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"
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 cosecreted 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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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADH:</td>
<td>antidiuretic hormone</td>
</tr>
<tr>
<td>ANF:</td>
<td>atrial natriuretic factor</td>
</tr>
<tr>
<td>AP-1:</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ATP:</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BNP:</td>
<td>brain natriuretic peptide</td>
</tr>
<tr>
<td>cAMP:</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT:</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>cDNA:</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP:</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNP:</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>DOCA:</td>
<td>deoxycorticosterone acetate</td>
</tr>
<tr>
<td>ET-1:</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>ir:</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>MAP kinase:</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK:</td>
<td>mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MHC:</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>mRNA:</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PCR:</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKC:</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RIA:</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>rRNA:</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SHR:</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SRE:</td>
<td>serum response element</td>
</tr>
<tr>
<td>SRF:</td>
<td>serum response factor</td>
</tr>
</tbody>
</table>
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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), natriodiatin (Nemer et al., 1994), and atriopeptin (Currie et al., 1984b). Some of these peptides (the atriopeptins) were later found to be arsial, 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., 1994b; Yamanaka et al., 1984; Zivin et al., 1994; 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_{1-96}), and the bioactive ANF_{97-126} (Flynn et al., 1983; Thibault et al., 1985). Although it was initially shown that ANF_{1-96} 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_{97-126} 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 ANF99-126 (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; kalikrein (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 ANF1-126 to ANF99-126. 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 (Rozensweig 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 α₁-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 α₁-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 α₁-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 α1-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 α1-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 α-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 α- and β-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, α-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 α1-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$_{2\alpha}$ (but not PGE$_2$ 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 β$_1$ (TGF-β$_1$) (Parker et al., 1991; Takahashi et al., 1994), acidic or basic fibroblast growth factor (Parker et al., 1990), α-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$_3$ (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., 1996; 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; lida 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; Douvell 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 (lida 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 (lida 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; Winquist et al., 1989; Schiebinger and Gomez-Sanchez, 1990; de Bold et al., 1991). perfused hearts (Mäntymäa 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 α-adrenergic and not β-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 α and β receptors, with a predominance of the β receptors (Schiebinger et al., 1987). Norepinephrine has varied effects on ANF secretion from cultured atrial cardiocytes (Gibbs, 1987b), and
decreases ANF secretion from perifused atrial strips (Inoue et al., 1988). Norepinephrine in
the presence of the β-adrenergic receptor antagonist propranolol increases ANF secretion
from atrial tissue and cultured adult atrial cardiocytes, suggesting that α₁-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 perifused right atria (Wong et al., 1988), but decreases ANF
release from perifused atrial strips (Inoue et al., 1988). Minced atria show no significant
secretory response to the α₁-adrenergic agonist phenylephrine (Lachance et al., 1986), while
ANF secretory rates from perifused 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 Glembocki, 1989; Sei and Glembocki, 1990). Others have shown
stimulated ANF release using the α₂-adrenergic agonist methoxamine on isolated atria
(Wong et al., 1988) and freshly isolated adult atrial cardiocytes (Hayashi et al., 1988). The
β-adrenergic agonist isoproterenol has been shown to increase ANF secretion rates from
freshly isolated adult atrial cardiocytes (Gibbs, 1987b) and perifused 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 perifused atrial
strips (Lachance et al., 1986; Inoue et al., 1988). Stimulation of β-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
Glembocki, 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 β-adrenergic receptor antagonist propranolol, suggesting the involvement of both α and β adrenergic receptors.

The discrepancies from one report to another may be due to marked differences in the experimental conditions. In some reports of β-adrenergic stimulation of ANF secretion, researchers have used paced left atria (Schiebinger et al., 1987; Schiebinger, 1989). These same researchers reported that β-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 β-adrenergic stimulation of ANF secretion is dependent on Ca\(^{2+}\) fluxes from external and internal Ca\(^{2+}\) sources (Schiebinger, 1989), it is possible that the exaggerated Ca\(^{2+}\) transients caused by pacing may influence the effects of β-adrenergic agonists. Another report, using spontaneously beating whole atria, showed that isoproterenol, a β-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 α₁-adrenergic receptor subtype. Precise pharmacological experiments further determined that it is the α₁-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⁺-K⁺-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 α₁-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 α₁-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).

\textit{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 \textit{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., 1999). 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 (Winquist 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⁺ reabsorption from the collecting duct, and possible inhibition of Na⁺ 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 micropertusion 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⁺ 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⁺ 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 (Steinhelper, 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; Steinhelper, 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; Steinhelper, 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; Thuerauf 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; Thuerauf et al., 1994), and is conserved in other species (Seilhamer et al., 1989; Steinhelper, 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; Thuerauf 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-β (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 ET₆ receptor in vascular smooth muscle cells (Eguchi et al., 1994); the ET₆ 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 (Shubert 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 Bion/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), CaCl₂, (2.5) NaH₂PO₄ (1.18), MgSO₄.7H₂O.
(1.18), NaHCO₃ (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% O₂/5% CO₂ and pre-heated at 30°C was dripped onto the cotton thread at a rate of 3 mL/min (see figure 2). The perifusate 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 perifused, given a basal tension of 200 mg, and were left to equilibrate for one hour. The perifusate 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. Perifusate 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 perifusion. In this manner, atria were bathed in the modified KRBB described above, and
were oxygenated with 95%O₂/5% CO₂ 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 10⁻⁷ 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), CaCl₂·2H₂O (2.54), NaH₂PO₄·H₂O (1.36), MgCl₂·6H₂O (1.16), NaHCO₃ (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 µg/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 µm 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 cava, 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

**Inflow (3 mL/min, 30°C)**

![Diagram of atrial cannulation](image)

- **Sup. vena cava**
- **Inf. vena cava**
- **RA**
- **Ventr.**
- **Pressure transducer**
- **Organ bath (30°C)**
- **Outflow**
- **Radioimmunoassay**

**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% O₂/5% CO₂) was
infused by a programmable solvent delivery module (Model 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% O₂/5%
CO₂ 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 intre-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 µL 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 (10⁻⁶ 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 µmol/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 µmol/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_{18} 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 µL 10 mM HCl for 20 min, to which 225 µl RIA buffer (see below) was subsequently added; 100 µl were used for BNP RIA. Extraction efficiency of BNP was 77±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_{99-126} (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 µL of ANF standard or samples was added to 100 µL of 1:30,000 dilution of antiserum raised against ANF_{99-126} (Peninsula Laboratories, Belmont, CA) and left at 4°C for 4 hours. Following this incubation period, 100 µL (10,000 cpm) of iodinated ANF_{99-126} (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 µL 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 γ-counter. For BNP RIA, the same procedure was carried out, except that iodinated BNP_{64-95} (Peninsula Laboratories, Belmont, CA) and anti-rat antiserum raised against BNP_{64-95} (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_3citrate) 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 NaPO₄, 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 SalI 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/SalI 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 [α-32P]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 quantified by scanning laser densitometry, using an LKB Ultroscan 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 28S 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): GAGAGTCGACACTGTTGGCAAGTTTGTCG
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 MgCl₂, 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): CUACUAUCUAUATCGGCACCATGCACCTCTCC

2) Upstream primer (649-664): CAUCAUCAUCAUTCCCTCCCCTCCCCTCCCCAAATA

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 of 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 MgSO4, 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 MgCl2) 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 Quiagen DNA isolation
procedure (Quiagen, Chatsworth, CA), labelled with [α-32P]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

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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±7 mg to 180±24 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±11 pg/mL/min to 743±28 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 (10⁻⁷mol/L) caused developed tension to increase over one hour from 43±12 to 194±31 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±4.8 bpm to 145.0±5.0 bpm (n=4, P<0.05 vs. 0 minutes and vehicle). Resting tension rose from the basal 200±0 mg load to 545±103 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±25 pg/mL/min to 395±45 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±24 fg/mL min to 417±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±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±16% increase in c-fos mRNA levels (n=4, P<0.025), and a 70±5% increase in Egr-1 mRNA levels (n=4, P<0.01), compared to control levels. C-myc mRNA levels were elevated 69±28% (n=6, P<0.05) over control levels after 4 hours of stretch. A 201±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±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

- **Resting Tension (g)**
  - ○ = Stretch
  - ▲ = Basal

- **Beating Rate (bpm)**

- **Developed Tension (g)**

Time (min)
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

![Graph showing irANF (pg/ml) over time (min). The graph compares Stretch and Basal conditions.](image)

- **Stretch**
- **Basal**

Time (min):
- 0
- 20
- 40
- 60
- 80
- 100
- 120
- 140
- 160
- 180
- 200
- 220
- 240

irANF (pg/ml):
- 0
- 200
- 400
- 600
- 800
- 1000

**Symbols:**
- **•** Stretch
- **□** Basal

Notes:
- **★** Significantly different from Basal
- **†** Significantly different from 0 min
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

![Graph showing time course of irBNP levels with stretch and basal conditions](image)

- **Y-axis**: irBNP (fg/ml)
- **X-axis**: TIME (min)
- **Legend**:
  - Stretch
  - Basal

The graph illustrates the time course of irBNP levels following a stretch event. The data points indicate a decrease in irBNP levels over time, with a notable drop following the stretch.
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=8). Endothelin-1 was applied at time 0 as indicated by the arrows. *P<0.05, **P<0.01 vs 0 minutes (by ANOVA).
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} \text{ 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).
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

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<th>Basal Stretch</th>
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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±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).
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±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.
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±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±6 min⁻¹ to 190±5 min⁻¹ (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±44 pg/mL to a maximum of 1135±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±0.13 pg/mL to a peak of 4.10±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±0.004 mmHg to 1.38±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±5 min⁻¹ to 176±13 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±35 pg/mL, and peaked after 60 minutes at 976±120 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±0.27 pg/mL, peaking at 3.95±0.93 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±15% increase in BNP mRNA levels after 1.5 hours of stimulation (n=5, P<0.01). This increased further, peaking at a 133±20% increase after 6 hours (n=5, P<0.001), and remained significantly elevated for the remainder of the period of stimulation (+105±12% after 8 hours; n=5, P<0.01). Egr-1 mRNA levels were increased by 92±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±25% increase in c-myc mRNA levels after 1.5 hours (n=5, P<0.05), which remained significantly elevated after 4 hours (+82±34%; n=5, P<0.05). BNP mRNA levels were increased by 10³ M ET-1 after 4 hours of stimulation (+ 83±20%, n=5, P<0.001)). The increase peaked at +120±56% after 6 hours (n=5, P<0.05), and decreased thereafter, although it was still significantly higher than controls after 8 hours (+49±11%, n=5, P<0.05). ET-1 also stimulated Egr-1 mRNA levels after
4 hours (+134±33%, 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±18 vs controls after 8 hours (n=5, P<0.05).

Perfused right atria were stimulated with phenylephrine at doses of 10 μmol/L, 50 μmol/L, or 100 μmol/L. Phenylephrine at all doses caused a significant sustained increase in intra-atrial pressure and heart rate (Fig. 19). Beating rate increased from 145±18 to 209±15 min⁻¹ at 2 hours with 10 μmol/L phenylephrine (n=5, P<0.001 vs. 0 minutes and vehicle), from 134±11 to 223±14 min⁻¹ at 2 hours with 50 μmol/L phenylephrine (n=6, P<0.001 vs. 0 minutes and vehicle), and from 149±4 to 213±15 min⁻¹ at 1.5 hours with 100 μmol/L phenylephrine (n=5, P<0.001 vs. 0 minutes and vehicle). Intra-atrial pressure increased from 0.56±0.02 to 1.22±0.27 mmHg at 1 hour with 10 μmol/L phenylephrine (n=5, P<0.001 vs. 0 minutes and vehicle), from 0.51±0.04 to 1.57±0.14 mmHg at 15 minutes with 50 μmol/L phenylephrine (n=6, P<0.001 vs. 0 minutes and vehicle), and from 0.52±0.05 to 1.28±0.25 mmHg at 1 hour with 100 μmol/L phenylephrine (n=5, P<0.001 vs. 0 minutes and vehicle). Prazosin (10 μmol/L) added at the same time as 50 μmol/L phenylephrine for 6 hours inhibited the increase in developed tension (Fig. 19; P<0.05 vs. 50 μmol/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±64 to 522±53 pg/mL at 1.5 hours with 10 μmol/L phenylephrine (n=5, P<0.01 vs. vehicle), from 266±25 to 702±108 pg/mL at 1.5 hours with 50 μmol/L phenylephrine (n=6, P<0.001 vs. 0 minutes and vs. vehicle), and from 317±34 to 854±134 pg/mL at 1.5 hours with 100 μmol/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±0.10 to 3.59±0.52 pg/mL at 1.5 hours with 50 μmol/L phenylephrine (n=6, P<0.01 vs. 0 minutes and P<0.001 vs. vehicle), and from 2.24±0.15 to 3.86±55 pg/mL at 1 hour with 100 μmol/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 μmol/L phenylephrine, and reached basal levels after 5 and 6 hours of stimulation with 10 and 50 μmol/L phenylephrine, respectively. Phenylephrine-stimulated irBNP secretion remained above basal levels throughout the period of stimulation. Prazosin added at the same time as 50 μmol/L phenylephrine for 6 h prevented the increase in irANF and irBNP secretion (Fig. 20; P<0.05 vs. 50 μmol/L phenylephrine, P>0.10 vs. vehicle) When total secretion during the period of stimulation is calculated (Table 1), 10 μmol/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 μmol/L phenylephrine for 6 hours led to a 49±13% increase in relative ANF mRNA levels (n=5, P<0.001) and a 135±18% increase in relative BNP mRNA levels (n=5, P<0.001). After 8 hours a 77±19% increase in relative BNP mRNA levels was measured (n=7, P<0.01). Relative Egr-1 mRNA levels were increased by 81±26% after 4 hours (n=5, P<0.01), 167±26% after 6 hours (n=5, P<0.001), and 40±11% after 8 hours (n=7, P<0.05). Increases in relative c-myc mRNA levels were observed after 4 hours (+49±14%, n=5, P<0.01), and after 6 hours (+53±7%, n=5, P<0.001). Prazosin added at the same time as 50 μmol/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 μmol/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=intratra-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. ‡P<0.001 vs. time 0 (by ANOVA).
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).
Figure 14: Effect of endothelin-1 on atrial contraction rate (A) and intra-atrial pressure (B) of isolated right atria. HR=heart rate; IAP=intracardiac 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

A

Rate of Contraction (min⁻¹)

ET-1

0 1 2 3 4 5 6 7 8

B

Intra-atrial pressure (mmHg)

IAP

0 1 2 3 4 5 6 7 8

Time (h)
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).
Figure 16: Ratios of irANF to irBNP secreted in response to (A) stretch or (B) endothelin-1 (10^{-8} \text{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).
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.
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±SEM of the percent change from unstimulated controls. *P<0.05, †P<0.01, ‡P<0.001 vs. vehicle (by ANOVA).
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 μM ascorbic acid, ○, n=5), 10 μM phenylephrine (●, n=4), 50 μM phenylephrine (■, n=6), 100 μM phenylephrine (▲, n=5), or 50 μM phenylephrine with 10 μM prazosin (□, n=5) at time 0, as indicated by the arrow. Data are mean±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.
Figure 20: Effect of phenylephrine on (A) irANF and (B) irBNP secretion from isolated right atria. Atria were perfused with vehicle (100 µM ascorbic acid, ○, n=5), 10 µM phenylephrine (●, n=4), 50 µM phenylephrine (■, n=6), 100 µM phenylephrine (▲, n=5), or 50 µM phenylephrine with 10 µM prazosin (♦, n=5) at time 0, as indicated by the arrow. Data are mean±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).
Figure 21: Ratios of irANF to irBNP secreted in response to phenylephrine (50 and 100 μM, n=9). Phenylephrine was applied at time 0, as indicated by the arrow. Data are mean±SE. *P<0.05 vs. 0 min and 3-8 h, †P<0.05 vs. 15 min.
Table 1: Total irANF and irBNP secretion.

<table>
<thead>
<tr>
<th></th>
<th>irANF (ng/8 h)</th>
<th>irBNP (ng/8 h)</th>
</tr>
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<tbody>
<tr>
<td>Vehicle (n=5)</td>
<td>312±26</td>
<td>5.14±0.22</td>
</tr>
<tr>
<td>Phenylephrine (10 µM) (n=5)</td>
<td>536±75*</td>
<td>5.65±0.42</td>
</tr>
<tr>
<td>Phenylephrine (50 µM) (n=5)</td>
<td>690±90†</td>
<td>6.93±0.21‡</td>
</tr>
<tr>
<td>Phenylephrine (100 µM) (n=6)</td>
<td>767±59†</td>
<td>6.88±0.27‡</td>
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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 μM phenylephrine (PE), or 50 μM phenylephrine with 10 μM 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.
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 μM 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±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.
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$_{99-126}$ and BNP$_{64-106}$ (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$_{1-126}$) is present in the atrial cardiocytes (Flynn et al., 1985a; 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 perifused 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 perifusate 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+ 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 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 (Gd³⁺), 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 Ca\textsuperscript{2+} transport may not be involved in regulating stretch-induced ANF secretion, there exists conclusive evidence for the involvement of Ca\textsuperscript{2+} transport from the sarcoplasmic reticulum. Experiments using isolated rat atria have shown that ryanodine, a blocker of sarcoplasmic reticulum Ca\textsuperscript{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 Ca\textsuperscript{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 Ca\textsuperscript{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 Ca\textsuperscript{2+}-binding peptides Annexin V and VI as granule-associated proteins (Doubell et al., 1991) suggests their involvement and a possible role for Ca\textsuperscript{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 Ca\textsuperscript{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 bisphosphate (IP$_2$), and inositol-1,4,5(tris)phosphate (IP$_3$) accumulation (von Harsdorf et al., 1989). IP$_3$ binds to receptors on the sarcoplasmic reticulum membrane, and causes release of Ca$^{2+}$ from the sarcoplasmic reticulum (Berridge, 1993). Since release of Ca$^{2+}$ 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$_3$ is probably at least partially involved in mediating this process. The IP$_3$ receptor on the sarcoplasmic reticulum is ryanodine-insensitive (Berridge, 1993), which suggests that Ca$^{2+}$ release from intracellular stores in response to stretch is due in part to a signalling molecule that is distinct from IP$_3$, or may be due to Ca$^{2+}$-induced Ca$^{2+}$ 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$_4$) (Dassouli et al., 1993). Importantly, in the cardiocyte cultures, the stretch-induced increase in IPs is not affected by depolarization by high K$^+$ (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$_3$ 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 IP₃ 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 IP₃ 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 β cells (Schubart et al., 1982; Brown et al., 1985; Howell and Tyhurst, 1986; Ashcroft and Hughes, 1990) and secretion of thromboxane A2 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 PGF₂. Increases in plasma ANF due to volume loading can be partially reduced by indomethacin, a cyclooxygenase inhibitor (Gardner and Schultz, 1990). PGF₂ 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 PGF₂. 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 PGF₂ may be an important mediator of stretch-induced ANF or BNP secretion. The actions of PGF₂ in the cardiocyte appear 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 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
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 discreet 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 (Kovacic-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; Sudo et al., 1989; Flynn et al., 1989; Seilhamer et al., 1989; Steinhelper, 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, α myosin heavy chain, and α skeletal actin gene expression (Sadoshima et al., 1992a). A Gd³⁺-sensitive stretch-activated ion channel has been characterized in neonatal ventricular cardiocytes, but blocking it with Gd³⁺ 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
al., 1991; LaPointe and Sitkins, 1993; Glembotski 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 TC (Blenis, 1993; Crews and Erikson, 1993). The c-fos protein has been implicated in the regulation of the ANF and BNP promoters (Kovacic-Miliojevic and Gardner, 1992; McBride et al., 1993; Kovacic-Miliojevic 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 TC 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\textsuperscript{ROS}, 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 \textit{in vivo} or \textit{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 \textit{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\textsuperscript{3+}-sensitive stretch-activated channels or on Ca\textsuperscript{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 \textit{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 Glembotski, 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 ET₄ 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 α, β, and γ. The α-subunits are generally considered to define the post-G-protein interaction (Hepler and Gilman, 1992). There are three families of G-protein α-subunits; those to which the ET₄ receptor is coupled is of the pertussis toxin-insensitive Gq type, which is in turn coupled to phospholipase Cβ (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, Ca\textsuperscript{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 Ca\textsuperscript{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 Ca\textsuperscript{2+} channel antagonists (Schiebinger and Gomez-Sanchez, 1990; Sei and Gilembotski, 1990; Irons et al., 1992; Doubell and Thibault, 1994). However, another report has shown that diltiazem, a blocker of L-type voltage-sensitive Ca\textsuperscript{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 Ca\textsuperscript{2+} in cultured neonatal cardiocytes eliminates ET-1-stimulated secretion (Doubell and Thibault, 1994). There appears to be no requirement for mobilization of Ca\textsuperscript{2+} for ET-1-stimulated ANF release, since blocking Ca\textsuperscript{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 Ca\textsuperscript{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 Ca\textsuperscript{2+}-independent (Steinberg et al., 1995), which suggests that a
Ca\textsuperscript{2+}-dependent component, probably mediated by calmodulin, and a Ca\textsuperscript{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; Schriebinger 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 ET₄ 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 (Dobelle 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 IP₃ (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 (Illo 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 aortocaval 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\textsubscript{A} 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 α₁-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 α₁-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 IP₃ and diacylglycerol, and possibly via a PKC-dependent pathway. In all models studied, stimulation of ANF secretion by α₁-adrenergic agonists depends on influx of extracellular Ca²⁺ (Sei and Glembski, 1990; Schiebinger et al., 1992; Ambler and Leite, 1994). In isolated atria, initiation, but not maintenance, of phenylephrine-induced ANF secretion depends on extracellular Ca²⁺ influx, and is independent from a ryanodine-sensitive intracellular Ca²⁺ compartment (Schiebinger et al., 1992). In cultured neonatal atrial cardiocytes, blocking either extracellular Ca²⁺ influx and ryanodine-sensitive channels partially inhibited phenylephrine-induced ANF secretion (Sei and Glembski, 1990). In cultured adult atrial cardiocytes, a similar dependency on extracellular and intracellular Ca²⁺ can be observed (Ambler and Leite, 1994); in addition, the α₁-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 Glombotski, 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 discreet 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., 1966; 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 α₁-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 α₁-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 α₁-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 (Maggio 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 Glembotski, 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-atrical 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 discordant 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 Heuglen 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 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 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 α1-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\textsuperscript{ras}, 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 \(G_{aq}\) is not normally coupled to ras, the activation of ras may occur through the β 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 Gα-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 Gαq-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 α1-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 α1-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 (Thuerauf 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; Thuerauf 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 α (but not β) 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 α subtype of adrenergic receptors, but not those specific
for β-adrenergic receptors. A similar response to norepinephrine is found in adult ventricular cardiocytes (Ikedo 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 α-adrenergic receptor activation (Iwaki et al., 1990). In vivo, infusion of α-adrenergic agonists, but not β-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 α-adrenergic stimulation contrasts with those of c-fos and c-jun in similar experiments, which respond to both α and β agonists (Iwaki et al., 1990; Moalic et al., 1992; Brand et al., 1993). Therefore, the transcriptional response of atrial tissue to α₁-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-Ruhrkorff 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 (Thuerauf 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-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 α-MHC transcription (Gupta et al., 1991), thus suggesting that Egr-1 links ET-1 stimulation to changes in α-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; Thuerauf 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 α₁-adrenergic receptors is dependent on distinct secretory pathways. It is possible that different granule populations, containing distinct proportions or 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 α₁-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.
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.
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