MAPPING THE CELLULAR MECHANISMS REGULATING ATRIAL NATRIURETIC PEPTIDE SECRETION

PANU TASKINEN

Department of Pharmacology and Toxicology

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Abstract

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are cardiac hormones, which are involved in the regulation of blood pressure and fluid homeostasis. The major determinant for ANP and BNP release are atrial and ventricular wall stretch, but also some vasoactive factors such as endothelin-1 (ET-1) can enhance cardiac hormone secretion. The mechanical stretch rapidly activates multiple signal transduction pathways in cardiac cells, but the cellular mechanisms mediating stretch-induced ANP secretion are still unknown. The aim of the present study was to examine the cellular mechanisms of autocrine/paracrine factors and stretch-induced ANP secretion.

Genistein, a potent protein tyrosine kinase (PTK) inhibitor, rapidly increased cardiac contractile force and ANP secretion in perfused rat heart. This effect of genistein may be unrelated to the inhibition of PTKs since this stimulation was blocked by a L-type calcium channel antagonist and Ca²⁺/calmodulin-dependent protein kinase II inhibitor. Pregnancy hormone relaxin increased heart rate and ANP secretion in perfused spontaneously beating heart, suggesting that relaxin may have a role in modulating cardiac function. Cellular mechanisms of atrial wall stretch-induced ANP secretion were also studied. This enhanced secretion was blocked by sarcoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin and PTK inhibitor lavendustin A, indicating that thapsigargin sensitive Ca²⁺ pools and activation of PTK or PTK cascade have an important role in the regulation of stretch-secretion coupling. In addition, protein phosphatase inhibitor okadaic acid accelerated stretch-induced ANP secretion, suggesting that precise balance of protein kinase and phosphatase activity plays a role in mechanical stretch-induced ANP secretion. Finally interactions of endothelial factors regulating ANP exocytosis were studied. The potent nitric oxide synthase inhibitor L-NAME increased basal and atrial wall stretch-induced ANP secretion in the presence of ET-1, suggesting that nitric oxide may tonically inhibit ANP secretion.

Keywords: atrial natriuretic peptide, mechanical stretch, hormone secretion, cellular signaling
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Panu Taskinen
Abbreviations

Ang II  angiotensin II
ANOVA analysis of variance of repeated measurements
ANP atrial natriuretic peptide
ANP$_x$ ANP receptor subtype
BNP B-type natriuretic peptide
CaM calmodulin
CaM kinase calcium/calmodulin-dependent protein kinase
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
CNP C-type natriuretic peptide
DAG 1,2-diacylglycerol
DMSO dimethylsulfoxide
ERK extracellular signal regulated kinase
ET endothelin
ET$_x$ endothelin receptor subtype
FAK focal adhesion tyrosine kinase
Gd$^{3+}$ gadolinium
G proteins guanine nucleotide-binding proteins
GDP guanosine diphosphate
GTP guanosine trisphosphate
HPLC high performance liquid chromatography
IP$_3$ inositol 1,4,5-trisphosphate
IR immunoreactive
MAPK mitogen-activated protein kinase
MEK MAPK/ERK kinase
NEP neutral endopeptidase
NO nitric oxide
NOS nitric oxide synthase
PI-3 kinase phosphatidylinositol 3-kinase
PIP$_2$ phosphatidylinositol 4,5-bisphosphate
PIP$_3$ phosphatidylinositol 3,4,5-trisphosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phosphoinositide phospholipase A₂</td>
</tr>
<tr>
<td>PLC</td>
<td>phosphoinositide phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phosphoinositide phospholipase D</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium</td>
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<tr>
<td>PP</td>
<td>protein phosphatase</td>
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<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
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<tr>
<td>RAP</td>
<td>right atrial pressure</td>
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<tr>
<td>RPTK</td>
<td>receptor protein tyrosine kinase</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RYR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SA</td>
<td>stretch-activated</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SERCA</td>
<td>smooth endoplasmic reticulum calcium</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>serine / threonine</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
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<tr>
<th>Greek Letters</th>
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<td>λ</td>
<td>lambda</td>
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List of original papers

This thesis is based on the following articles, which are referred to in the text by Roman numerals:


IV Taskinen P, Toth M, Vuolteenaho O, Magga J & Ruskoaho H. Inhibition of atrial wall stretch-induced cardiac hormone secretion by lavendustin A, a potent tyrosine kinase inhibitor. Endocrinology (in press).

V Taskinen P, Vuolteenaho O & Ruskoaho H. Nitric oxide inhibits atrial natriuretic peptide secretion in isolated perfused rat hearts in the presence of endothelin-1. Revised manuscript for Eur J Pharmacol.
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Original papers
1. Introduction

Besides a blood pumping function, the mammalian heart is also known as an endocrine organ. Atrial natriuretic peptide (ANP) and brain or B-type natriuretic peptide (BNP) are members of the natriuretic hormone family released by cardiac cells (Ruskoaho 1992, Davidson & Struthers 1994). ANP is mainly produced by atrial myocytes while BNP is produced by both the atria and ventricles. Both peptides are involved in the regulation of blood pressure and fluid homeostasis (Wilkins et al. 1997, Levin et al. 1998). Atrial wall stretching is the predominant stimulus for the secretion of ANP (Lang et al. 1985, Ruskoaho 1992). Wall stretch appears also to be a potent stimulus for ventricular ANP release (Kinnunen et al. 1992) and for the secretion of BNP from the ventricles (Kinnunen et al. 1993) and atria (Mäntymaa et al. 1993) in vitro. The primary sensors for stretch-dependent cardiac hormone secretion may be cardiac myocytes, but other cell types, including endothelial and endocardial cells may also be involved in the activation of cardiac hormone release.

A number of different cell types have transduction systems that convert externally applied mechanical forces to signals that regulate cellular function (Watson 1991). Cardiac myocytes have the ability to sense mechanical stretch and convert it into intracellular signals, which lead e.g. to hypertrophy (Sadoshima & Izumo 1997) or secretion of ANP from atrium (Lang et al. 1985, Ruskoaho 1992). Mechanical stretch rapidly activates multiple cellular signaling pathways in myocytes (Sadoshima & Izumo 1993a). These second messenger pathways include e.g. phospholipid system and protein kinase C (PKC) which are thought to be associated in ANP secretion from myocytes (Ruskoaho 1992). Although it is well known that mechanical forces have many effects on the structure and function of cells, little is known of how the mechanical stimuli are converted into intracellular signals and cardiac hormone secretion. The purpose of this study was to examine the mechanism regulating natriuretic peptide release, particularly those pathways that are involved in atrial wall stretch-induced natriuretic peptide exocytosis.
2. Review of literature

2.1. Natriuretic peptides and receptors

2.1.1. Natriuretic peptide family

There are three members in the natriuretic peptide family, ANP, BNP and C-type natriuretic peptide (CNP) (Wilkins et al. 1997, Levin et al. 1998) (Table 1). De Bold and co-workers (1981) first demonstrated that atrial extracts contain a substance which produced natriuresis and diuresis. Soon after that the ANP molecule was purified and sequenced (Flynn et al. 1983, Atlas et al. 1984). Some years later two other major new peptides of this family were discovered. BNP was originally isolated from porcine brain (Sudoh et al. 1988) but it was reported to be more abundant in cardiac atria and ventricles than in the central nervous system (Minamino et al. 1988, Hosoda et al. 1991, for review.

Table 1. Comparison of some properties of natriuretic peptides.

<table>
<thead>
<tr>
<th></th>
<th>Atrial natriuretic peptide (ANP)</th>
<th>B-type natriuretic peptide (BNP)</th>
<th>C-type natriuretic peptide (CNP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biologically active forms</td>
<td>– ANP-28</td>
<td>– BNP-32 (humans)</td>
<td>– CNP-22</td>
</tr>
<tr>
<td>Major storage forms</td>
<td>– proANP</td>
<td>– BNP-32 (humans)</td>
<td>– CNP-53</td>
</tr>
<tr>
<td>Major tissue distribution</td>
<td>– atrial myocytes</td>
<td>– ventricular myocytes</td>
<td>– central nervous system</td>
</tr>
<tr>
<td>Major cardiovascular effects</td>
<td>– natriuresis, diuresis</td>
<td>– vasodilatation</td>
<td>– vasodilatation</td>
</tr>
<tr>
<td></td>
<td>– inhibition of renin,</td>
<td>– inhibition of renin,</td>
<td>– inhibition of proliferation of</td>
</tr>
<tr>
<td></td>
<td>aldosterone, vasopressin</td>
<td>aldosterone, vasopressin</td>
<td>vascular smooth muscle cells</td>
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<tr>
<td></td>
<td>and endothelin release</td>
<td>and endothelin release</td>
<td>inhibition of aldosterone,</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>vasopressin and endothelin release</td>
</tr>
<tr>
<td>Major regulator of release</td>
<td>– atrial wall stretch</td>
<td>– atrial and ventricular</td>
<td>– ANP and BNP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wall stretch</td>
<td>– cytokines and growth factors</td>
</tr>
<tr>
<td>Elimination</td>
<td>– C-receptors</td>
<td>– C-receptors</td>
<td>– C-receptors</td>
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<tr>
<td></td>
<td>– Neutral endopeptidases</td>
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</table>
CNP was also firstly identified in porcine brain (Sudoh et al. 1990) but it has been reported to be produced by endothelial cells (Suga et al. 1992a, Suga et al. 1993, for review see Chen & Burnett 1998).

It is now well established that natriuretic peptides are polypeptide hormones produced in a regulated manner (for review see Ruskoaho 1992, Yandle 1994, Wilkins et al. 1997, Levin et al. 1998). Each natriuretic peptide is encoded by a specific gene. ANP is mainly synthesized in the atria of normal adult heart, where ANP mRNA levels make up as much as 1-3 % of all mRNA. Ventricular expression is next in abundance, although the level is much lower than in the atrium (Gardner et al. 1986). In addition, a number of extracardiac tissues, including the central nervous system, lung, adrenal, kidney and vasculature contain ANP mRNA or proANP-like immunoreactivity (Gardner et al. 1986, for review see Ruskoaho 1992). BNP is also produced in the heart, although the ratios of atrial to ventricular expression are more in the range of 3:1 rather than the 30:1 seen for ANP (Gerbes et al. 1994). Significant levels of CNP gene expression are found in the central nervous system, anterior pituitary gland, kidney and in endothelial cells of the vasculature (Suga et al. 1992a, Suga et al. 1993, Chen & Burnett 1998).

ANP and BNP are stored in atrial myocytes in storage granules that are morphologically similar to those known to store other polypeptide hormones. The translation product of ANP gene is preproANP, from which proANP is formed by cleavage of signal peptide. PreproANP is transported unprocessed through the Golgi complex to secretory granules and thus 126-amino acid proANP is the major storage form (Vuolteenaho et al. 1985). On the contrary, the major storage form of BNP in the heart is cleaved mature peptide (Saito et al. 1989). Thus, this part of ANP processing differs from that of most other endocrine peptides, which are stored as bioactive peptide hormones. Finally, ANP-peptide is released by exocytosis to the interstitial fluid (Page et al. 1986). During or soon after its release proANP$_{1-126}$ is further split into an amino-terminal fragment (ANP$_{1-98}$, N-terminal ANP) and the biologically active hormone, the carboxy-terminal peptide (ANP$_{99-126}$, ANP). In some studies proteases from serum were suggested to convert proANP$_{1-126}$ to ANP$_{99-126}$ (Bloch et al. 1985, Itoh et al. 1987, for review see Ruskoaho 1992). However, ANP$_{99-126}$ is also released from isolated perfused hearts (Lang et al. 1985, Ruskoaho et al. 1986a) and atria (Vuolteenaho et al. 1985) in the absence of blood components. Therefore, the activation of proANP$_{1-126}$ probably either occurs intracellularly or simultaneously during release from the atrial myocyte, when an extracellular stimulus initiates secretion, possibly by simultaneously changing the activity of the processing enzyme(s) (for review see Ruskoaho 1992). Some researchers suggest that the N-terminal ANP is further processed to release three peptides (proANPs 1-30, 31-67 and 79-98) which may have either renal or vasorelaxant actions, or both (Vesely et al. 1994). Despite extensive study the mechanisms of proteolytic processing of ANP are not well understood.

The biologically active forms of natriuretic peptides share a common structural motif, consisting of a 17-amino acid loop formed by an intramolecular disulphide linkage between two cysteine residues (for review see Ruskoaho 1992, Yandle 1994, Levin et al. 1998). This disulphide loop and varying N- and C-terminal extensions are essential for biological activity of natriuretic peptides. The biologically active form of ANP is a 28-amino acid peptide, which amino acid sequence is highly homologous between species. BNP contains characteristic 17-amino acid structure to the natriuretic peptide family, but
the sequence variability between species is large. The carboxy-terminal tail commonly found in ANP and BNP is completely lost in CNP. Like ANP, the amino acid sequence of CNP is highly homologous between species. The biologically active form of BNP is 32-amino acid peptide, whereas CNP is found in two forms, 22- and 53-amino acid peptides (Chen & Burnett 1998).

### 2.1.2. Natriuretic peptide receptors

Biological effects of natriuretic peptides are mediated by cell membrane receptors. Three subtypes of natriuretic peptide receptors have been described: ANP_A, ANP_B and ANP_C receptors (for review see Maack 1992, Anand-Srivastava & Trachte 1993). ANP_A and ANP_B receptors are guanylyl cyclases through which the ligands induce the production of cyclic guanosine monophosphate (cGMP). The ANP_A receptor is thought to mediate many of the effects of ANP and BNP (Maack 1992, Davidson & Struthers 1994) while CNP acts via ANP_B receptors (Koller et al. 1991, Chen & Burnett 1998). ANP_C receptor is a clearance receptor, which may signal through alternative pathways (Anand-Srivastava et al. 1990, Levin 1993). Besides the capacity to activate guanylyl cyclases, these receptors seem to activate the phosphoinositide second messenger system (Maack 1992). The rank order of potency of binding (and stimulation of cGMP production for types ANP_A and ANP_B receptors) for the receptors is as follows: ANP_A receptor = ANP ≥ BNP > CNP; ANP_B receptor = CNP >> ANP ≥ BNP; ANP_C receptors = ANP > CNP > BNP (Koller et al. 1991, Suga et al. 1992b). No high-affinity guanylyl-cyclase-linked receptor that is specific for BNP has been identified. The properties of natriuretic peptides are predominantly mediated through increases of cGMP in target cells. These include cGMP-dependent protein kinases (PKG), cGMP-gated ion channels and cGMP-regulated phosphodiesterases (Lincoln & Cornwell 1993, de Bold et al. 1996).

Several studies have revealed natriuretic peptide receptors in many tissues, including blood vessels, kidney, adrenal gland, heart, lung and central nervous system (for review see Anand-Srivastava & Trachte 1993). ANP binding sites in the heart have been found in cardiac myocytes, atrial and ventricular endocardium and cells of conductive system (Anand-Srivastava & Trachte 1993). In the heart, both myocytes and fibroblasts are capable of expressing the natriuretic peptide receptor genes (Lin et al. 1995). There are no differences in distribution of ANP_A and ANP_B receptors in the atrial and ventricular tissues of rat hearts. However, the amount of the ANP_C receptors is greater in the atria than in the ventricles (Nunez et al. 1992).

Since natriuretic peptides and receptors are expressed in cardiac tissue, they may have paracrine/autocrine effects. Especially the role of natriuretic peptide receptors in the regulation of cardiac function, such as feedback regulation of natriuretic peptide release and modulation of contractility of the heart is of interest. The production of cGMP in myocytes is stimulated by ANP and BNP via ANP_A receptors, whereas CNP is ineffective (Lin et al. 1995). In the heart, cGMP has a negative inotropic effect (Lohmann et al. 1991). In addition, ANP inhibits cardiac L-type Ca^{2+} channel activity via cGMP dependent mechanisms (Tohse et al. 1995). Leskinen and co-workers (1997a) have shown that ANP modulates its own release by ANP_A receptors in vivo whereas CNP, the
effects of which are mainly mediated by ANP$_B$ receptors, does not act as a feedback regulator of ANP release.

ANP is rapidly eliminated from blood; the half-life of ANP in the circulation is 1/2-5 minutes, depending upon species. The clearance of ANP involves two predominant mechanisms: enzymatic degradation and receptor-mediated uptake (see Maack 1992, Ruskoaho 1992, Levin et al. 1998). The majority of ANP is eliminated by ANP$_C$ receptor-mediated mechanisms in several tissues including lung, liver, kidney and intestine (Maack 1992). The binding of ANP to these receptors leads to the internalization and subsequent intralysosomal hydrolysis of the peptide. Natriuretic peptides are also cleared by neutral endopeptidase (NEP), an ectoenzyme with a broad substrate specificity and a wide tissue distribution, which includes lung and kidney. NEP inactivates all three natriuretic peptides; it has a greater affinity for CNP than for ANP and the affinity for BNP is much lower than that for the other two peptides (Kenny et al. 1993). Also other enzymes, such as angiotensin converting enzyme and kallikreins have been suggested to participate in the metabolism of ANP (for review see Ruskoaho 1992).

2.1.3. Physiological effects of natriuretic peptides

Natriuretic peptides are important regulators of blood pressure and fluid homeostasis. In vivo, ANP lowers blood pressure by decreasing cardiac output and reducing peripheral vascular resistance (Levin et al. 1998). Overexpression of the ANP gene lowers systemic blood pressure (Steinhelper et al. 1990) while the knock-out of ANP gene or ANP$_A$-receptor gene leads to hypertension (John et al. 1995, Lopez et al. 1995) and cardiac hypertrophy (Oliver et al. 1997). ANP and BNP cause natriuresis and diuresis both by renal hemodynamic and direct tubular actions (Levin et al. 1998). ANP vasodilates afferent arterioles and vasoconstricts efferent arterioles, leading to increased glomerular capillary hydrostatic pressure (Dunn et al. 1986). In consequence, intrarenal infusion of ANP increases sodium, potassium and phosphate excretion (Burnett et al. 1984).

Collectively, the natriuretic peptide family counterbalances the effects of the renin-angiotensin-aldosterone system (Espiner 1994, Wilkins et al. 1997, Levin et al. 1998). ANP and BNP have been shown to be physiological antagonists of the effects of angiotensin II (Ang II) on vascular tone, aldosterone secretion, renal-tubule sodium reabsorption, and vascular cell growth (Harris et al. 1987, Itoh et al. 1990, Wilkins et al. 1997, Levin et al. 1998). In addition, secretion of vasopressin (Obana et al. 1985) and endothelin-1 (ET-1) (Saigonmaa et al. 1990) are decreased by ANP.

In vasculature ANP inhibits the growth of endothelial and vascular smooth muscle cells (Itoh et al. 1990, 1992). CNP, like ANP, has antitrophic effects in cell-culture systems (Furya et al. 1991) which suggest that both peptides can directly inhibit the structural remodeling of blood vessels that occurs in response to hypertension and vascular injury. In addition, ANP acts as an antigrowth factor for cardiac fibroblast thereby modulating growth of the interstitial compartment in the development of cardiac hypertrophy (Cao & Gardner 1995).
2.2. Regulation of ANP secretion

2.2.1. Mechanical factors

The major determinant of ANP secretion is atrial wall stretch (Lang et al. 1985, Ruskoaho et al. 1986a), but several other factors, such as rate of contraction, hormones and vasoactive peptides influence the release of ANP (for review see Ruskoaho 1992, de Bold et al. 1996) (Fig. 1). In vitro atrial stretch and in vivo volume expansion increase ANP release and it has been shown that wall stretch and not pressure per se is a direct stimulator of ANP release from the atria (Lang et al. 1985, Ruskoaho et al. 1986a, Edwards et al. 1988). The predominant stimulus controlling the release of BNP from the atria and ventricles as well as ANP release from the ventricles also appears to be the myocyte stretch (Mäntymaa et al. 1993, Kinnunen et al. 1993). The primary sensors for stretch-dependent natriuretic peptide secretion may be cardiac myocytes or other cell types, including endothelial and endocardial cells, and fibroblasts. However, it has not been established whether wall stretch acts directly or via local factors such as ET-1, nitric oxide (NO), and Ang II liberated in response to distension.

Fig. 1. Some factors that have been shown to affect plasma ANP levels in vivo or stimulate ANP secretion in vitro.

An increase in heart rate and contractility are effective stimuli for ANP release both in vitro and in vivo (for review see Ruskoaho 1992). In isolated rat atria and in perfused rat hearts an increase in beating rate enhances ANP release (Schiebinger & Linden 1986, Doubell 1989). In humans both supraventricular (Tikkanen et al. 1985, Roy et al. 1987)
and ventricular tachycardia (Crozier et al. 1987) increases plasma ANP concentrations. Tachycardia seems to increase ANP release by mechanisms associated with hemodynamic changes, such as increased mean atrial pressure.

ANP secretion is also stimulated by hypoxia both in vivo and in vitro (Baertschi et al. 1986, Lew & Baertschi 1989a). Atrial stretch, tachycardia, increased sympathetic activity and metabolic changes may be the factors which mediate hypoxia-induced increase in ANP release. In isolated perfused rat hearts reduction of coronary flow produces an increase in ANP release and this response shows a positive correlation with the lactate to pyruvate ratio and a negative correlation with phosphorylation potential (Uusimaa et al. 1992a). Thus, ANP release may be modulated by changes in myocardial energy metabolism.

2.2.2. Neurohumoral factors and pressor hormones

Although increase in atrial wall stretch appears to be the major signal for the release of ANP, a variety of humoral factors have been implicated in the control of ANP secretion. Stimulation of both \( \alpha \)- and \( \beta \)-adrenoreceptors by adrenaline or noradrenaline has been reported to increase ANP secretion from the isolated perfused heart (Currie & Newman 1986, Toth et al. 1986), dispersed myocytes (Gibbs 1987) and cultured neonatal myocytes (Matsubara et al. 1988), although conflicting results exist (for review see Ruskoaho 1992). In addition, cholinergic stimulation by acetylcholine increases ANP release from the isolated perfused heart and cultured myocytes (Toth et al. 1986, Ruskoaho 1992).

Pressor hormones such as vasopressin and Ang II have also been shown to modulate ANP secretion. Infusion of vasopressin or Ang II in vivo increases plasma ANP levels in several species (Katsube et al. 1985, Edwards et al. 1986, Uehlinger et al. 1986, Inoue et al. 1988, Cases et al. 1992). The stimulatory effect of vasopressin on ANP release in vitro has been demonstrated only in isolated atria (Sonnenberg & Veres 1984), whereas no effect has been seen in other experimental models. Similarly, Ang II had no effect on ANP release in rat heart-lung preparation (Dietz 1988) or in cultured atrial myocytes (Glenbotski et al. 1991). On the contrary, in cardiac myocyte and nonmyocyte coculture Ang II has been shown to increase ANP and BNP production (Harada et al. 1997). However, this effect was not seen in myocyte culture alone, suggesting that it depends on the existence of nonmyocytes (Harada et al. 1997). Therefore, hemodynamic effects of pressor hormones may mediate the positive effects on ANP secretion (Steward et al. 1990, Ruskoaho 1992).

2.2.3. The endothelial factors, endothelin-1 and nitric oxide

Endothelial cells line the inner surface of blood vessels and the much-trabeculated cavity of the cardiac chambers. Endothelial cells sense the transmural pressure at the endothelial surface, and release of endothelium-derived vasoconstrictor and relaxant factors has been observed in response to mechanical stretch and rise in transmural pressure. Endothelins (ETs) are a family of three (ET-1, ET-2 and ET-3) regulatory peptides produced by
endothelial cells (Rubanyi & Polokoff 1994, Levin 1995). ET-1 was the first identified ET-peptide and it was found to be the most potent vasoconstrictor substance yet identified (Yanagisawa et al. 1988). ET-1 has been shown to be a potent ANP secretagogue in cultured rat atrial myocytes (Fukuda et al. 1988, Sei & Glembotski 1990, Lew & Baertschi 1989b, 1992, Gardner et al. 1991, Uusimaa et al. 1992b, Muir et al. 1993), isolated atria (Hu et al. 1988, Stasch et al. 1989, Winquist et al. 1989, Schiebinger & Gomez-Sanchez 1990) and isolated perfused rat heart (Mäntymaa et al. 1990, Pitkänen et al. 1991) as well as in vivo (Stasch et al. 1989, Garcia et al. 1990, Kohno et al. 1990). On the contrary, low doses of ET-1 have been reported to inhibit ANP secretion both in perfused rat hearts and in conscious unrestrained rats (Shirakami et al. 1993). The inhibitory effect of ET-1 on ANP secretion was almost abolished by simultaneous administration of indomethacin but not by methylene blue, suggesting that prostanoids, not NO, are involved in the ET-1-induced inhibition of ANP secretion (Shirakami et al. 1993).

Currently, two major ET receptor subtypes have been cloned, the ETA and ETB receptors, which are G-protein coupled (Rubanyi & Polokoff 1994, Levin 1995). Vasoconstriction induced by ET-1 is mainly mediated by ETA receptors but in some blood vessels also by ETB Receptors (Clozel et al. 1992). In the heart, both ETA and ETB mRNAs are found throughout the myocardium of the atria and ventricles (Hori et al. 1992). However, ETA receptors constitute approximately 91% of the total population of ET receptors in human right atrial myocytes (Molenaar et al. 1993). It has been shown that ET-stimulated secretion of natriuretic peptides in cultured myocytes is mediated by ETA receptors (Irons et al. 1993, Leite et al. 1994, Thibault et al. 1994).

In addition to ET, vascular endothelium has been shown to produce factors which can induce relaxation of vascular smooth muscle cells (Furchgott & Zawadzki 1980). This endothelium-derived relaxing factor, which was discovered to be nitric oxide (NO)(Palmer et al. 1987), is synthesized in endothelial cells from L-arginine by NO synthase (NOS)(Palmer et al. 1988, Palmer & Moncada 1989, for reviews see Moncada et al. 1991, Ånggård 1994). Three different NOSs have been identified, all of which are also present in the heart. Endothelial cells continuously release small amounts of NO, producing a basal level of vascular smooth muscle relaxation (Moncada et al. 1991, Calver et al. 1993, Ånggård 1994). In addition, endocardial cells and both vascular and cardiac myocytes can synthesize NO (Schulz et al. 1991, Schulz et al. 1992, Schulz & Triggle 1994). The most important physiological stimulus for the release of NO in the vasculature is the increase in shear stress (for review see Davies 1995), but also some physiological agents such as acetylcholine, bradykinin, thrombin and adenosine diphosphate regulate NO release (Nathan 1992). NO stimulates soluble guanylyl cyclase and increases vascular and cardiac muscle cGMP formation and exerts a negative inotropic effect on cardiac muscle cells similar to its relaxation of smooth muscle cells (Smith et al. 1991). Thus, by releasing NO endocardial cells may have a physiological role in the regulation of the function of the heart.

NO has been suggested to be involved in the regulation of ANP release. Coculture of aortic endothelial cells with atrial myocytes stimulates ANP release, and this release is inhibited by acetylcholine, known to evoke the release of NO (Lew & Baertschi 1989b). Removal of endocardium by saponin or infusion of inhibitors of NO significantly increases the release of ANP from isolated rat atria (Sanchez-Ferrer et al. 1990).
Furthermore, in the isolated rat heart competitive inhibition of NO synthesis with L-NMMA overcame the inhibitory effect of acetylcholine on ANP secretion, showing that NO release from endothelium negatively modulates ANP secretion (Melo & Sonnenberg 1996). In addition, in conscious rats basal plasma ANP concentrations are dose-dependently increased by L-NAME, an inhibitor of NOS (Leskinen et al. 1995). These results suggest that NO released from the endothelium may tonically inhibit the secretion of ANP from cardiac myocytes.

2.3. Signal transduction pathways in cardiac cells

2.3.1. Receptors and G-proteins

Hormones, growth factors and autocrine/paracrine factors act by binding to specific receptors to initiate diverse cellular events. These factors bind to membrane-associated receptors to trigger a cascade of secondary events, including the generation of diffusible intracellular second messengers (Davis et al. 1992a). Membrane receptors are linked to an intracellular effector system via intermediate transducing proteins. A family of these membrane-bound transducing proteins has been identified, which bind guanine nucleotides (guanosine triphosphate and guanosine diphosphate, GTP and GDP), and are hence termed G-proteins (Strader et al. 1994, Gudermann et al. 1997). Several hundred G-protein-coupled receptors have been cloned (Strader et al. 1994, Gudermann et al. 1997).

The G-proteins are located on the cytoplasmic surface of the cell membrane. Each G-protein is a heterotrimer containing α-, β- and γ-subunits and they are classified based upon the amino acid sequence similarity of their α-subunits (Strader et al. 1994, Gudermann et al. 1997). Upon stimulation by ligand-receptor complex, the G-protein exchanges a bound GDP molecule for GTP, and the β- and γ-subunits then dissociate from the Gα-subunit. Both GTP-bound α-subunits and βγ dimers are signaling molecules and modulate the activity of coupled effectors, such as enzymes, ion channels, and transporters, resulting in rapid alterations of second messenger (Sternweis & Smrcka 1992, Exton 1994, Clapham 1994, Birnbaumer & Birnbaumer 1995, Gudermann et al. 1997, Dolphin 1998). There is a wide diversity of G proteins involved in signal transduction (Simon et al. 1991). Main types include G_i proteins that inhibit adenylyl cyclase and activation of potassium channels, G_s proteins that stimulate adenylyl cyclase and activate Ca^{2+} channels, and G_q proteins that activate phospholipase C (PLC). G-protein-regulated macromolecules known to affect cardiac cellular responses include the enzymes adenylyl cyclase, PLC, phospholipase A_2 (PLA_2) and guanylyl cyclase (for review see Fleming et al. 1992, Johnson & Friedman 1993). Thus, it is evident that G proteins are essential links in the cascade of biochemical events that ensue when neurotransmitters and hormones interact with receptors on myocardial cells.

The efficacy of signal transduction systems may be modified in a number of ways. These include the modulation of receptor number and/or function, desensitization of post-receptor coupling systems, and antagonistic or synergistic interactions between parallel signaling systems (Davis et al. 1992a, Böhm et al. 1997). For example, in human heart
there are many receptor systems that regulate contractility and heart rate. In chronic heart failure a decrease in $\beta_1$-adrenoreceptor number and an increase in the functional activity of $G_i$ will lead to reduced physiologic responses of the failing heart to $\beta$-adrenergic stimulation (Brodde et al. 1995).

### 2.3.2. Protein kinases

Protein kinases regulate numerous biological processes. In the cardiac myocytes, these include the regulation of contraction, ion transport, fuel metabolism and gene expression and growth (for review see Sugden & Bogoyevich 1995). Protein kinases often participate in the transduction of a (extracellular) signal to a biological response. Binding of the regulatory molecule to its membrane receptor often changes the intracellular level of one of the second messengers, which then modulates the activity of protein kinase (Table 2). Many protein kinases require a change in phosphorylation state (phosphorylation or dephosphorylation) for activity. When the phosphorylated form of the enzyme is active, phosphorylation can be either permissive or modulatory. The majority of protein kinases transfer the $\gamma$-phosphate group from ATP to hydroxyl groups of serine/threonine residues (protein Ser/Thr kinases) or tyrosine residues (protein tyrosine kinases, PTKs) in proteins, including a change in activity or function of the substrate enzyme or protein. For many protein Ser/Thr kinases, the transduction process involves receptor activation followed by the synthesis or release of a second messenger. In contrast, the PTKs are often receptors themselves (Fantl et al. 1993) but the existence of nonreceptor PTKs is also well-established (Bolen 1993).

**Table 2. Second messenger-dependent protein kinases.**

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>Abbreviations</th>
<th>Activator</th>
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<tbody>
<tr>
<td>cyclic AMP-dependent protein kinase</td>
<td>PKA</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>cyclic GMP-dependent protein kinase</td>
<td>PKG</td>
<td>cyclic GMP</td>
</tr>
<tr>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase</td>
<td>CaM kinase</td>
<td>4(Ca$^{2+}$)-calmodulin complex</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>PKC</td>
<td>Ca$^{2+}$ and 1,2-diacylglycerol</td>
</tr>
</tbody>
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Processes that are reversibly controlled by protein phosphorylation require not only protein kinases but also protein phosphatases (PPs) (Hunter 1995). Target proteins are phosphorylated at specific sites by one or more protein kinases, and these phosphates are removed by specific PPs. The extent of phosphorylation on a particular site can be regulated by changing the activity of the protein kinases or phosphatases or both (Hunter 1995). The maximal activities of the protein kinases and phosphatases acting on a particular site must be in the controlled balance; otherwise, the protein would be either fully phosphorylated or completely dephosphorylated. Several different protein kinase or phosphatase inhibitors can be used to determine the physiological significance of the protein phosphorylation systems in various types of cells (Hidaka & Kobayashi 1992).
Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is an important component of several intracellular signaling pathways. Many cellular responses to the occupancy of membrane receptors include the hydrolysis of PIP$_2$ by PLC and the subsequent generation of second messengers inositol 1,4,5-trisphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) (for review see Nishizuka 1992, Divecha & Irvine 1995). PIP$_2$ is a precursor not only for the second messengers IP$_3$ and DAG, but also for the putative messenger phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$), which is produced by phosphatidylinositol 3-kinase (PI-3 kinase). PIP$_2$ is also known to serve as a regulator of many actin-binding proteins, and as a cofactor for an isoform of phospholipase D (PLD) (for review see Lee & Rhee 1995).

All PLC enzymes identified up to now are single polypeptides that can be divided into three types ($\beta$, $\gamma$, $\delta$)(Lee & Rhee 1995). It is generally accepted that there are at least two distinct mechanisms to link receptor occupancy to the activation of PLC isoenzymes. PLC-$\beta$ isoenzymes are activated by G proteins (Sternweis & Smrcka 1992, Exton 1994), while PLC-$\gamma$ isoenzymes are activated by receptor and nonreceptor PTKs (Lee & Rhee 1995). The activation mechanism of PLC-$\delta$ is not known at the present time. Cardiac myocytes predominantly express PLC-$\beta$ (Hansen et al. 1995).

IP$_3$, by mediating Ca$^{2+}$ mobilization, acts synergistically with DAG to activate PKC. Activation of PKC is thought to involve the redistribution of the enzyme from a cytosolic location in resting cells to a membrane-associated site during stimulation. A rise in internal Ca$^{2+}$ alone can bring about this redistribution and activation of PKC (Nishizuka 1992, Newton 1997). Isotope-labeling studies indicate that DAG is metabolized very rapidly either when it is produced endogenously or when it is added exogenously to cells (Nishizuka 1992). DAG may be phosphorylated to form phosphatidic acid, and then resynthesized back to phosphatidylinositol, or it can be hydrolyzed by DAG lipase to form arachidonic acid, which in turn can act as a second messenger. Thus, PKC is active for only a short time after the stimulation.

As mentioned earlier, in response to agonist, DAG is initially produced as a result of hydrolysis of inositol phospholipids (PIP$_2$). This DAG production is transient and it is frequently followed by a more sustained increase in the amount of DAG. This second phase of DAG formation results from hydrolysis of phosphatidylcholine. Phosphatidylcholine is degraded by PLC to produce DAG and cholinephosphate or by PLD to produce choline and phosphatic acid, which is further dephosphorylated to DAG by phosphomonoesterase (Nishizuka 1992). In addition, a potential role of free fatty acids as second messengers has been suggested (for review see Asaoka et al. 1992, Azzi et al. 1992, Nishizuka 1992). PLA$_2$ hydrolyzes phospholipids to liberate free fatty acids and lysophospholipids. Several cis unsaturated fatty acids, which are produced from phospholipids by the action of nonselective type of PLA$_2$, enhance the DAG-dependent activation of PKC, and allow PKC to exhibit nearly full activity in the presence of low amounts of Ca$^{2+}$. However, it is thought that the activity of PKC is increased initially by the increase in the concentrations of intracellular Ca$^{2+}$ and DAG. The activity of PKC may then be sustained, even after the concentration of intracellular Ca$^{2+}$ is no longer increased, if DAG and cis unsaturated fatty acids both become available (Asaoka et al. 1992, Nishizuka 1992).
PKC represents a structurally homologous group of proteins similar in size, structure and mechanism of activation. Several isoenzymes have been defined which are derived both from multiple genes and alternative splicing of a single RNA transcript (Azzi et al. 1992, Hug & Sarre 1993). PKC has been subdivided into three groups: 1) the conventional PKCs (α, β, γ) which are regulated by Ca\(^{2+}\), phosphatidylserine, and DAG, 2) the novel PKCs (δ, ε, η, θ) which are regulated by DAG and phosphatidylinerine, 3) the atypical PKCs (τ, λ, ζ) which are Ca\(^{2+}\) and DAG independent but are all dependent on phosphatidylserine (Sugden & Bogoyevitch 1995, Hofmann 1997, Newton 1997). At least five different isoforms of PKCs (α, β, δ, η and ζ isoforms) have been revealed in both adult and neonatal rat cardiac myocytes and four of these isoforms are Ca\(^{2+}\) independent subtypes (Kohout & Rogers 1993, Sugden & Bogoyevitch 1995).

PKC participates in one of the major signal transduction systems triggered by the external stimulation of cells by various ligands including hormones, neurotransmitters and growth factors (Azzi et al. 1992, Davis et al. 1992a, Nishizuka 1992). Several physiological functions have been assigned to PKC, including involvement in secretion and exocytosis, modulation of ion conductance, interaction and down-regulation of receptors, smooth muscle contraction, gene expression, and cell proliferation. The connection between PKC activation and its cellular responses is mediated by proteins, which become phosphorylated. The phosphorylated residues on the target proteins are serine and threonines and PKC is known as a Ser/Thr kinase. Several substrate proteins of PKC have been identified, including receptor proteins, membrane proteins such as Ca\(^{2+}\)-ATPase and Na\(^{+}/K^{+}\)-ATPase, contractile proteins such as myosin light chain, and enzymes such as myosin light chain kinase and guanylate cyclase (Azzi et al. 1992). PKC can phosphorylate and activate sarcoplasmic reticulum (SR) Ca\(^{2+}\) channels and it may also phosphorylate PLC to inactivate it. In addition, mitogen-activated protein kinase (MAPK) is a Ser/Thr-protein kinase, whose enzymatic activity requires phosphorylation of both threonine and tyrosine residues and it is activated by PKC (Andersson et al. 1990, Adams & Parker 1991).

### 2.3.2.2. Cyclic nucleotide-dependent protein kinases

In cardiac cells, stimulation of β-adrenergic receptors (β\(_1\)/β\(_2\)) on the sarcolemma by noradrenaline activates the stimulatory G\(_s\) protein by promoting the exchange of GDP for GTP. This reaction catalyzes the dissociation of the GTP-bound G\(_{s\alpha}\) subunit from G\(_{βγ}\). GTP-bound G\(_{s\alpha}\) then binds to and stimulates adenylyl cyclase, a membrane-bound enzyme that catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP) (Ishikawa & Homcy 1997). So far, at least nine isoforms of adenylyl cyclase are known, and types II-VII are also detected in cardiac tissue (Ishikawa & Homcy 1997). They all share the same membrane topology; tandem repetition of a six-transmembrane domain and a large cytoplasmic domain. Biochemical characterization of the enzyme obtained from various tissues demonstrated that there were two distinct subtypes: calmodulin (CaM)-sensitive and -insensitive isoforms. Types V and VI represent the major adenylyl cyclase isoforms in the heart. These isoforms are insensitive to both CaM and G\(_{βγ}\) subunits (Ishikawa & Homcy 1997).
cAMP acts as an intracellular second messenger. Severalfold increases in cAMP levels can occur within a millisecond-to-second time frame of hormonal stimulation. cAMP, in turn, activates several target molecules, primarily cyclic AMP-dependent protein kinase (protein kinase A, PKA) by dissociating its regulatory subunit from the catalytic subunit leading to a phosphorylation cascade and activation of multiple proteins (Walsh & van Patten 1994). PKA is a member of Ser/Thr kinases. PKA phosphorylates at least the L-type Ca\textsuperscript{2+} channel, phospholamban and troponin I, and causes, for example, an increase in Ca\textsuperscript{2+} entry into the cardiocytes. β-adrenergic effects on both the rate and force of cardiac contraction are mediated by PKA (for review see Sugden & Bogoyevitch 1995). The activating effects of G\textsubscript{sa} rapidly reverse when agonist occupancy of the receptor ceases and cAMP is rapidly catabolized to 5′AMP by phosphodiesterases (for review see Tang & Gilman 1992, Ishikawa & Homy 1997). PKA is then inactivated by reassociation of the catalytic subunit with the regulatory subunit and phosphorylated proteins are rapidly dephosphorylated by specific phosphatases. Participation of adenylyl cyclase and cAMP in biological processes in intact cells can be studied by treatment with permeating analogues of cAMP or with agonist that directly activate the catalytic domain of adenylyl cyclase; for example forskolin and its homologues.

PKGs constitute a second group of cyclic nucleotide-dependent protein Ser/Thr kinases (Francis & Corbin 1994, Sugden & Bogoyevitch 1995). They are involved in the mediation of the effects of NO and natriuretic peptides which activate guanylyl cyclase (Chinkers & Garbers 1991, Maack 1992, Anand-Srivastava & Trachte 1993, Anderson et al. 1994), thereby increasing intracellular concentration of cGMP. The increased cGMP activates PKG, which in turn phosphorylates a number of smooth muscle proteins, including the myosin light chain leading further to the relaxation of smooth muscle and vasodilatation.

### 2.3.2.3. Calcium/calmodulin-dependent protein kinases

Ca\textsuperscript{2+} acts as an intracellular second messenger of large varieties in species and is involved in cellular processes ranging from contraction and secretion to gene expression (Gnegy 1993). Cells contain a number of Ca\textsuperscript{2+}-binding proteins, and in most cell types, the major Ca\textsuperscript{2+}-binding protein is CaM. CaM is a ubiquitous, multifunctional Ca\textsuperscript{2+}-binding protein that binds four Ca\textsuperscript{2+} ions (Gnegy 1993). This complex of 4(Ca\textsuperscript{2+})-CaM activates downstream targets and acts as Ca\textsuperscript{2+}-dependent regulator of cyclic nucleotide metabolism, Ca\textsuperscript{2+}-transport, protein phosphorylation-dephosphorylation cascades, ion transport, cytoskeletal function, and cell proliferation. CaM activates isoenzymes of enzymes such as adenylyl cyclase, cyclic nucleotide phosphodiesterase, Ca\textsuperscript{2+}-ATPase, Mg\textsuperscript{2+}-ATPase, calcineurin, NOS, and several protein kinases (for review see Gnegy 1993).

CaM also activates a class of proteins called Ca\textsuperscript{2+}/CaM-dependent protein kinases (CaM kinases). These kinases are a structurally related group of enzymes that constitute a subfamily within the larger family of protein Ser/Thr kinases (Hanks & Quinn 1991). Currently recognized members of the CaM kinases are phosphorylase kinase, myosin light chain kinase and CaM kinase Ia/Ib, II, III and IV (Schulman 1993, Braun &
CaM kinase II is a multifunctional protein kinase which is activated by CaM-mediated autophosphorylation, thus, CaM-binding removes the restraint of an autoinhibitory domain on autophosphorylation (Schulman 1993, Braun & Schulman 1995). CaM kinase II is also present in the heart (Mayer et al. 1994, Sugden & Bogoyevitch 1995, Braun & Schulman 1995). CaM kinase II has the ability to phosphorylate and alter the function of a variety of substrates including cardiac Ca²⁺-ATPase, IP₃ receptor, NOS, caldesmon, myosin light chain kinase, phospholamban, PLA₂, cardiac ryanodine receptor (RYR) and troponin I (Braun & Schulman 1995). This indicates that CaM kinase II has a central role in the regulation of cardiac excitation-contraction coupling, ion handling, and vascular smooth muscle contraction.

2.3.2.4. Protein tyrosine kinases

Characteristic feature of many receptors is that they have intrinsic PTK activity and belong to the receptor protein tyrosine kinase (RPTK) family (Fantl et al. 1993, van der Geer & Hunter 1994). Such receptors are coupled to the regulation of many cellular programs, such as growth and differentiation. Most known ligands for RPTKs are soluble proteins such as growth factors, but membrane-bound proteins as well as extracellular matrix proteins may also activate RPTKs (Fantl et al. 1993, van der Geer & Hunter 1994). Ligand binding to the extracellular domain of RPTK is followed by receptor dimerization, stimulation of PTK activity and autophosphorylation. This leads to downstream activation of a number of common signaling molecules such as PLC-γ, PI-3 kinase, GTPase-activating protein, pp60-src, p21ras, raf-1 kinase and MAPKs, and S6 ribosomal kinases (Carpenter 1992, Fantl et al. 1993, van der Geer & Hunter 1994). Another class of PTKs is nonreceptor PTKs which represent a collection of cellular enzymes that are grouped together because of their lack of extracellular sequences (Bolen 1993). A number of nonreceptor PTKs have been found to be associated with other cell surface proteins and shown to be capable of facilitating cell surface initiated signal transduction much like the class of RPTKs.

A number of PTKs have been shown to be activated by neuropeptides and mechanical forces. Bombesin, vasopressin and ET-1 have been shown to stimulate tyrosine phosphorylation (Zachary et al. 1992, Koide et al. 1992). In Dictyostelium cells tyrosine phosphorylation of actin correlates with changes in cell shape (Howard et al. 1993). Focal adhesion kinase (FAK) is a cytoplasmic PTK, specifically located at the focal adhesion, which has been shown to be phosphorylated and activated by a number of growth factors and integrin-dependent cell adhesion. Stretching of mesangial cells has been shown to stimulate FAK (Hamasaki et al. 1995). In fetal lung cell mechanical stretch caused activation of Src, nonreceptor type of PTKs, within 5 min (Liu et al. 1996). Since cardiac myocytes are also targets for the action of growth factors (Schneider & Parker 1990), PTKs may have some functional role in the regulation of cardiac cells.
MAPKs are a group of important mediators that transduce extracellular signals to intracellular responses. At least three different MAPK isoforms have recently been described in mammalian cells, including extracellular signal-regulated kinase (ERK), p38 kinase and stretch-activated protein kinase (SAPK), also known as c-Jun N-terminal protein kinase (Bokemeyer et al. 1996, Force et al. 1996). Recent developments have extensively characterized the ERK cascade, which is the best studied MAPK signaling cascade. ERK1/ERK2 are the forms of MAPKs expressed in the heart (Sugden & Bogoyevitch 1995). In addition, SAPK is a new subgroup of MAPKs also present in cardiac cells (Kyriakis et al. 1994, Force et al. 1996) activated in response to extracellular stimuli binding both to tyrosine kinase receptors or G-protein-coupled receptors. A common feature of all MAPK isoforms is the requirement for phosphorylation of both threonine and tyrosine regulatory sites by a specific upstream protein kinase for activation (Anderson et al. 1990). The ERK family member of MAPKs is activated by MEK (MAPK kinase / ERK kinase). In response to extracellular stimuli MAPKs regulate the transcriptional activity of several transcription factors via phosphorylation of either stimulatory or inhibitory regulatory sites. PTK receptors, G-protein coupled receptors and cytokine receptors were shown to be capable of activating the ERK cascade (for review see Bokemeyer et al. 1996). Ang II, ET-1 and fibroblast growth factor have been shown to be potent stimulators of ERK in cardiac myocytes (Sadoshima et al. 1993, Bogoyevitch et al. 1994, Lazou et al. 1994).

2.3.3. Ionized calcium as a second messenger

Ionized calcium is the most common signal transduction element in cells (for review see Davis et al. 1992a, Petersen et al. 1994, Clapham 1995). Changes in intracellular Ca\(^{2+}\) concentration can control specialized functions like excitability, contraction, exocytosis, metabolism and gene expression. In most cells, elevations in intracellular Ca\(^{2+}\) arise from Ca\(^{2+}\) entry via Ca\(^{2+}\) channels in the surface membrane, or Ca\(^{2+}\) discharge from internal stores, or both. In the beating heart Ca\(^{2+}\) homeostasis uses 20-25% of total energy output of myocytes (Langer 1992). Homeostasis is maintained by Ca\(^{2+}\) pumps in the sarcolemma and the SR, and by the sarcolemmal Na\(^{+}\)-Ca\(^{2+}\) exchanger. Unlike many other second-messenger molecules, Ca\(^{2+}\) cannot be metabolized, so cells tightly regulate intracellular levels through numerous binding and specialized extrusion proteins.

Normal intracellular Ca\(^{2+}\) levels are 10,000-fold lower than the concentration found extracellularly (100 nM vs. 2 mM). At least two mechanisms exist to pump Ca\(^{2+}\) out of the cell, one involving a Ca\(^{2+}\)-ATPase, and another a Na\(^{+}\)/Ca\(^{2+}\) exchanger, which couples the cellular uptake of Na\(^+\) to Ca\(^{2+}\) extrusion. Of these, the Na\(^{+}\)/Ca\(^{2+}\) exchanger has the largest transport capacity (Doohan & Rasmussen 1993). In cells Ca\(^{2+}\) is stored to the SR and mitochondria. Ca\(^{2+}\) pumps in the SR membrane (smooth endoplasmic reticulum calcium pumps, SERCA pumps) use ATP to pump Ca\(^{2+}\) ions into the SR, where they are sequestered by buffer molecules (Lytton & Nikam 1992, Clapham 1995). Ca\(^{2+}\) ion can also be removed to the extracellular space by plasma membrane Ca\(^{2+}\) pumps (PMCA.
pumps). Both SERCA and PMCA pumps are ATPases. The SERCA pumps are products of three different genes. SERCA2 pumps are expressed in cardiac (Grover & Khan 1992) and slow-twitch skeletal muscle, while SERCA1 pumps are expressed in fast-twitch skeletal muscle (Lytton & Nigam 1992, Clapham 1995). Intracellular Ca²⁺-binding proteins can be classified as trigger or buffer proteins (see Lytton & Nikam 1992, Clapham 1995). Trigger proteins, e.g. CaM, change their conformation upon binding Ca²⁺ and modulate effector molecules such as enzymes and ion channels. Ca²⁺ storage in SR is achieved by the low-affinity, high-capacity Ca²⁺-binding protein, e.g. in cardiac cells it is called calsequestrin.

The transduction of electrical signals to cellular function is initiated by the opening of voltage-gated Ca²⁺ channels in response to depolarization of the surface membrane. Ca²⁺ penetrates the plasma membrane through the Ca²⁺-channels which could be voltage-, receptor-, second messenger- or mechanically operated or tonically active (Tsien & Tsien 1990, Spedding & Paoletti 1992, Balke & Gold 1992). L-type voltage-operated Ca²⁺ channels are high-voltage activated channels (Miller 1992) and they are the major pathway for voltage-gated Ca²⁺ entry involved in the activation of contraction in heart and smooth muscle, and in the control of transmitter release from endocrine cells. T-type Ca²⁺ channels are low-voltage activated Ca²⁺ channels and the most obvious function of T-type channels is to support pacemaker activity (Balke & Gold, 1992). N-type and P-type voltage-operated Ca²⁺ channels are found in neurons and second messenger-operated Ca²⁺ channels in neutrophils, platelets and lymphocytes. Receptor-operated Ca²⁺ channels open in direct response to the binding of an external ligand and tonically active Ca²⁺ channels help to set resting Ca²⁺ level in muscle cells. The mechanically operated channels include stretch-activated and stretch-inactivated ion channels (Tsien & Tsien 1990, Spedding & Paoletti 1992).

IP₃ and ryanodine receptors represent the two principal intracellular Ca²⁺ channels responsible for mobilizing stored Ca²⁺ (see Taylor & Marshall 1992, Berridge 1993, Tsunoda 1993, Ehrlich et al. 1994). Some cells either have ryanodine-sensitive Ca²⁺ stores or IP₃-sensitive stores, whereas e.g. atrial cells contain both. In response to many stimuli both IP₃ and DAG are formed by the hydrolysis of an inositol lipid precursor stored in the plasma membrane. This IP₃ acts as an intracellular second messenger by binding to the specialized tetrameric IP₃ receptor that spans the endoplasmic reticular membrane and by triggering release of Ca²⁺ from the endoplasmic reticulum. IP₃ is the only known physiological activator of the IP₃ receptor, and Ca²⁺ is the only known physiological inhibitor since high cytoplasmic Ca²⁺ concentration decreases the binding of IP₃ to its own receptor (Ehrlich et al. 1994).

RYR is the other major intracellular Ca²⁺ channel and the RYRs identified in cardiac cells are called RYR2 (for review see Coronado et al. 1994, Meissner 1994). The plant alkaloid ryanodine opens the channels at nanomolar concentrations but closes them at micromolar doses. SR Ca²⁺ stores can be depleted by ryanodine. The activity of the RYR channel is strongly enhanced by adenosine nucleotides, caffeine and Ca²⁺ itself and inhibited by procaine (Tsien & Tsien 1990, Coronado et al. 1994). RYR, when activated by Ca²⁺-release agents, induces a release of Ca²⁺ from the primary Ca²⁺-binding protein of SR, calsequestrin. Excitation-contraction coupling is initiated when depolarization permits Ca²⁺ to enter the sarcoplasm through voltage-dependent Ca²⁺ channels in the sarcolemma, releasing a large quantity of Ca²⁺ from the SR (Callewaert 1992). This is
called Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Ca\(^{2+}\) interacts with troponin C and produces a conformational change in the regulatory complex of muscle filament, which moves tropomyosin away and allows actin and myosin to form cross-bridges and thus initiate contraction (Valdeolmillos \textit{et al.} 1989). Relaxation occurs when Ca\(^{2+}\) dissociates from the contractile apparatus and is resequestered (Calwewaert 1992, Barry & Bridge 1993). In cardiac cells RYRs may be up- or downregulated by the phosphorylation of protein kinases such as CaM kinase, PKA and PKC (Coronado \textit{et al.} 1994).

2.4. The cellular and molecular response of cardiac myocytes to mechanical stress

Although mechanical stretch activates multiple second messenger systems, it is unknown which molecules are directly activated by stretch and which molecules are indirectly activated by other upstream modulators. A mechanosensitive molecule is assumed to have some interaction with the plasma membrane in order to sense tension of the membrane. One candidate for mechanosensor are stretch-activated (SA) ion channels, which are suggested to interact directly with cytoskeleton and thus can sense the cell stretch (Hamill & McBride 1992, Sackin 1995). SA channels have been identified e.g. in vascular endothelial (Lansman \textit{et al.} 1987, for review see Davies 1995) and smooth muscle (Davis \textit{et al.} 1992b) cells as well as in atrial (Sadoshima \textit{et al.} 1992, Kim 1993) and ventricular myocytes (Craelius 1993, Ruknudin \textit{et al.} 1993). These include potassium channels, nonselective cation channels, and stretch-inactivated channels (Sadoshima \textit{et al.} 1992, Sigurdson \textit{et al.} 1992, Ruknudin \textit{et al.} 1993, Sackin 1995). Opening of SA channels causes an increase in intracellular Ca\(^{2+}\) concentration because some of these channels are permeable to Ca\(^{2+}\). Nonselective SA channels in the heart have been shown to be blocked by Gd\(^{3+}\) (Sadoshima \textit{et al.} 1992) and stretch-induced rise in the intracellular Ca\(^{2+}\) concentration has been shown to be decreased by Gd\(^{3+}\) in cardiac cells (Sigurdson \textit{et al.} 1992). Gd\(^{3+}\) has also been shown to block the stretch-induced increase in BNP mRNA levels in isolated superfused atrium, suggesting that Gd\(^{3+}\) -sensitive stretch-activated channels may be involved in stretch-induced hypertrophic responses (Laine \textit{et al.} 1996). In contrast, Sadoshima and co-workers have shown (1992) that treatment of myocytes with Gd\(^{3+}\) does not affect stretch-induced immediate-early gene expression or stretch-induced increase in the rate of protein synthesis. However, these results do not deny the possibility that a mechano-sensitive channel, which is Gd\(^{3+}\) insensitive, may work as a mechanotransducer.

Another candidate for mechanosensors are integrins, transmembrane receptors that couple components of the extracellular matrix with the actin cytoskeleton (Wang \textit{et al.} 1993, Parson 1996). Cytoskeleton may form a complex with membrane proteins such as ion channels, adenyl cyclase, and Na\(^+\)/H\(^+\) exchanger and regulate their responsiveness to external forces (Watson 1991). Multiple integrins are expressed in the heart (Baldwin & Buck 1994). Integrins are suggested to interact e.g. with FAK, which further interacts with various signaling molecules (for review see Sadoshima & Izumo 1997). Studies by Sadoshima and co-workers (1992) argue against the possibility that SA cation channels,
actin microfilaments, microtubules, integrins or contractile activity are necessary for the stretch-induced immediate-early gene induction.

Also G protein-linked receptors are likely to be involved in mechanotransduction. Activation may be secondary to flow-mediated binding of a known ligand, or the receptor may itself be uniquely mechanosensitive. Strong evidence for G protein responses to shear stress implicates endothelial receptors as putative flow sensors (for review see Davies 1995).

2.4.1. Signal transduction pathways activated by mechanical stretch

In cardiac cells mechanical stimuli-induced signal transduction is characterized by activation of multiple second messenger systems (Vandenburgh 1992, Sadoshima & Izumo 1993a, for review see Sadoshima & Izumo 1997). In cultured neonatal cardiac myocytes mechanical stretch causes activation of phospholipases C, D, and A2; tyrosine kinases; p21ras; Raf-1; MAPKs and their activators; SAPK; 90-kDa S6; PKC; and probably other molecules as well (Sadoshima & Izumo 1993a, Sadoshima et al. 1993, Yamazaki et al. 1995, Komuro et al. 1995). Phosphatidylinositol turnover has been shown to be increased by mechanical stimuli in cardiac myocytes (von Harsdorf et al. 1988, 1989, Sadoshima & Izumo 1993a) and mechanical stretch also activates phospholipases within minutes (Sadoshima & Izumo 1993a) leading to the generation of various lipid-derived second messengers such as IP₃, DAG, arachidonic acids, and phosphatidic acid. PKC activity and concentration has been reported to increase during development of left ventricular hypertrophy induced by pressure overload in rat hearts (Gu & Bishop 1994). In addition, mechanical stretch could increase Ang II secretion leading to the activation of phospholipases (Sadoshima & Izumo 1993b). In vitro mechanical stress stimulates the activation of all components of the Raf-MEK-ERK signaling cascade in neonatal cardiac myocytes (Sadoshima & Izumo 1993a, Yamazaki et al. 1995). When cardiac myocytes of neonatal rats cultured on a deformable silicone dish were stretched, activity of SAPK was increased (Komuro et al. 1996). Activation of SAPK by stretch is relatively slow (15 min) compared with ERK activation (5 min) (Komuro et al. 1996, Sadoshima & Izumo 1997). Recently, hypotonic cell swelling of cardiac myocytes has also been shown to activate ERKs and SAPK (Sadoshima et al. 1996). Thus, the activation of these pathways may be a common signaling mechanism in response to different types of increased membrane tension in cardiac myocytes.

RPTKs have transmembrane segments, and some of the nonreceptor-type PTKs are anchored to the inner surface of cell membranes. Thus it is possible that membrane stretch directly causes conformational changes of tyrosine kinases, thereby activating them. Sadoshima & Izumo (1993a) have shown that mechanical stretch of cardiac myocytes causes a rapid increase in phosphotyrosine content of protein such as p42, p44, p60, p70, p85, p120, and p170 within 1 minute. A significant increase in tyrosine phosphorylation can be observed in 5 s (Sadoshima et al. 1996). Thus, the activation of PTKs appears to be one of the earliest cellular responses observed in response to mechanical stretch. Hypotonic swelling-induced immediate-early gene expression was abolished by PTK inhibitors but not by inhibitors of PKC, PLC, or Ang II antagonists.
(Sadoshima et al. 1996). In contrast, stretch-induced immediate-early gene induction was inhibited by inhibitors of PTK, PKC and PLC, as well as by Ang II antagonists (Sadoshima & Izumo 1993a, Sadoshima et al. 1993). Thus although hypotonic cell swelling and linear stretch activate separate signaling mechanisms, tyrosine kinase activation is required for both stimuli (Sadoshima & Izumo 1997). However, the mechanism of tyrosine kinase activation by mechanical stress is still far from clear.

2.4.1.1. Cellular signals controlling stretch-induced ANP secretion

The major determinant of ANP secretion is myocyte stretch (Ruskoaho 1992, de Bold et al. 1996). A rapid and complete inhibition of stretch-induced ANP secretion was observed after cellular ATP depletion (Page et al. 1991), thus emphasizing the energy-dependence of the secretory event. When considering the stretch-dependency of ANP secretory response the SA cation channels could be the most obvious candidates initiating the stretch-secretion coupling. In support of this, Laine and co-workers (1994) found that Gd³⁺, a known blocker of SA channels, dose-dependently inhibited stretch-induced ANP secretion in superfused atrium.

2.4.1.2. Protein kinases

Several lines of evidence support the concept that PKC activation may promote ANP secretion from the heart. In the atrial and ventricular myocytes PKC is present in both membrane and cytosolic fractions (Kuo et al. 1984, Yuan & Sen 1986). Stimulation of phosphatidylinositol turnover and formation of IP₃ was noted when right atria were dilated (von Harsdorff et al. 1988, 1989). Tumor promoting phorbol esters, which activate intracellular PKC, have been shown to stimulate basal ANP secretion in cultured myocytes (Matsubara et al. 1988, Iida and Page 1988, 1989, Shields & Glembotski 1989) and in perfused hearts (Ruskoaho et al. 1985, 1986b, for review see Ruskoaho 1992). Ruskoaho et al. (1990) have shown that activity of PKC appears to positively regulate stretch-induced ANP release. When 12-O-tetradecanoyl-phorbol-13-acetate (TPA), tumor promoting phorbol ester known to stimulate PKC, was added to the perfusion fluid, a dose-dependent augmentation of stretch-induced ANP release was observed. In addition, inactive phorbol ester 4α-PDD had no effect (Ruskoaho et al. 1990). It is expected that hormones and neurotransmitters that activate phosphoinositide hydrolysis in heart cells would influence stretch-mediated ANP secretion. In fact, ET-1, α-adrenergic agonists, Ang II and vasopressin have been shown to augment the atrial stretch-induced ANP release both in vivo and in vitro (Ruskoaho 1992). PKC inhibitor H-7 has been reported to decrease secretagogue-induced release of ANP in isolated atria (Ishida et al. 1988). In addition, H-7 decreased atrial stretch-induced ANP secretion (Page et al. 1990) but conflicting results exists (Ishida et al. 1988, Ruskoaho 1992). ANP secretion produced by passive left ventricular wall stretch was inhibited by PKC inhibitor staurosporine (Kinnunen et al. 1993). These results indicate that PKC may mediate a part of the stretch-secretion coupling of ANP.
The role of other second messengers in the regulation of stretch-dependent ANP secretion is still far from clear. In the isolated perfused rat heart infusion of forskolin, a compound that increases cAMP concentration and then intracellular Ca\(^{2+}\), dose-dependently inhibited stretch-induced ANP release (Ruskoaho et al. 1990). Stretch-dependent ANP secretion can also be inhibited by 8-chlorophenylthio-cAMP and caffeine in noncontracting rat atria (Page et al. 1990) and by isoprenaline pretreatment in contracting rat atria (Agnoletti et al. 1992), further suggesting that cAMP may be a negative modulator of ANP secretion.

2.4.1.3. Intracellular calcium

Several studies have addressed the importance of cytosolic Ca\(^{2+}\) as a possible intracellular messenger mediating stretch-induced ANP secretion. Atrial stretch and the rate of contractions, factors known to modulate ANP secretion (Ruskoaho 1992), are linked to changes in intracellular Ca\(^{2+}\). Processing of ANP to the released hormone seems to be dependent on Ca\(^{2+}\) (Ito et al. 1988). In addition, ANP granules inside the cells contain high concentration of Ca\(^{2+}\) (Somlyo et al. 1988, Thibault & Doubell 1992), further supporting the possibility of a connection between the intracellular Ca\(^{2+}\) level and ANP secretion. ANP secretion is also known to be dependent on several neurotransmitters and humoral agents, which induce phosphoinositide hydrolysis and the formation of IP\(_3\), eventually leading to Ca\(^{2+}\) mobilization from intracellular stores. ANP secretion has been shown to be dependent on several protein kinase activities and many of these kinases are Ca\(^{2+}\)-dependent (Ruskoaho 1992).

Experiments have revealed both positive and negative modulation of stretch-induced ANP secretion by cellular Ca\(^{2+}\) (for review see Ruskoaho 1992). In Ca\(^{2+}\)-depleted hearts in the absence of spontaneous contractility total ANP release by stretch was partially suppressed (Ito et al. 1988). Left atrial pressure-induced increase in ANP release in the isolated perfused heart preparation was significantly attenuated by low Ca\(^{2+}\), nifedipine, CaM antagonist W-7 and ryanodine (Katoh et al. 1990). Stretch-induced ANP secretion was also inhibited by CaM-binding drug in isolated rat atria (Page et al. 1990). Pretreatment by ryanodine, which inhibits the release of Ca\(^{2+}\) from the SR, inhibited stretch-induced ANP secretion in isolated rat atria (Kuroski-de Bold & de Bold, 1991). In addition, Laine and co-workers (1994) reported that ryanodine inhibited stretch-induced ANP secretion both in contracting and noncontracting superfused atrium. These results show that internal Ca\(^{2+}\) stores are involved in the stretch-secretion coupling of ANP release.

In contrast, Ruskoaho and co-workers (1990) have shown that in the perfused rat heart preparation Bay K8644, a compound that increases the concentration of intracellular Ca\(^{2+}\), dose-dependently inhibited stretch-stimulated ANP release. In addition, a dose-dependent decrease in the rate of ANP secretion in isolated rat atria was noted at higher extracellular Ca\(^{2+}\) levels (Page et al. 1991). Ca\(^{2+}\) also inhibited ANP secretion from osmotically stretched neonatal atrial myocytes (Greenwald et al. 1988). Stretch-induced ANP release in isolated rat atrial preparation was independent of extracellular Ca\(^{2+}\) (Kuroski-de Bold & de Bold, 1991, de Bold et al. 1996) or ryanodine-sensitive Ca\(^{2+}\)-
release (Page et al. 1990). Similarly, ANP release from isolated rat atria induced by stretching was not inhibited by depolarization with KCl or a low concentration of external Ca²⁺ (Agnoletti et al. 1992). L-type Ca²⁺ channel blockers nifedipine (Deng & Lang 1992) and diltiazem (Laine et al. 1994) did not decrease stretch-induced ANP secretion, suggesting that the mechanotransduction may be mediated by other than voltage-gated Ca²⁺ channel. Thus, experiments by using various Ca²⁺ modulating agents suggest both negative and positive modulation of stretch-stimulated ANP release by intracellular Ca²⁺ concentration. These conflicting results concerning the effect of Ca²⁺ on stretch-induced ANP secretion may be explained by methodological differences including the species used, the age of the myocytes and the preparation technique of isolated atrial preparation. The rate of contractions, which is affected by changes in Ca²⁺ homeostasis, may also modulate the ANP secretory responses. This may explain the differences between the observations of studies where isolated spontaneously beating or paced organ preparations or arrested or slowly beating cell cultures are used. Furthermore, even under identical experimental conditions Ca²⁺ may have a dual effect on cellular ANP release by stretch; an initial transient stimulation followed by more marked inhibition was noted as intracellular Ca²⁺ was increased (Page et al. 1991).

2.4.1.4. Endothelium-derived factors, endothelin-1 and nitric oxide

Most of the ET produced by endothelial cells is released into the basolateral site (Wagner et al. 1992) and the circulating levels of the ETs are very low (Simson 1993). Therefore, ET must be considered rather as a local autocrine or paracrine factor than as a circulating hormone. Recently it has been suggested that endothelial cells to some extent store ET-1 (Macarthur et al. 1994, McClellan et al. 1994), and when endothelial cells in culture are stretched, ET-1 can be released rapidly (Macarthur et al. 1994). Thus, it has been speculated that ET-1 may be the mediator of stretch-induced ANP secretion. Mäntymaa and co-workers (1990) used a modification of the perfused heart preparation which permitted distension of right atrium. They found that ET-1 dose-dependently increased stretch-induced ANP secretion. In addition, it has been reported that in neonatal atrial cell cultures the response to ET administration in the presence of cyclic stretch was significantly greater than either cyclic stretch or ET alone (Gardner et al. 1991). Leskinen and co-workers (1997b) determined the effects of selective ET receptor antagonists on both baseline and atrial stretch-induced ANP and NT-ANP release in conscious rats. Volume load-induced ANP and NT-ANP release was reduced by the ETₐ receptor antagonist BQ-123 and the mixed ETₐ/ETₐ receptor antagonist bosentan. These results agree with the observation that passive immunization with ET-1 antiserum decreases volume load-induced ANP secretion in anesthetized rats (Fyhrquist et al. 1993). The effect of ET on ANP secretion seems to be mediated by ETₐ receptors since ETₐ receptor antagonists inhibit ET-stimulated secretion of ANP in cultured atrial myocytes (Irons et al. 1993, Leite et al. 1994, Thibault et al. 1994).

In addition to ET, vascular endothelium as well as endocardial cells have been shown to produce endothelium-derived relaxing factor. Several actions of NO on myocardial contractile performance have been reported, including mediation of cytokine (Finkel et al.
1992) and cholinergic responses (Balligand et al. 1993), modulation of β-adrenergic inotropic responses (Balligand et al. 1993) and changes in myocardial relaxation, force-frequency relationship and Frank-Starling response (Smith et al. 1991, Brady et al. 1993, Prendengast 1997). The role of NO in the regulation of stretch-induced cardiac hormone secretion is not clear. A potent inhibitor of NOS, L-NAME (Rees et al. 1990), has been recently reported to increase volume expansion-induced ANP secretion in conscious rats (Leskinen et al. 1995), suggesting that NO may negatively influence ANP secretion in vivo. In perfused rat atria, the inhibition of NO activity has been shown to restore the normal response to stretch in the presence of acetylcholine (Skvorak & Dietz 1997).
3. Aims of the research

The aim of the present study was to examine the cellular signaling mechanisms of atrial wall stretch-induced ANP release. Moreover, the role of peptide hormones and autocrine and/or paracrine factors on basal and stretch-induced ANP secretion were studied. Isolated perfused rat heart models were used.

The specific aims of the present study were:

1. to study the effects of genistein, a potent tyrosine kinase inhibitor, on cardiac function and ANP secretion.
2. to study the effects of relaxin, a reproductive hormone of the insulin-like growth factor family, on ANP secretion.
3. to study the role of intracellular Ca\(^{2+}\) pools in mechanotransduction and ANP secretion.
4. to study the cellular signaling mechanisms of atrial stretch-induced ANP and BNP secretion.
5. to study the role of the endothelium-derived factors, endothelin-1 and nitric oxide, on atrial wall stretch-induced ANP secretion.
4. Materials and methods

4.1. Materials

The chemicals used in this study were (Table 3.): genistein and lavendustin A methyl ester (Research Biochemicals Inc., Natick, MA, USA), diltiazem hydrochloride (Orion, Espoo, Finland), KN-62 (1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) and H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide) (Seikagaku Incorporation, Tokyo, Japan); staurosporine, ML-9 (1-[5-iodonaphthalene-1-sulfonyl]-H-hexahydro-1,4-diazepine), okadaic acid, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), thapsigargin and L-arginine (Sigma Chemical Co., St. Louis, MO, USA); L-NAME hydrochloride (Fluka Chemie AG, Buchs, Switzerland). Human recombinant gene-2 relaxin was a generous gift from Genentech Inc (San Francisco, CA, USA). Bosentan (Ro 47-0203; 4-tert-butyl-N-(6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyridimin-4-yl)-benzene-sulfonamide) was generously supplied by Dr. Martine Clozel from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Synthetic peptides were purchased from Peninsula Laboratories (St. Helens, UK) and heparin from Leiras (Turku, Finland). All other chemicals were obtained from Sigma Chemical Co.

Human recombinant gene-2 relaxin, L-NAME, bosentan and L-arginine were dissolved in 0.9 % saline. Genistein, lavendustin A, staurosporine, H-89, okadaic acid and TPA were dissolved in dimethylsulfoxide (DMSO) and KN-62 in DMSO and HCl and ML-9 in ethanol. Final concentration of each solvent was less than 0.03 %. Addition of an appropriate concentration of each solvent caused no significant change in hemodynamic variables or immunoreactive ANP (IR-ANP) concentration in the perfusate.
Table 3. Drugs used in the present studies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effects</th>
<th>Referens</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>Endothelin receptor antagonist</td>
<td>Clozel et al. 1994</td>
<td>V</td>
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<tr>
<td>Diltiazem</td>
<td>L-type calcium channel antagonist</td>
<td>Chaffman &amp; Brogden 1985</td>
<td>I</td>
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<tr>
<td>Genistein</td>
<td>Tyrosine kinase inhibitor</td>
<td>Akiyama et al. 1987</td>
<td>I</td>
</tr>
<tr>
<td>H-89</td>
<td>cAMP-dependent protein kinase inhibitor</td>
<td>Hidaka &amp; Kobayashi 1992</td>
<td>II, IV</td>
</tr>
<tr>
<td>KN-62</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II inhibitor</td>
<td>Hidaka &amp; Kobayashi 1992</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nitric oxide synthase inhibitor</td>
<td>Rees et al. 1990</td>
<td>V</td>
</tr>
<tr>
<td>ML-9</td>
<td>Myosin light chain kinase inhibitor</td>
<td>Hidaka &amp; Kobayashi 1992</td>
<td>IV</td>
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<tr>
<td>Okadaic acid</td>
<td>Protein phosphatase 1 and A2 inhibitor</td>
<td>Cohen et al. 1990</td>
<td>IV</td>
</tr>
<tr>
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<td>Endothelin ET(_B)-receptor agonist</td>
<td>Rubanyi &amp; Polokoff 1994</td>
<td>V</td>
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<tr>
<td>Staurosporine</td>
<td>Protein kinase C inhibitor</td>
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<td>Thapsigargin</td>
<td>Sarcoplasmic reticulum Ca(^{2+})-ATPase inhibitor</td>
<td>Thastrup 1990</td>
<td>III</td>
</tr>
</tbody>
</table>

4.2. Animals

Male 2-month-old Sprague-Dawley rats (250-350g) from the colony of the Center of Experimental Animals at the University of Oulu, Finland, were used. The rats were maintained on rat chow ad libitum in a room with controlled 40 % humidity, temperature of 22 °C, and a 12 h light and 12 h dark cycle. The experimental designs were approved by the Animal Experimentation Committee of the University of Oulu.

4.3. Isolated perfused heart preparation

Normal Langendorff and modified atrial stretch preparation were used in this study (Ruskoaho et al. 1986, 1990, Mantymaa et al. 1993). The blood of the rats was anticoagulated with heparin (500 units/kg body weight, i.p.) and they were decapitated 20 min later. The abdominal cavity was quickly opened, the diaphragm was transected, lateral incisions were made along both sides of the rib cage, and the heart was immediately cooled with perfusion fluid (4-10 °C). The aorta was cannulated superior to the aortic valve and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH was 7.40, equilibrated with 95 % O\(_2\)/5 % CO\(_2\) at 37 °C. The composition of the buffer was as follows (mmol/L): NaCl 113.8, NaHCO\(_3\) 22.0, KCl 4.7, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\)\(_4\)7H\(_2\)O 1.1, CaCl\(_2\)\(_2\)H\(_2\)O 2.5 and glucose 11.0.

Variations in perfusion pressure arising from changes in coronary vascular resistance were recorded on a Grass polygraph (model 7 D, Grass Instruments Co., Quincy, Massachusetts) with a pressure transducer (model MP-15, Micron Instruments, Los Angeles) situated on a side arm of the aortic cannula. The isometric force of contraction
was recorded by a strain gauge transducer (model FT03, Grass Instruments) connected to the Grass polygraph. The hearts were submitted to a resting tension of 2 g. Heart rate was counted from the contractions by the Grass tachograph and if needed, increased 15-20 % above the spontaneous beating rate by using a Grass stimulator (model S88, 10 V, 0.5 msec).

In atrial stretch experiments (Fig. 2) the right atrial pressure was recorded on a Grass polygraph via a cannula (PE-60) in the inferior vena cava connected to a pressure transducer (model MP-15). A glass cannula was inserted into the pulmonary artery for the collection of perfusate. The right atrial pressure was kept constant at the desired level by adjusting the level of the pulmonary artery cannula tip. During the equilibration period (40 - 60 min) the hearts were perfused with a peristaltic pump (Miniplus 3, model 312, Gilson, Villiers, France) at a flow rate of 7 mL/min and then switched to constant flow of 5 mL/min.

**Fig. 2. Schematic representation of perfused rat heart model.**

### 4.4. Experimental protocols

In the first series of experiments after a 60 min equilibration time a 10 min control period was followed by the addition of vehicle or drugs into the aortic perfusion cannula as a continuous infusion via an infusion pump (Secan PSA 55, Skyelectronics S.A., Grenoble, France) at a rate of 0.5 mL/min for 30 min (Fig. 3A). In the second series of experiments
after a 60 min equilibration time drugs were infused into the perfusate during atrial wall stretch. After a 10 min control period, a continuous infusion of vehicle or drugs was made for 41 min and right atrial stretch was superimposed for 10 min after 25 min vehicle or drug infusion by elevating the level of the pulmonary artery cannula tip (Fig. 3B). In the third series of experiments a 40 min equilibration time was followed by a 10 min control period. After that right atria were stretched for 2 h by elevating the level of the pulmonary artery cannula. The coronary venous effluents were collected at 1 or 2 min intervals, placed immediately on dry ice and stored at -20 °C until assayed. Each heart was only used for one experiment.

**EXPERIMENTAL DESIGN**

![Experimental Design Diagram]

Fig. 3. Experimental protocols.

### 4.5. Radioimmunoassay of ANP and BNP

ANP and BNP concentrations were determined by radioimmunoassay (RIA) from the unextracted (ANP) or extracted (BNP) perfusate samples, as previously described (Vuolteenaho et al. 1985, Kinnunen et al. 1993). Perfusate BNP was extracted by Sep-Pak C_{18} cartridges, and eluates were redissolved in RIA buffer. The BNP perfusate extracts and unextracted perfusate samples were incubated in duplicates of 100 µL with the rabbit ANP antiserum (final dilution, 1:100,000) or the rabbit BNP antiserum (final dilution, 1:50,000). Synthetic rat ANP_{99-126} and rat BNP_{51-95}, ranging from 0 to 160 fmol/tube, were incubated as standard. The ANP tracer was rat ¹²⁵I-ANP_{99-126} and the
BNP tracer was prepared by chloramine-T-iodination of synthetic rat Tyr\(^0\)-BNP\(_{51-95}\), followed by Sephadex G-25 gel filtration and reverse phase high performance liquid chromatography (HPLC) purification. After incubation for 48 hours at 8 °C, the immunocomplexes were precipitated with goat antiserum against rabbit gammaglobulin in the presence of 8 % polyethylene glycol, followed by centrifugation at 3000 g for 30 min. The sensitivity of the assay was 1.0 and 0.5 fmol/tube. The 50 % displacement of the standard curves was at 6 and 5 fmol/tube. The intra-assay and inter-assay variations in both assays were less than 10 % and 15 %, respectively. Serial dilutions of the perfusate showed parallelism to the synthetic ANP and BNP standards. The molecular forms of ANP-like and BNP-like immunoreactive material secreted by the perfused rat hearts were determined by HPLC analyses as described earlier (Kinnunen et al. 1993). The ANP and BNP immunoreactivities in the perfusate were almost completely due to processed, active ANP\(_{99-126}\) and BNP\(_{77-106}\) material.

### 4.6. Statistical analysis

The results are expressed as mean ± SEM. The data were analyzed with one- or two-way analysis of variance (ANOVA). The statistical significance of the difference between two groups was determined with Student's \(t\)-test. Differences at the 95 % level were considered statistically significant.
5. Results

Perfused isolated rat heart preparations were used to study the mechanisms of cardiac hormone secretion. Normal Langendorff (I-V) and modified atrial stretch preparation (III-V) were used. The perfused rat heart preparation was stable for the period of time which was used in these studies (90 to 160 minutes). The basal contractile force was 2.0±0.1 g, perfusion pressure 26±1 mm Hg and IR-ANP concentration was 146±9 fmol/L before vehicle or drug infusions (n=439). The basal heart rate in non-paced hearts was 221±8 (n=92) and 318±4 beats/min in paced hearts (n=376). When vehicle was infused, heart rate, contractile force and perfusion pressure remained constant. A small (30 to 40 %) decrease in the perfusate IR-ANP concentration towards the end of the experiment was noted during vehicle infusion, as previously described (Ruskoaho et al. 1990).

Right atrial pressure was varied by manipulation of atrial afterload by means of adjustment of the cannula leading into the pulmonary artery. The basal atrial pressure was 1.5±0.1 mm Hg (n=217). When the level of pulmonary artery cannula tip was elevated, right atrial pressure increased immediately. To compare the ANP secretion during atrial wall stretch in the presence of drug or vehicle infusion, the ratio of IR-ANP secreted during the atrial stretch to the rate of IR-ANP secreted to the perfusate before atrial distension was calculated in each distension experiment. This change in IR-ANP secretion to the perfusion fluid was then related to the increase in right atrial pressure. The changes in right atrial pressure and cardiac function were similar in vehicle- and drug-treated hearts during the distension experiments unless otherwise mentioned. A slight increase in contractile force and perfusion pressure was noted during atrial stretch. However, these increases in contractile force (0.1-0.4 g) and perfusion pressure (3-5 mm Hg) were similar in all experimental groups during the distension. Thus, it was possible to separate direct action of drugs on IR-ANP release at the level of the myocyte itself from potential indirect secretory effects caused by chemically induced changes in the contraction frequency and force.
5.1. Effects of genistein on cardiac function and ANP secretion (I)

5.1.1. Spontaneously beating hearts

The identification of growth factors in the developing and adult heart suggests a functional role for peptide growth factors in cardiac muscle (for review see Schneider & Parker 1990). The synthesis of inhibitors of PTKs has allowed a new pharmacological approach to study the role of signal transduction mechanisms in the actions of peptide growth factors. Effects of genistein, a PTK inhibitor on cardiac function and ANP secretion in isolated spontaneously beating and paced rat heart preparation were examined. In the first series of experiments vehicle or genistein were added to the aortic perfusion cannula in spontaneously beating hearts. Addition of 22 and 37 µmol/L genistein for 30 min caused a 1.7-fold and 2.2-fold increase in the contractile force while the concentration of 11 µmol/L had no effect. Genistein also produced a gradual, dose-dependent vasoconstriction (1.5- to 2-fold increase). There were few changes in heart rate during genistein infusion, yet genistein at a concentration of 22 µmol/L slightly but significantly decreased the heart rate by 15%. Addition of genistein to the perfusate produced a sustained, dose-dependent increase in IR-ANP secretion (max 2.3-fold increase).

5.1.2. Paced hearts

In the second set of experiments heart rate was held constant by pacing throughout the experiments. Genistein at the concentration of 37 µmol/L was added to the perfusion fluid. The positive inotropic and coronary vasoconstrictor effects of genistein were slightly greater (14 % and 37 %; p<0.001) in the paced than in the spontaneously beating rat hearts. Infusion of lavendustin A methyl ester (26 µmol/L), another structurally different PTK inhibitor (Onoda et al. 1989, 1990), to the perfusate produced a 1.2-fold increase in contractile force. However, this positive inotropic effect of lavendustin A was approximately only 1/7 of that of genistein.

In paced hearts the addition of genistein (37 µmol/L) to the perfusion fluid caused a rapid but transient increase in IR-ANP secretion (maximally from 126±9 to 175±27 fmol/L, p<0.001). During continuous genistein infusion, the maximum increase in perfusate IR-ANP concentration was seen after 12 min of infusion, thereafter the perfusate IR-ANP concentration returned to its control value.

5.1.3. Cellular mechanisms of genistein-induced cardiac effects

In the course of analyzing signaling pathways leading to synthesis and secretion of ANP, genistein unexpectedly had a positive inotropic effect at low concentrations (below 40 µmol/L). Because intracellular Ca²⁺ plays an important role in excitation-contraction coupling in myocardial and vascular smooth muscle cells as well as in ANP secretion, the
influences of diltiazem, a Ca\textsuperscript{2+} channel antagonist (Chaffman & Brogden 1985), and KN-62, a potent inhibitor of CaM kinase II (Hidaka & Kobayashi 1992), with genistein were tested.

Infusion of diltiazem alone at a concentration of 3 µmol/L decreased the contractile force by 17 % (p<0.01) but had no effect on the perfusion pressure, while KN-62 alone (1.5 µmol/L) had no effects on cardiac function. Addition of diltiazem, KN-62 or their combination to the perfusion fluid attenuated the genistein-induced positive inotropic effect by 52 %, 34 % or 59 % (p<0.001). Diltiazem also reduced the genistein-induced increase in perfusion pressure (20 % decrease), while KN-62 had no effect.

Addition of diltiazem to the perfusate led to a rapid decrease in basal IR-ANP secretion, while KN-62 had no effect. Diltiazem completely blocked the genistein-induced IR-ANP secretion. KN-62 significantly decreased 83 % and delayed the genistein-induced increase in IR-ANP secretion (p<0.02). The combination of diltiazem and KN-62 also completely blocked genistein-induced increase in IR-ANP secretion from isolated perfused paced rat hearts.

5.2. Effects of relaxin on ANP secretion and cardiac function (II)

5.2.1. Cardiac function and ANP secretion

Relaxin is a reproductive hormone (Bryant-Greenwood & Schwabe 1994) that has been shown to be a potent chronotropic and inotropic agent both \textit{in vitro} and \textit{in vivo} (Kakouris \textit{et al.} 1993). Administration of relaxin to the perfusate at a concentration of 1.5, 3 and 10 nmol/L for 30 min increased heart rate by 8, 16 and 20 % (p<0.001) (Fig. 4). The heart rate began to increase 1 min after the initiation of infusion and the maximum increase was obtained after 5-10 min of infusion (Fig. 4). Relaxin had no significant effect on contractile force or perfusion pressure. Addition of relaxin to the perfusate induced a dose-dependent increase in ANP secretion (Fig. 4). At a concentration of 10 nmol/L relaxin produced a gradual, 1.5-fold increase in IR-ANP secretion (from 148±25 to 228±40 fmol/L, p<0.001) (Fig. 4).
Fig. 4. Effects of relaxin (Rlx) on heart rate and IR-ANP secretion in spontaneously beating isolated perfused rat heart. After a 10-min control period, as shown by the arrows, vehicle or relaxin were added to the perfusion fluid. Values are expressed as mean ± SEM.

5.2.2. Cellular mechanisms of relaxin-induced cardiac effects

To study the signal transduction pathways mediating relaxin-induced increase in heart rate and ANP secretion, relaxin (10 nmol/L) in combination with PKC inhibitor staurosporine (30 nmol/L), PKA inhibitor H-89 (100 nmol/L) or CaM kinase II inhibitor KN-62 (3 µmol/L) were infused to the perfusate. Infusion of staurosporine or H-89 alone had no effect on cardiac function. Administration of KN-62 to the perfusion fluid decreased heart rate by 28 % and slightly but significantly increased perfusion pressure from 23±1 to 36±5 mm Hg (p<0.05). Administration of staurosporine with relaxin to the perfusion fluid decreased the relaxin-induced positive chronotrophic effect by 70 %. When H-89 was given together with relaxin, heart rate initially increased comparable to that observed with relaxin infusion alone but after 10 min of combined H-89 and relaxin infusion, the heart rate returned close to its control value. When given together with relaxin, KN-62 significantly attenuated the relaxin-induced positive chronotrophic effect by 35 %. None of the protein kinase inhibitors influenced basal IR-ANP secretion. Staurosporine and H-89 almost completely blocked relaxin-induced increase in IR-ANP secretion (p<0.001), while KN-62 had no effect.
5.3. Effect of thapsigargin on ANP secretion (III)

5.3.1. Basal ANP secretion

The effects of thapsigargin, a specific inhibitor of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (Thastrup 1990) which leads to the depletion of intracellular Ca\(^{2+}\) stores, on basal and atrial stretch-induced ANP secretion were studied. First thapsigargin was infused alone to the perfusate in order to find out the concentration of the drug which would minimally influence myocardial contractility and coronary vasculature. Addition of 300 nmol/L thapsigargin to the perfusate caused a 1.5-fold increase in perfusion pressure and 1.2-fold increase in contractile force (p<0.001). Furthermore, concentrations lower than 100 nmol/L had no effect on cardiac function. At high concentrations (1-10 µmol/L) thapsigargin induced a transient increase in contractile force followed later by myocardial depression, as reported previously (Vigne et al. 1992). Addition of thapsigargin at the concentrations of 300 nmol/L to the perfusate induced 1.3-fold increase in IR-ANP release (p<0.001). In contrast, no difference was observed in the baseline secretion when isolated rat hearts were perfused with 30 or 100 nmol/L thapsigargin. The concentrations of 30 and 100 nmol/L were thus chosen for further experiments.

5.3.2. Stretch-induced ANP secretion

To examine the role of Ca\(^{2+}\) pools in stretch-activated ANP release intracellular Ca\(^{2+}\) stores were depleted by starting the infusion of thapsigargin 25 min prior to the elevation of the right atrial pressure. A significant decrease (32 %, p<0.05) in perfusate IR-ANP levels was seen during infusion of 30 nmol/L of thapsigargin and addition of 100 nmol/L completely blocked stretch-activated ANP secretion (p<0.001)(Fig. 5). Furthermore, during thapsigargin infusion, the relationship between changes in IR-ANP and in right atrial pressure shifted dose-dependently to the right (Fig. 5).
5.4. Effects of protein kinase inhibitors on stretch-induced ANP and BNP secretion (IV)

5.4.1. Effect of lavendustin A

The role of PTKs in ANP secretion was studied by using the potent PTK inhibitor lavendustin A (Onoda et al. 1989, 1990). Firstly lavendustin A was infused to the perfusion fluid with TPA, a tumor-promoting phorbol ester known to activate PKC and increase ANP secretion. Lavendustin A (1.3 µmol/L) failed to decrease TPA (46 nmol/L) induced ANP secretion indicating, that lavendustin A at the concentrations used had no effect on PKC.

Atrial wall stretch during the vehicle infusion induced a 2.9-fold increase in the perfusate IR-ANP concentration (Fig. 6). Lavendustin A at the concentrations of 0.5 and 1.3 µmol/L decreased atrial wall stretch induced ANP secretion by 53 % and 68 % (p<0.001)(Fig. 6). During lavendustin A infusion, the relationship between changes in the perfusate IR-ANP concentration and right atrial pressure shifted to the right (Fig. 6).
Next, the effect of lavendustin A on the atrial wall stretch-stimulated BNP secretion was studied. Right atrial wall stretch induced a 2.2-fold increase in IR-BNP secretion from the perfused rat heart, and this secretion was significantly decreased by lavendustin A at the concentration of 1.3 µmol/L (50 % decrease, p<0.05).

5.4.2. Effect of okadaic acid

Reversible protein phosphorylation is a critical component of the signal transduction mechanisms by which extracellular signals regulate cellular processes. Okadaic acid, a specific and potent PP1 and PPA2 inhibitor (Cohen et al. 1990, Hunter 1995), had no effect on basal or maximal ANP secretion induced by atrial stretch at the concentration of 100 nmol/L (Fig. 7). On the other hand, the ANP secretory response to right atrial wall stretch appears significantly earlier. The relationship between changes in the perfusate IR-ANP concentration and right atrial pressure shifted to the left during the first 3 min of wall stretch (Fig. 7).
5.4.3. Effects of other protein kinase inhibitors

In order to further characterize the selectivity of the action of lavendustin A on wall stretch-induced ANP secretion, PKC inhibitor staurosporine (100 nmol/L), CaM kinase II inhibitor KN-62 (1.5 μmol/L), PKA inhibitor H-89 (100 nmol/L) and myosin light chain kinase inhibitor ML-9 (1 μmol/L) were infused to the perfusate. None of these inhibitors were capable of decreasing statistically significantly the right atrial wall stretch-induced increase in IR-ANP secretion. The calculated increases in ANP secretion corresponding to the 3 mm Hg increase in right atrial pressure were 2.27-, 2.82-, 2.50-, 2.38, and 2.77-fold for staurosporine, KN-62, H-89, ML-9 and control groups, respectively.

5.4.4. Sustained increase in atrial wall stretch

In a separate series of experiments, the effects of inhibitors of PTK and PKC on ANP secretion stimulated by a sustained increase in atrial wall stretch were studied. The right atria were stretched for 2 hours during infusion of lavendustin A, staurosporine or their combination. Cardiac contractile force, perfusion pressure and rate of contraction did not differ between the vehicle- and drug-infused groups. During continuous atrial wall stretch, the maximum increase of ANP secretion was seen after 10 min distension. Thereafter the peptide levels gradually decreased towards the end of the experimental period but remained elevated compared with those in the unstretched hearts (at 2 hrs, p<0.05). Administration of staurosporine at the concentration of 30 nM had no effect on
the wall stretch-induced ANP secretion, but lavendustin A (1 µM) either alone or in combination with staurosporine (30 nM) inhibited significantly sustained stretch-induced increase in perfusate ANP levels. Thus, under these experimental conditions lavendustin A consistently inhibited ANP secretion in response to both acute and sustained increase in right atrial wall stretch.

5.5. Interaction of nitric oxide and endothelin in ANP secretion (V)

5.5.1. Basal ANP secretion

5.5.1.1. Effects of L-NAME and L-arginine

In addition to synthetizing ET-1, vascular endothelium as well as endocardial cells has been shown to produce NO (Moncada et al. 1991, Schulz et al. 1991). Endothelial-derived NO has a physiological role in the regulation of heart function. Role of NO in ANP secretion was studied by infusing potent NOS inhibitor L-NAME or L-arginine, a substrate for NOS to the perfusate. The addition of L-NAME (300 µmol/L) or L-arginine (100 mmol/L) alone to the perfusate for 30 min had no effects on perfusate ANP concentration or cardiac function, except that L-arginine infusion slightly increased contractile force by 6 % (p<0.001), probably due to the high osmolality of the L-arginine solution used for infusion.

5.5.1.2. Interaction of L-NAME and endothelin-1

Next, the influence of NO in ET-1-induced ANP and BNP secretion was studied. ET-1 alone at the concentration of 2.0 nmol/L produced a sustained increase in IR-ANP secretion (46 % increase, p<0.001), whereas concentration of 0.5 nmol/L had no statistically significant effect. However, when ET-1 at the dose of 0.5 nmol/L was infused in combination with L-NAME (100 and 300 µmol/L), a dose-dependent increase in the perfusate IR-ANP concentration was noted (20 % and 85 % increase, p<0.001)(Fig. 8). L-NAME 300 µmol/L combined with ET-1 induced a 108 % increase in IR-BNP concentration when compared to the infusion of ET-1 alone (p<0.001)(Fig. 8).
Administration of ET-1 alone to the perfusion fluid for 30 min induced a gradual, dose-dependent vasoconstriction and slightly increased the contractile force, as reported earlier (Mäntymaa et al. 1990). When infused in combination with ET-1 (0.5 nmol/L), L-NAME augmented ET-1-induced vasoconstriction (100 µmol/L, 20 % increase, NS; 300 µmol/L, 112 % increase, p<0.001), but had no effect on contractile force. These effects of L-NAME on ANP secretion and perfusion pressure were reduced by the administration of L-arginine (100 mmol/L) to the perfusate.

### 5.5.2. Atrial stretch-induced ANP secretion

#### 5.5.2.1. Interaction of L-NAME and endothelin-1

Continuous atrial stretch for 10 min resulted in a 1.7-fold increase in IR-ANP concentration in the coronary venous effluent of the perfused rat heart (p<0.001). L-NAME (300 µmol/L), L-arginine (100 mmol/L) or ET-1 (0.2 or 0.5 nmol/L) alone had no influence on stretch-induced ANP release. When L-NAME was infused in the presence of ET-1, a significantly greater amount of ANP was released from the heart during the first 5 min of atrial stretch (L-NAME plus ET-1 0.2 nmol/L, 25 % increase, NS; L-NAME plus ET-1 0.5 nmol/L, 37 % increase, p<0.01)(Fig. 9). Furthermore, during the combined infusion of L-NAME and ET-1, the relationship between changes in the perfusate IR-ANP concentration and right atrial pressure shifted to the left (Fig. 9).
Fig. 9. A and B: Effect of L-NAME on atrial wall stretch-induced secretion of IR-ANP in the presence of ET-1 in the isolated perfused paced rat hearts. After a 10 min control period ET-1 (O) at the concentration of 0.2 (A) or 0.5 nmol/L (B) or ET-1 with L-NAME 300 µmol/L (●) was added to the perfusate. The right atrium was distended for 10 min (horizontal line) by elevating the pulmonary artery cannula tip. C and D: Effect of L-NAME on the relation between the change of right atrial pressure (RAP) and changes in IR-ANP secretion. ANP stretch indicates ANP secretion during the first 5 minutes of stretching, ANP control before stretching. Values are expressed as mean±SEM. *p<0.05 (Student's t-test, unpaired).

ET-1 at the concentrations of 0.2 and 0.5 nmol/L increased perfusion pressure by 35 % and 37 % (p<0.001), while L-NAME and L-arginine had no effect. When L-NAME was combined with ET-1 (0.5 mmol/L), a transient change both in the contractile force (16 % increase, p<0.001) and perfusion pressure (119 % increase, p<0.001) was observed during atrial stretch experiments.
5.5.2.2. Effects of bosentan and sarafotoxin 6C

The mechanism for increased NO formation in response to atrial wall stretch may be either direct or mediated by autocrine/paracrine factor(s) such as ET-1 liberated by stretch which could stimulate NOS by activating endothelin ET$_B$-receptors (Moncada et al. 1991, Winegrad 1997). This latter hypothesis was tested by studying the effects of a potent endothelin ET$_{A/B}$-receptor antagonist bosentan (1 µmol/L) and endothelin ET$_B$-receptor agonist sarafotoxin 6C (100 nmol/L) on atrial wall stretch-stimulated increase in IR-ANP secretion. At this concentration bosentan decreases the right atrial stretch-induced increases in BNP mRNA levels (Magga et al. 1997) in perfused rat heart. The dose of sarafotoxin 6C was about 100-fold higher than that of sarafotoxin 6B, known to activate ET$_A$-receptors and ANP secretion under these experimental conditions (Pitkänen et al. 1991). Bosentan and sarafotoxin 6C had no effect on ANP secretion or cardiac function.
6. Discussion

6.1. Effects of genistein on cardiac function

Cardiac myocytes have been shown to be targets for the action of peptide growth factors. In cultured cardiac muscle cells, peptide growth factors induce features of myocardial cell hypertrophy and regulate a number of genes, including α-and β-myosin heavy chains, cardiac and skeletal α-actin and ANP (Parker et al. 1990a,b). The presence of several peptide growth factors in the developing and adult heart (for review see Schneider & Parker 1990) suggest a functional role of these peptides in cardiac muscle. Furthermore, altered production of growth factors accompanies myocardial hypertrophy and smooth muscle cell proliferation in coronary artery stenosis, supporting a potential role of peptide growth factors in cardiac or vascular growth associated with these pathological states (Schneider & Parker 1990). In addition, second messenger cascades activated by mechanical stress on cardiac myocytes are very similar to those evoked by growth factors or cytokines (Komuro & Yazaki 1993, Yamasaki et al. 1995, Sadoshima & Izumo 1997). The identification and synthesis of inhibitors of PTKs have allowed a new pharmacological approach for studying the role of signal transduction mechanisms in the actions of peptide growth factors.

In the present study the effects of genistein, a PTK inhibitor (Akiyama et al. 1987, Levitzki 1992) on cardiac function and ANP secretion were examined in perfused rat heart model. Present results show that genistein dose-dependently increased myocardial contractile force and coronary vascular tone and was a potent stimulator of ANP secretion. The finding that genistein, a compound widely used to examine the role of signal transduction mechanisms in actions of growth factors, increased cardiac contractile force was unexpected. Several growth factors have been found to stimulate phosphatidylinositol hydrolysis and increase intracellular Ca\(^{2+}\) levels (Mohammadi et al. 1992, Peters et al. 1992) whereas inhibitors of PTK inhibit phosphatidylinositol turnover, and therefore IP\(_3\) and Ca\(^{2+}\) release caused by growth factors (Gaudette et al. 1990, Hill et al. 1990).

It is possible that genistein-induced increase in contractile force, ANP secretion and vascular tone by affecting a process (or processes) that may not be related to its PTK inhibitory activity, such as changing cytosolic free Ca\(^{2+}\) concentration. Diltiazem, an L-type Ca\(^{2+}\) channel antagonist (Chaffman & Brodgen 1985), and KN-62, an inhibitor of
CaM kinase II (Hidaka & Kobayashi 1992), inhibited the inotropic and ANP secretory effects of genistein, showing that it either directly or indirectly modulates those cellular processes which regulate the intracellular concentration of Ca\(^{2+}\). Thus, voltage-operated Ca\(^{2+}\) channels may be involved in the actions of inhibitors of PTKs in cardiac myocytes, although a possible action through PTK inhibition by genistein on intracellular Ca\(^{2+}\) concentration cannot be excluded. However, the concentration range over which cardiac effects were seen is similar to that reported to competitively inhibit ATP binding to the catalytic domain of PTKs (Akiyama et al. 1987). Several reports have also shown that the ability of genistein to inhibit responses to growth factors correlates with a decrease in the level of tyrosine phosphorylation (Levitzki 1992). Further, genistein in the same concentration range as used in our study inhibits the cell proliferation, DNA synthesis and proto-oncogene gene expression induced by growth factors (Lyall et al. 1989, Levitski 1992). Genistein at much higher concentrations (185 µmol/L) transiently increases cytoplasmic free Ca\(^{2+}\) concentrations in isolated rat hepatocytes (Tomonaga et al. 1992). More recently, genistein has been demonstrated to increase the sensitivity of cardiac ion channels to ß-adrenergic receptor stimulation (Hool et al. 1998) and to inhibit the Ca\(^{2+}\) current in rat ventricular myocytes (Katsube et al. 1998) or even have a dual effect on Ca\(^{2+}\) currents in atrial myocytes (Wang & Lipsius 1998). Lavendustin A, another structurally different PTK inhibitor (Onoda et al. 1989, 1990) also acutely increased cardiac contractile force, but this effect was more than seven times smaller than that of genistein. Differences in genistein-induced ANP secretion were also noted in two different experimental models; when compared to spontaneously beating hearts, the genistein-induced ANP secretory response was smaller and more transient in paced hearts. This rate-dependence of genistein-induced ANP secretion also implies the involvement of intracellular Ca\(^{2+}\) in ANP secretion. Thus, the present results suggest that genistein introduces Ca\(^{2+}\) into the heart cells, mainly from extracellular pools, by mechanisms not yet identified and that this increased free intracellular Ca\(^{2+}\) leads to the stimulation of ANP secretion. Since genistein and lavendustin A have been shown to be selective inhibitors of PTKs without any activity against PKA, PKC, myosin light chain kinase or serine kinases (for reviews see Hidaka & Kobayashi 1992, Levitzki 1992), these results are consistent with the interpretation that an endogenous PTK (or PTKs) might participate either directly or indirectly in the regulation of myocardial function.

6.2. Effect of relaxin on ANP secretion

Relaxin is classically regarded as a pregnancy hormone involved in regulation of myometrial activity and tissue remodeling of the reproductive system (Bryant-Greenwood & Schwabe 1994), but recently, novel target sites have been found, including the atrium of the heart. High levels of relaxin-binding sites are detected in the atrium of newborn rats (Osheroff et al. 1992, Osheroff & Ho 1993). In addition, atrial myocytes have been shown to secrete detectable amounts of relaxin, suggesting that it may act via autocrine/paracrine routes to regulate cardiac function (Taylor & Clark 1994). Relaxin has been shown to be a potent chronotropic and inotropic agent in isolated rat atrium (Kakouris et al. 1992, Ward et al. 1992) and it can increase heart rate in vivo and in vitro (Parry et al.
1990, Ward et al. 1992, Thomas & Vanden 1993). On the other hand, the effect of relaxin on contractile force appears to depend upon the model used. Unlike studies performed in isolated paced left atrium (Kakouris et al. 1992, Ward et al. 1992), relaxin failed to increase contractile force in spontaneously beating isolated heart, consistently with an earlier report (Thomas & Vanden 1993). These observations are in agreement with the findings that relaxin-binding sites have been found in the atrium but not in ventricles (Osheroff et al. 1992, Osheroff & Ho 1993).

The present results show that in the isolated heart preparation relaxin increased the heart rate and induced a slowly developing rise in perfusate ANP concentrations. Under these experimental conditions, relaxin is one of the most potent ANP secretagogues on a molar basis (Ruskoaho 1992). In fact, from the other peptide compounds tested in the perfused rat heart preparation only ET-1 (effective at the concentrations of 0.9-2.7 nmol/L) has comparable activity in stimulating ANP secretion (Mäntymaa et al. 1990). Since specific binding sites having an affinity similar to that found in the uterus have been demonstrated in the rat atrium (Osheroff et al. 1992), it is likely that the relaxin-induced increase in ANP exocytosis is a receptor-mediated response. Although increases in heart rate are known to stimulate ANP release in vitro (Ruskoaho 1992), the relaxin-induced ANP exocytosis is unlikely to be mediated by the chronotropic effect, since chronotropic and secretory responses showed an entirely different time-course pattern. The heart rate reached its maximum values within 3-4 minutes, whereas perfusate IR-ANP concentrations began to increase 10 minutes after starting the relaxin infusion.

The ANP secretory and chronotropic effects of relaxin appear to be mediated by a number of intracellular signal transduction pathways. An important mechanism for regulating cellular physiology involves activation of phosphoinositide hydrolysis with subsequent production of IP3 and DAG. The IP3 released into the cytoplasm mobilizes Ca2+ from internal stores (Berridge 1993), whereas DAG activates PKC (Nishizuka 1992). Previously, translocation of PKC from the cytosol to the cellular membrane by relaxin has been described in myometrial cells and also this effect was similar to that induced by phorbol esters (Kalbag et al. 1991). Relaxin has been shown to elevate intracellular cAMP concentrations in myometrial, endometrial glandular epithelial and anterior pituitary cells, although there is no consensus that cAMP is an obligatory second messenger for relaxin in these tissues (Bryant-Greenwood & Schwabe 1994). Relaxin enhances the L-type Ca2+ current in single cells isolated from sinoatrial node of rabbit heart (Han et al. 1994). This increase in response to relaxin is dependent on both cAMP accumulation and PKA activation. Previously relaxin has also been reported to induce Ca2+-efflux from myometrial cells (Rao & Sanborn 1986, Ginsburg et al. 1988).

Several neurohumoral agents or pharmacological drugs that increase cAMP content or activate PKC in cardiac cells have been shown to stimulate ANP exocytosis (Ruskoaho 1992). When adenyllyl cyclase was activated by forskolin, both basal and phorbol ester-stimulated ANP secretion were increased from isolated perfused rat heart (Ruskoaho et al. 1986b). In the present study PKC inhibitor staurosporine and PKA inhibitor H-89 (Hidaka & Kobayashi 1992) almost completely blocked the relaxin-induced ANP release from perfused rat heart, while CaM kinase II inhibitor KN-62 had no effect on ANP secretion. We also found that the relaxin-induced increase in heart rate was attenuated by staurosporine and H-89, suggesting that PKC and PKA may be involved both in the chronotropic and ANP secretory effects of relaxin. These results agree with those of Han and
co-workers (1994), who found that a peptide inhibitor of PKA prevented the effects of relaxin on cardiac pacemaker cells. Interestingly, the time course of the ANP secretory effect of relaxin resembled that caused by TPA, a phorbol ester known to activate PKC under similar experimental conditions (Ruskoaho et al. 1985, Ruskoaho et al. 1986b). Further, KN-62 attenuated the chronotropic effect of relaxin but failed to decrease the relaxin-induced ANP release. Thus, the increased Ca\(^{2+}\)-influx and resulting activation of Calmodulin kinase II appears to be involved in the positive chronotropic effects of relaxin, whereas the PKC and PKA systems may be of greater significance in the ANP secretory response.

6.3. Mechanical stretch-induced ANP secretion

A number of different cell types have transduction systems that convert externally applied mechanical forces to signals that regulate cellular function (Watson 1991). For example, during every myocardial contraction, each cardiac cell shortens against the load imposed by the adjoining cells and develops force. Mechanical loading of myocytes also increases protein synthesis and induces the expression of specific genes (Komuro & Yazaki 1993) as well as being a potent stimulus for the secretion of ANP (Lang et al. 1985, Ruskoaho 1992). The cellular mechanisms by which mechanical forces regulate myocardial function are, however, uncertain, although there is evidence that several factors may be involved, including SA ion channels and several protein kinases (Komuro & Yazaki 1993, Sadoshima & Izumo 1993a, 1997).

6.3.1. Role of thapsigargin-sensitive intracellular calcium pools

Changes in cytosolic free Ca\(^{2+}\) concentration constitute an important element of signal transduction in various cells (Clapham 1995). Several studies have addressed also the importance of intracellular Ca\(^{2+}\) concentration in stretch-induced ANP secretion. However, experiments by using various Ca\(^{2+}\) modulating agents such as nifedipine, caffeine and ryanodine have suggested both positive and negative modulation of stretch-stimulated ANP release by Ca\(^{2+}\) (Ruskoaho 1992).

SR is the major intracellular Ca\(^{2+}\) store in cardiac cells. It is sensitive to caffeine and ryanodine and has an important role in excitation-contraction coupling. Thapsigargin is a selective inhibitor of SERCA pumps and it is widely used as pharmacological tool for studying importance of these pumps (Thastrup 1990, Lytton & Nigam 1992). Recent studies have provided evidence for the presence of an IP\(_3\)- and thapsigargin-sensitive intracellular Ca\(^{2+}\) pool in atrial cells (Vigne et al. 1992, Negretti et al. 1993, Lewartowski et al. 1994). Vigne and co-workers (1992) showed that in atrial cells thapsigargin at the concentrations of 0.1-10 \(\mu\)mol/L increased intracellular Ca\(^{2+}\) concentration in a manner that was independent of the presence of external Ca\(^{2+}\) and of the production of inositol phosphates. In agreement with the observation that thapsigargin rapidly raises intracellular Ca\(^{2+}\) concentration in cardiac cells (Vigne et al. 1992) and vascular cells (Thastrup 1990), the present results show that addition of thapsigargin to the perfusate at the con-
centrations above 100 nmol/L increases contractile force and ANP secretion and produces coronary vasoconstriction in the perfused rat heart preparation, suggesting that mobilization of Ca^{2+} from IP_3-sensitive intracellular stores may be involved in this response.

In order to examine the role of intracellular Ca^{2+} pools in mechanotransduction of cardiac myocytes low doses of thapsigargin (30 and 100 nmol/L) were infused to the perfusate for 25 min to deplete the thapsigargin-sensitive Ca^{2+} stores. Present results show that thapsigargin completely blocked stretch-activated ANP exocytosis from right atrial myocytes. This finding suggests that the stretch-induced increase in ANP secretion is linked to its capacity to mobilize a thapsigargin-sensitive intracellular Ca^{2+} pool. Present data, together with those from previous studies, also support the conclusion that there is a principal difference between the mechanical stretch- and agonist-induced ANP secretion, because thapsigargin has no influence on ANP secretion stimulated by ET-1 in cardiac cell cultures (Doubell & Thibault 1994).

6.3.2. Role of protein kinases

Mechanical stretch of cardiac myocytes in vitro causes an activation of multiple second messenger systems including PTKs and PKC (Sadoshima & Izumo 1993a). Peptide growth factors act by binding to and activating specific receptors with intrinsic PTK activity (Ullrich & Schlessinger 1990, Van der Geer & Hunter 1994). RPTKs have transmembrane segments, and some of the nonreceptor-type PTKs, such as Src family tyrosine kinases, are anchored to the inner surface of cell membranes. Thus it is possible that membrane stretch directly causes conformational change of tyrosine kinases, thereby activating them. Mechanical stretch caused activation of Src within 5 min in fetal lung cells (Liu et al. 1996) and an increase in tyrosine phosphorylation of FAK in mesangial cells (Hamasaki et al. 1995). In cardiac myocytes, mechanical stretch causes a significant increase in phosphotyrosine content of proteins, such as p42 and p44, within one minute (Sadoshima & Izumo 1993a). In cultured neonatal myocytes phorbol esters, ET-1 (Bogoyevitch et al. 1993) and mechanical stretch (Sadoshima & Izumo 1993a, 1997) have been shown to stimulate tyrosine phosphorylation of MAPKs, a family of related Ser/Thr kinases which activities are dependent on phosphorylation of both tyrosine and threonine residues (Anderson et al. 1990).

Several natural and synthetic PTK inhibitors have been used to study the physiological and pathophysiological role of these kinases. These inhibitors at micromolar concentrations have been shown to inhibit cell proliferation, DNA synthesis, proto-oncogene gene expression and phosphatidylinositol turnover caused by several growth factors (Lyall et al. 1989, Margolis et al. 1989, Hill et al. 1990, Levitzki 1992). Lavendustin A, a competitive inhibitor of ATP binding to the catalytic domain of PTKs (Onoda et al. 1989, 1990), was used to study the potential role of PTKs in regulation of mechanical stretch-induced ANP and BNP secretion. Present results show that lavendustin A dose-dependently decreased right atrial wall stretch-induced cardiac hormone secretion, while PKC inhibitor staurosporine, PKA, CaM kinase II and myosin light chain kinase inhibitors failed to block stretch-induced ANP secretion. Some PTK inhibitors may also inhibit other protein kinases (Levitzki 1992, Hidaka & Kobayashi 1992), e.g. PKC, which has a
central role in the regulation of ANP secretion (Ruskoaho 1992). Previously, PKC inhibitors have been reported to either decrease (Page et al. 1991) or have no effect (Ishida et al. 1988) on stretch-induced ANP secretion in isolated rat atria preparations. In addition, PKC inhibitor staurosporine at a low concentration (10 nmol/L) was a potent inhibitor of ANP secretion produced by passive left ventricular wall stretch, suggesting that a PKC dependent pathway may play an important role in the regulation of ventricular stretch-stimulated ANP exocytosis (Kinnunen et al. 1993). However, it is unlikely that the effects of lavendustin A were non-specific because at the concentration used (maximally 1.3 µmol/L) lavendustin A had no effects on basal cardiac hormone secretion or cardiac function, and this inhibition occurred at the concentrations similar to or even below those shown to inhibit the activities of PTKs in vitro (Onoda et al. 1989, 1990). Furthermore, lavendustin A failed to decrease TPA-induced ANP secretion, indicating that lavendustin A has no influence on PKC-mediated responses in this experimental model.

The mechanisms of tyrosine kinase activation by mechanical wall stress as well as the following activation of downstream signaling pathways are yet unclear. There may be hierarchy in protein kinase activation induced by mechanical forces and some protein kinases may have a regulatory role and some may have an obligatory role. In agreement with our results, hypotonic swelling-induced *c-fos* gene expression was abolished by PTK inhibitors but not by inhibitors of PKC and PLC (Sadoshima et al. 1996, Sadoshima & Izumo 1997) while stretch-induced induction was inhibited by inhibitors of PTKs, PKC and PLC. Thus, although hypotonic cell swelling and linear stretch activate separate signaling mechanisms, PTK activation is required for *c-fos* induction by both stimuli. PKC and PTK activities may both also be involved in coupling cardiac overload to alterations in atrial BNP synthesis, since lavendustin A and staurosporine inhibited stretch-induced increase in atrial BNP concentrations in perfused rat hearts (Magga et al. 1997b). Thus, although mechanical stretch activates multiple signaling mechanisms in the heart, specific protein kinase pathways seem to be important for different cellular processes, and of those pathways, PTK activity appears to be required for wall stretch-induced ANP and BNP exocytosis. It remains to be determined, however, which tyrosine kinases are responsible for wall stretch-induced cardiac hormone secretion.

### 6.3.3. Role of protein phosphatases

The finding that okadaic acid accelerated ANP secretion suggests that PPs may play a regulatory role in mechanical stretch-induced cardiac hormone exocytosis from atrial myocytes, possibly by dephosphorylating signaling molecules activated by PTKs. Several components of ERK1/ERK2 pathways are subject to regulation by PPA2, which causes dephosphorylation of threonine and inhibition of kinase activity (Bokemeyer et al. 1996). Previously okadaic acid has been shown to inhibit PPs in the heart at the same concentration range as was used in our studies (Neumann et al. 1993). Our finding that okadaic acid, a potent inhibitor of PPA2 and a strong inhibitor of PP1 (Cohen et al. 1990), can accelerate wall stretch-induced ANP secretion suggests that wall stretch-induced ANP secretion may involve activation of PTK pathway modulated by MAPK/ERK pathways, although many other possibilities also exist. Nevertheless, because the only targets
Fig. 16. A hypothetical model of cellular mechanisms of ANP secretion. PKC, protein kinase C; PLC, phospholipase C; DAG, diacylglycerol; G, guanine nucleotide-binding protein.
of okadaic acid are the catalytic subunits of PPA2 and PP1 (Cohen et al. 1990, Hunter 1995), these enzymes appear to play a significant role in atrial wall stretch-induced ANP secretion. Furthermore, the findings that okadaic acid accelerated and lavendustin A significantly decreased ANP secretion show that a balance between PTK and PP activities plays a major role in mechanical stretch-induced ANP exocytosis. On the basis of these studies one may suggest that tyrosine kinase or kinase cascade is required for the induction of cardiac hormone secretion by mechanical stretch, while the PKC and phospholipid system seems to be more important in agonist-induced hormone release. A hypothetical model of cellular mechanisms by which some protein kinases and phosphatases as well as thapsigargin-sensitive Ca\(^{2+}\) pool may regulate mechanical wall stretch- and segretagogue-induced ANP exocytosis is shown in Fig. 10.

6.3.4. Role of the endothelial factors

Previous studies have shown that mechanical stretch causes release of factor(s) to the culture medium, which in turn induces c-fos expression and activates MAPKs (Sadoshima & Izumo 1993a). Endogenous paracrine/autocrine factors such as Ang II and ET-1 liberated in response to mechanical stretch rather than direct stretch appear to be responsible for the activation of cardiac gene expression in neonatal ventricular myocytes (Yamasaki et al. 1995, Sadoshima & Izumo 1997). Several studies have demonstrated that ET-1 and Ang II signal through the PTK-dependent mechanism (Force et al. 1991, Simonson & Herman 1993). However, because the release of ANP by mechanical stretch takes place in the presence of treatment with an Ang II antagonist, losartan (Leskinen et al. 1997b), it is unlikely that Ang II is involved in mediating the wall stretch-induced ANP secretion observed in the present studies.

Recent studies suggest that cardiac contractile performance may be influenced by NO released by endothelial cells, analogous to vascular endothelial regulation of vessel tone and blood flow. Several actions of NO on myocardial contractile performance have been reported, including mediation of cytokine (Finkel et al. 1992) and cholinergic responses (Balligand et al. 1993), modulation of β-adrenergic inotropic responses (Balligand et al. 1993) and changes in myocardial relaxation, force-frequency relationship and Frank-Starling response (Smith et al. 1991, Brady et al. 1993, Prendengast et al. 1997). Our results showed that potent NOS inhibitor L-NAME (Rees et al. 1990) alone had no effect on basal contractile force or perfusion pressure, but it increased coronary perfusion pressure in the presence of ET-1, known to release NO in several models (Rubanyi & Polokoff 1994). These results agree with previous studies suggesting that vasoconstrictor influence is required for basal NO release (Adeagbo et al. 1994) and that cardiac myocytes do not alter myocardial contractility and synthesize appreciable amounts of constitutive NO in unstimulated conditions (Amrani et al. 1992, Balligand et al. 1993, Weyrich et al. 1994). Although there is significant evidence that NO may act on myocardial cells to regulate contractility, its role in the regulation of cardiac hormone exocytosis is less clear.

In support of the hypothesis that NO may be involved in the regulation of ANP release, bovine aortic cells, when placed in co-culture with rat atrial myocytes, stimulate the release of ANP and this release could be inhibited by acetylcholine (Lew & Baertschi
Infusion of substances eliminating the action of NO (methylene blue, oxyhemoglobin or hydroquinone) also significantly increased the basal release of ANP from isolated rat atria (Sanchez-Ferrer et al. 1990). NO blocking agents have also been reported to overcome the inhibitory effect of acetylcholine on ANP secretion in vitro (Melo et al. 1996, Skvorak & Dietz 1997). We found that both L-NAME as well as L-arginine, a substrate for NOS, alone had no effects on basal ANP secretion in the perfused rat heart. This lack of effect may be due to the fact that under these experimental conditions coronary vessels are maximally dilated (Mäntymaa et al. 1990) and the basal production of NO is therefore minimal (Davies 1995). These results are consistent with those in the perfused atria, in which stimulation of NO production with acetylcholine or inhibition of NO had no significant effect on ANP secretion (Skvorak & Dietz 1997).

Leskinen and co-workers (1995) noted that in conscious rats the elevation of plasma ANP levels in response to acute volume expansion with 0.9 % saline was greater in the presence of L-NAME than in the control group, suggesting that NO may have a regulatory role in mechanical stretch-induced ANP secretion in vivo. In the perfused rat atria, the inhibition of NO activity has been shown to restore the normal response to stretch in the presence of acetylcholine (Skvorak & Dietz 1997). In the present study neither NOS inhibitor L-NAME or L-arginine alone had any effect on stretch-induced ANP secretion. In the presence of ET-1, L-NAME significantly increased right atrial wall stretch-induced ANP secretion. Since L-arginine partially reversed the effects of L-NAME on acute volume load-induced ANP release (Leskinen et al. 1995), NO released from the endothelium appears to inhibit tonically the secretion of ANP from cardiac myocytes in the presence of ET-1. Although cardiac cells themselves are capable of producing NO (Kelly et al. 1996, Winegrad 1997), this chemical signal may still originate from other cell types closely surrounding the myocytes in the atrium wall, including endothelial and endocardial cells.

In the perfused rat heart preparation ET-1 increases both basal and atrial wall stretch-induced ANP secretion (Mäntymaa et al. 1990, Shirakami et al. 1993). One possibility is that the atrial wall stretch could increase ET-1 release, which then acts on atrial myocytes to stimulate ANP release. Recently it has been shown that endothelial cells may to some extent contain stores of ET (MacClellan et al. 1993, Macarthur et al. 1994), which after its release can regulate the contractility of the heart (MacClellan et al. 1993). Local wall stretch caused by atrial stretch could release this stored ET, which may then participate in the regulation of ANP and BNP release. In cultured myocytes, the effect of ET-1 on ANP secretion has been shown to be mediated by endothelin ET\(_A\)-receptors (Thibault et al. 1994). Recently, the potent endothelin ET\(_{AB}\)-receptor antagonist bosentan (Clozel et al. 1994) has been shown to significantly inhibit acute volume load-induced ANP secretion in conscious rats (Leskinen et al. 1997b). Present results show that bosentan had no effect on acute atrial wall stretch-induced ANP in the isolated perfused rat heart. These results are not consistent with recent in vivo studies (Leskinen et al. 1997b). The reason for these discrepant results is not clear, but one possibility is that after a one-hour stabilization period endothelial cells may not be able to produce, store and liberate ET-1 sufficiently, as suggested previously (Macarthur et al. 1994).

One possibility is that ET-1 regulates atrial stretch-induced cardiac hormone release via stimulation of ET\(_B\)-receptors, which leads to release of NO, and prostacyclin. To examine this question further, we used sarafotoxin 6C, a potent and selective endothelin
ET\textsubscript{B}-receptor agonist (Bax & Saxena 1994), to stimulate NOS activity during atrial stretch. Sarafotoxin 6C had no effects on basal or stretch-induced ANP secretion, supporting the important role of endothelin ET\textsubscript{A}-receptors in ANP secretion. Taken together, these results show that the atrial wall stretch-induced ANP release under these experimental conditions appears to occur independently of ET-1 and implicate that the mechanism for NO formation is local wall stretch rather than the release of ET-1 and consequent stimulation of endothelin ET\textsubscript{B}-receptors. These experiments, however, do not exclude the possibility that other autocrine and/or paracrine factors are released which may be capable of stimulating wall stretch-induced cardiac hormone secretion. A hypothetical model of mechanisms by which ET-1 and NO may regulate ANP release is shown in Fig. 11.

Fig. 11. A hypothetical model of interaction of nitric oxide (NO) and endothelin-1 (ET-1) on ANP secretion. NOS, NO synthase; ET\textsubscript{X}, endothelin receptor subtype; big ET-1, proendothelin-1, cGMP, cyclig guanosine monophosphate.
7. Summary and conclusions

1. The present study shows that PTK inhibitor genistein acutely increased cardiac contractile force, perfusion pressure and ANP secretion to the perfusate. Diltiazem, an L-type Ca\(^{2+}\) channel antagonist, and KN-62, an inhibitor of CaM kinase II, inhibited the inotropic and ANP secretory effects of genistein, showing that genistein either directly or indirectly modulates those cellular processes which regulate the intracellular concentration of Ca\(^{2+}\).

2. ANP secretion and heart rate were increased by relaxin, indicating that this pregnancy hormone may act via autocrine/paracrine routes to regulate cardiac function. The ANP secretory effect appears to be mediated by PKC and PKA since selective protein kinase inhibitors blocked the relaxin-stimulated ANP secretion.

3. Thapsigargin, a selective inhibitor of sarcoplasmic reticulum Ca\(^{2+}\) adenosine triphosphatase, completely blocked stretch-activated ANP exocytosis from right atrial myocytes. This finding suggests that the stretch-induced increase in ANP secretion is linked to its capacity to mobilize a thapsigargin-sensitive intracellular Ca\(^{2+}\) pool.

4. Atrial wall stretch-induced ANP and BNP secretion were markedly decreased by lavendustin A, a potent PTK inhibitor. This dose-dependent inhibition of wall stretch-induced increase in cardiac hormone secretion occurred at the concentrations similar to or even below those shown to inhibit the activities of PTKs in vitro, suggesting that PTKs have an essential role in stretch-secretion coupling.

5. Okadaic acid accelerated atrial wall stretch-induced ANP secretion. Because the only targets of okadaic acid are the catalytic subunits of PP 1 and A2, these enzymes appear to play a significant role in atrial wall stretch-induced ANP secretion.

6. NO may act as a regulator of basal and stretch-induced cardiac hormone release since the NOS inhibitor L-NAME enhanced ANP secretion in the presence of ET-1. The endothelin ETA/B-receptor antagonist bosantan, and endothelin ET\(_B\)-receptor agonist sarafotoxin 6C failed to influence basal or atrial stretch-induced increase in ANP secretion. Therefore, under these experimental conditions ET-1 or stimulation of ET\(_B\)-receptors are not the mediators of acute atrial wall stretch-induced ANP secretion.
8. References


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