-1638.

de Wardener, H.E., Mills, I.H., Clapham, W.F., and Hayter, C.J. (1961). Studies on the efferent mechanism of the sodium diuresis which follows the administration of intravenous saline in the dog. *Clin. Sci.* 21, 249-258.

De Young, M.B., Keller, J.C., Graham, R.M., and Wildey, G.M. (1994). Brefeldin A defines distinct pathways for atrial natriuretic factor secretion in neonatal rat atrial and ventricular myocytes. *Circ. Res.* 74, 33-40.

Decock, J.B., Gillespie-Brown, J., Parker, P.J., Sugden, P.H., and Fuller, S.J. (1994). Classical, novel and atypical isoforms of PKC stimulate ANF- and TRE/AP-1-regulated-promoter activity in ventricular cardiomyocytes. *Febs Let.* 356, 275-278.

Deftos, L.J., Burton, D.W., and Brandt, D.W. (1993). Parathyroid hormone-like protein is a secretory product of atrial myocytes. *J. Clin. Invest.* 92, 727-735.

Dietz, J.R. (1984). Release of natriuretic factor from rat heart-lung preparation by atrial distension. *Am. J. Physiol.* 247, R1093-R1096.

Dillingham, M.A. and Anderson, R.J. (1986). Inhibition of vasopressin action by atrial natriuretic factor. *Science* 231, 1572-1573.

Donckier, J., Hanet, C., Galanti, L., Stoleru, L., Van Mechelen, H., Robert, A., Ketelslegers, J.M., and Pouleur, H. (1992). Low-dose endothelin-1 potentiates volume-induced secretion of atrial natriuretic factor. *Am. J. Physiol.* 263, H939-H944.

Doubell, A., Bester, A., and Thibault, G. (1991). Annexins V and VI: Major calcium-dependent atrial secretory granule-binding proteins. *Hypertension* 18, 648-656.

Doubell, A.F. and Thibault, G. (1994). Calcium is involved in both positive and negative modulation of the secretory system for ANP. *Am. J. Physiol.* 266, H1854-H1863.

Drexler, H., Hranze, J., Finckh, M., Lu, W., Just, H., and Lang, R.E. (1989). Atrial natriuretic peptide in a rat model of cardiac failure Atrial and ventricular mRNA, atrial content, plasma levels, and effect of volume loading. *Circulation* 79, 620-633.

Dubé, G.R., Kuroski-de Bold, M.L., and de Bold, A.J. (1993). Post-translational processing of atrial natriuretic factor by adult rat atrial cardiocytes in culture. *Can. J. Physiol. Pharmacol.* 71, 497-505.

Dunn, B.R., Ichikawa, I., Pfeffer, J.M., Troy, J.L., and Brenner, B.M. (1986). Renal and systemic hemodynamic effects of synthetic atrial natriuretic peptide in the anesthetized rat. *Circ. Res.* 59, 237-246.

Dzau, V.J., Baxter, J.D., Cantin, M., de Bold, A.J., Ganten, D., Gross, K., Husain, A., Inagami, T., Menard, J., Poole, S., Robertson, J.I.S., Tang, J., and Yamamoto, K. (1987). Nomenclature for atrial peptides. *N. Engl. J. Med.* 316, 1278-1279.

Dzau, V.J. (1988). Contributions of neuroendocrine and local autocrine-paracrine mechanisms to the pathophysiology and pharmacology of congestive heart failure. *Am. J. Cardiol.* 62, 76E-81E.

Edwards, B.S., Schwab, T.R., Zimmerman, R.S., Heublein, D.M., Jiang, N., and Burnett, J.C., Jr. (1986). Cardiovascular, renal, and endocrine response to atrial natriuretic peptide in angiotensin II mediated hypertension. *Circ. Res.* 59, 663-667.

Edwards, B.S., Zimmerman, R.S., Schwab, T.R., Heublein, D.M., and Burnett, J.C., Jr. (1988). Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor. *Circ. Res.* 62, 191-195.

Eguchi, S., Hirata, Y., Imai, T., and Marumo, F. (1994). C-type natriuretic peptide upregulates vascular endothelin type B receptors. *Hypertension* 23, 936-940.

Eid, H., Kuroski-de Bold, M.L., Chen, J.H., and de Bold, A.J. (1994). Epicardial mesothelial cells synthesize and secrete endothelin. *J. Cardiovasc. Pharmacol.* 24, 715-720.

Eid, H. and de Champlain, J. (1988). Increased inositol monophosphate production in cardiovascular tissues of DOCA-salt hypertensive rats. *Hypertension* 12, 122-128.

Emori, T., Hirata, Y., Imai, T., Eguchi, S., Kanno, K., and Marumo, F. (1993). Cellular mechanism of natriuretic peptides-induced inhibition of endothelin-1 biosynthesis in rat endothelial cells. *Endocrinology* 133, 2474-2480.

Engel, J.D., Beug, H., LaVail, J.H., Zenke, M.W., Mayo, K., Leonard, M.W., Foley, K.P., Yang, Z., Kornhauser, J.M., Ko, J., and et al (1992). cis and trans regulation of tissue-specific transcription. *J. Cell Sci.* 16, 21-31.

Eskay, R., Zukowska-Grojec, Z., Haass, M., Dave, J.R., and Zamir, N. (1986). Circulating atrial natriuretic peptides in conscious rats: regulation of release by multiple factors. *Science* 232, 636-639.

Evers, A.S., Dunkel, C.G., Saffitz, J.E., and Needleman, P. (1987). Exaggerated atrial arachidonate metabolism in rabbit left ventricular myocardial infarction. *J. Clin. Invest.* 79, 155-162.

Fenoy, F.J., Quesada, T., García-Salom, M., Romero, J.C., and Salazar, F.J. (1989). Hemodynamic effects of chronic infusion of rANP in renal hypertensive rats. *Am. J. Physiol.* 256, H1393-H1398.

Field, L.J. (1988). Atrial natriuretic factor-SV40 T antigen transgenes produce tumors and cardiac arrhythmias in mice. *Science* 239, 1029-1033.

Fioretto, P., Sambataro, M., Cipollina, M.R., Giorato, C., Carraro, A., Opocher, G., Sacerdoti, D., Brocco, E., Morocutti, A., Mantero, F., and et al. (1992). Role of atrial natriuretic peptide in the pathogenesis of sodium retention in IDDM with and without glomerular hyperfiltration. *Diabetes* 41, 936-945.

Fischer, T.A., Haass, M., Dietz, R., Willenbrock, R.C., Saggau, W., Lang, R.E., and Kubler, W. (1991). Transcription, storage and release of atrial natriuretic factor in the failing human heart. *Clin. Sci.* 80, 285-291.

Flückiger, J.P., Waeber, B., Matsueda, G., Delaloye, B., Nussberger, J., and Brunner, H.R. (1986). Effect of atriopeptin III on hematocrit and volemia of nephrectomized rats. *Am. J. Physiol.* 251, H880-H883.

Flynn, T.G., de Bold, M.L., and de Bold, A.J. (1983). The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. *Biochem. Biophys. Res. Commun.* 117, 859-865.

Flynn, T.G., Davies, P.L., Kennedy, B.P., de Bold, M.L., and de Bold, A.J. (1985a). Alignment of rat cardionatrin sequences with the preprocardionatrin sequence from complementary DNA. *Science* 228, 323-325.

Flynn, T.G., de Bold, A.J., de Bold, M.L., Davies, P.L., and Kennedy, B.P. (1985b). Main forms of immunoreactive cardionatrin in atrial extracts and in atrial specific granules. *Biochem. Soc. Trans.* 13, 1141

Flynn, T.G., Brar, A., Tremblay, L., Sarda, I., Lyons, C., and Jennings, D.B. (1989). Isolation and characterization of iso-rANP, a new natriuretic peptide from rat atria [published erratum appears in *Biochem Biophys Res Commun* 1989 Sep 15;163(2):1189]. *Biochem. Biophys. Res. Commun.* 161, 830-837.

Focaccio, A., Volpe, M., Ambrosio, G., Lembo, G., Pannain, S., Rubattu, S., Enea, I., Pignalosa, S., and Chiariello, M. (1993). Angiotensin II directly stimulates release of atrial natriuretic factor in isolated rabbit hearts. *Circulation* 87, 192-198.

Forssmann, W.G., Hock, D., Lottspeich, F., Henschen, A., Kreye, V., Christmann, M., Reinecke, M., Metz, J., Carlquist, M., and Mutt, V. (1983). The right auricle of the heart is an endocrine organ. Cardiodilatin as a peptide hormone candidate. *Anat. Embryol.* 168, 307-313.

Fujioka, S., Tamaki, T., Fukui, K., Okahara, T., and Abe, Y. (1995). Effects of a synthetic human atrial natriuretic polypeptide on regional blood flow in rats. *Eur. J. Pharmacol.* 109, 301-304.

Fukuda, Y., Hirata, Y., Yoshimi, H., Kojima, T., Kobayashi, Y., Yanagisawa, M., and Masaki, T. (1988). Endothelin is a potent secretagogue for atrial natriuretic peptide in cultured rat atrial myocytes. *Biochem. Biophys. Res. Commun.* 155, 167-172.

Fukuda, Y., Hirata, Y., Taketani, S., Kojima, T., Oikawa, S., Nakazato, H., and Kobayashi, Y. (1989). Endothelin stimulates accumulations of cellular atrial natriuretic peptide and its messenger RNA in rat cardiocytes. *Biochem. Biophys. Res. Commun.* 164, 1431-1436.

Fuller, F., Porter, J.G., Arlsten, A.E., Miller, J., Schilling, J.W., Scarborough, R.M., Lewicki, J.A., and Schenk, D.B. (1988). Atrial natriuretic peptide clearance receptor. Complete sequence and functional expression of cDNA clones. *J. Biol. Chem.* 263, 9395-9401.

Furuya, M., Takehisa, M., Minamitake, Y., Kitajima, Y., Hayashi, Y., Ohnuma, N., Ishihara, T., Minamino, N., Kangawa, K., and Matsuo, H. (1990). Novel natriuretic peptide, CNP, potently stimulates cyclic GMP production in rat cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 170, 201-208.

Furuya, M., Yoshida, M., Hayashi, Y., Ohnuma, N., Minamino, N., Kangawa, K., and Matsuo, H. (1991). C-type natriuretic peptide is a growth inhibitor of rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 177, 927-931.

Furuya, M., Aisaka, K., Miyazaki, T., Honbou, N., Kawashima, K., Ohno, T., Tanaka, S., Minamino, N., Kangawa, K., and Matsuo, H. (1993). C-type natriuretic peptide inhibits intimal thickening after vascular injury. *Biochem. Biophys. Res. Commun.* 193, 248-253.

Furuya, M., Miyazaki, T., Honbou, N., Kawashima, K., Ohno, T., Tanaka, S., Kangawa, K., and Matsuo, H. (1995). C-type natriuretic peptide inhibits intimal thickening after vascular injury. *Ann. N. Y. Acad. Sci.* 748, 517-523.

Fuhrquist, F., Sirvio, M.L., Helin, K., Saijonmaa, O., Metsarinne, K., Paakkari, I., Jarvinen, A., and Tikkanen, I. (1993). Endothelin antiserum decreases volume-stimulated and basal plasma concentration of atrial natriuretic peptide. *Circulation* 88, 1172-1176.

Garcia, R., Cantin, M., Thibault, G., Ong, H., and Genest, J. (1982). Relationship of specific granules to the natriuretic and diuretic activity of rat atria. *Experientia* 38, 1071-1073.

Garcia, R., Thibault, G., Nutt, R.F., Cantin, M., and Genest, J. (1984). Comparative vasoactive effects of native and synthetic atrial natriuretic factor (ANF). *Biochem. Biophys. Res. Commun.* 119, 685-688.

Garcia, R., Thibault, G., Gutkowska, J., Hamet, P., Cantin, M., and Genest, J. (1985). Effect of chronic infusion of synthetic atrial natriuretic factor (ANF 8-33) in conscious two-kidney, one-clip hypertensive rats. *Proc. Soc. Exp. Biol. Med.* 178, 155-159.

Gardner, D.G., Deschepper, C.F., Ganong, W.F., Hane, S., Fiddes, J., Baxter, J.D., and Lewicki, J. (1986a). Extra-atrial expression of the gene for atrial natriuretic factor. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6697-6701.

Gardner, D.G., S.Hane, D.Trachewsky, D.Schenk, and J.D.Baxter (1986b). Atrial natriuretic peptide mRNA is regulated by glucocorticoids in vivo. *Biochem. Biophys. Res. Commun.* **139**, 1047-1054.

Gardner, D.G., Deschepper, C.F., and Baxter, J.D. (1987a). The gene for the atrial natriuretic factor is expressed in the aortic arch. *Hypertension* **9**, 103-106.

Gardner, D.G., Gertz, B.J., and Hane, S. (1987b). Thyroid hormone increases rat atrial natriuretic peptide messenger ribonucleic acid accumulation in vivo and in vitro. *Mol. Endocrinol.* **1**, 260-265.

Gardner, D.G., Vlasuk, G.P., Baxter, J.D., Fiddes, J.C., and Lewicki, J.A. (1987c). Identification of atrial natriuretic factor gene transcripts in the central nervous system of the rat. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2175-2179.

Gardner, D.G., Gertz, B.J., Deschepper, C.F., and Kim, D.Y. (1988). Gene for the rat atrial natriuretic peptide is regulated by glucocorticoids in vitro. *J. Clin. Invest.* **82**, 1275-1281.

Gardner, D.G., Newman, E.D., Nakamura, K.K., and Nguyen, K.P. (1991). Endothelin increases the synthesis and secretion of atrial natriuretic peptide in neonatal rat cardiocytes. *Am. J. Physiol.* **261**, E177-E182.

Gardner, D.G., Wirtz, H., and Dobbs, L.G. (1992). Stretch-dependent regulation of atrial peptide synthesis and secretion in cultured atrial cardiocytes. *Am. J. Physiol.* **263**, E239-E244.

Gardner, D.G. and Schultz, H.D. (1990). Prostaglandins regulate the synthesis and secretion of the atrial natriuretic peptide. *J. Clin. Invest.* **86**, 52-59.

Gauer, O.H., Henry, J.P., and Seiker, H.O. (1961). Cardiac receptors and fluid volume control. *Prog. Cardiovasc. Dis.* **4**, 1-26.

Genovesi, S., Protasoni, G., Assi, C., Golin, R., Stella, A., and Zanchetti, A. (1990). Interactions between the sympathetic nervous system and atrial natriuretic factor in the control of renal functions. *J. Hypertens.* 8, 703-710.

Gerrard, J.M., Friesen, L.L., McCrea, J.M., Israels, S.J., and Robinson, P. (1985). Platelet protein phosphorylation. *Adv. Exp. Med. Biol.* 192, 235-248.

Gibbs, D.M. (1987a). Noncalcium-dependent modulation of in vitro atrial natriuretic factor release by extracellular osmolality. *Endocrinology* 120, 194-197.

Gibbs, D.M. (1987b). Beta-adrenergic control of atrial natriuretic factor secretion from dispersed rat atrial myocytes. *Regul. Pept.* 19, 73-78.

Glembotski, C.C., Irons, C.E., Krown, K.A., Murray, S.F., Sprengle, A.B., and Sei, C.A. (1993). Myocardial alpha-thrombin receptor activation induces hypertrophy and increases atrial natriuretic factor gene expression. *J. Biol. Chem.* 268, 20646-20652.

Goetz, K.L., Wang, B.C., Geer, P.G., Leadley RJ, J.r., and Reinhardt, H.W. (1986). Atrial stretch increases sodium excretion independently of release of atrial peptides. *Am. J. Physiol.* 250, R946-R950.

Golomb, E., Friedman, E., Abassi, Z.A., Trachewsky, D., and Keiser, H.R. (1993). Rare renal transcription of the atrial natriuretic factor gene in rats: Demonstration by polymerase chain reaction. *Am. J. Hypertens.* 6, 867-872.

Granger, J.P., Opgenorth, T.J., Salazar, J., Romero, J.C., and Burnett Jr., J.C. (1986). Long-term hypotensive and renal effects of atrial natriuretic peptide. *Hypertension* 8, 1112-1116.

Greenberg, B.D., Bencen, G.H., Seilhamer, J.J., Lewicki, J.A., and Fiddes, J.C. (1984). Nucleotide sequence of the gene encoding human atrial natriuretic factor precursor. *Nature* 312, 656-658.

Greenwald, J.E., Apkon, M., Hruska, K.A., and Needleman, P. (1989). Stretch-induced atriopeptin secretion in the isolated rat myocyte and its negative modulation by calcium. *J. Clin. Invest.* 83, 1061-1065.

Greenwald, J.E., Ritter, D., Tetens, E., and Rotwein, P.S. (1992). Renal expression of the gene for atrial natriuretic factor. *Am. J. Physiol.* 263, F974-F978.

Grépin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T., and Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell. Biol.* 14, 3115-3129.

Grépin, C., Robitaille, L., Antakly, T., and Nemer, M. (1995). Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation. *Mol. Cell. Biol.* 15, 4095-4102.

Gruver, C.L., DeMayo, F., Goldstein, M.A., and Means, A.R. (1993). Targeted developmental overexpression of calmodulin induces proliferative and hypertrophic growth of cardiomyocytes in transgenic mice. *Endocrinology* 133, 376-388.

Gu, X. and Bishop, S.P. (1994). Increased protein kinase C and isozyme redistribution in pressure-overload cardiac hypertrophy in the rat. *Circ. Res.* 75, 926-931.

Gunning, M.E., Ballermann, B.J., Silva, P., Brenner, B.M., and Zeidel, M.L. (1988). Characterization of ANP receptors in rabbit inner medullary collecting duct cells. *Am. J. Physiol.* 255, F324-F330.

Gupta, M.P., Gupta, M., Zak, R., and Sukhatme, V.P. (1991). Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac alpha-myosin heavy chain gene. *J. Biol. Chem.* 266, 12813-12816.

Gulowska, J., Thibault, G., Januszewicz, P., Cantin, M., and Genest, J. (1984). Direct radioimmunoassay of atrial natriuretic factor. *Biochem. Biophys. Res. Commun.* 122, 593-601.

Haass, M., Fischer, T.A., Hänze, J., Saggau, W., Lang, R.E., and Dietz, R. (1990). Atrial natriuretic peptide mRNA in patients with heart disease. *Am. J. Hypertens.* 3, 234-236.

Hagiwara, N., Masuda, H., Shoda, M., and Irisawa, H. (1992). Stretch-activated anion currents of rabbit cardiac myocytes. *J. Physiol. (London)* 456, 285-302.

Hama, N., Itoh, H., Shirakami, G., Suga, S., Komatsu, Y., Yoshimasa, T., Tanaka, I., Mori, K., and Nakao, K. (1994). Detection of C-type natriuretic peptide in human circulation and marked increase of plasma CNP level in septic shock patients. *Biochem. Biophys. Res. Commun.* 198, 1177-1182.

Hanford, D.S., Thuerauf, D.J., Murray, S.F., and Glembotski, C.C. (1994). Brain natriuretic peptide is induced by  $\alpha_1$ -adrenergic agonists as a primary response gene in cultured rat cardiac myocytes. *J. Biol. Chem.* 269, 26227-26233.

Hannan, R.D., Stennard, F.A., and West, A.K. (1994). Localization of c-myc protooncogene expression in the rat heart in vivo and in the isolated, perfused heart following treatment with norepinephrine. *Biochim. Biophys. Acta* 1217, 281-290.

Harris, P.J., Thomas, D., and Morgan, T.O. (1987). Atrial natriuretic peptide inhibits angiotensin-stimulated proximal tubular sodium and water reabsorption. *Nature* 326, 697-698.

Hasebe, N., Hittinger, L., Kohin, S., Shen, Y.T., Graham, R.M., and Vatner, S.F. (1995). Effects of hypertrophy on left atrial and ventricular compliance and plasma ANF levels in conscious dogs. *Am. J. Physiol.* 268, Pt 2:H781-7.

Hasegawa, K., Fujiwara, H., Itoh, H., Nakao, K., Fujiwara, T., Imura, H., and Kawai, C. (1991). Light and electron microscopic localization of brain natriuretic peptide in relation to atrial natriuretic peptide in porcine atrium. Immunohistochemical study using specific monoclonal antibodies. *Circulation* 84, 1203-1209.

Hasegawa, K., Fujiwara, H., Doyama, K., Miyamae, M., Fujiwara, T., Suga, S., Mukoyama, M., Nakao, K., Imura, H., and Sasayama, S. (1993a). Ventricular expression of brain natriuretic peptide in hypertrophic cardiomyopathy. *Circulation* 88, 372-380.

Hasegawa, K., Fujiwara, H., Doyama, K., Mukoyama, M., Nakao, K., Fujiwara, T., Imura, H., and Kawai, C. (1993b). Ventricular expression of atrial and brain natriuretic peptides in dilated cardiomyopathy. An immunohistochemical study of the endomyocardial biopsy specimens using specific monoclonal antibodies. *Am. J. Pathol.* 142, 107-116.

Hayashi, J., Ohni, M., Manabe, H., and Watanabe, Y. (1988). Biochemical mechanism of release of atrial natriuretic polypeptide. *Jap. Circ. J.* 52, 1421-1424.

- Healy, D.P. and Fanestil, D.D. (1986). Localization of atrial natriuretic peptide binding sites within the rat kidney. *Am. J. Physiol.* **250**, F573-F578.
- Heikinheimo, M., Scandrett, J.M., and Wilson, D.B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Developmental Biology* **164**, 361-373.
- Henrich, W.L., McAlister, E.A., Smith, P.B., Lipton, J., and Campbell, W.B. (1987). Direct inhibitory effect of atriopeptin III on renin release in primate kidney. *Life Sci.* **41**, 259-264.
- Hepler, J.R. and Gilman, A.G. (1992). G proteins. *Trends Biochem. Sci.* **17**, 383-387.
- Herman, W.H. and Simonson, M.S. (1995). Nuclear signaling by endothelin-1. A Ras pathway for activation of the c-fos serum response element. *J. Biol. Chem.* **270**, 11654-11661.
- Hibbs, R.G. and Ferrans, V.J. (1969). An ultrastructural and histochemical study of rat atrial myocardium. *Am. J. Anat.* **124**, 251-279.
- Hintze, T.H., Currie, M.G., and Needleman, P. (1985). Atriopeptins: renal-specific vasodilators in conscious dogs. *Am. J. Physiol.* **248**, H587-H591.
- Hintze, T.H. (1988). Significance of renal vasodilation after administration of atrial natriuretic factor in the conscious dog. *Hypertension* **12**, 143-151.
- Hirai, S., Izumi, Y., Higa, K., Kaibuchi, K., Mizuno, K., Osada, S., Suzuki, K., and Ohno, S. (1994). Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKC delta. *EMBO J.* **13**, 2331-2340.
- Hirata, Y., Ishii, M., Sugimoto, T., Matsuoka, H., Kangawa, K., and Matsuo, H. (1985). The effects of human atrial 28-amino acid peptide on systemic and renal hemodynamics in anesthetized rats. *Circ. Res.* **57**, 634-639.
- Hirata, Y., Suzuki, Y., Suzuki, E., Hayakawa, H., Kimura, K., Goto, A., Omata, M., Minamino, N., Kangawa, K., and Matsuo, H. (1994). Mechanisms underlying the augmented responses of

deoxycorticosterone acetate-salt hypertensive rats to neutral endopeptidase inhibitors. *J. Hypertens.* 12, 367-374.

Hirose, S., Akiyama, F., Shinjo, M., Ohno, H., and Murakami, K. (1985). Solubilization and molecular weight estimation of atrial natriuretic factor receptor from bovine adrenal cortex. *Biochem. Biophys. Res. Commun.* 130, 574-579.

Holmes, S.J., Espiner, E.A., Richards, A.M., Yandle, T.G., and Frampton, C. (1993). Renal, endocrine, and hemodynamic effects of human brain natriuretic peptide in normal man. *J. Clin. Endocrin. Metab.* 76, 91-96.

Holtz, J., Stewart, D.J., Elsner, D., and Bassenge, E. (1986). In vivo atrial peptide-venodilation: minimal potency relative to nitroglycerin in dogs. *Life Sci.* 39, 2177-2184.

Holtz, J., Sommer, O., and Bassenge, E. (1987). Inhibition of sympathoadrenal activity by atrial natriuretic factor in dogs. *Hypertension* 9, 350-354.

Horio, T., Kohno, M., and Takeda, T. (1992). Effects of arginine vasopressin, angiotensin II and endothelin-1 on the release of brain natriuretic peptide in vivo and in vitro. *Clin. Exp. Pharmacol. Physiol.* 19, 575-582.

Horio, T., Kohno, M., and Takeda, T. (1993). Cosecretion of atrial and brain natriuretic peptides stimulated by endothelin-1 from cultured rat atrial and ventricular cardiocytes. *Metab. : Clin. & Exp.* 42, 94-96.

Hosoda, K., Nakao, K., Mukoyama, M., Saito, Y., Jougasaki, M., Shirakami, G., Suga, S., Ogawa, Y., Yasue, H., and Imura, H. (1991). Expression of brain natriuretic peptide gene in human heart -production in the ventricle. *Hypertension* 17, 1152-1156.

Howell, S.L. and Tyhurst, M. (1986). The cytoskeleton and insulin secretion. *Diabetes-Metabolism Reviews* 2, 107-123.

Hoyer, J., Distler, A., Haase, W., and Gogelein, H. (1994).  $Ca^{2+}$  influx through stretch-activated cation channels activates maxi  $K^{+}$  channels in porcine endocardial endothelium. *Proc. Natl. Acad. Sci. U. S. A.* 91, 2367-2371.

- Hu, J.R., Berninger, U.G., and Lang, R.E. (1988). Endothelin stimulates atrial natriuretic peptide (ANP) release from rat atria. *Eur. J. Pharmacol.* 158, 177-178.
- Hu, R.M., Levin, E.R., Pedram, A., and Frank, H.J. (1992). Atrial natriuretic peptide inhibits the production and secretion of endothelin from cultured endothelial cells. Mediation through the C receptor. *J. Biol. Chem.* 267, 17384-17389.
- Huang, C.L., Lewicki, J., Johnson, L.K., and Cogan, M.G. (1985). Renal mechanism of action of rat atrial natriuretic factor. *J. Clin. Invest.* 75, 769-773.
- Huxley, V.H., Tucker, V.L., Verburg, K.M., and Freeman, R.H. (1987). Increased capillary hydraulic conductivity induced by atrial natriuretic peptide. *Circ. Res.* 60, 304-307.
- Iida, H., Barron, W.M., and Page, E. (1988). Monensin turns on microtubule-associated translocation of secretory granules in cultured rat atrial myocytes. *Circ. Res.* 62, 1159-1170.
- Iida, H. and Shibata, Y. (1994). Phasic secretion of newly synthesized atrial natriuretic factor from unstimulated atrial myocytes in culture. *Circ. Res.* 74, 659-668.
- Ikeda, U., Tsuruya, Y., and Yaginuma, T. (1991). Alpha 1-adrenergic stimulation is coupled to cardiac myocyte hypertrophy. *Am. J. Physiol.* 260, H953-H956.
- Imada, T., Takayanagi, R., and Inagami, T. (1988). Atrioactivase, a specific peptidase in bovine atria for the processing of pro-atrial natriuretic factor. Purification and characterization. *J. Biol. Chem.* 263, 9515-9519.
- Inoue, H., Hashimoto, K., and Ota, Z. (1988). In vitro release of immunoreactive atrial natriuretic peptide from the rat atria. *Acta Med. Okayama* 42, 61-67.
- Irons, C.E., Sei, C.A., Hidaka, H., and Glembotski, C.C. (1992). Protein kinase C and calmodulin kinase are required for endothelin-stimulated atrial natriuretic factor secretion from primary atrial myocytes. *J. Biol. Chem.* 267, 5211-5216.

Irons, C.E., Murray, S.F., and Glembotski, C.C. (1993a). Identification of the receptor subtype responsible for endothelin-mediated protein kinase C activation and atrial natriuretic factor secretion from atrial myocytes. *J. Biol. Chem.* 268, 23417-23421.

Irons, C.E., Sei, C.A., and Glembotski, C.C. (1993b). Regulated secretion of atrial natriuretic factor from cultured ventricular myocytes. *Am. J. Physiol.* 264, H282-H285.

Ishida, A., Tanahashi, T., Okumura, K., Hashimoto, H., Ito, T., Ogawa, K., and Satake, T. (1988). A calmodulin antagonist (W-7) and a protein kinase C inhibitor (H-7) have no effect on atrial natriuretic peptide release induced by atrial stretch. *Life Sci.* 42, 1659-1667.

Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Taniguchi, K., and Marumo, F. (1991). Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ. Res.* 69, 209-215.

Ito, H., Hirata, Y., Adachi, S., Tanaka, M., Tsujino, M., Koike, A., Nogami, A., Murumo, F., and Hiroe, M. (1993). Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes. *J. Clin. Invest.* 92, 398-403.

Ito, H., Hiroe, M., Hirata, Y., Fujisaki, H., Adachi, S., Akimoto, H., Ohta, Y., and Marumo, F. (1994). Endothelin ET<sub>A</sub> receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload. *Circulation* 89, 2198-2203.

Itoh, H., Nakao, K., Katsuura, G., Morii, N., Shiono, S., Sakamoto, M., Sugawara, A., Yamada, T., Saito, Y., Matsushita, A., and et, a.l. (1986). Centrally infused atrial natriuretic polypeptide attenuates exaggerated salt appetite in spontaneously hypertensive rats. *Circ. Res.* 59, 342-347.

Itoh, H., Pratt, R.E., and Dzau, V.J. (1990). Atrial natriuretic polypeptide inhibits hypertrophy of vascular smooth muscle cells. *J. Clin. Invest.* 86, 1690-1697.

Itoh, H., Pratt, R.E., and Dzau, V.J. (1991). Interaction of atrial natriuretic polypeptide and angiotensin II on protooncogene expression and vascular cell growth. *Biochem. Biophys. Res. Commun.* 176, 1601-1609.

- Itoh, H., Pratt, R.E., Ohno, M., and Dzau, V.J. (1992). Atrial natriuretic polypeptide as a novel antigrowth factor of endothelial cells. *Hypertension* 19, 758-761.
- Iwaki, K., Sukhatme, V.P., Shubeita, H.E., and Chien, K.R. (1990). Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. *fos/jun* expression is associated with sarcomere assembly; *Egr-1* induction is primarily an alpha 1-mediated response. *J. Biol. Chem.* 265, 13809-13817.
- Izumo, S., Nadal-Ginard, B., and Mahdavi, V. (1988). Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc. Natl. Acad. Sci. U. S. A.* 85, 339-343.
- Jackson, T., Allard, M.F., Sreenan, C.M., Doss, L.K., Bishop, S.P., and Swain, J.L. (1990). The c-myc proto-oncogene regulates cardiac development in transgenic mice. *Mol. Cell. Biol.* 10, 3709-3716.
- Jahnel, U., Duwe, E., Pfennigsdorf, S., and Nawrath, H. (1994). On the mechanism of action of phenylephrine in rat atrial heart muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.* 349, 408-415.
- Jamieson, J.D. and Palade, G.E. (1964). Specific granules in atrial muscle cells. *J. Cell Biol.* 23, 151-172.
- Jarygin, C., Hanze, J., and Lang, R.E. (1994). Gene expression of atrial natriuretic peptide in rat papillary muscle. Rapid induction by mechanical loading. *Febs Let.* 346, 185-188.
- Jiao, J.H. and Baertschi, A.J. (1993). Neural control of the endocrine rat heart. *Proc. Natl. Acad. Sci. U. S. A.* 90, 7799-7803.
- Jiao, J.H. and Baertschi, A.J. (1995). Synergistic regulation of ANF in isolated rat hearts. *Am. J. Physiol.* 268, Pt 2):H1405-11.
- John, S.W., Krege, J.H., Oliver, P.M., Hagaman, J.R., Hodgins, J.B., Pang, S.C., Flynn, T.G., and Smithies, O. (1995). Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science* 267, 679-681.

Jones, L.G., Rozich, J.D., Tsutsui, H., and Cooper, G., 4th (1992). Endothelin stimulates multiple responses in isolated adult ventricular cardiac myocytes. *Am. J. Physiol.* 263, Pt 2):H1447-54.

Kabayama, Y., Fukamachi, Y., Kamisaki, T., and Tanaka, H. (1987). Stimulation by volume expansion of atrial natriuretic peptide release from perfused rat heart. *Peptides* 8, 257-260.

Kangawa, K., Tawaragi, Y., Oikawa, S., Mizuno, A., Sakuragawa, Y., Nakazato, H., Fukuda, A., Minamino, N., and Matsuo, H. (1984). Identification of rat gamma atrial natriuretic polypeptide and characterization of the cDNA encoding its precursor. *Nature* 312, 152-155.

Kangawa, K., Fukuda, A., and Matsuo, H. (1985). Structural identification of beta- and gamma-human atrial natriuretic polypeptides. *Nature* 313, 397-400.

Katoh, S., Toyama, J., Aoyama, M., Miyamoto, N., Seo, H., Matsui, N., Kodama, I., and Yamada, K. (1990). Mechanisms of atrial natriuretic peptide (ANP) secretion by rat hearts perfused in vitro—Ca<sup>2+</sup>(+)-dependent signal transduction for ANP release by mechanical stretch. *Jap. Circ. J.* 54, 1283-1294.

Kennedy, B.P., Marsden, J.J., Flynn, T.G., de Bold, A.J., and Davies, P.L. (1984). Isolation and nucleotide sequence of a cloned cardionatin cDNA. *Biochem. Biophys. Res. Commun.* 122, 1076-1082.

Kim, D. (1992). A mechanosensitive K<sup>+</sup> channel in heart cells. Activation by arachidonic acid. *J. Gen. Physiol.* 100, 1021-1040.

Kim, D. (1993). Novel cation-selective mechanosensitive ion channel in the atrial cell membrane. *Circ. Res.* 72, 225-231.

Kim, D. and Fu, C. (1993). Activation of a nonselective cation channel by swelling in atrial cells. *J. Membrane Biol.* 135, 27-37.

Kinnunen, P., Vuolteenaho, O., and Ruskoaho, H. (1993). Mechanisms of atrial and brain natriuretic peptide release from rat ventricular myocardium: effect of stretching. *Endocrinology* 132, 1961-1970.

- Kisch, B. (1956). Electron microscopy of the atrium of the heart I. Guinea pig. *Exp. Med. Surg.* 14, 99-112.
- Kisch, B. (1963). A significant electron microscopic difference between the atria and the ventricles of the mammalian heart. *Exp. Med. Surg.* 21, 193-221.
- Kita, T., Kida, O., Yokota, N., Eto, T., Minamino, N., Kangawa, K., Matsuo, H., and Tanaka, K. (1991). Effects of brain natriuretic peptide-45, a circulating form of rat brain natriuretic peptide, in spontaneously hypertensive rats. *Eur. J. Pharmacol.* 202, 73-79.
- Knowlton, K.U., Baracchini, E., Ross, R.S., Harris, A.N., Henderson, S.A., Evans, S.M., Glembotski, C.C., and Chien, K.R. (1991). Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during alpha-adrenergic stimulation of neonatal rat ventricular cells. Identification of cis sequences within an embryonic and a constitutive contractile protein gene which mediate inducible expression. *J. Biol. Chem.* 266, 7759-7768.
- Knowlton, K.U., Michel, M.C., Itani, M., Shubeita, H.E., Ishihara, K., Brown, J.H., and Chien, K.R. (1993). The alpha 1A-adrenergic receptor subtype mediates biochemical, molecular, and morphologic features of cultured myocardial cell hypertrophy. *J. Biol. Chem.* 268, 15374-15380.
- Knowlton, K.U., Rockman, H.A., Itani, M., Vovan, A., Seidman, C.E., and Chien, K.R. (1995). Divergent pathways mediate the induction of ANF transgenes in neonatal and hypertrophic ventricular myocardium. *J. Clin. Invest.* 96, 1311-1318.
- Kohno, M., Yasunari, K., Yokokawa, K., Murakawa, K., Horio, T., and Takeda, T. (1991). Inhibition by atrial and brain natriuretic peptides of endothelin-1 secretion after stimulation with angiotensin II and thrombin of cultured human endothelial cells. *J. Clin. Invest.* 87, 1999-2004.
- Kohno, M., Horio, T., Yokokawa, K., Kurihara, N., and Takeda, T. (1992a). C-type natriuretic peptide inhibits thrombin- and angiotensin II-stimulated endothelin release via cyclic guanosine 3',5'-monophosphate. *Hypertension* 19, 320-325.
- Kohno, M., Yokokawa, K., Horio, T., Yasunari, K., Murakawa, K., and Takeda, T. (1992b). Atrial and brain natriuretic peptides inhibit the endothelin-1 secretory response to angiotensin II in porcine aorta. *Circ. Res.* 70, 241-247.

Kohno, M., Horio, T., Ikeda, M., Yokokawa, K., Fukui, T., Yasunari, K., Murakawa, K., Kurihara, N., and Takeda, T. (1993). Natriuretic peptides inhibit mesangial cell production of endothelin induced by arginine vasopressin. *Am. J. Physiol.* 264, F678-F683.

Kohtz, D.S., Dische, N.R., Inagami, T., and Goldman, B. (1989). Growth and partial differentiation of presumptive human cardiac myoblasts in culture. *J. Cell Biol.* 108, 1067-1078.

Kojima, M., Minamino, N., Kangawa, K., and Matsuo, H. (1989). Cloning and sequence analysis of cDNA encoding a precursor for rat brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* 159, 1420-1426.

Kojima, M., Minamino, N., Kangawa, K., and Matsuo, H. (1990). Cloning and sequence analysis of a cDNA encoding a precursor for rat C-type natriuretic peptide (CNP). *FEBS Lett.* 276, 209-213.

Kolbeck-Ruhmkorff, C., Horban, A., and Zimmer, H.G. (1993). Effect of pressure and volume overload on proto-oncogene expression in the isolated working rat heart. *Cardiovasc. Res.* 27, 1998-2004.

Koller, K.J., Lowe, D.G., Bennett, G.L., Minamino, N., Kangawa, K., Matsuo, H., and Goeddel, D.V. (1991). Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* 252, 120-123.

Koller, P.T., Grekin, R.J., and Nicklas, J.M. (1987). Paradoxical response of plasma atrial natriuretic hormone to pericardiocentesis in cardiac tamponade. *Am. J. Cardiol.* 59, 491-492.

Komuro, I., Kurabayashi, M., Takaku, F., and Yazaki, Y. (1988). Expression of cellular oncogenes in the myocardium during the developmental stage and pressure-overloaded hypertrophy on the rat heart. *Circ. Res.* 62, 1075-1079.

Komuro, I., Kaida, T., Shibasaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F., and Yazaki, Y. (1990). Stretching cardiac myocytes stimulates protooncogene expression. *J. Biol. Chem.* 265, 3595-3598.

Komuro, I., Katoh, Y., Kaida, T., Shibasaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. (1991). Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. Possible role of protein kinase C activation. *J. Biol. Chem.* 266, 1235-1268.

Komuro, I. and Yazaki, Y. (1993). Control of cardiac gene expression by mechanical stress. *Annu. Rev. Physiol.* 55, 55-75.

Kondo, Y., Imai, M., Kangawa, K., and Matsuo, H. (1986). Lack of direct action of alpha-human atrial natriuretic polypeptide on the in vitro perfused segments of Henle's loop isolated from rabbit kidney. *Pflüegers Arch.* 406, 273-278.

Koseki, C., Hayashi, Y., Torikai, S., Furuya, M., Ohmura, N., and Imai, M. (1986). Localization of binding sites of  $\alpha$ -rat atrial natriuretic polypeptide in rat kidney. *Am. J. Physiol.* 250, F210-F216.

Kovacic-Milivojevic, B., Schultz, H.D., and Gardner, D.G. (1991). Arachidonic acid metabolites regulate the secretion of atrial natriuretic peptide in cultured rat atrial cardiocytes. *Can. J. Physiol. Pharmacol.* 69, 1493-1499.

Kovacic-Milivojevic, B. and Gardner, D.G. (1992). Divergent regulation of the human atrial natriuretic peptide gene by c-jun and c-fos. *Mol. Cell. Biol.* 12, 292-301.

Kovacic-Milivojevic, B. and Gardner, D.G. (1993). Regulation of the human atrial natriuretic peptide gene in atrial cardiocytes by the transcription factor AP-1. *Am. J. Hypertens.* 6, 258-263.

Kovacic-Milivojevic, B. and Gardner, D.G. (1995). Fra-1, a Fos gene family member that activates atrial natriuretic peptide gene transcription. *Hypertension* 25, 679-82.

Kretzner, L., Blackwood, E.M., and Eisenman, R.N. (1992). Myc and Max proteins possess distinct transcriptional activities. *Nature* 359, 426-429.

Kuchel, O., Debinski, W., Racz, K., Buu, N.T., Garcia, R., Cusson, J.R., Larochelle, P., Cantin, M., and Genest, J. (1987). An emerging relationship between peripheral sympathetic nervous activity and atrial natriuretic factor. *Life Sci.* 40, 1545-1551.

Kudo, T. and Baird, A. (1984). Inhibition of aldosterone production in the adrenal glomerulosa by atrial natriuretic factor. *Nature* 312, 756-757.

- Kuliawat, R. and Arvan, P. (1992). Protein targeting via the "constitutive-like" secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. *J. Cell Biol.* 118, 521-529.
- Kuno, T., Andresen, J.W., Kamisaki, Y., Waldman, S.A., Chang, L.Y., Saheki, S., Leitman, D.C., Nakane, M., and Murad, F. (1986). Co-purification of an atrial natriuretic factor receptor and particulate guanylate cyclase from rat lung. *J. Biol. Chem.* 261, 5817-5823.
- Kuraja, I.J., Tanner, J.K., and Woodcock, E.A. (1990). Endothelin stimulates phosphatidylinositol turnover in rat right and left atria. *Eur. J. Pharmacol.* 189, 299-306.
- Kuroski-de Bold, M.L., Pulido, O., Dubé, G., Fernandez, B.E., and de Bold, A.J. (1992). Morphological and biochemical studies on the subcellular distribution of A- and B-type natriuretic factors in the rat heart. *FASEB J.* 6(Pt 1), A1234(Abstract)
- Kuroski-de Bold, M.L. and de Bold, A.J. (1991). Stretch-secretion coupling in atrial cardiocytes. Dissociation between atrial natriuretic factor release and mechanical activity. *Hypertension* 18(Suppl.3), III-169-III-178.
- Kurtz, A., Della Bruna, R., Pfeilschifter, J., Taugner, R., and Bauer, C. (1986). Atrial natriuretic peptide inhibits renin release from juxtaglomerular cells by a cGMP-mediated process. *Proc. Natl. Acad. Sci. U. S. A.* 83, 4769-4773.
- Lachance, D., Garcia, R., Gutkowska, J., Cantin, M., and Thibault, G. (1986). Mechanisms of release of atrial natriuretic factor. I. Effect of several agonists and steroids on its release by atrial minces. *Biochem. Biophys. Res. Commun.* 135, 1090-1098.
- Ladenson, P.W., Bloch, K.D., and Seidman, J.G. (1988). Modulation of atrial natriuretic factor by thyroid hormone: messenger ribonucleic acid and peptide levels in hypothyroid, euthyroid, and hyperthyroid rat atria and ventricles. *Endocrinology* 123, 652-657.
- Laine, M., Arjamaa, O., Vuolteenaho, O., Ruskoaho, H., and Weckstrom, M. (1994a). Block of stretch-activated atrial natriuretic peptide secretion by gadolinium in isolated rat atrium. *J. Physiol.* 480, 553-561.

- Laine, M., Weckström, M., Vuolteenaho, O., and Arjamaa, O. (1994b). Effect of ryanodine on atrial natriuretic peptide secretion by contracting and quiescent rat atrium. *Pflüegers Arch.* 426, 276-283.
- LaMorte, V.J., Thorburn, J., Absher, D., Spiegel, A., Brown, J.H., Chien, K.R., Feramisco, J.R., and Knowlton, K.U. (1994).  $G_q$ - and Ras-dependent pathways mediate hypertrophy of neonatal rat ventricular myocytes following  $\alpha_1$ -adrenergic stimulation. *J. Biol. Chem.* 269, 13490-13496.
- Lang, C.C., Choy, A.M.J., Turner, K., Tobin, R., Coutie, W., and Struthers, A.D. (1993). The effect of intravenous saline loading on plasma levels of brain natriuretic peptide in man. *J. Hypertens.* 11, 737-741.
- Lang, R.E., Thölken, H., Ganten, D., Luft, F.C., Unger, T., and Dohlemann, D. (1985). Atrial natriuretic factor: a circulating hormone stimulated by volume loading. *Nature* 314, 264-266.
- LaPointe, M.C., Wu, J.P., Greenberg, B., and Gardner, D.G. (1988). Upstream sequences confer atrial-specific expression on the human atrial natriuretic factor gene. *J. Biol. Chem.* 263, 9075-9078.
- LaPointe, M.C. and Sitkins, J.R. (1993). Phorbol ester stimulates the synthesis and secretion of brain natriuretic peptide from neonatal rat ventricular cardiocytes: a comparison with the regulation of atrial natriuretic factor. *Mol. Endocrinol.* 7, 1284-1296.
- Lappe, R.W., Smits, J.F.M., Todt, J.A., Debets, J.J.M., and Wendt, R.L. (1985a). Failure of atriopeptin II to cause arterial vasodilation in the conscious rat. *Circ. Res.* 56, 606-612.
- Lappe, R.W., Todt, J.A., and Wendt, R.L. (1985b). Mechanism of action of vasoconstrictor responses to atriopeptin II in conscious SHR. *Am. J. Physiol.* 249(6 Pt 2), R781-R786.
- Larivière, R., Deng, L.Y., Day, R., Sventek, P., Thibault, G., and Schiffrin, E.L. (1995). Increased endothelin-1 gene expression in the endothelium of coronary arteries and endocardium in the DOCA-salt hypertensive rat. *J. Mol. Cell. Cardiol.* 27, 2123-2131.
- Lattion, A.L., Michel, J.B., Arnauld, E., Corvol, P., and Soubrier, F. (1986). Myocardial recruitment during ANF mRNA increase with volume overload in the rat. *Am. J. Physiol.* 251, H890-H896.

- Laxson, D.D., Dai, X.Z., Schwartz, J.S., and Bache, R.J. (1988). Effects of atrial natriuretic peptide on coronary vascular resistance in the intact awake dog. *J. Am. Col. Cardiol.* 11, 624-629.
- Leach, K.L., Ruff, V.A., Wright, T.M., Pessin, M.S., and Raben, D.M. (1991). Dissociation of protein kinase C activation and sn-1,2-diacylglycerol formation. Comparison of phosphatidylinositol- and phosphatidylcholine-derived diglycerides in alpha-thrombin-stimulated fibroblasts. *J. Biol. Chem.* 266, 3215-3221.
- Ledsome, J.R., Wilson, N., Courneya, C.A., and Rankin, A.J. (1985). Release of atrial natriuretic peptide by atrial distension. *Can. J. Physiol. Pharmacol.* 63, 739-742.
- Lee, R.T., Bloch, K.D., Pfeffer, J.M., Pfeffer, M.A., Neer, E.J., and Seidman, C.E. (1988). Atrial natriuretic factor gene expression in ventricles of rats with spontaneous biventricular hypertrophy. *J. Clin. Invest.* 81, 431-434.
- Leite, M.F., Page, E., and Ambler, S.K. (1994). Regulation of ANP secretion by endothelin-1 in cultured atrial myocytes: desensitization and receptor subtype. *Am. J. Physiol.* 267, H2193-H2203.
- Leitman, D.C., Andresen, J.W., Kuno, T., Kamisaki, Y., Chang, J.K., and Murad, F. (1986). Identification of multiple binding sites for atrial natriuretic factor by affinity cross-linking in cultured endothelial cells. *J. Biol. Chem.* 261, 11650-11655.
- Leskinen, H., Vuolteenaho, O., and Ruskoaho, H. (1995). Endothelin receptor antagonists inhibit volume load-induced atrial natriuretic peptide release in vivo. *Circulation* 92, 1-220(Abstract)
- Li, Q. and Gardner, D.G. (1994). Negative regulation of the human atrial natriuretic peptide gene by 1,25-dihydroxyvitamin D3. *J. Biol. Chem.* 269, 4934-4939.
- Lopez, M.J., Wong, S.K.-F., Kishimoto, I., DuBois, S., Mach, V., Friesen, J., Garbers, D.L., and Beuve, A. (1995). Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for natriuretic peptide. *Nature* 378, 65-68.
- Lynch, D.R., Venable, J.C., and Snyder, S.H. (1988). Enkephalin convertase in the heart: similar disposition to atrial natriuretic factor. *Endocrinology* 122, 2683-2691.

- Maack, T., Marion, D.N., Camargo, M.J., Kleinert, H.D., Laragh, J.H., Vaughan ED, J.r., and Atlas, S.A. (1984). Effects of auriculin (atrial natriuretic factor) on blood pressure, renal function, and the renin-aldosterone system in dogs. *Am. J. Med.* 77, 1069-1075.
- Maack, T., Suzuki, M., Almeida, F.A., Nussenzveig, D., Scarborough, R.M., McEnroe, G.A., and Lewicki, J.A. (1987). Physiological role of silent receptors of atrial natriuretic factor. *Science* 238, 675-678.
- Maack, T. (1992). Receptors of atrial natriuretic factor. *Annu. Rev. Physiol.* 54, 11-27.
- Magga, J., Marttila, M., Mäntymaa, P., Vuolteenaho, O., and Ruskoaho, H. (1994). Brain natriuretic peptide in plasma, atria, and ventricles of vasopressin- and phenylephrine-infused conscious rats. *Endocrinology* 134, 2505-2515.
- Maki, M., Takayanagi, R., Misono, K.S., Pandey, K.N., Tibbetts, C., and Inagami, T. (1984). Structure of rat atrial natriuretic factor precursor deduced from cDNA sequence. *Nature* 309, 722-724.
- Malek, A. and Izumo, S. (1992). Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. *Am. J. Physiol.* 263, Pt 1):C389-96.
- Mangat, H. and de Bold, A.J. (1993). Stretch-induced atrial natriuretic factor release utilizes a rapidly depleting pool of newly synthesized hormone. *Endocrinology* 133, 1398-1403.
- Manning, P.T., Schwartz, D., Katsube, N.C., Holmberg, S.W., and Needleman, P. (1985). Vasopressin-stimulated release of atriopeptin: endocrine antagonists in fluid homeostasis. *Science* 229, 395-397.
- Marcu, K.B., Harris, L.J., Stanton, L.W., Erikson, J., Watt, R., and Croce, C.M. (1983). Transcriptionally active c-myc oncogene is contained within NIARD, a DNA sequence associated with chromosome translocations in B-cell neoplasia. *Proc. Natl. Acad. Sci. U. S. A.* 80, 519-523.
- Marcu, K.B., Bossone, S.A., and Patel, A.J. (1992). myc function and regulation. *Annu. Rev. Biochem.* 61, 809-860.

Marie, J.-P., Guillemot, H., and Hatt, P.-Y. (1976). Le degré de granulation des cardiocytes auriculaires. Étude planimétrique au cours de différents apports d'eau et de sodium chez le Rat. *Pathol. Biol.* 24, 549-554.

Martinez-Palomo, A. and Bencosme, S.A. (1966). Changes in the content of myocardial specific granules in adrenal regeneration hypertension. *Fed. Proc. Fed. Am. Soc. Exp.* 25, 476a(Abstract)

Matsubara, H., Hirata, Y., Yoshimi, H., Takata, S., Takagi, Y., Iida, T., Yamane, Y., Umeda, Y., Nishikawa, M., and Inada, M. (1987a). Effects of steroid and thyroid hormones on synthesis of atrial natriuretic peptide by cultured atrial myocytes of rat. *Biochem. Biophys. Res. Commun.* 145, 336-343.

Matsubara, H., Hirata, Y., Yoshimi, H., Takata, S., Takagi, Y., Yamane, Y., Umeda, Y., Nishikawa, M., and Inada, M. (1987b). Ventricular myocytes from neonatal rats are more responsive to dexamethasone than atrial myocytes in synthesis of atrial natriuretic peptide. *Biochem. Biophys. Res. Commun.* 148, 1030-1038.

Matsubara, H., Hirata, Y., Yoshimi, H., Takata, S., Takagi, Y., Umeda, Y., Yamane, Y., and Inada, M. (1988). Role of calcium and protein kinase C in ANP secretion by cultured rat cardiocytes. *Am. J. Physiol.* 255, H405-H409.

Matsubara, H., Yamamoto, J., Hirata, Y., Mori, Y., Oikawa, S., and Inada, M. (1990). Changes of atrial natriuretic peptide and its messenger RNA with development and regression of cardiac hypertrophy in renovascular hypertensive rats. *Circ. Res.* 66, 176-184.

Mägert, H.J., Appelhans, H., Gassen, H.G., and Forssmann, W.G. (1990). Nucleotide sequence of a porcine prepro atrial natriuretic peptide (ANP) cDNA. *Nucl. Acids Res.* 18, 6704

Mäntymaa, P., Leppäluoto, J., and Ruskoaho, H. (1990). Endothelin stimulates basal and stretch-induced atrial natriuretic peptide secretion from the perfused rat heart. *Endocrinology* 126, 587-595.

Mäntymaa, P., Vuolteenaho, O., Marttila, M., and Ruskoaho, H. (1993). Atrial stretch induces rapid increase in brain natriuretic peptide but not in atrial natriuretic peptide gene expression in vitro. *Endocrinology* 133, 1470-1473.

- McBride, K., Robitaille, L., Tremblay, S., Argentin, S., and Nemer, M. (1993). fos/jun repression of cardiac-specific transcription in quiescent and growth-stimulated myocytes is targeted at a tissue-specific cis element. *Mol. Cell. Biol.* 13, 600-612.
- McClellan, G., Weisberg, A., Rose, D., and Winegrad, S. (1994). Endothelial cell storage and release of endothelin as a cardioregulatory mechanism. *Circ. Res.* 75, 85-96.
- McDonough, P.M., Brown, J.H., and Glembotski, C.C. (1993). Phenylephrine and endothelin differentially stimulate cardiac PI hydrolysis and ANF expression. *Am. J. Physiol.* 264, H625-H630.
- McDonough, P.M., Stella, S.L., and Glembotski, C.C. (1994). Involvement of cytoplasmic calcium and protein kinases in the regulation of atrial natriuretic factor secretion by contraction rate and endothelin. *J. Biol. Chem.* 269, 9466-9472.
- McMurray, J.J., Ray, S.G., Abdullah, I., Dargie, H.J., and Morton, J.J. (1992). Plasma endothelin in chronic heart failure. *Circulation* 85, 1374-1379.
- Meloche, S., Ong, H., Cantin, M., and De Lean, A. (1986). Affinity cross-linking of atrial natriuretic factor to its receptor in bovine adrenal zona glomerulosa. *J. Biol. Chem.* 261, 1525-1528.
- Mendez, R.E., Dunn, B.R., Troy, J.L., and Brenner, B.M. (1986). Modulation of the natriuretic response to atrial natriuretic peptide by alterations in peritubular Starling forces in the rat. *Circ. Res.* 59, 605-611.
- Mendez, R.E., Pfeffer, J.M., Ortola, F.V., Bloch, K.D., Anderson, S., Seidman, J.G., and Brenner, B.M. (1987). Atrial natriuretic peptide transcription, storage, and release in rats with myocardial infarction. *Am. J. Physiol.* 253, H1449-H1455.
- Mercadier, J.J., Samuel, J.L., Michel, J.B., Zongazo, M.A., de la Bastie, D., Lompré, A., Wisnewsky, C., Rappaport, L., Levy, B., and Schwartz, K. (1989). Atrial natriuretic factor gene expression in rat ventricle during experimental hypertension. *Am. J. Physiol.* 257, H979-H987.
- Metzler, C.H. and Ramsay, D.J. (1989). Physiological doses of atrial peptide inhibit angiotensin II-stimulated aldosterone secretion. *Am. J. Physiol.* 256, R1155-R1159.

Michener, M.L., Gierse, J.K., Seetharam, R., Fok, K.F., Olins, P.O., Mai, M.S., and Needleman, P. (1986). Proteolytic processing of atriopeptin prohormone. *Mol. Pharmacol.* 30, 552-557.

Miller-Hance, W.C., LaCorbière, M., Fuller, S.J., Evans, S.M., Lyons, G., Schmidt, C., Robbins, J., and Chien, K.R. (1993). In vitro chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. *J. Biol. Chem.* 268, 25244-25252.

Minamino, N., Aburaya, M., Kojima, M., Miyamoto, K., Kangawa, K., and Matsuo, H. (1993). Distribution of C-type natriuretic peptide and its messenger RNA in rat central nervous system and peripheral tissue. *Biochem. Biophys. Res. Commun.* 197, 326-335.

Misono, K.S., Fukumi, H., Grammer, R.T., and Inagami, T. (1984). Rat atrial natriuretic factor: complete amino acid sequence and disulfide linkage essential for biological activity. *Biochem. Biophys. Res. Commun.* 119, 524-529.

Moalic, J.M., Moazami-Goudarzi, K., Thiem, N.V., Delcayre, C., Bercovici, J., Mouas, C., and Swynghedauw, B. (1992). Hormonal induction of c-fos and HSP68 mRNAs on an isolated coronary perfused adult rat heart. *Arch. Int. Physiol. Biochim. Biophys.* 100, 165-170.

Moe, G.W., Stopps, T.P., Angus, C., Forster, C., de Bold, A.J., and Armstrong, P.W. (1989). Alterations in serum sodium in relation to atrial natriuretic factor and other neuroendocrine variables in experimental pacing-induced heart failure. *J. Am. Col. Cardiol.* 13, 173-179.

Mollenhauer, H.H., Morre, D.J., and Rowe, L.D. (1990). Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta* 1031, 225-246.

Mori, N., Singer-Sam, J., Lee, C.Y., and Riggs, A.D. (1986). The nucleotide sequence of a cDNA clone containing the entire coding region for mouse X-chromosome-linked phosphoglycerate kinase. *Gene* 45, 275-280.

Mori, Y., Nishikawa, M., Matsubara, H., Takagi, T., Toyoda, N., Oikawa, S., and Inada, M. (1990). Stimulation of rat atrial natriuretic peptide (rANP) synthesis by triiodothyronine and thyroxine (T<sub>4</sub>): T<sub>4</sub> as a prohormone in synthesizing rANP. *Endocrinology* 126, 466-471.

- Mori, Y., Nishikawa, M., Matsubara, H., Toyoda, N., Masaki, H., Yonemoto, T., Takagi, T., and Inada, M. (1991). Regulation of atrial natriuretic hormone production by triiodothyronine in cultured rat atrial myocytes. *Acta Endocrinol.* 125, 694-699.
- Morise, T., Takeuchi, Y., Okamoto, S., and Takeda, R. (1991). Stimulation of atrial natriuretic peptide secretion and synthesis by Na-K-ATPase inhibitors. *Biochem. Biophys. Res. Commun.* 176, 875-881.
- Morishita, R., Gibbons, G.H., Pratt, R.E., Tomita, N., Kaneda, Y., Ogihara, T., and Dzau, V.J. (1994). Autocrine and paracrine effects of atrial natriuretic peptide gene transfer on vascular smooth muscle and endothelial cellular growth. *J. Clin. Invest.* 94, 824-829.
- Morita, H., Nishida, Y., Motochigawa, H., Kangawa, K., Minamino, N., Matsuo, H., and Hosomi, H. (1989). Effects of brain natriuretic peptide on renal nerve activity in conscious rabbits. *Am. J. Physiol.* 256, R792-R796.
- Muir, T.M., Hair, J., Inglis, G.C., Dow, J.W., Lindop, G.B., and Leckie, B.J. (1993). Hormonal control of atrial natriuretic peptide synthesis and secretion from cultured atrial myocytes. *J. Mol. Cell. Cardiol.* 25, 509-518.
- Mukherjee, D.P., McTiernan, C.F., and Sen, S. (1993). Myotrophin induces early response genes and enhances cardiac gene expression. *Hypertension* 21, 142-148.
- Mukoyama, M., Nakao, K., Saito, Y., Ogawa, Y., Hosoda, K., Suga, S., Shirakami, G., Jougasaki, M., and Imura, H. (1990). Increased human brain natriuretic peptide in congestive heart failure. *N. Engl. J. Med.* 323, 757-758.
- Mukoyama, M., Nakao, K., Hosoda, K., Suga, S., Saito, Y., Ogawa, Y., Shirakami, G., Jougasaki, M., Obata, K., Yasue, H., Kambayashi, Y., Inoue, K., and Imura, H. (1991). Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. *J. Clin. Invest.* 87, 1402-1412.
- Munger, K.A., Sugiura, M., Takahashi, K., Inagami, T., and Badr, K.F. (1991). A role for atrial natriuretic peptide in endothelin-induced natriuresis. *J. Am. Soc. Nephrol.* 1, 1278-1283.

- Murphy, K.M., McLaughlin, L.L., Michener, M.L., and Needleman, P. (1985). Autoradiographic localization of atriopeptin III receptors in rat kidney. *Eur. J. Pharmacol.* **111**, 291-292.
- Nachshon, S., Zamir, O., Matsuda, Y., and Zamir, N. (1995). Effects of ANP receptor antagonists on ANP secretion from adult rat cultured atrial myocytes. *Am. J. Physiol.* **268**, E428-E432.
- Nakagawa, O., Ogawa, Y., Itoh, H., Suga, S., Komatsu, Y., Kishimoto, I., Nishino, K., Yoshimasa, T., and Nakao, K. (1995). Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. *J. Clin. Invest.* **96**, 1280-1287.
- Nakamura, S., Naruse, M., Naruse, K., Kawana, M., Nishikawa, T., Hosoda, S., Tanaka, I., Yoshimi, T., Yoshihara, I., Inagami, T., Uemura, H., and Demura, H. (1991). Atrial natriuretic peptide and brain natriuretic peptide coexist in the secretory granules of human cardiac myocytes. *Am. J. Hypertens.* **4**, 909-912.
- Nakao, K., Ogawa, Y., Suga, S., and Imura, H. (1992). Molecular biology and biochemistry of the natriuretic peptide system. I: Natriuretic peptides. *J. Hypertens.* **10**, 907-912.
- Nakayama, K., Ohkubo, H., Hirose, T., Inayama, S., and Nakanishi, S. (1984). mRNA sequence for human cardiodilatin-atrial natriuretic factor precursor and regulation of precursor mRNA in rat atria. *Nature* **310**, 699-701.
- Nanot, J. and Le Douarin, G. (1970). Étude au microscope électronique du myocarde du fœtus de Souris: influence de l'adrénaline et de la noradrénaline sur la teneur en granules spécifiques. *C. R. Soc. Biol.* **164**, 890-893.
- Naruse, M., Higashida, T., Naruse, K., Shibasaki, T., Demura, H., Inagami, T., and Shizume, K. (1987). Coronary hemodynamics and cardiac beating modulate atrial natriuretic factor release from isolated Langendorff-perfused rat hearts. *Life Sci.* **41**, 421-427.
- Nazario, B., Hu, R.M., Pedram, A., Prins, B., and Levin, E.R. (1995). Atrial and brain natriuretic peptides stimulate the production and secretion of C-type natriuretic peptide from bovine aortic endothelial cells. *J. Clin. Invest.* **95**, 1151-1157.

- Nemer, M., Chamberland, M., Sirois, D., Argentin, S., Drouin, J., Dixon, R.A., Zivin, R.A., and Condra, J.H. (1984). Gene structure of human cardiac hormone precursor, pronatriodilatin. *Nature* **312**, 654-656.
- Newman, T.M. and Severs, N.J. (1993). Stretch and anesthetic dependency of atrial natriuretic peptide release demonstrated by an ultrastructural assay. *Journal of Cellular Physiology* **155**, 240-247.
- Neyses, L., Nouskas, J., and Vetter, H. (1991). Inhibition of endothelin-1 induced myocardial protein synthesis by an antisense oligonucleotide against the early growth response gene-1. *Biochem. Biophys. Res. Commun.* **181**, 22-27.
- Neyses, L., Nouskas, J., Luyken, J., Fronhoffs, S., Oberdorf, S., Pfeifer, U., Williams, R.S., Sukhatme, V.P., and Vetter, H. (1993). Induction of immediate-early genes by angiotensin II and endothelin-1 in adult rat cardiomyocytes. *J. Hypertens.* **11**, 927-934.
- Nguyen, P.V., Parent, A., Deng, L.Y., Fluckiger, J.P., Thibault, G., and Schiffrin, E.L. (1992). Endothelin vascular receptors and responses in deoxycorticosterone acetate-salt hypertensive rats. *Hypertension* **19**, 1198-11104.
- Nguyen, T.T., Lazure, C., Babinski, K., Chretien, M., Ong, H., and De Lean, A. (1989). Aldosterone secretion inhibitory factor: a novel neuropeptide in bovine chromaffin cells. *Endocrinology* **124**, 1591-1593.
- Nishikimi, T., Miura, K., Minamino, N., Takeuchi, K., and Takeda, T. (1994). Role of endogenous atrial natriuretic peptide on systemic and renal hemodynamics in heart failure rats. *Am. J. Physiol.* **267**, H182-H186.
- Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-614.
- Noda, M., Katoh, T., Takuwa, N., Kumada, M., Kurokawa, K., and Takuwa, Y. (1994). Synergistic stimulation of parathyroid hormone-related peptide gene expression by mechanical stretch and angiotensin II in rat aortic smooth muscle cells. *J. Biol. Chem.* **269**, 17911-17917.

Noël, R. and Morin, G. (1929). Contribution à l'étude cytologique du noeud de Tawara et du faisceau de His. *Bull. Histol. Appl. Physiol. Pathol. Tech. Microsc.* 6, 119-130.

Nomura, T., Kurokawa, H., Kanda, S., Mizukawa, K., Sasaki, J., and Otsuka, N. (1988). Exocytotic features of rat specific atrial granules. *Acta Med. Okayama* 42, 201-206.

Northridge, D.B., McMurray, J., Ray, S., Jardine, A., and Dargie, H.J. (1989). Release of atrial natriuretic factor after pericardiocentesis for malignant pericardial effusion. *BMJ* 299, 603-604.

Ogawa, T., Linz, W., Stevenson, M., Bruneau, B.G., Kuroski-de Bold, M.L., Chen, J.H., Eid, H., Schölkens, B.A., and de Bold, A.J. (1996). Evidence for load-dependant and load-independent determinants of cardiac natriuretic peptide production. *Circulation* (in press)

Ogawa, Y., Nakao, K., Mukoyama, M., Hosoda, K., Shirakami, G., Arai, H., Saito, Y., Suga, S., Jougasaki, M., and Imura, H. (1991). Natriuretic peptides as cardiac hormones in normotensive and spontaneously hypertensive rats. The ventricle is a major site of synthesis and secretion of brain natriuretic peptide. *Circ. Res.* 69, 491-500.

Ogawa, Y., Itoh, H., Tamura, N., Suga, S., Yoshimasa, T., Uehira, M., Matsuda, S., Shiono, S., Nishimoto, H., and Nakao, K. (1994). Molecular cloning of the complementary DNA and gene that encode mouse brain natriuretic peptide and generation of transgenic mice that overexpress the brain natriuretic peptide gene. *J. Clin. Invest.* 93, 1911-1921.

Oikawa, S., Imai, M., Ueno, A., Tanaka, S., Noguchi, T., Nakazato, H., Kangawa, K., Fukuda, A., and Matsuo, H. (1984). Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. *Nature* 309, 724-726.

Oikawa, S., Imai, M., Inuzuka, C., Tawaragi, Y., Nakazato, H., and Matsuo, H. (1985). Structure of dog and rabbit precursors of atrial natriuretic polypeptides deduced from nucleotide sequence of cloned cDNA. *Biochem. Biophys. Res. Commun.* 132, 892-899.

Okazaki, T., Ando, K., Igarashi, T., Ogata, E., and Fujita, T. (1992). Conserved mechanism of negative gene regulation: by extracellular calcium. Parathyroid hormone gene versus atrial natriuretic polypeptide gene. *J. Clin. Invest.* 89, 1268-1273.

- Onwochei, M.O. and Rapp, J.P. (1988). Biochemically stimulated release of atrial natriuretic factor from heart-lung preparation of Dahl rats. *Proc. Soc. Exp. Biol. Med.* 188, 395-404.
- Oppenorth, T.J., Burnett, J.C., Jr., Granger, J.P., and Scriven, T.A. (1986). Effects of atrial natriuretic peptide on renin secretion in nonfiltering kidney. *Am. J. Physiol.* 250, F798-F801.
- Osol, G., Halpern, W., Tesfamariam, B., Nakayama, K., and Weinberg, D. (1986). Synthetic atrial natriuretic factor does not dilate resistance-sized arteries. *Hypertension* 8, 606-610.
- Ostlund, E., Bloom, G., Adams-Ray, J., Ritzen, M., Siegman, M., Nordenstam, H., Lishajko, F., and von Euler, U.S. (1960). Storage and release of catecholamines, and the occurrence of a specific submicroscopic granulation in hearts of cyclostomes. *Nature* 186, 324-325.
- Ota, K., Kimura, T., Shoji, M., Inoue, M., Sato, K., Ohta, M., Yamamoto, T., Tsunoda, K., Abe, K., and Yoshinaga, K. (1992). Interaction of ANP with endothelin on cardiovascular, renal, and endocrine function. *Am. J. Physiol.* 262, E135-E141.
- Page, E., Goings, G.E., Power, B., and Upshaw-Earley, J. (1986). Ultrastructural features of atrial peptide secretion. *Am. J. Physiol.* 251, H340-H348.
- Page, E., Goings, G.E., Power, B., and Upshaw-Earley, J. (1990). Basal and stretch-augmented natriuretic peptide secretion by quiescent rat atria. *Am. J. Physiol.* 259, C801-C818.
- Page, E., Upshaw-Earley, J., Goings, G.E., and Hanck, D.A. (1991). Inhibition of atrial peptide secretion at different stages of the secretory process:  $\text{Ca}^{2+}$  dependence. *Am. J. Physiol.* 261, C1162-C1172.
- Page, E., Upshaw-Earley, J., and Goings, G. (1992). Permeability of rat atrial endocardium, epicardium, and myocardium to large molecules. Stretch-dependent effects. *Circ. Res.* 71, 159-173.
- Page, E., Upshaw-Earley, J., and Goings, G.E. (1994). Localization of atrial natriuretic peptide in caveolae of in situ atrial myocytes. *Circ. Res.* 75, 949-954.
- Palade, G.E. (1961). Secretory granules in the atrial myocardium. *Anat. Rec.* 139, 262

Parker, T.G., Chow, K.L., Schwartz, R.J., and Schneider, M.D. (1990). Differential regulation of skeletal alpha-actin transcription in cardiac muscle by two fibroblast growth factors. *Proc. Natl. Acad. Sci. U. S. A.* 87, 7066-7070.

Parker, T.G., Chow, K.L., Schwartz, R.J., and Schneider, M.D. (1991). TGF-beta 1 and fibroblast growth factors selectively up-regulate tissue-specific fetal genes in cardiac muscle cells. *Ciba Foundation Symposium* 157, 152-160.

Paul, A.K., Marala, R.B., Jaiswal, R.K., and Sharma, R.K. (1987). Coexistence of guanylate cyclase and atrial natriuretic factor receptor in a 180-kD protein. *Science* 235, 1224-1226.

Pegram, B.L., Kardon, M.B., Trippodo, N.C., Cole, F.E., and MacPhee, A.A. (1985). Atrial extract: hemodynamics in Wistar-Kyoto and spontaneously hypertensive rats. *Am. J. Physiol.* 249, H265-H271.

Pegram, B.L., Trippodo, N.C., Natsume, T., Kardon, M.B., Frohlich, E.D., Cole, F.E., and MacPhee, A.A. (1986). Hemodynamic effects of atrial natriuretic hormone. *Fed. Proc.* 45, 2382-2386.

Perrella, M.A., Schwab, T.R., O'Murchu, B., Redfield, M.M., Wei, C.M., Edwards, B.S., and Burnett, J.C., Jr. (1992). Cardiac atrial natriuretic factor during evolution of congestive heart failure. *Am. J. Physiol.* 262, H1248-H1255.

Peters, J.P. (1952). The problems of cardiac edema. *Am. J. Med.* 12, 66-76.

Peterson, L.N., De Rouffignac, C., Sonnenberg, H., and Levine, D.Z. (1987). Thick ascending limb response to dDAVP and atrial natriuretic factor in vivo. *Am. J. Physiol.* 252, F374-F381.

Pirola, C.J., Wang, H.M., Strgacich, M.I., Kamyar, A., Cercek, B., Forrester, J.S., Clemens, T.L., and Fagin, J.A. (1994). Mechanical stimuli induce vascular parathyroid hormone-related protein gene expression in vivo and in vitro. *Endocrinology* 134, 2230-2236.

Pollack, P.S., Houser, S.R., Budjak, R., and Goldman, B. (1994). c-myc gene expression is localized to the myocyte following hemodynamic overload in vivo. *J. Cell. Biochem.* 54, 78-84.

- Proudfoot, N.J. and Brownlee, G.G. (1974). Sequence at the 3' end of globin mRNA shows homology with immunoglobulin light chain mRNA. *Nature* 252, 359-362.
- Puceat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L.L., and Brown, J.H. (1994). Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J. Biol. Chem.* 269, 16938-16944.
- Quadt, J.F., Voss, R., and ten Hoor, F. (1982). Prostacyclin production of the isolated pulsatingly perfused rat aorta. *J. Pharm. Meth.* 7, 263-270.
- Raine, A.E., Erne, P., Burgisser, E., Müller, F.B., Bolli, P., Burkart, F., and Bühler, F.R. (1986). Atrial natriuretic peptide and atrial pressure in patients with congestive heart failure. *N. Engl. J. Med.* 315, 533-537.
- Rankin, A.J., Ledsome, J.R., Keeler, R., and Wilson, N. (1987). Extracted and nonextracted atrial natriuretic peptide in rabbits during tachycardia. *Am. J. Physiol.* 253, R696-R700.
- Ransone, L.J. and Verma, I.M. (1990). Nuclear proto-oncogenes fos and jun. *Annu. Rev. Cell. Biol.* 6, 539-557.
- Rashtchian, A., Buchman, G.W., Schuster, D.M., and Berninger, M.S. (1992). Uracil DNA glycosylase mediated cloning of polymerase chain reaction-amplified DNA: application to genomic and cDNA cloning. *Anal. Biochem.* 206, 91-97.
- Rayner, T.E., Chen, B.N., McLoughlin, J.W., Menadue, M.F., Norman, R.J., and Oliver, J.R. (1993). Prostaglandin F2 alpha mediates platelet-activating factor-stimulated atrial natriuretic factor release from the isolated rat heart. *Endocrinology* 133, 1108-1115.
- Reis, L.C., Ramalho, M.J., Favaretto, A.L., Gutkowska, J., McCann, S.M., and Antunes-Rodrigues, J. (1994). Participation of the ascending serotonergic system in the stimulation of atrial natriuretic peptide release. *Proc. Natl. Acad. Sci. U. S. A.* 91, 12022-12026.
- Rhodes, C.J. and Halban, P.A. (1987). Newly synthesized proinsulin-insulin and stored insulin are released from pancreatic B-cells predominantly via a regulated, rather than constitutive, pathway. *J. Cell Biol.* 105, 145-153.

- Richards, A.M., Crozier, I.G., Holmes, S.J., Espiner, E.A., Yandle, T.G., and Frampton, C. (1993). Brain natriuretic peptide: natriuretic and endocrine effects in essential hypertension. *J. Hypertens.* **11**, 163-170.
- Riegger, A.J. (1991). Role of neuroendocrine mechanisms in the pathogenesis of heart failure. *Basic Res. Cardiol.* **86 Suppl 3**, 125-131.
- Riegger, A.J. (1994). Neuroendocrine excitation in heart failure. *Br. Heart J.* **72**, S28-S30.
- Robbins, R.J. and Swain, J.L. (1992). C-myc protooncogene modulates cardiac hypertrophic growth in transgenic mice. *Am. J. Physiol.* **262**, H590-H597.
- Rocha, A.S. and Kudo, L.H. (1990a). Factors governing sodium and chloride transport across the inner medullary collecting duct. *Kidney Int.* **38**, 654-667.
- Rocha, A.S. and Kudo, L.H. (1990b). Atrial peptide and cGMP effects on NaCl transport in inner medullary collecting duct. *Am. J. Physiol.* **259**, F258-F268.
- Rockman, H.A., Ross, R.S., Harris, A.N., Knowlton, K.U., Steinhilper, M.E., Field, L.J., Ross, J., Jr., and Chien, K.R. (1991). Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8277-8281.
- Rosenzweig, A., Halazonetis, T.D., Seidman, J.G., and Seidman, C.E. (1991). Proximal regulatory domains of rat atrial natriuretic factor gene. *Circulation* **84**, 1256-1265.
- Rosenzweig, A. and Seidman, C.E. (1991). Atrial natriuretic factor and related peptide hormones. *Annu. Rev. Biochem.* **60**, 229-255.
- Roy, R.N., Pang, S.C., and Flynn, T.G. (1992). Expression of natriuretic peptide genes in cardiac tissues of hypertensive rats. *Mol. Cell. Endocrinol.* **86**, R1-R5.
- Roy, R.N. and Flynn, T.G. (1990). Organization of the gene for iso-rANP, a rat B-type natriuretic peptide. *Biochem. Biophys. Res. Commun.* **171**, 416-423.

- Ruskoaho, H., Vakkuri, O., Arjamaa, O., Vuolteenaho, O., and Leppäluoto, J. (1989a). Pressor hormones regulate atrial-stretch-induced release of atrial natriuretic peptide in the pithed rat. *Circ. Res.* **64**, 482-492.
- Ruskoaho, H., Vakkuri, O., Arjamaa, O., Vuolteenaho, O., and Leppäluoto, J. (1989b). Pressor hormones regulate atrial stretch-induced release of atrial natriuretic peptides in the pithed rat. *Circ. Res.* **64**, 482-492.
- Ruskoaho, H., Vuolteenaho, O., and Leppäluoto, J. (1990). Phorbol esters enhance stretch-induced atrial natriuretic peptide secretion. *Endocrinology* **127**, 2445-2455.
- Rychlik, W., Spencer, W.J., and Rhoads, R.E. (1990). Optimization of the annealing temperature for DNA amplification in vitro. *Nucl. Acids Res.* **18**, 6409-6412.
- Rychlik, W. and Rhoads, R.E. (1989). A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. *Nucl. Acids Res.* **17**, 8543-8551.
- Saavedra, J.M., Correa, F.M., Plunkett, L.M., Israel, A., Kurihara, M., and Shigematsu, K. (1986). Binding of angiotensin and atrial natriuretic peptide in brain of hypertensive rats. *Nature* **320(6064)**, 758-760.
- Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T.J., and Izumo, S. (1992a). Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J. Biol. Chem.* **267**, 10551-10560.
- Sadoshima, J., Takahashi, T., Jahn, L., and Izumo, S. (1992b). Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9905-9909.
- Sadoshima, J., Xu, Y., Slayter, H.S., and Izumo, S. (1993). Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* **75**, 977-984.
- Sadoshima, J., Qiu, Z., Morgan, J.P., and Izumo, S. (1995). Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein

kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of  $\text{Ca}^{2+}$ -dependent signaling. *Circ. Res.* 76, 1-15.

Sadoshima, J. and Izumo, S. (1993a). Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J.* 12, 1681-1692.

Sadoshima, J. and Izumo, S. (1993b). Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ. Res.* 73, 413-423.

Saito, Y., Nakao, K., Arai, H., Nishimura, K., Okumura, K., Obata, K., Takemura, G., Fujiwara, H., Sugawara, A., Yamada, T., Itoh, H., Mukoyama, M., Hosoda, K., Kawai, C., Ban, T., Yasue, H., and Imura, H. (1989a). Augmented expression of atrial natriuretic polypeptide gene in ventricle of human failing heart. *J. Clin. Invest.* 83, 298-305.

Saito, Y., Nakao, K., Itoh, H., Yamada, T., Mukoyama, M., Arai, H., Hosoda, K., Shirakami, G., Suga, S., Minamino, N., and et, a.l. (1989b). Brain natriuretic peptide is a novel cardiac hormone. *Biochem. Biophys. Res. Commun.* 158, 360-368.

Saito, Y., Nakao, K., Itoh, H., Yamada, T., Mukoyama, M., Arai, H., Hosoda, K., Shirakami, G., Suga, S., Jougasaki, M., Morishika, S., and Imura, H. (1989c). Detection and characterization of endothelin-1-like immunoreactivity in rat plasma. *Biochem. Biophys. Res. Commun.* 163, 1512-1516.

Samson, W.K. (1985). Atrial natriuretic factor inhibits dehydration and hemorrhage-induced vasopressin release. *Neuroendocrinology* 40, 277-279.

Saper, C.B., Standaert, D.G., Currie, M.G., Schwarts, D., Geller, D.M., and Needleman, P. (1985). Atriopeptin-immunoreactive neurons in the brain: presence in cardiovascular regulatory areas. *Science* 227, 1047-1049.

Sarda, I.R., de Bold, M.L., and de Bold, A.J. (1989). Optimization of atrial natriuretic factor radioimmunoassay. *Clin. Biochem.* 22, 11-15.

- Scarborough, R.M., Schenk, D.D., McEnroe, G.A., Arfsten, A., Kang, L., Schwartz, K., and Lewicki, J.A. (1986). Truncated atrial natriuretic peptide analogs. Comparison between receptor binding and stimulation of cyclic GMP accumulation in cultured vascular smooth muscle cells. *J. Biol. Chem.* **261**, 12960-12964.
- Schenk, D.B., Phelps, M.N., Porter, J.G., Fuller, F., Cordell, B., and Lewicki, J.A. (1987). Purification and subunit composition of atrial natriuretic peptide receptor. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1521-1525.
- Schiebinger, R.J., Baker, M.Z., and Linden, J. (1987). Effect of adrenergic and muscarinic cholinergic agonists on atrial natriuretic peptide secretion by isolated rat atria Potential role of the autonomic nervous system in modulating atrial natriuretic peptide secretion. *J. Clin. Invest.* **80**, 1687-1691.
- Schiebinger, R.J. (1989). Calcium, its role in isoproterenol-stimulated atrial natriuretic peptide secretion by superfused rat atria. *Circ. Res.* **65**, 600-606.
- Schiebinger, R.J., Parr, H.G., and Cragoe, E.J., Jr. (1992). Calcium: its role in alpha 1-adrenergic stimulation of atrial natriuretic peptide secretion. *Endocrinology* **130**, 1017-1023.
- Schiebinger, R.J. and Cragoe, E.J., Jr. (1993). Ouabain. A stimulator of atrial natriuretic peptide secretion and its mechanism of action. *Circ. Res.* **72**, 1035-1043.
- Schiebinger, R.J. and Gomez-Sanchez, C.E. (1990). Endothelin: a potent stimulus of atrial natriuretic peptide secretion by superfused rat atria and its dependency on calcium. *Endocrinology* **127**, 119-125.
- Schiebinger, R.J. and Greening, K.M. (1992). Interaction between stretch and hormonally stimulated atrial natriuretic peptide secretion. *Am. J. Physiol.* **262**, H78-H83.
- Schiebinger, R.J. and Linden, J. (1986a). The influence of resting tension on immunoreactive atrial natriuretic peptide secretion by rat atria superfused in vitro. *Circ. Res.* **59**, 105-109.
- Schiebinger, R.J. and Linden, J. (1986b). Effect of atrial contraction frequency on atrial natriuretic peptide secretion. *Am. J. Physiol.* **251**, H1095-H1099.

Schubart, U.K., Erlichman, J., and Fleischer, N. (1982). Insulin release and protein phosphorylation: possible role of calmodulin. *Fed. Proc.* 41, 2278-2282.

Schulz, S., Singh, S., Bellet, R.A., Singh, G., Tubb, D.J., Chin, H., and Garbers, D.L. (1989). The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* 58, 1155-1162.

Schunkert, H., Jahn, L., Izumo, S., Apstein, C.S., and Lorell, B.H. (1991). Localization and regulation of c-fos and c-jun protooncogene induction by systolic wall stress in normal and hypertrophied rat hearts. *Proc. Natl. Acad. Sci. U. S. A.* 88, 11480-11484.

Sealey, J.E., Kirshman, J.E., and Laragh, J.H. (1969). Natriuretic activity in plasma and urine of salt-loaded man and sheep. *J. Clin. Invest.* 48, 2210-2224.

Sei, C.A., Irons, C.E., Sprenkle, A.B., McDonough, P.M., Brown, J.H., and Glembotski, C.C. (1991). The alpha-adrenergic stimulation of atrial natriuretic factor expression in cardiac myocytes requires calcium influx, protein kinase C, and calmodulin-regulated pathways. *J. Biol. Chem.* 266, 15910-15916.

Sei, C.A., Hand, G.L., Murray, S.F., and Glembotski, C.C. (1992). The cosecretional maturation of atrial natriuretic factor by primary atrial myocytes. *Mol. Endocrinol.* 6, 309-319.

Sei, C.A. and Glembotski, C.C. (1990). Calcium dependence of phenylephrine-, endothelin-, and potassium chloride-stimulated atrial natriuretic factor secretion from long term primary neonatal rat atrial cardiocytes. *J. Biol. Chem.* 265, 7166-7172.

Seidah, N.G., Cromlish, J.A., Hamelin, J., Thibault, G., and Chrétien, M. (1986). Homologous IRCM-serine protease 1 from pituitary, heart atrium and ventricle: a common pro-hormone maturation enzyme? *Biosci. Rep.* 6, 835-844.

Seidman, C.E., Bloch, K.D., Klein, K.A., Smith, J.A., and Seidman, J.G. (1984a). Nucleotide sequences of the human and mouse atrial natriuretic factor genes. *Science* 226(4679), 1206-1209.

Seidman, C.E., Duby, A.D., Choi, E., Graham, R.M., Haber, E., Homcy, C., Smith, J.A., and Seidman, J.G. (1984b). The structure of rat preproatrial natriuretic factor as defined by a complementary DNA clone. *Science* 225(4659), 324-326.

Seidman, C.E., Bloch, K.D., Zisfein, J., Smith, J.A., Haber, E., Homcy, C., Duby, A.D., Choi, E., Graham, R.M., and Seidman, J.G. (1985). Molecular studies of the atrial natriuretic factor gene. *Hypertension* 7, 131-134.

Seidman, C.E., Wong, D.W., Jarcho, J.A., Bloch, K.D., and Seidman, J.G. (1988). Cis-acting sequences that modulate atrial natriuretic factor gene expression. *Proc. Natl. Acad. Sci. U. S. A.* 85, 4104-4108.

Seidman, C.E., Schmidt, E.V., and Seidman, J.G. (1991). cis-dominance of rat atrial natriuretic factor gene regulatory sequences in transgenic mice. *Can. J. Physiol. Pharmacol.* 69, 1486-1492.

Seilhamer, J.J., Arfsten, A., Miller, J.A., Lundquist, P., Scarborough, R.M., and Lewicki, J.A. (1989). Human and canine gene homologs of porcine brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* 165, 650-658.

Shapiro, J.T., DeLeonardis, V.M., Needleman, P., and Hintze, T.H. (1986). Integrated cardiac and peripheral vascular response to atriopeptin 24 in conscious dogs. *Am. J. Physiol.* 251, H1292-H1297.

Shichiri, M., Hirata, Y., Ando, K., Emori, T., Ohta, K., Kimoto, S., Ogura, M., Inoue, A., and Marumo, F. (1990). Plasma endothelin levels in hypertension and chronic renal failure. *Hypertension* 15, 493-496.

Shields, P.P., Dixon, J.E., and Glembotski, C.C. (1988). The secretion of atrial natriuretic factor-(99-126) by cultured cardiac myocytes is regulated by glucocorticoids. *J. Biol. Chem.* 263, 12619-12628.

Shields, P.P. and Glembotski, C.C. (1989). Regulation of atrial natriuretic factor-(99-126) secretion from neonatal rat primary atrial cultures by activators of protein kinases A and C. *J. Biol. Chem.* 264, 9322-9328.

Shimonaka, M., Saheki, T., Hagiwara, H., Ishido, M., Nogi, A., Fujita, T., Wakita, K., Inada, Y., Kondo, J., and Hirose, S. (1987). Purification of atrial natriuretic peptide receptor from bovine lung. Evidence for a disulfide-linked subunit structure. *J. Biol. Chem.* 262, 5510-5514.

Shirakami, G., Nakao, K., Saito, Y., Magaribuchi, T., Mukoyama, M., Arai, H., Hosoda, K., Suga, S., Mori, K., and Imura, H. (1993). Low doses of endothelin-1 inhibit atrial natriuretic peptide secretion. *Endocrinology* 132, 1905-1912.

Shubeita, H.E., McDonough, P.M., Harris, A.N., Knowlton, K.U., Glembotski, C.C., Brown, J.H., and Chien, K.R. (1990). Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J. Biol. Chem.* 265, 20555-20562.

Shubeita, H.E., Martinson, E.A., Van Bilsen, M., Chien, K.R., and Brown, J.H. (1992). Transcriptional activation of the cardiac myosin light chain 2 and atrial natriuretic factor genes by protein kinase C in neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci. U. S. A.* 89, 1305-1309.

Sigurdson, W., Ruknudin, A., and Sachs, F. (1992). Calcium imaging of mechanically induced fluxes in tissue-cultured chick heart: role of stretch-activated ion channels. *Am. J. Physiol.* 262, H1110-H1115.

Singhal, P.C., DeCandido, S., Satriano, J.A., Schlondorff, D., and Hays, R.M. (1989). Atrial natriuretic peptide and nitroprusside cause relaxation of cultured rat mesangial cells. *Am. J. Physiol.* 257, C86-C93.

Skvorak, J.P., Nazian, S.J., and Dietz, J.R. (1995). Endothelin acts as a paracrine regulator of stretch-induced atrial natriuretic peptide release. *Am. J. Physiol.* 269, R1093-R1098.

Smith, H.W. (1957). Salt and water volume receptors. *Am. J. Med.* 23, 623-652.

Snoeckx, L.H., Contard, F., Samuel, J.L., Marotte, F., and Rappaport, L. (1991). Expression and cellular distribution of heat-shock and nuclear oncogene proteins in rat hearts. *Am. J. Physiol.* 261, H1443-H1451.

- Sonnenberg, H., Cupples, W.A., de Bold, A.J., and Veress, A.T. (1982). Intrarenal localization of the natriuretic effect of cardiac atrial extract. *Can. J. Physiol. Pharmacol.* **60**, 1149-1152.
- Sonnenberg, H., Honrath, U., Chong, C.K., and Wilson, D.R. (1986). Atrial natriuretic factor inhibits sodium transport in medullary collecting duct. *Am. J. Physiol.* **250**, F963-F966.
- Sonnenberg, H. and Veress, A.T. (1984). Cellular mechanism of release of atrial natriuretic factor. *Biochem. Biophys. Res. Commun.* **124**, 443-449.
- Sosa-Lucero, J.C., de la Iglesia, F.A., Lumb, G., Berger, J.M., and Bencosme, S.A. (1969). Subcellular distribution of catecholamines and specific granules in rat heart. *Lab. Invest.* **21**, 19-26.
- Sprenkle, A.B., Murray, S.F., and Glembotski, C.C. (1995). Involvement of multiple *cis* elements in basal- and  $\alpha$ -adrenergic agonist-inducible atrial natriuretic factor transcription. Roles for serum response elements and an SP-1-like element. *Circ. Res.* **77**, 1060-1069.
- Standaert, D.G., Cechetto, D.F., Needleman, P., and Saper, C.B. (1987). Inhibition of the firing of vasopressin neurons by atriopeptin. *Nature* **329**, 151-153.
- Starksen, N.F., Simpson, P.C., Bishopric, N., Coughlin, S.R., Lee, W.M., Escobedo, J.A., and Williams, L.T. (1986). Cardiac myocyte hypertrophy is associated with c-myc protooncogene expression. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8348-8350.
- Stasch, J.P., Hirth-Dietrich, C., Kazda, S., and Neuser, D. (1989). Endothelin stimulates release of atrial natriuretic peptides in vitro and in vivo. *Life Sci.* **45**, 869-875.
- Steardo, L. and Nathanson, J.A. (1987). Brain barrier tissues: end organs for atriopeptins. *Science* **235**(4787), 470-473.
- Steinberg, S.F., Goldberg, M., and Rybin, V.O. (1995). Protein kinase C diversity in the heart. *J. Mol. Cell. Cardiol.* **27**, 141-153.
- Steinhilper, M.E. (1993). Structure, expression, and genomic mapping of the mouse natriuretic peptide type-B gene. *Circ. Res.* **72**, 984-992.

Stevens, T.L., Burnett, J.C., Jr., Kinoshita, M., Matsuda, Y., and Redfield, M.M. (1995). A functional role for endogenous atrial natriuretic peptide in a canine model of early left ventricular dysfunction. *J. Clin. Invest.* 95, 1101-1108.

Stewart, D.J., Cernacek, P., Costello, K.B., and Rouleau, J.L. (1992). Elevated endothelin-1 in heart failure and loss of normal response to postural change. *Circulation* 85, 510-517.

Stewart, J.M., Wang, J., Zeballos, G.A., Ochoa, M., Schustek, M., and Hintze, T.H. (1993). Role of tachycardia and V wave wall stress in the release of ANF during volume loading. *Am. J. Physiol.* 264, H217-H223.

Stingo, A.J., Clavell, A.L., Aarhus, L.L., and Burnett, J.C., Jr. (1992a). Cardiovascular and renal actions of C-type natriuretic peptide. *Am. J. Physiol.* 262, H308-H312.

Stingo, A.J., Clavell, A.L., Heublein, D.M., Wei, C.M., Pittelkow, M.R., and Burnett, J.C., Jr. (1992b). Presence of C-type natriuretic peptide in cultured human endothelial cells and plasma. *Am. J. Physiol.* 263, H1318-H1321.

Sudoh, T., Kangawa, K., Minamino, N., and Matsuo, H. (1988). A new natriuretic peptide in porcine brain. *Nature* 332, 78-81.

Sudoh, T., Maekawa, K., Kojima, M., Minamino, N., Kangawa, K., and Matsuo, H. (1989). Cloning and sequence analysis of cDNA encoding a precursor for human brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* 159, 1427-1434.

Sudoh, T., Minamino, N., Kangawa, K., and Matsuo, H. (1990). C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem. Biophys. Res. Commun.* 168, 863-870.

Suga, S., Nakao, K., Hosoda, K., Mukoyama, M., Ogawa, Y., Shirakami, G., Arai, H., Saito, Y., Kambayashi, Y., Inouye, K., and Imura, H. (1992a). Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* 130, 229-239.

Suga, S., Nakao, K., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., and Imura, H. (1992b). Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-beta. Possible existence of "vascular natriuretic peptide system". *J. Clin. Invest.* 90, 1145-1149.

Suga, S., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., Yoshimasa, T., and Nakao, K. (1993). Cytokine-induced C-type natriuretic peptide (CNP) secretion from vascular endothelial cells--evidence for CNP as a novel autocrine/paracrine regulator from endothelial cells. *Endocrinology* 133, 3038-3041.

Sugawara, A., Nakao, K., Sakamoto, M., Morii, N., Yamada, T., Itoh, H., Shiono, S., Imura, H., Ogawa, K., Ito, T., Hashimoto, H., Ito, Y., Ohno, O., Tsuboi, H., Takasu, N., Tanahashi, T., and Satake, T. (1986). Plasma atrial natriuretic factor in congestive heart failure. *Lancet* 1, 106

Sugawara, M. (1987). Ultrastructural evidence for exocytotic release of atrial natriuretic polypeptide in the rat atrial muscle with the use of the tannic acid method. *Biomed. Res.* 8(1), 9-17.

Sukhatme, V.P., Cao, X.M., Chang, L.C., Tsai-Morris, C.H., Stamenkovich, D., Ferreira, P.C., Cohen, D.R., Edwards, S.A., Shows, T.B., Curran, T., Le Beau, M.M., and Adamson, E.D. (1988). A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell* 53, 37-43.

Suva, L.J., Winslow, G.A., Wettenhall, R.E., Hammonds, R.G., Moseley, J.M., Diefenbach-Jagger, H., Rodda, C.P., Kemp, B.E., Rodriguez, H., Chen, E.Y., and et al (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* 237, 893-896.

Suzuki, E., Hirata, Y., Kohmoto, O., Sugimoto, T., Hayakawa, H., Matsuoka, H., Kojima, M., Kangawa, K., Minamino, N., and Matsuo, H. (1992). Cellular mechanisms for synthesis and secretion of atrial natriuretic peptide and brain natriuretic peptide in cultured rat atrial cells. *Circ. Res.* 71, 1039-1048.

Suzuki, E., Hirata, Y., Hayakawa, H., Omata, M., Kojima, M., Kangawa, K., Minamino, N., and Matsuo, H. (1993). Evidence for C-type natriuretic peptide production in the rat kidney. *Biochem. Biophys. Res. Commun.* 192, 532-538.

Svanegaard, J., Johansen, J.B., Thayssen, P., and Haghfelt, T. (1993). Neurohormonal systems during progression of heart failure: a review. *Cardiology* 83, 21-29.

Swedberg, K., Eneroth, P., Kjekshus, J., and Wilhelmsen, L. (1990). Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. CONSENSUS Trial Study Group. *Circulation* 82, 1730-1736.

Takahashi, N., Calderone, A., Izzo, N.J., Jr., Maki, T.M., Marsh, J.D., and Colucci, W.S. (1994). Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes. *J. Clin. Invest.* 94, 1470-1476.

Takayanagi, R., Inagami, T., Snajdar, R.M., Imada, T., Tamura, M., and Misono, K.S. (1987). Two distinct forms of receptors for atrial natriuretic factor in bovine adrenocortical cells. Purification, ligand binding, and peptide mapping. *J. Biol. Chem.* 262, 12104-12113.

Tanaka, I., Misono, K.S., and Inagami, T. (1984). Atrial natriuretic factor in rat hypothalamus, atria and plasma: determination by specific radioimmunoassay. *Biochem. Biophys. Res. Commun.* 124, 663-668.

Tarjan, E., Denton, D.A., and Weisinger, R.S. (1988). Atrial natriuretic peptide inhibits water and sodium intake in rabbits. *Regul. Pept.* 23, 63-75.

Tawaragi, Y., Fuchimura, K., Nakazato, H., Tanaka, S., Minamino, N., Kangawa, K., and Matsuo, H. (1990). Gene and precursor structure of porcine C-type natriuretic peptide. *Biochem. Biophys. Res. Commun.* 172, 627-632.

Tawaragi, Y., Fuchimura, K., Tanaka, S., Minamino, N., Kangawa, K., and Matsuo, H. (1991). Gene and precursor structures of human C-type natriuretic peptide. *Biochem. Biophys. Res. Commun.* 175, 645-651.

Thibault, G., Garcia, R., Cantin, M., and Genest, J. (1983a). Atrial natriuretic factor. Characterization and partial purification. *Hypertension* 5(2 Pt 2), 175-180.

Thibault, G., Garcia, R., Seidah, N.G., Lazure, C., Cantin, M., Chretien, M., and Genest, J. (1983b). Purification of three rat atrial natriuretic factors and their amino acid composition. *Febs Let.* 164, 286-290.

- Thibault, G., Garcia, R., Cantin, M., Genest, J., Lazure, C., Seidah, N.G., and Chretien, M. (1984). Primary structure of a high Mr form of rat atrial natriuretic factor. *Febs Let.* 167, 352-356.
- Thibault, G., Lazure, C., Schiffrin, E.L., Gutkowska, J., Chartier, L., Garcia, R., Seidah, N.G., Chretien, M., Genest, J., and Cantin, M. (1985). Identification of a biologically active circulating form of rat atrial natriuretic factor. *Biochem. Biophys. Res. Commun.* 130, 981-986.
- Thibault, G., Garcia, R., Gutkowska, J., Bilodeau, J., Lazure, C., Seidah, N.G., Chrétien, M., Genest, J., and Cantin, M. (1987). The propeptide Asn<sup>1</sup>-Tyr<sup>126</sup> is the storage form of rat atrial natriuretic factor. *Biochem. J.* 241, 265-272.
- Thibault, G., Lazure, C., Chretien, M., and Cantin, M. (1989). Molecular heterogeneity of pro-atrial natriuretic factor. *J. Biol. Chem.* 264, 18796-18802.
- Thibault, G., Charbonneau, C., Bilodeau, J., Schiffrin, E.L., and Garcia, R. (1992). Rat brain natriuretic peptide is localized in atrial granules and released into the circulation. *Am. J. Physiol.* 263, R301-R309.
- Thibault, G., Doubell, A.F., Garcia, R., Larivière, R., and Schiffrin, E.L. (1994). Endothelin-stimulated secretion of natriuretic peptides by rat atrial myocytes is mediated by endothelin A receptors. *Circ. Res.* 74, 470.
- Thorburn, A., Thorburn, J., Chen, S.Y., Powers, S., Shubeita, H.E., Feramisco, J.R., and Chien, K.R. (1993). HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. *J. Biol. Chem.* 268, 2244-2249.
- Thorburn, A. (1994). Ras activity is required for phenylephrine-induced activation of mitogen-activated protein kinase in cardiac muscle cells. *Biochem. Biophys. Res. Commun.* 205, 1417-1422.
- Thorburn, J., Frost, J.A., and Thorburn, A. (1994a). Mitogen-activated protein kinases mediate changes in gene expression, but not cytoskeletal organization associated with cardiac muscle cell hypertrophy. *J. Cell Biol.* 126, 1565-1572.

Thorburn, J., McMahon, M., and Thorburn, A. (1994b). Raf-1 kinase activity is necessary and sufficient for gene expression changes but not sufficient for cellular morphology changes associated with cardiac myocyte hypertrophy. *J. Biol. Chem.* 269, 30580-30586.

Thorburn, J. and Thorburn, A. (1994). The tyrosine kinase inhibitor, genistein, prevents alpha-adrenergic-induced cardiac muscle cell hypertrophy by inhibiting activation of the Ras-MAP kinase signaling pathway. *Biochem. Biophys. Res. Commun.* 202, 1586-1591.

Thören, P., Mark, A.L., Morgan, D.A., O'Neill, T.P., Needleman, P., and Brody, M.J. (1986). Activation of vagal depressor reflexes by atriopeptins inhibits renal sympathetic nerve activity. *Am. J. Physiol.* 251, H1252-H1259.

Thuerauf, D.J., Hanford, D.S., and Glembotski, C.C. (1994). Regulation of rat brain natriuretic peptide transcription. A potential role for GATA-related transcription factors in myocardial cell gene expression. *J. Biol. Chem.* 269, 17772-17775.

Tokola, H., Uusimaa, P.A., Taskinen, T., Hassinen, I.E., and Ruskoaho, H. (1991). Effect of hypo-osmolality on atrial natriuretic peptide gene expression in neonatal cultured cardiomyocytes. *Acta Physiol. Scand.* 143, 223-224.

Tokola, H., Salo, K., Vuolteenaho, O., and Ruskoaho, H. (1994). Basal and acidic fibroblast growth factor-induced atrial natriuretic peptide gene expression and secretion is inhibited by staurosporine. *Eur. J. Pharmacol.* 267, 195-206.

Trillo, A., Martinez Palomo, A., and Bencosme, S.A. (1966). Estudio ultraestructural del musculo cardiaco en relacion a los granulos especificos II. Estudio comparativo. *Archivos Del Instituto De Cardiologia De Mexico* 36, 45-57.

Trillo, A. and Bencosme, S.A. (1965). La ultraestructura de las celulas musculares cardiacas con referencia special a los granulos especificos. I. El miocardio de la rana. *Archivos Del Instituto De Cardiologia De Mexico* 35, 803-817.

Trippodo, N.C. and Barbee, R.W. (1987). Atrial natriuretic factor decreases whole-body capillary absorption in rats. *Am. J. Physiol.* 252, R915-R920.

- Urbain, R., Michel, J.B., Bouveret, P., Wisnewsky, C., Schwartz, K., and Mercadier, J.J. (1989). Left ventricular accumulation of messenger ribonucleic acid coding for the natriuretic atrial factor in various experimental models of cardiac hypertrophy in rats. *Arch. Mal. Coeur.* 82, 1089-1092.
- Uusimaa, P.A., Hassinen, I.E., Vuolteenaho, O., and Ruskoaho, H. (1992). Endothelin-induced atrial natriuretic peptide release from cultured neonatal cardiac myocytes: the role of extracellular calcium and protein kinase-C. *Endocrinology* 130, 2455-2464.
- van der Stolpe, A. and Jamison, R.L. (1988). Micropuncture study of the effect of ANP on the papillary collecting duct in the rat. *Am. J. Physiol.* 254, F477-F483.
- Van Heugten, H.A.A., Bezstarosti, K., Dekkers, D.H., and Lamers, J.M.J. (1993). Homologous desensitization of the endothelin-1 receptor mediated phosphoinositide response in cultured neonatal rat cardiomyocytes. *J. Mol. Cell. Cardiol.* 25, 41-52.
- Vandlen, R.L., Arcuri, K.E., and Napier, M.A. (1985). Identification of a receptor for atrial natriuretic factor in rabbit aorta membranes by affinity cross-linking. *J. Biol. Chem.* 260, 10889-10892.
- Vardas, P.E., Travill, C.M., Williams, T.D., Ingram, A.M., Lightman, S.L., and Sutton, R. (1988). Effect of dual chamber pacing on raised plasma atrial natriuretic peptide concentrations in complete atrioventricular block. *Br. Med. J. [Clin. Res.]* 296, 94
- Vari, R.C., Freeman, R.H., Davis, J.O., Villarreal, D., and Verburg, K.M. (1986). Effect of synthetic atrial natriuretic factor on aldosterone secretion in the rat. *Am. J. Physiol.* 251, R48-R52.
- Vesely, D.L., Norris, J.S., Walters, J.M., Jespersen, R.R., and Baeyens, D.A. (1987). Atrial natriuretic prohormone peptides 1-30, 31-67, and 79-98 vasodilate the aorta. *Biochem. Biophys. Res. Commun.* 148, 1540-1548.
- Vesely, D.L. (1992). Peptides from the N-terminus of the atrial natriuretic factor prohormone enhance guanylate cyclase activity and increase cyclic GMP levels in a wide variety of tissues. *Molecular & Cellular Biochemistry* 109, 43-50.
- Vesely, D.L., Douglass, M.A., Dietz, J.R., Gower, W.R., Jr., McCormick, M.T., Rodriguez-Paz, G., and Schocken, D.D. (1994). Three peptides from the atrial natriuretic factor prohormone amino terminus

lower blood pressure and produce diuresis, natriuresis, and/or kaliuresis in humans. *Circulation* 90, 1129-1140.

Vlasuk, G.P., Miller, J., Bencen, G.H., and Lewicki, J.A. (1986). Structure and analysis of the bovine atrial natriuretic peptide precursor gene. *Biochem. Biophys. Res. Commun.* 136, 396-403.

Vollmar, A.M., Gerbes, A.L., Nemer, M., and Schulz, R. (1993). Detection of C-type natriuretic peptide (CNP) transcript in the rat heart and immune organs. *Endocrinology* 132, 1872-1874.

Volpe, M., Mele, A.F., De Luca, N., Golino, P., Bondiolotti, G., Camargo, M.J., Atlas, S.A., and Trimarco, B. (1986). Carotid baroreceptor unloading decreases plasma atrial natriuretic factor in hypertensive patients. *J. Hypertens. [Suppl]* 4, S519-S522.

von Harsdorf, R., Lang, R.E., Fullerton, M., and Woodcock, E.A. (1989). Myocardial stretch stimulates phosphatidylinositol turnover. *Circ. Res.* 65, 494-501.

Wada, A., Tsutamoto, T., Matsuda, Y., and Kinoshita, M. (1994). Cardiorenal and neurohumoral effects of endogenous atrial natriuretic peptide in dogs with severe congestive heart failure using a specific antagonist for guanylate cyclase-coupled receptors. *Circulation* 89, 2232-2240.

Walsh, K.P., Williams, T.D., Canepa-Anson, R., Pitts, E., Lightman, S.L., and Sutton, R. (1987). Effects of endogenous atrial natriuretic peptide released by rapid atrial pacing in dogs. *Am. J. Physiol.* 253, R599-R604.

Walsh, K.P., Williams, T.D.M., Wilder, R., Pitts, E., Lightman, S.L., and Sutton, R. (1988). Decline of atrial natriuretic peptide release in dogs during sustained rapid cardiac pacing. *Clin. Sci.* 74, 567-570.

Wang, D.L., Chen, J.J., Shin, N.L., Kao, Y.C., Hsu, K.H., Huang, W.Y., and Liew, C.C. (1992). Endothelin stimulates cardiac alpha- and beta- myosin heavy chain gene expression. *Biochem. Biophys. Res. Commun.* 183, 1260-1265.

Wangler, R.D., Breuhaus, B.A., Otero, H.O., Hastings, D.A., Holzman, M.D., Saneii, H.H., Sparks HV, J.r., and Chimoskey, J.E. (1985). Coronary vasoconstrictor effects of atriopeptin II. *Science* 230, 558-561.

- Wei, C.M., Heublein, D.M., Perrella, M.A., Lerman, A., Rodeheffer, R.J., McGregor, C.G., Edwards, W.D., Schaff, H.V., and Burnett, J.C., Jr. (1993). Natriuretic peptide system in human heart failure. *Circulation* 88, 1004-1009.
- Wei, Y.F., Rodi, C.P., Day, M.L., Wiegand, R.C., Needleman, L.D., Cole, B.R., and Needleman, P. (1987). Developmental changes in the rat atriopeptin hormonal system. *J. Clin. Invest.* 79, 1325-1329.
- Weidmann, P., Hellmueller, B., Uehlinger, D.E., Lang, R.E., Gnaedinger, M.P., Hasler, L., Shaw, S., and Bachmann, C. (1986). Plasma levels and cardiovascular, endocrine, and excretory effects of atrial natriuretic peptide during different sodium intakes in man. *J. Clin. Endocrin. Metab.* 62, 1027-1036.
- Weir, M.L., Honrath, U., Flynn, T.G., and Sonnenberg, H. (1994). Lack of biologic activity or specific binding of amino-terminal pro-ANP segments in the rat. *Regul. Pept.* 53, 111-122.
- Weisinger, R.S., Blair-West, J.R., Denton, D.A., and Tarjan, E. (1992). Central administration of atrial natriuretic peptide suppresses sodium and water intake of sheep. *Brain Res.* 579, 113-118.
- Wildey, G.M., Fischman, A.J., Margolies, M.N., Graham, R.M., and Horncy, C.J. (1990). Phosphorylation state of pro-atrial natriuretic factor in rat atrial secretory granules. *Endocrinology* 127, 2839-2848.
- Winqvist, R.J., Faison, E.P., Waldman, S.A., Schwartz, K., Murad, F., and Rapoport, R.M. (1984). Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. *Proc. Natl. Acad. Sci. U. S. A.* 81, 7661-7664.
- Winqvist, R.J., Scott, A.L., and Vlasuk, G.P. (1989). Enhanced release of atrial natriuretic factor by endothelin in atria from hypertensive rats. *Hypertension* 14, 111-114.
- Wong, N.L., Wong, E.F., Au, G.H., and Hu, D.C. (1988). Effect of alpha- and beta-adrenergic stimulation on atrial natriuretic peptide release in vitro. *Am. J. Physiol.* 255, E260-E264.
- Wu, J., LaPointe, M.C., West, B.L., and Gardner, D.G. (1989). Tissue-specific determinants of human atrial natriuretic factor gene expression in cardiac tissue. *J. Biol. Chem.* 264, 6472-6479.

Wu, J.P., Deschepper, C.F., and Gardner, D.G. (1988). Perinatal expression of the atrial natriuretic factor gene in rat cardiac tissue. *Am. J. Physiol.* 255, E388-E396.

Wu, J.P., Kovacic-Milivojevic, B., LaPointe, M.C., Nakamura, K., and Gardner, D.G. (1991). Cis-active determinants of cardiac-specific expression in the human atrial natriuretic peptide gene. *Mol. Endocrinol.* 5, 1311-1322.

Wypij, D.M. and Harris, R.B. (1988). Atrial granules contain an amino-terminal processing enzyme of atrial natriuretic factor. *J. Biol. Chem.* 263, 7079-7086.

Wypij, D.M. and Harris, R.B. (1992). Characterization of homogeneous atrial granule serine proteinase, a candidate processing enzyme of pro-atrial natriuretic factor. *Life Sci.* 50, 523-531.

Yamamoto, M., Harm, S.C., Grasser, W.A., and Thiede, M.A. (1992). Parathyroid hormone-related protein in the rat urinary bladder: a smooth muscle relaxant produced locally in response to mechanical stretch. *Proc. Natl. Acad. Sci. U. S. A.* 89, 5326-5330.

Yamanaka, M., Greenberg, B., Johnson, L., Seilhamer, J., Brewer, M., Friedeman, T., Miller, J., Atlas, S., Laragh, J., Lewicki, J., and Fiddes, J. (1984). Cloning and sequence analysis of the cDNA for the rat atrial natriuretic factor precursor. *Nature* 309, 719-722.

Yamazaki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., Kadowaki, T., Nagai, R., and Yazaki, Y. (1993). Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. *J. Biol. Chem.* 268, 12069-12076.

Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Hiroi, Y., Mizuno, T., Maemura, K., Kurihara, H., Aikawa, R., Takano, H., and Yazaki, Y. (1996). Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J. Biol. Chem.* 271, 3221-3228.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332, 411-415.

Yang, X.C. and Sachs, F. (1989). Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science* 243, Pt 1:1068-71.

- Ye, H., Wolf, R.A., Kurz, T., and Corr, P.B. (1994). Phosphatidic acid increases in response to noradrenaline and endothelin-1 in adult rabbit ventricular myocytes. *Cardiovasc. Res.* 28, 1828-1834.
- Yokota, N., Aburaya, M., Yamamoto, Y., Kato, J., Kitamura, K., Kida, O., Eto, T., Minamino, N., Kangawa, K., Matsuo, H., and Tonaka, K. (1990). Increased plasma brain natriuretic peptide levels in DOCA-salt hypertensive rats: relation to blood pressure and cardiac concentration. *Biochem. Biophys. Res. Commun.* 173, 632-638.
- Yokota, N., Bruneau, B.G., Kuroski-de Bold, M.L., and de Bold, A.J. (1994). Atrial natriuretic factor significantly contributes to the mineralocorticoid escape phenomenon. Evidence for a guanylate cyclase-mediated pathway. *J. Clin. Invest.* 94, 1938-1946.
- Yokota, N., Bruneau, B.G., Fernandez, B.E., Kuroski-de Bold, M.L., Piazza, L.A., Eid, H., and de Bold, A.J. (1995). Dissociation of cardiac hypertrophy, myosin heavy chain isoform expression, and natriuretic peptide production in DOCA-salt rats. *Am. J. Hypertens.* 8, 301-310.
- Yoshimura, M., Yasue, H., Morita, E., Sakaino, N., Jougasaki, M., Kurose, M., Mukoyama, M., Saito, Y., Nakao, K., and Imura, H. (1991). Hemodynamic, renal, and hormonal responses to brain natriuretic peptide infusion in patients with congestive heart failure. *Circulation* 84, 1581-1588.
- Yoshizumi, M., Kurihara, H., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., and Yazaki, Y. (1989). Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. *Biochem. Biophys. Res. Commun.* 161, 859-864.
- Yukimura, T., Ito, K., Takenaga, T., Yamamoto, K., Kangawa, K., and Matsuo, H. (1984). Renal effects of a synthetic alpha-human atrial natriuretic polypeptide (alpha-hANP) in anesthetized dogs. *Eur. J. Pharmacol.* 103, 363-366.
- Yunge, L., Benchimol, S., and Cantin, M. (1980). Ultrastructural cytochemistry of atrial muscle cells. VIII Radioautographic study of synthesis and migration of proteins. *Cell Tissue Res.* 207, 1-11.
- Zeller, R., Bloch, K.D., Williams, B.S., Arceci, R.J., and Seidman, C.E. (1987). Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes. Dev.* 1, 693-698.

Zhou, M.D., Sucov, H.M., Evans, R.M., and Chien, K.R. (1995). Retinoid-dependent pathways suppress myocardial cell hypertrophy. *Proc. Natl. Acad. Sci. U. S. A.* 92, 7391-7395.

Zimmerman, R.S., Schirger, J.A., Edwards, B.S., Schwab, T.R., Heublein, D.M., and Burnett, J.C., Jr. (1987). Cardio-renal-endocrine dynamics during stepwise infusion of physiologic and pharmacologic concentrations of atrial natriuretic factor in the dog. *Circ. Res.* 61, 63-69.

Zimmerman, R.S., Martinez, A.J., MacPhee, A.A., and Barbee, R.W. (1990). Atrial natriuretic factor blocks the pressor action of endothelin. *J. Cardiovasc. Pharmacol.* 16, 865-870.

Zivin, R.A., Condra, J.H., Dixon, R.A., Seidah, N.G., Chretien, M., Nemer, M., Chamberland, M., and Drouin, J. (1984). Molecular cloning and characterization of DNA sequences encoding rat and human atrial natriuretic factors. *Proc. Natl. Acad. Sci. U. S. A.* 81, 6325-6329.

Zongazo, M.A., Carayon, A., Masson, F., Maistre, G., Noe, E., Eurin, J., Barthelemy, C., Komajda, M., and Legrand, J.C. (1991). Effects of arginine vasopressin and extracellular osmolarity on atrial natriuretic peptide release by superfused rat atria. *Eur. J. Pharmacol.* 209, 45-55.

Zukowska-Grojec, Z., Haass, M., Kopin, I.J., and Zamir, N. (1986). Interactions of atrial natriuretic peptide with the sympathetic and endocrine systems in the pithed rat. *J. Pharmacol. & Exptl. Ther.* 239, 480-487.