ADRENOMEDULLIN AS A REGULATOR OF CARDIAC FUNCTION

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium of the Department of Pharmacology and Toxicology, on June 16th, 2000, at 12 noon.
To my family
Abstract

Adrenomedullin (AM) is a 52-amino acid peptide which is produced in many tissues, including adrenal medulla, lung, kidney and heart. Intravenous administration of AM causes a long-lasting hypotensive effect, accompanied with an increase in the cardiac output in experimental animals. This study was aimed to examine whether AM has any direct effects on myocardial function. In addition to the myocardial contractility, the effects of AM on coronary vascular tone and A-type natriuretic peptide (ANP) release from atria and B-type natriuretic peptide (BNP) gene expression in the ventricles were studied in the perfused rat heart preparation.

In spontaneously beating hearts, AM had no effects on the heart rate, but dose-dependently increased the developed tension (DT) with an EC50 of 7 x 10^{-11} nmol/L, reflecting a potent positive inotropic effect. The lower the initial resting tension, the higher was the elevation in DT. In paced hearts, a protein kinase A inhibitor, H-89, had no effect on AM-induced inotropic effect, and AM did not increase the cAMP content of the ventricular myocardium. In contrast, the inhibitors of sarcoplasmic reticulum Ca^{2+} stores, ryanodine and thapsigargin, as well as a protein kinase C inhibitor, staurosporine, significantly attenuated the inotropic response to AM. L-type Ca^{2+} channel blocker, diltiazem, also suppressed the AM-induced elevation in DT. Moreover, AM increased the duration of myocyte action potentials between 10 mV and -50 mV in isolated rat atria, consistent with an increase in L-type Ca^{2+} channel current during the plateau.

Inotropic effect of endothelin-1 (ET-1), another locally acting peptide, was enhanced by inhibiting the myocardial nitric oxide (NO) synthesis by N\textsubscript{\omega}-nitro-L-arginine methyl ester (L-NAME) in perfused rat heart. The AM-induced inotropic action was unaltered by L-NAME treatment. When AM and ET-1 were administrated in combined infusion, the inotropic response was significantly smaller than that following the infusion of the peptides alone. This attenuated response was more than overcome by infusion of L-NAME, although the individual responses to AM and ET-1 were not modulated by L-NAME at the doses used in the combination. Consistent with its vasodilator action, AM dose-dependently dilated the coronary arteries of the perfused heart. The effect of AM was not dependent on NO under basal conditions or in coronary arteries constricted with ET-1. Furthermore, AM enhanced the stretch-induced release of ANP from the right atrium, but did not affect the ventricular BNP expression induced by ET-1.

In conclusion, AM exerts regulatory actions on the heart by increasing cardiac contractility, dilating coronary arteries and modulating stretch-induced ANP release. The inotropic effect of AM was independent of cyclic AMP, but may involve activation of protein kinase C, Ca^{2+} influx through L-type Ca^{2+} channels and the release of Ca^{2+} from the sarcoplasmic reticulum. Endogenous NO production did not modulate the inotropic effect of AM, although the effect of ET-1 was suppressed. Combined administration of AM and ET-1 produced a weak inotropic response most likely because of a potentiated synthesis of NO. Finally, AM had a coronary vasodilator effect and augmented the stretch-induced ANP release in the right atrium.

Keywords: myocardial contraction, signaling, coronary arteries, natriuretic peptides
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Oulu, May 2000

Pietari Kinnunen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropin</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AM</td>
<td>adrenomedullin</td>
</tr>
<tr>
<td>ANP</td>
<td>A-type natriuretic peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AP-2</td>
<td>activator protein-2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine8-vasopressin</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>[Ca\textsuperscript{2+}]\textsubscript{i}</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>3',5'-cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CGRP\textsubscript{8-37}</td>
<td>calcitonin gene-related peptide\textsubscript{8-37}</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium-induced calcium release</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>CRLR</td>
<td>calcitonin receptor-like receptor</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DT</td>
<td>developed tension</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>GC</td>
<td>guanylyl cyclase</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>G\textsubscript{s}</td>
<td>stimulatory G-protein</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
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</table>
IL interleukin
IP$_3$ inositol-1,4,5-triphosphate
ir immunoreactive
K$_{ATP}$ channels ATP-sensitive potassium channels
L-NAME N$^\omega$-nitro-L-arginine methyl ester
LPS lipopolysaccharide
MAPK mitogen-activated protein kinases
mRNA messenger ribonucleic acid
NF-IL6 nuclear factor-IL6
NO nitric oxide
PAMP proadrenomedullin N-terminal 20-peptide
PDGF platelet-derived growth factor
PKA cAMP-dependent protein kinase
PKC protein kinase C
PKG cGMP-dependent protein kinase
PLB phospholamban
PLC phospholipase C
preproAM preproadrenomedullin
proAM proadrenomedullin
RAMP receptor activity modifying protein
RBF renal blood flow
RIA radioimmunoassay
RT resting tension
RT-PCR reverse transcriptase polymerase chain reaction
RyR ryanodine receptor
sGC soluble guanylyl cyclase
SR sarcoplasmic reticulum
T$_3$ thyroid hormone
TGF-$\beta$ transforming growth factor-$\beta$
TNF tumour necrosis factor
TnC troponin C
TnI troponin I
TnT troponin T
TPA 12-O-tetradecanoyl-phorbol-13-acetate
VSMC vascular smooth muscle cell
List of original papers

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


IV Kinnunen, P., Szokodi, I. & Ruskoaho, H. Adrenomedullin reverses the pressor response to ET-1 independently of NO in rat coronary circulation. Submitted for publication.

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Original papers
1. Introduction

The basic function of the heart is to act as a pump which ejects blood from the left ventricle to be driven forward throughout the body, ultimately to reach the peripheral circulation. There, in the capillaries, oxygen is removed to nourish the various organs and tissues of the body, forming a basis for the organ function. (Opie 1998) Thus, any disease that worsens the pumping function may lead to failure of the heart and thus an inadequate blood and oxygen supply to the peripheral tissues. Congestive heart failure (CHF) is a syndrome that is caused by volume or pressure overload (e.g. aortic regurgitation or essential hypertension, respectively) of the heart, or by a primary myocardial disease (e.g. ischemic cardiomyopathy) (Cohn 1996). CHF is highly lethal; the 5-year survival rates are only 25 % and 38 % for men and women, respectively (Ho et al. 1993). Therefore, research has been focused on searching new regulatory mechanisms of the circulation that could eventually be used in the prevention and treatment of CHF.

In 1993, Kitamura et al. discovered a novel vasoactive peptide, adrenomedullin (AM), from human pheochromocytoma extracts. AM was found to have a potent hypotensive effect when infused into the circulation of anesthetized rats. (Kitamura et al. 1993a) To date, numerous investigations have revealed that AM plays an important role in the physiological regulation of circulation (for review, see Charles et al. 1999, Samson 1999) as well as in pathophysiological disease states like CHF (for review, see Eto et al. 1999). Since intravenous infusion of AM causes, in addition to the hypotension, an increase in cardiac output (see e.g. Fukuhara et al. 1995), the present study was aimed to examine whether AM has any direct effects on myocardial contractility. Furthermore, the effects of AM on the coronary artery tone and cardiac hormone production were investigated.
2. Review of the literature

2.1. Adrenomedullin

2.1.1. Discovery and structure

AM is a 52-amino acid peptide that was discovered in platelet bioassay by monitoring the ability of human pheochromocytoma tissue extracts to stimulate rat platelet 3',5'-cyclic adenosine monophosphate (cAMP) levels (Kitamura et al. 1993a). Structurally AM was observed to share some homology with the calcitonin gene-related peptide (CGRP) superfamily (see Fig. 1B). However, the sequence homology with human CGRP, amylin and calcitonin is not high, although they share a six residue ring structure formed by an intramolecular disulfide linkage and the C-terminal amide structure. Different from other peptides of the family, AM was reported to have a 14-residue amino terminal extension. In addition to the ability to increase platelet cAMP, AM appeared to have a potent, long lasting hypotensive action when infused in anesthetized rats (Kitamura et al. 1993a).

The cDNA encoding the precursor of human (Kitamura et al. 1993b), porcine (Kitamura et al. 1994a) and rat (Sakata et al. 1993) AM has been cloned. The human AM gene which was later shown to be located at a single locus on chromosome 11 and to consist of four exons and three introns (Ishimitsu et al. 1994a), encodes a 185-residue protein (preproadrenomedullin, preproAM), first 21 residues of which are thought to be a signal peptide (Kitamura et al. 1993b) (see Fig. 1A). It is likely that cleavage of signal peptide generates a 164-amino acid proadrenomedullin (proAM) peptide, which includes the AM sequence. In addition to AM, proadrenomedullin contains a unique 20 amino acid peptide, termed proadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura et al. 1993b) that was also shown to be a biologically active peptide with its hypotensive action (Kitamura et al. 1994b). In comparison with the human peptide, mature rat AM has two amino acid deletions and six substitutions (see Fig. 1B) (Sakata et al. 1993), and porcine AM only one substitution (Kitamura et al. 1994a).
2.1.2. Distribution

Although originally discovered from pheochromocytoma tissue (Kitamura et al. 1993a), AM has been found in several tissues by means of radioimmunoassay (RIA) of plasma and tissue extracts, immunohistochemical studies and by detecting tissue AM mRNA. Following the discovery, the distribution of AM was investigated in human tissues (Kitamura et al. 1993a). Highest concentration of immunoreactive (ir-) AM was found in...
adrenal medulla, but it was also detected in plasma, lung and kidney. Later, a more specific and sensitive RIA for AM was established in the same laboratory and ir-AM was again detected in high concentration in adrenal medulla, relatively high concentrations being also present in heart atrium, lung, pancreas and small intestine (Ichiki et al. 1994). Smaller amounts of ir-AM were observed in heart ventricle, aorta, kidney, liver, spleen, brain and thyroid gland. Similarly to man, in rat tissues, highest concentrations of AM are found in adrenal gland, lung and heart atrium (Sakata et al. 1994). In an immunohistochemical study, human, rat and porcine tissues displayed a similar interspecies distribution of AM (Washimine et al. 1995). AM immunostaining was present in adrenal medulla, stomach, pancreatic islets, small intestine, colon and choroid plexus in all three species, in addition to which rat and human anterior pituitary showed intense staining. In contrast to RIA studies, no AM was detected in lung, kidney and heart. However, later reports demonstrated clearly positive immunohistochemical findings for AM in human (Jougasaki et al. 1995a, Asada et al. 1999) and canine (Jougasaki et al. 1995b, Jougasaki et al. 1997) heart, human lung tissue (Martinez et al. 1995, Asada et al. 1999) and human (Asada et al. 1999), rat (Chini et al. 1997) and canine (Jougasaki et al. 1995c) kidney. The AM peptide level appears to be higher in cardiac atrial than in ventricular tissue (Jougasaki et al. 1995a, Jougasaki et al. 1995b, Jougasaki et al. 1997, Asada et al. 1999). In the kidney, AM immunostaining is found in the glomeruli, cortical distal tubules and medullary collecting duct cells (Jougasaki et al. 1995c, Chini et al. 1997, Asada et al. 1999).

In agreement with the results of RIA- and immunohistochemical studies, human AM mRNA is highly expressed in the adrenal medulla, but equally high levels are also found in cardiac ventricle, lung and kidney (Kitamura et al. 1993b). Since the concentration of ir-AM in ventricle, lung and kidney was reported to be less than 1 % of that in adrenal medulla (Kitamura et al. 1993a), the AM biosynthesized in these tissues may be rapidly released into the blood or eliminated. However, ir-AM concentration of human plasma samples collected during cardiac catheterization in various sites did not confirm that these organs are the main sources of circulating AM (Nishikimi et al. 1994), suggesting that AM is preferably synthesized to act as a local autocrine/paracrine hormone. Yet, it was later suggested by a similar study that the heart could contribute somewhat to the plasma level of AM (Hirayama et al. 1999a). By means of in situ hybridization of rat and mouse tissues AM mRNA was observed, in addition to the tissues above, in endometrium, epithelial cells lining the uterus, ovarian corpus luteum and follicles, epithelial cells lining the bronchioles, female rat posterior pituitary and submandibular gland (Cameron & Fleming 1998). More specific investigations have shown the presence of AM mRNA and peptide also in rat adrenal zona glomerulosa (Kapas et al. 1998), human brain tissues including thalamus, hypothalamus, pons, cortex, cerebellum and medulla oblongata (Satoh et al. 1995, Takahashi et al. 1997), human (Jimenez et al. 1999) and rat (Jimenez et al. 1999, Pewitt et al. 1999) prostate, gastrointestinal tissues (Sakata et al. 1998) and neuroendocrine cells of gastric mucosa (Mulder et al. 1996, Tajima et al. 1999) and pancreas of various species (Martinez et al. 1996).

Cultured vascular endothelial cells (EC) actively synthesize and secrete AM, the mRNA level being at least 20 times higher than that of adrenal gland, lung and atrium (Sugo et al. 1994a). Endothelial synthesis of AM was confirmed by detecting its mRNA in porcine aortic endothelial scrape by reverse transcriptase-polymerase chain reaction (RT-
The AM expression level was, however, not as high in endothelium in situ (Nishimura et al. 1997) as in cultured ECs (Sugo et al. 1994a). Vascular smooth muscle cells (VSMC) also produce and secrete AM, but the secretion rate was estimated to be only one fifth or sixth of that of ECs (Sugo et al. 1994b, Isumi et al. 1998a). Purified rat cardiac ventricular myocytes (Nishimori et al. 1997, Horio et al. 1998) and nonmyocytes (Horio et al. 1998) actively release AM and express the AM gene. AM is also produced by various other cells, such as glomerular mesangial cells (Chini et al. 1997, Owada et al. 1997), juxtaglomerular granular cells (Jensen et al. 1997), tracheal and urinary bladder epithelial cells (Nishimura et al. 1997), adrenal medullary (Katoh et al. 1994) and chromaffin (Kobayashi et al. 1999) cells, monocytes and macrophages (Kubo et al. 1998a, Nakayama et al. 1999), astrocytes (Takahashi et al. 2000), several insulin-producing cell lines (Martinez et al. 1996) and fibroblasts (Isumi et al. 1998b).

2.1.3. Regulation of synthesis and secretion

The substances affecting AM synthesis and secretion have been systematically studied in rat vascular ECs (Isumi et al. 1998a) and VSMCs (Sugo et al. 1995a). Among the cytokines and growth factors, interleukin (IL) -1α and -1β, tumor necrosis factor (TNF)-α and -β, and lipopolysaccharide (LPS) consistently increase AM mRNA and ir-AM in both cell types (Sugo et al. 1994b, Sugo et al. 1995a, Sugo et al. 1995b, Isumi et al. 1998a). In vascular ECs, bovine serum albumin, thrombin, and to a lesser extent glucocorticoids, aldosterone, sex hormones, thyroid hormone (T₃), norepinephrine, isoproterenol and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) also increased the ir-AM content of the culture medium, whereas fetal calf serum and transforming growth factor-β₁ (TGF-β₁) were the most potent inhibitors of AM secretion (Isumi et al. 1998a). EC synthesis and secretion of AM has also been reported to be stimulated (Chun et al. 1997) or inhibited (Shinoki et al. 1998) by shear stress. In VSMCs, AM secretion was increased also by angiotensin II, bradykinin, substance P, endothelin-1 (ET-1), adrenaline and TPA (Sugo et al. 1995a). Differently from EC culture, thrombin strongly inhibited the AM secretion, which was also somewhat decreased by interferon-γ and vasoactive intestinal polypeptide. Interestingly, cAMP related compounds, such as forskolin and 8-bromo-cAMP significantly decreased the ir-AM level, suggesting that AM may suppress its own release by a feedback mechanism in VSMCs. Hayashi et al. reported that hyperglycemia stimulates AM synthesis and release in rat aorta and cultured VSMC in protein kinase C (PKC) -mediated mechanism (Hayashi et al. 1999), which may explain the elevated plasma level of AM observed in hyperglycemic patients (Hayashi et al. 1997). Synthesis and secretion of AM in VSMCs is stimulated by oxidative stress induced by superoxide dismutase inhibitor (Ando et al. 1998). TPA, retinoic acid, LPS and interferon-γ strongly augmented the production of AM in a mouse macrophage cell line, suggesting that macrophage-derived AM may be one of the major sources of circulating peptide, especially in cases of sepsis and inflammation (Kubo et al. 1998a). In cultured cardiac myocytes, AM synthesis and secretion is stimulated, in addition to IL-1β, TNF-α (Horio et al. 1998) and dexamethasone (Nishimori et al. 1997), also by hypoxia (Cormier-Regard et al. 1998,
Increased AM mRNA level induced by dexamethasone and T₃ in vascular ECs and VSMCs (Imai et al. 1995) and by dexamethasone in cardiac myocytes (Nishimori et al. 1997) was shown to be due to increased transcription rate rather than decreased degradation of the mRNA. AM is cosecreted with catecholamines in cultured bovine adrenal medullary cells in nicotine receptor-mediated way (Katoh et al. 1994). In bovine adrenal chromaffin cells, AM synthesis and secretion is increased by cAMP- analogs and prevented by protein kinase A (PKA) inhibitors (Kobayashi et al. 1999).

The 5'-flanking region of the human AM gene contains a number of possible binding sites for transcriptional regulation factors (Ishimitsu et al. 1994a). Rat AM promoter shows 60% identity with the human gene (Hattori et al. 1999). Serial deletion analysis of the 5'-flanking region in human aortic ECs indicated that three sites corresponding to TATA box, activator protein-2 (AP-2) and nuclear factor-IL6 (NF-IL6), respectively, are functionally significant for the promoter activity of the human AM gene (Ishimitsu et al. 1998). Since AP-2 is assumed to mediate transcriptional activation mediated by PKC and cAMP (Imagawa et al. 1987), and AM is able to elevate intracellular cAMP (Kitamura et al. 1993a), it is possible that a feedback mechanism exists for AM expression. NF-IL6 has been shown to be induced by the stimulation with LPS, TNF-α and IL-1 (Akira et al. 1990, Kiehntopf et al. 1995), which may explain the augmented synthesis of AM by these substances (Shoji et al. 1995, Sugo et al. 1995a, Isumi et al. 1998a, Elsasser et al. 1999). In the rat, potential binding sites for transcription factor nuclear factor-kB (NF-kB) are expected to participate in the mediation of the responses to those cytokines (Hattori et al. 1999). Cormier-Regard et al. observed two potential hypoxia-inducible factor-1 (HIF-1) consensus binding sites in the mouse AM promoter. Transfection studies in adult rat cardiac myocytes revealed that the intact 5'-flanking sequence of the AM gene was capable of mediating a hypoxia-inducible increase in transcription, which was lost by mutation of the potential HIF-1 binding sites. (Cormier-Regard et al. 1998) Furthermore, AM promoter binds HIF-1 protein when incubated with nuclear extract from mouse HL-1 cardiomyocytes cultured under hypoxic conditions (Nguyen & Claycomb 1999). In vivo hypoxia is also a strong stimulus for AM gene expression in several tissues (Hofbauer et al. 2000). Moreover, focal brain ischemia induced a rapid, 20-fold increase in AM mRNA in rats (Wang et al. 1995), confirming the sensitivity of AM production to hypoxia.

### 2.1.4. Mechanisms of action and AM receptors

Inoue 1994), which initiates various biological cellular responses. There is evidence that
the increased cAMP levels are due to AM-induced activation of cholera toxin-sensitive
stimulatory G-protein, $G_s$ (Eguchi et al. 1994a, Shimekake et al. 1995). Eguchi et al. could
demonstrate that specific AM receptors exist and functionally couple to adenyl cyclase
(AC) in rat cultured vascular smooth muscle cells (Eguchi et al. 1994b, Fig. 2). Kureishi et al.
showed that the relaxation of porcine coronary arterial strips by AM involves a
decrease in $[Ca^{2+}]_{i}$, but also a decrease in the $Ca^{2+}$-sensitivity of the contractile apparatus,
which may result from the activation of the $G_s$-cAMP system (Kureishi et al. 1995).
Similarly, a fall in $[Ca^{2+}]_{i}$ of porcine renal artery smooth muscle cells was observed in
parallel with cell relaxation (Seguchi et al. 1995). Although AM may cause vasorelaxation
by stimulating AC of VSMCs, which is generally regarded as an endothelium-independent
mechanism of vasodilatation, the vasorelaxation in response to AM has also been shown to
include the release of potent vasodilator nitric oxide (NO) derived from the vascular
endothelium (see Table 2, Fig. 2). In endothelial cells, AM possesses functional
receptor(s), the activation of which probably also lead, in addition to accumulation of
cAMP, to IP$_3$-mediated mobilization of $[Ca^{2+}]_{i}$. This is associated with an increase in
intracellular cGMP concentration, which can be blocked by NOS inhibitor, suggesting that
the latter mechanism releases NO from the endothelium. (Shimekake et al. 1995) There
are also reports showing that the vasodilatory action of AM could be partially mediated
via ATP-sensitive potassium ($K_{ATP}$) ion channels (Lang et al. 1997, Sabates et al. 1997,
Sakai et al. 1998). The renal vasodilatory action of AM was not, however, blocked with
glibenclamide, a blocker of $K_{ATP}$ ion channels (Miura et al. 1995). AM also decreases the
release of noradrenaline from adrenergic nerves elicited by transmural electrical
stimulation, simultaneously inhibiting the rise in perfusion pressure in isolated canine
mesenteric arteries (Fujioka et al. 1999). In rats, however, AM failed to inhibit
noradrenaline overflow from mesenteric periarterial nerve endings, whereas PAMP
exerted a significant inhibition and is thought to dilate vessels by that mechanism
(Shimosawa et al. 1995).

Fig. 2. Hypothetical signaling pathways underlying the vasorelaxant effect of adrenomedullin
(AM). EC: endothelial cells, VSMC: vascular smooth muscle cells, PLC: phospholipase C, IP$_3$:
inositol-1,4,5-triphosphate, $K_{ATP}$: ATP-sensitive potassium channel, NO: nitric oxide, NOS: NO
synthase, $[Ca^{2+}]_{i}$: intracellular calcium concentration.
It has not been possible to determine whether some or all of the actions of AM are mediated through specific AM receptors, since CGRP, a related peptide, shares many of the biological actions and binding sites with AM, and CGRP$_{8-37}$, a CGRP receptor antagonist, has variably been shown to inhibit AM-induced biological actions (see e.g. Table 2). Owji et al. reported specific binding sites for AM in rat tissues; the highest level of binding was present in the heart, but also lung, spleen, liver, soleus, gastrocnemius and diaphragm showed significant intensity of binding sites. AM binding in the lung was not competed with CGRP, CGRP$_{8-37}$ and calcitonin, but in the heart CGRP was capable of slightly inhibiting AM binding. In both tissues, AM was shown to inhibit CGRP binding, although at low potency. Together with cross-linking studies, these results suggest two binding sites for AM, which have different pharmacological properties and molecular weight from each other and from CGRP receptors. (Owji et al. 1995)

An orphan receptor widely distributed in rat tissues, G10D (Harrison et al. 1993), was identified as AM receptor (Kapas et al. 1995). When transfected to COS-7 cells, this receptor bound AM with high affinity and mediated the cAMP response to AM but not to CGRP. A corresponding human receptor was also cloned, the expression of which is high in the heart, skeletal muscle, liver, pancreas, stomach, spleen, lymph node, bone marrow, adrenal gland and thyroid (Hanze et al. 1997). Kennedy et al., however, reported conflicting results. These putative rat and human AM receptors did not mediate the cAMP response and no specific binding of AM was detected in their studies in COS-7 cells expressing these receptors. (Kennedy et al. 1998) An orphan canine receptor RDC-1 that is expressed most abundantly in the heart and kidney (Libert et al. 1989), appeared to function as CGRP receptor (Kapas & Clark 1995). As this receptor is transfected to COS-7 cells, CGRP is a most effective agonist to elevate the cellular level of cAMP, but it is also responsive to AM. (Kapas & Clark 1995) Others could not, however, confirm that RDC-1 induces binding or responses to CGRP when expressed in cells (McLatchie et al. 1998). An orphan canine receptor RDC-1 that is expressed most abundantly in the heart and kidney (Libert et al. 1989), appeared to function as CGRP receptor (Kapas & Clark 1995). As this receptor is transfected to COS-7 cells, CGRP is a most effective agonist to elevate the cellular level of cAMP, but it is also responsive to AM. (Kapas & Clark 1995) Others could not, however, confirm that RDC-1 induces binding or responses to CGRP when expressed in cells (McLatchie et al. 1998).

Indeed, more recently, it has also been suggested that RDC-1 represents an orphan chemokine receptor rather than a CGRP or an AM receptor (Heesen et al. 1998). In addition, a calcitonin receptor-like receptor (CRLR) has been cloned from cDNA libraries of rat (Njuki et al. 1993, Chang et al. 1993) and human (Fluhmann et al. 1995) cerebellum. CRLR binds both CGRP and AM (Aiyar et al. 1996, Han et al. 1997) and is expressed predominantly in the lung and heart and to a lesser degree in the kidney, pancreas and spleen, while other tissues show very low expression of CRLR (Njuki et al. 1993, Fluhmann et al. 1995, Aiyar et al. 1996). Interestingly, McLatchie et al. demonstrated that depending on the receptor-activity modifying protein (RAMP) protein subtype expressed in the tissue, CRLR is either a CGRP or an AM receptor (for review, see Foord & Marshall, 1999). Expression of RAMP1 with CRLR generates a CGRP receptor whereas RAMP2 is needed to generate an AM receptor. RAMP1 was shown to be highly expressed in human heart, pancreas and skeletal muscle, while the most abundant expression of RAMP2 was present in heart, placenta, lung and skeletal muscle. (McLatchie et al. 1998) In rabbit aortic ECs transfected with cDNA encoding RAMP1, CGRP stimulated cAMP production, but not in the non-transfected cells. Endogenous AM receptor of those cells was not affected by transfection of any RAMP subtype. (Muff et al. 1998) Human aortic and umbilical vein ECs and aortic smooth muscle cells express both CRLR and RAMP2, which acts as functional cAMP-elevating AM receptor (Kamitani et al. 1999). All of these putative AM receptors are present in the rat heart, and more
specifically, in the cardiac myocyte. RDC-1 receptor is the most abundant followed by CRLR, while G10D is expressed at relatively low levels. (Autelitano 1998) Taken together, it is not clear which of these receptors mediates the biological effects of AM. Development of selective ligands for these receptors will be crucial for clarifying the authentic AM receptors responsible for different actions of AM.

Table 1. Documentation on the candidate receptors mediating the effects of AM and CGRP.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Suggested ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>G10D</td>
<td>AM (Kapas et al. 1995), AM does not activate (Kennedy et al. 1998)</td>
</tr>
<tr>
<td>RDC-1</td>
<td>CGRP and AM (Kapas &amp; Clark 1995), CGRP does not activate (McLatchie et al. 1998)</td>
</tr>
<tr>
<td>CRLR</td>
<td>CGRP family does not activate (Chang et al. 1993, Fluhmann et al. 1995)</td>
</tr>
<tr>
<td></td>
<td>CGRP and AM (Han et al. 1997, Aiyar et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>CGRP, if coexpressed with RAMP1 (McLatchie et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>AM, if coexpressed with RAMP2 (McLatchie et al. 1998)</td>
</tr>
</tbody>
</table>


2.1.5. Biological effects of AM

AM circulates in plasma of healthy humans in a low picomolar range (2-19 pmol/l, Kitamura et al. 1993a, Ichiki et al. 1994, Kitamura et al. 1994c, Sato et al. 1995, Lewis et al. 1998, Kitamura et al. 1998). Thus, although the plasma AM seems not to consist totally of the mature AM but also of the non-amidated inactive form (Kitamura et al. 1998), it may at least partially function in an endocrine way in the tissues where its receptors are expressed. AM is produced in many tissues, but no individual organ could be shown to contribute to its concentration in plasma under physiological conditions (Nishikimi et al. 1994). It is considered that AM detected in plasma may be generated by spillover from the vascular ECs (Sugo et al. 1994a) and VSMSs (Sugo et al. 1994b). Furthermore, since the receptors of AM are regularly expressed in the same tissues as the peptide itself, AM is commonly regarded as a local autocrine/paracrine effector. Studies with different truncated forms of AM have shown that the six-membered ring structure (Lippton et al. 1994, Eguchi et al. 1994a, Heaton et al. 1995, Nossaman et al. 1996, Belloni et al. 1998), as well as the C-terminal amidation (Eguchi et al. 1994a) are essential in generation of the biological actions of AM. Interestingly, the pure ring structure (amino acids 15-22) of AM increases systemic arterial pressure, which is opposite to the effect of intact AM (Champion et al. 1996).

In response to intravenous administration of AM into experimental animals, the hypotensive action is usually accompanied by an increase in heart rate (HR) and/or cardiac output (CO) (Ishiyama et al. 1993, Takahashi et al. 1994, Parkes 1995, He et al. 1995, Fukuhara et al. 1995, Hjelmqvist et al. 1997, Parkes & May 1997, Charles et al. 1997, Saita et al. 1998a, Lainchbury et al. 2000). In rabbits, the hypotensive action of AM is also associated with a rise in renal sympathetic nerve activity and plasma noradrenaline, suggesting that the cardiostimulatory effect is due to a reflex enhancement of cardiac function (Fukuhara et al. 1995). Sympathetic nerve activity was also increased in rats (Takahashi et al. 1994, Saita et al. 1998a). However, an equivalent decrease in blood pressure by sodium nitroprusside increases HR and sympathetic nerve activity to a significantly greater degree than AM in rabbits (Fukuhara et al. 1995) and to a lesser degree in rats (Saita et al. 1998a), reflecting that AM may modulate the autonomic reflexes, although differentially between species. In conscious sheep, AM induces a large (40-50%) increase in CO and simultaneously a rise in peak aortic flow, which suggests...
that AM could also have a direct inotropic effect (Parkes 1995, Charles et al. 1997). Autonomic ganglion blockade by hexamethonium enhances the systemic hypotensive action of AM in sheep and increases the cardiac output to a similar extent as in the control animals, while no tachycardia is observed under those conditions. This result suggests that the autonomic baroreflexes can partially maintain the blood pressure by increasing HR, but also that AM has a direct inotropic effect. (Parkes & May 1997) This hypothesis was not supported by the results of Perret et al., which describe a mild negative inotropic effect for AM in isolated perfused rabbit heart (Perret et al. 1993). In agreement, Ikenouchi et al. found that AM and a related peptide, CGRP, have concentration-dependent negative inotropic actions mediated by NO-3',5'-cyclic guanosine monophosphate (cGMP) system and a decrease of \([\text{Ca}^{2+}]\) in rabbit isolated cardiac myocytes. Human AM also failed to influence the contractility of isolated porcine atrial and ventricular myocardial trabeculae, whereas human CGRP was able to stimulate the contraction force of both, and human amylin that of atrial tissue (Saetrum et al. 1999). However, a recent study showed that AM has a small positive inotropic effect on rat papillary muscle preparation (Ihara et al. 2000).

2.1.5.2 Renal effects

Intravenous administration of AM has been reported to affect the parameters of renal function others than renal blood flow (RBF). In rats, slight increases in urinary sodium excretion and glomerular filtration rate (GFR) were observed (Hirata et al. 1995). Nagaya et al. found an increase in rat urinary sodium excretion and urinary volume (Nagaya et al. 1999a). In humans, AM increases urine volume, urinary sodium and potassium excretion and creatinine clearance at an infusion dose of 50 ng/kg/min (Nagaya et al. 2000a), whereas doses of 2 and 8 ng/kg/min do not alter the sodium excretion, although both markedly decrease the blood pressure (Lainchbury et al. 1997). Similarly in sheep, despite a modest postinfusion decrease in urinary sodium excretion, AM induced no changes in the indexes of renal function, while the cardiovascular actions of AM were significant, showing that the threshold concentration of AM for the vascular actions is lower than that for the renal effects (Charles et al. 1997).
Table 2. Characteristics of the regional vasodilatory actions of AM.

<table>
<thead>
<tr>
<th>Vascular tissue</th>
<th>Species</th>
<th>Adm.</th>
<th>Threshold dose or *EC50</th>
<th>P</th>
<th>Involvement of cAMP</th>
<th>NO</th>
<th>Inhibition by CGRP8-37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenteric</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuki et al. (1993)</td>
<td>rat</td>
<td>B</td>
<td>0.01 nM, ~1 nM*</td>
<td>&lt;</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td>Berthiaume et al. (1995)</td>
<td>rat</td>
<td>B</td>
<td>0.01 nM</td>
<td>ND</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Hindquarter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feng et al. (1994)</td>
<td>rat</td>
<td>B</td>
<td>0.01 nmol</td>
<td>=</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>Pulmonary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heaton et al. (1995)</td>
<td>rat</td>
<td>B</td>
<td>0.5 nmol</td>
<td>&lt;</td>
<td>ND</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pinto et al. (1996)</td>
<td>g.-pig</td>
<td>T</td>
<td>5 nM, ~10 nM*</td>
<td>=</td>
<td>ND</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Nossaman et al. (1996)</td>
<td>rat</td>
<td>B</td>
<td>0.03 nmol</td>
<td>&lt;</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>Renal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ebara et al. (1994)</td>
<td>dog</td>
<td>I</td>
<td>0.8 ng/kg/min</td>
<td>No</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hirata et al. (1995)</td>
<td>rat</td>
<td>B</td>
<td>1 nM</td>
<td>ND</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Miura et al. (1995)</td>
<td>dog</td>
<td>I</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Elhawary et al. (1995)</td>
<td>rat</td>
<td>I</td>
<td>0.001 nmol/kg/min</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Haynes &amp; Cooper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Majid et al. (1996)</td>
<td>dog</td>
<td>I</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hayakawa et al. (1999)</td>
<td>rat</td>
<td>B</td>
<td>0.01 nM</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Coronary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzeroth et al. (1995)</td>
<td>rat</td>
<td>I</td>
<td>1 nM, 3 nM*</td>
<td>&lt;</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td>Sabates et al. (1997)</td>
<td>dog</td>
<td>B</td>
<td>1 µg</td>
<td>=</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>Yoshimoto et al. (1998)</td>
<td>pig</td>
<td>T</td>
<td>1 nM, 27.6 nM*</td>
<td>&lt;</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Hindlimb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Champion et al. (1997)</td>
<td>cat</td>
<td>B</td>
<td>0.01 nmol</td>
<td>=</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cerebral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lang et al. (1997)</td>
<td>rat</td>
<td>T</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Mori et al. (1997)</td>
<td>rat</td>
<td>T</td>
<td>1 nM, 1.6 nM*</td>
<td>&lt;</td>
<td>ND</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hayakawa et al. (1999)</td>
<td>rat</td>
<td>T</td>
<td>0.1 nM</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Yoshimoto et al. (1998)</td>
<td>rat</td>
<td>T</td>
<td>1 nM, 2.4 nM*</td>
<td>&gt;</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Adm.: way of administration, ND: not determined, B: bolus injection, I: infusion, T: topical application, g.-pig: guinea-pig, P: potency on a molar basis compared with CGRP. < denotes a lower, > higher and, = equal potency. Studies where the involvement of cAMP, NO or CGRP receptors was determined are shown.

Intrarenal infusion of AM in dogs dose-dependently increased urine flow, urinary sodium and potassium excretion as well as GFR (Ebara et al. 1994). This result was reproduced by other investigators in rats (Elhawary et al. 1995) and dogs (Jougasaki et al. 1995c, Majid et al. 1996), except that in rats AM did not influence the potassium excretion. The renal effects of AM were not blocked by CGRP8-37. (Elhawary et al. 1995)
Miura et al. reported that these renal actions were attenuated with nitric oxide synthase (NOS) inhibitor treatment when the increases were compared in terms of absolute change, but not if expressed as percentage increases (Miura et al. 1995). Majid et al. also suggested that NO may mediate the diuretic and natriuretic action of AM (Majid et al. 1996). It was reported that renal neutral endopeptidase may cleave AM since an inhibitor of this enzyme potentiated the natriuretic actions of AM (Lisy et al. 1998). The diuresis and natriuresis in response to AM seem to be due to a direct tubular action, since they are produced by a low dose of AM which does not affect the GFR which only contributes to the actions at higher concentrations (Jougasaki et al. 1995c).

Although circulating AM may have minor effects on the function of kidneys, the locally produced peptide is thought to be an important regulator of diuresis and natriuresis. Indeed, AM mRNA measured by RT-PCR is localized in glomerulus, cortical collecting duct, outer medullary collecting duct and inner medullary collecting duct, and AM selectively elevates cAMP in these microdissected nephron segments (Owada et al. 1997).

2.1.5.3. Effects on hormone systems

AM elevates plasma renin concentration in conscious sheep (Parkes & May 1995, Parkes & May 1997, Charles et al. 1997) and plasma renin activity in conscious rabbits (Fukuhara et al. 1995) and in humans (Lainchbury et al. 2000). This may partially be a consequence of renal vasodilation but on the other hand, AM has been shown to stimulate renin secretion in perfused rat kidney and mouse cultured juxtaglomerular granular cells (Jensen et al. 1997). In rat anterior pituitary cells AM inhibits the basal and corticotropin releasing hormone-stimulated secretion of adrenocorticotropin (ACTH, Samson et al. 1995). Consistently, in conscious sheep, intravenous infusion of AM decreased the plasma level of ACTH (Parkes & May 1995, Parkes & May 1997). There is also evidence that AM may regulate the secretion of aldosterone in the zona glomerulosa of adrenal cortex (for review, see Lumbers et al. 1999). The first study showed that AM inhibits the aldosterone secretion stimulated by angiotensin II or potassium in dispersed rat adrenal zona glomerulosa cells, but not if stimulated by ACTH (Yamaguchi et al. 1995). This result has been reproduced in rat (Mazzocchi et al. 1996a, Andreis et al. 1997a) and human (Andreis et al. 1997b, Belloni et al. 1998, Andreis et al. 1998) zona glomerulosa cells. In vivo, subcutaneous injections of AM inhibited the increase in aldosterone concentration of rat plasma and adrenal cortex induced by a sodium-deficient diet and bilateral nephrectomy (Yamaguchi et al. 1996). In humans, angiotensin II-induced aldosterone secretion was also inhibited by intravenous AM (Petrie et al. 2000). However, AM has been shown to stimulate aldosterone secretion in intact capsules (Kapas & Hinson 1996) and intact perfused rat adrenal preparation (Mazzocchi et al. 1996b). Similarly, Kapas et al. suggested that local production of AM in the adrenal cortex stimulates the secretion of aldosterone in zona glomerulosa cells in a cAMP mediated manner (Kapas et al. 1998).

AM also appears to influence the natriuretic peptide hormone system. In neonatal rat cultured cardiac myocytes, AM led to a dose-dependent increase in cAMP accumulation and subsequent inhibition of A-type natriuretic peptide (ANP) gene expression and
secretion (Sato et al. 1997). In that experiment, ANP production remained constant in the cells treated for 16 h with 100 nmol/l AM but increased simultaneously 2.5-fold in the control cells. Others could not, however, demonstrate any change in basal ANP secretion in the same experimental model, but the release stimulated by ET-1 was significantly enhanced by AM (Horio et al. 1999). Kaufman & Deng studied the effect of AM on the atrial secretion of ANP. AM at a concentration of 175 nmol/l did not influence the basal release of ANP to the perfusion fluid of rat isolated atrium, but suppressed the ANP release stimulated by atrial distension by 28 %. (Kaufman & Deng 1998) In sheep (Charles et al. 1997), human (Lainchbury et al. 1997, Nagaya et al. 2000a) and rat (Nagaya et al. 1999a) intravenous infusion of AM does not significantly change the plasma levels of ANP and B-type natriuretic peptide (BNP).

Intravenous AM increased plasma noradrenaline levels in rabbits (Fukuhara et al. 1995) and humans (Nagaya et al. 2000a, Lainchbury et al. 2000), but no changes in adrenaline levels were induced. This may reflect the increased sympathetic discharge due to the hypotension, because AM seems to have no direct effect on adrenal catecholamine release (Houchi et al. 1996, Masada et al. 1999). Insulin secretion of rat isolated pancreatic islets is dose-dependently reduced by AM, and AM antibody is able to increase insulin release in the absence of exogenous AM. Furthermore, intravenous AM delays the plasma insulin response to oral glucose tolerance test in rats. (Martinez et al. 1996) AM has been shown to stimulate growth hormone secretion in normal rat pituitary cells as well as in GH3 pituitary tumor cells (Nakamura et al. 1998). AM also inhibits the release of ET-1 in VSMCs, a fact that may partially contribute to the influence of AM on vascular tone (Kohno et al. 1995a).

2.1.5.4. Effects on the central nervous system

Since AM is present in the brain, it has been assumed to have biological functions in the central nervous system. Indeed, intracerebroventricular (icv) administration of AM suppresses water drinking (Murphy & Samson 1995, Charles et al. 1998), salt appetite (Samson & Murphy 1997) and food intake (Taylor et al. 1996) in rat, whereas administration of AM antibodies results in increased salt and water intake (Samson & Murphy 1997). In addition, central AM inhibits gastric emptying (Martinez et al. 1997) as well as ethanol (Kaneko et al. 1998) and reserpine-induced (Clementi et al. 1998) gastric injury. Icv administration of AM also causes an increase in systemic blood pressure, HR and sympathetic discharge in rats (Takahashi et al. 1994, Samson et al. 1998, Saita et al. 1998b) and rabbits (Matsumura et al. 1999). There are also studies that failed to demonstrate these central effects of AM on hemodynamic parameters (Murphy & Samson 1995, Parkes & May 1995, Charles et al. 1998). However, in vitro recordings from area postrema neurons show excitation in response to AM (Allen & Ferguson 1996) and microinjection of AM to that area induces an increase in systemic blood pressure (Allen et al. 1997). In sheep, icv AM induced rises in plasma ANP, AM, ACTH and cortisol levels, but did not affect the plasma renin activity or levels of aldosterone and arginine8-vasopressin (AVP) (Charles et al. 1998). Hyperosmolality and hypovolemia-induced but
not basal AVP release in rats has been reported to be inhibited by icv administration of AM (Yokoi et al. 1996). Release of oxytocin to plasma, in turn, is dose-dependently increased by icv AM in rats, and the increase is associated with induction of c-fos protein and gene expression in the oxytocin producing cells of the paraventricular and supraoptic nuclei in hypothalamus (Serino et al. 1999).

2.1.5.5. Effects on cell growth and division

AM inhibits the angiotensin II- and fetal bovine serum-induced increase in protein synthesis of cultured neonatal rat cardiomyocytes, and moreover, anti-AM antibody significantly enhances the basal and stimulated protein synthesis, showing that AM may act on myocytes as an autocrine or a paracrine factor modulating the cardiac growth (Tsuruda et al. 1998). Under ET-1-stimulated conditions, however, AM slightly increases the protein synthesis of cultured cardiac myocytes (Horio et al. 1999). Furthermore, an early report suggested that in cardiac myocytes AM induces expression of c-fos and activation of activator protein-1 (AP-1), which are considered to be involved in the process of cardiac hypertrophy (Sato & Autelitano 1995). In cardiac fibroblast cultures, AM consistently decreases protein synthesis and proliferation (Tsuzuura et al. 1999, Horio et al. 1999).

AM has been shown to suppress the basal and mitogen-induced proliferation of cultured glomerular mesangial cells (Chini et al. 1995, Segawa et al. 1996, Chini et al. 1997, Michibata et al. 1998), ECs (Michibata et al. 1998) and VSMCs (Kano et al. 1996, Chini et al. 1997, Michibata et al. 1998) via the cAMP pathway. In mesangial cells and VSMCs, especially the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) -stimulated proliferation and accompanying increases in cellular MAPK activity seem to be markedly, but the corresponding actions of ET-1 only slightly, inhibited by AM (Chini et al. 1997). However, AM decreases the production of ET-1 in both mesangial cells (Kohno et al. 1996a) and VSMCs (Kohno et al. 1995a), which may complement the antiproliferative action of AM. The mesangial cell proliferation induced by transmural pressure is also inhibited by AM (Osajima et al. 1999). Michibata et al. reported that neutralizing antibody against AM significantly enhances the proliferation of vascular ECs (Michibata et al. 1998). AM also stimulates apoptosis of mesangial cells (Parameswaran et al. 1999a), which, as well as the antiproliferative effect, is blocked by p38 MAPK inhibitor (Parameswaran et al. 1999b). Furthermore, AM is a potent inhibitor of PDGF and fetal calf serum induced migration of rat VSMC (Horio et al. 1995), and angiotensin II (Kohno et al. 1997) and lysophosphatidylcholine-induced (Kohno et al. 1998) migration of human coronary artery smooth muscle cells.

Growth promoting effects of AM have also been observed. AM was demonstrated to stimulate the proliferation of VSMCs via protein tyrosine kinase-mediated MAPK activation (Iwasaki et al. 1998). AM also protects ECs against apoptotic death (Kato et al. 1997, Shichiri et al. 1999). Mitogenic activity of AM has also been reported in fibroblast cell lines (Withers et al. 1996), human tumor cell lines (Miller et al. 1996), rat osteoblasts (Cornish et al. 1997) and human oral keratinocytes (Kapas et al. 1997).
2.1.5.6. Other effects

AM inhibits the acetylcholine- and histamine-induced bronchoconstriction in vivo (Kanazawa et al. 1994). AM also potentiates the bronchodilative effects of vasoactive intestinal polypeptide, isoprenaline and theophylline (Kanazawa et al. 1996). Moreover, AM inhibits the antigen-induced bronchoconstriction and airway microvascular leakage (Ohbayashi et al. 1999). AM is produced by tracheal epithelial and smooth muscle cells, but in vitro it had almost no effects on the contractions of these smooth muscles (Nishimura et al. 1997). In tracheal rings and bronchial strips, AM also failed to affect the acetylcholine-induced constriction (Pinto et al. 1996). Thus, it is still not clear whether or not AM is an epithelium derived-relaxing factor in the bronchus or bronchioles. In the stomach, AM inhibits the acid secretion (Rossowski et al. 1997) and in isolated pancreatic acini it inhibits cholecystokinin-induced amylase secretion (Tsuchida et al. 1999).

2.1.6. AM in pathophysiological conditions

AM production has been reported to be changed in a number of diseases. The plasma level of AM is elevated in patients with cardiovascular diseases (for review, see Eto et al. 1999) such as essential hypertension (Ishimitsu et al. 1994b, Kitamura et al. 1994c, Kohno et al. 1996b, Kato et al. 1999), CHF (Nishikimi et al. 1995, Jougasaki et al. 1995a, Jougasaki et al. 1996, Kobayashi et al. 1996a, Hirayama et al. 1999b), acute myocardial infarction (Kobayashi et al. 1996b, Yoshitomi et al. 1998, Miyao et al. 1998), hypertrophic cardiomyopathy (Hamada et al. 1998) and mitral stenosis with pulmonary hypertension (Nishikimi et al. 1997a, Yamamoto et al. 1998). In humans (Jougasaki et al. 1995a) and dogs (Jougasaki et al. 1997) with CHF the cardiac AM immunostaining seems to be elevated, suggesting that the dysfunctioning heart may contribute to the increased plasma levels of AM. Ventricular AM mRNA and ir-AM are also increased in experimental heart failure (Willenbrock et al. 1999), cardiac pressure (Shimokubo et al. 1995, Romppanen et al. 1997, Yoshihara et al. 2000) and volume (Nishikimi et al. 1997b, Yoshihara et al. 2000) overload and myocardial infarction (Nagaya et al. 2000b). Left ventricular AM synthesis correlates positively with the degree of left ventricular hypertrophy caused by aortic banding in rats (Morimoto et al. 1999). On the other hand, it was noticed that the induction in AM synthesis is clearly weaker than that of ANP following aortic banding or post-infarction failure in rat left ventricle (Kaiser et al. 1998), and left ventricular AM mRNA levels of spontaneously hypertensive rats do not differ from the levels of the control strain (Magga et al. 1999). Furthermore, increased aortic pressure resulted in a decreased concentration of AM mRNA in the left ventricle (Magga et al. 1998). A recent study shows that AM, its receptor and RAMP2 mRNAs are parallely elevated in ischemic heart failure (Oie et al. 2000). Catheterization studies support the AM secretory role of the heart since there is a significant increase in plasma AM between coronary sinus and aorta
of patients with CHF (Jougasaki et al. 1996, Nishikimi et al. 1997b). However, as a diagnostic marker for early CHF, AM does not seem to be useful (Daggubati et al. 1997). An elevated level of AM after acute myocardial infarction is predictive of death in a two-year follow-up (Richards et al. 1998, Nagaya et al. 1999b), but this relation is far weaker than for N-terminal BNP (Richards et al. 1998). In addition to cardiovascular diseases, AM is also increased in the plasma of patients with hyperglycemia (Hayashi et al. 1997), acute asthma (Kohno et al. 1996c), primary aldosteronism (Kato et al. 1995b), septic shock (Hirata et al. 1996, Nishio et al. 1997), renal failure (Ishimitsu et al. 1994b, Sato et al. 1995, Kubo et al. 1998b, Kubo et al. 1998c), pre-eclampsia (Di Iorio et al. 1998) and in humans with physiological stress conditions like pregnancy (Di Iorio et al. 1997) and orthostatic head-up tilting (Rössler et al. 1999).

There are investigations of the effects of AM on diseased organisms. AM decreases the blood pressure of spontaneously hypertensive rats (He et al. 1995, Khan et al. 1997a). Delivery of human AM gene to the same strain of animals also induces a hypotensive action (Chao et al. 1997). In an L-NAME-induced model of pre-eclampsia in rats, AM lowers blood pressure (Makino et al. 1999). In two-kidney, one-clip model of renovascular hypertension in rats AM reduces the blood pressure simultaneously decreasing the plasma renin activity and aldosterone concentration (Khan et al. 1997b). In rats (Nagaya et al. 1999a), sheep (Rademaker et al. 1997) or humans (Nagaya et al. 2000a) with CHF, intravenous infusion of AM results in beneficial hemodynamic and renal effects as a decrease in ventricular preload and afterload and an increase in CO, diuresis and natriuresis. Pulmonary hypertension and right ventricular hypertrophy induced by monocrotaline are lessened by chronic infusion of AM in rats (Yoshihara et al. 1998).

### 2.2. Regulation of cardiac contractility

#### 2.2.1. Excitation-contraction coupling

The overall process by which depolarization of the sarcolemma causes Ca\(^{2+}\) release into the myoplasm and Ca\(^{2+}\) binding to regulatory sites to regulate crossbridge cycling is termed excitation-contraction coupling (E-C coupling). The intracellular contractile event is initiated when the depolarization of cardiac myocyte sarcolemma opens the voltage-dependent L-type Ca\(^{2+}\) channels, which triggers a Ca\(^{2+}\) influx to the cytosol (see e.g. Clapham 1995, Hobai & Levy 1999, Fig. 3). The L-type Ca\(^{2+}\) channels are localized in the sarcolemma and in the invaginations of the sarcolemma, T-tubules (see e.g. Berne and Levy 1993), in close functional association with clustered Ca\(^{2+}\) release channels of sarcoplasmic reticulum (SR) (Sham et al. 1995), called ryanodine receptors (RyR). Activation of RyR by calcium influx is followed by an amplified release of Ca\(^{2+}\) ions from the SR calcium stores, a phenomenon known as calcium-induced calcium release (CICR) that was initially characterized in skinned cardiac myocyte preparation (Fabio & Fabio 1979, Fabio 1985). According to the present knowledge, CICR causes local increases in
[Ca^{2+}]_i, so called calcium sparks, which can be visualized by confocal imaging (Cheng et al. 1993). (for review, see Wier & Balke 1999, Niggli 1999)

The contraction is triggered when Ca^{2+} ion binds to the troponin complex, specifically N-terminal domain of troponin C (TnC), which is a two-domain protein with a central role in the initiation of crossbridge cycling (for review, see Parmacek & Leiden 1991). TnC has two specific binding sites for Ca^{2+}, and occupation of the site with lower affinity results in contraction (Pan & Solaro 1987). Binding of Ca^{2+} produces conformational changes in the distant regions of the TnC molecule, leading to interaction with TnI, another member of the troponin complex (Parmacek & Leiden 1991). Troponin I (Tnl) is a protein that under low [Ca^{2+}]_i conditions inhibits the interaction of actin (thin myofilament) and myosin (thick myofilament). Interaction of Tnl with TnC is followed by moving the tropomyosin molecule to allow the crossbridges to attach and to produce force (see e.g. Opie 1995). In crossbridges (myosin heads), ATP hydrolysis is associated with the interaction of actin and myosin head, and phosphate release initiates the power stroke. ADP is then substituted by a new ATP, which detaches the crossbridges from actin and allows the next cycle to begin. (Rayment et al. 1993)
E-C coupling is affected by several intracellular molecules. Extracellular receptor agonists can activate intracellular second messengers (see Fig. 3.), which may have an influence on the E-C coupling process, resulting in either decreased or increased contraction, negative or positive inotropic effect, respectively. The relationship between the free calcium concentration and force production is very steep (see e.g. Opie 1995). Therefore, in order to induce a positive inotropic action, a receptor agonist must increase the [Ca\(^{2+}\)], or alternatively, sensitize the contractile element to calcium, which means that the response of the myofilaments to a given level of occupancy of Ca\(^{2+}\) binding sites on TnC is increased (Endoh 1998).

Cyclic nucleotides, cAMP and cGMP, are well established second messengers that may mediate positive and negative inotropic responses, respectively. cAMP is produced from adenosine triphosphate by AC that is coupled to sarcolemmal receptors, e.g. \(\beta\)-adrenergic receptor (Steinberg 1999). cAMP subsequently activates a multisubstrate enzyme PKA, the main targets of which in cardiac myocytes are L-type Ca\(^{2+}\) channel, phospholamban (PLB) and TnI (for review, see e.g. Katz 1990, Walsh & van Patten 1994, see Fig. 3.). PLB is a sarcoplasmic membrane protein which in phosphorylated form stimulates the Ca\(^{2+}\) ATPase that is responsible for pumping Ca\(^{2+}\) into SR Ca\(^{2+}\) stores (Fujii et al. 1987). By phosphorylating TnI, PKA enhances the interaction between TnI and actin, thus decreasing the sensitivity of the contractile apparatus to Ca\(^{2+}\) (Venema & Kuo 1993). However, this potential negative inotropic effect is normally overcome by a marked increase in [Ca\(^{2+}\)], due to stimulation of Ca\(^{2+}\) influx through L-type calcium channels, for example under the influence of \(\beta\)-adrenergic agonist (Katz 1990).

Phosphoinositide hydrolysis by phospholipase C (PLC) results in intracellular accumulation of IP\(_3\) and DAG (see Lee & Rhee 1995, Fig. 3.). IP\(_3\) has a receptor on SR and may release Ca\(^{2+}\) from the SR stores (for review, see e.g. Berridge 1993), but the role of this molecule as an inductor of [Ca\(^{2+}\)], in cardiomyocytes is probably less important than in non-excitable cells (see e.g. Clapham 1995). DAG is known to activate PKC, which is a structurally homologous group of isoenzymes with divergent intracellular actions (Sugden & Bogoyevitch 1995). Pi et al. demonstrated by using a light-sensitive caged DAG compound that DAG has a positive inotropic action which can be abolished by chelerytrine, a PKC inhibitor (Pi et al. 1997). PKC may enhance the contraction by activating the L-type Ca\(^{2+}\) channel (Lacerda et al. 1988). PKC is also capable of activating the Na\(^{+}-H^+\)-exchanger of cardiac myocytes, which may lead to intracellular alkalinization and increased sensitivity of myofilaments to calcium (see e.g. Karmazyn et al. 1999). However, it has been shown that PKC directly phosphorylates TnI, leading to decreased activity of actomyosin Mg\(^{2+}\)-ATPase, the molecular basis of contraction (Venema & Kuo 1993). PKC also seems to inhibit the Ca\(^{2+}\)-ATPase of SR (Rogers et al. 1990). The significance of these findings in the regulation of contractility remains to be solved.
2.2.2. Role of the autonomic nervous system

The autonomic nervous system is the main extrinsic regulator of myocardial contractile function. Peripheral autonomic neurons receive afferent inputs from mechanosensory and chemosensory nerve endings located in cardiovascular and pulmonary tissues as well as efferent inputs from central neurons. Based on these input signals two reciprocally acting efferent peripheral neurone systems, sympathetic and parasympathetic, augment and suppress the cardiac indices, respectively. (for review, see e.g. Horackova & Armour 1995)

Sympathetic responses are generated by the release of noradrenaline from the sympathetic nerve endings and by the activation of adrenergic receptors that follows. β-adrenergic receptors are the predominant type in the myocardium. (see e.g. Berne & Levy 1993, Steinberg 1999) They are coupled to intracellular AC to increase the cAMP content in the cardiac myocytes, which is followed by a positive inotropic effect (Robison et al. 1965, Steinberg 1999). α-adrenergic receptors also exist in cardiomyocytes and their stimulation may lead to a positive inotropic response in a PLC-PKC mediated manner (Otani et al. 1990). Sensitization of the myofilaments to Ca^{2+} has also been observed (Endoh & Blinks 1988). However, the effect of α-adrenergic agonists on contractility has been relatively inconsistent (Opie 1998).

Cardiac parasympathetic signals through the vagal nerve lead to a decreased force of contraction. Stimulation of myocardial muscarinergic receptors by acetylcholine has for a long time been known to be coupled to increased intracellular cGMP (George et al. 1970). Recent data, however, suggest that the increase is secondary to enhanced synthesis of NO and not a direct receptor activated stimulation of guanylyl cyclase (GC) (see Section 2.2.5.2.).

2.2.3. Frank-Starling mechanism

The Frank-Starling relation is an important intrinsic regulatory mechanism of myocardial contractility. It was discovered in 1895 by Frank that the greater the preload, the greater the force generated by frog cardiac muscle. In 1914 Starling was able to demonstrate the same phenomenon in canine heart-lung preparation by elevating either right atrial pressure or aortic resistance. (see e.g. Berne & Levy 1993)

The response of myocardium to an increased sarcomere length is biphasic (for review, see Kentish 1999). A slowly developing increase in the force of contraction follows the abrupt response to the stretch. (Parmley & Chuck 1973) The intracellular events underlying the Frank-Starling relation are largely unknown. However, since the rapid phase is not accompanied with a rise in [Ca^{2+}], it is generally supposed that stretching increases the calcium affinity of TnC by a yet unknown mechanism (Allen & Kentish 1988). In contrast, the slow phase of the Frank-Starling mechanism is associated with an increase in [Ca^{2+}], (Allen & Kurihara 1982), but no clear model to explain the reasons behind it exists to date. Kentish & Wroze showed that the slow force increase is not due to a rise in diastolic [Ca^{2+}], which could theoretically have led to an increased
sequestration of Ca\(^{2+}\) by SR (Kentish & Wrzosek 1998). Interestingly, recent reports suggested that the slow rise in contractility could result from an indirect increase in intracellular pH, namely caused by stretch-induced sequential release of autocrine/paracrine factors angiotensin II and ET-1, and subsequent activation of sarcolemmal Na\(^+\)-H\(^+\)-exchanger (Cingolani et al. 1998, Alvarez et al. 1999). However, the mechanism how the exchanger mediates the slow response remains obscure.

### 2.2.4. Circulating hormones

Circulating hormones can also have an impact on the myocardial performance. Adrenomedullary hormones, adrenaline and noradrenaline, exert effects via cardiac myocyte \(\alpha\) and \(\beta\)-adrenergic receptors. It is likely, however, that under normal conditions the circulating catecholamines have only minor effects on contractility compared with the influence of the sympathetic nervous system, which is usually activated in parallel with the adrenomedullary hormone release. (Berne & Levy 1993) The hearts of hyperthyroid rats show increased contractile parameters compared with hearts of euthyroid rats (Kolar et al. 1992), but thyroid hormones exert also acute positive inotropic effects, e.g. in isolated rat heart (Segal et al. 1996). Pancreatic hormones, insulin and glucagon (Farah 1983), as well as circulating insulin-like growth factor (Cittadini et al. 1998), have a direct inotropic effect on myocardial contraction, but the physiological significance of these actions is not known.

### 2.2.5. Autocrine/paracrine factors

The finding that disruption of endocardial endothelium of isolated cat papillary muscles results in a shorter contraction with a lower peak force (Brutsaert et al. 1988) was the early evidence suggesting that myocardial cells synthesize and secrete different substances that are able to regulate the cardiac function. Thereafter, cross-talk among the different cell types has been recognized to be of considerable importance and a great number of reports have described the autocrine/paracrine mechanisms in the regulation of cardiac contractility. (for review, see Shah 1996, Winegrad 1997).

#### 2.2.5.1. ET-1

ET-1 is a 21-amino acid peptide that was originally isolated from the supernatant of porcine aortic ECs when searching for the protease-sensitive mediators of endothelium-derived vasoconstriction (Yanagisawa et al. 1988). Mature ET-1 is formed by enzymatic processing of precursor proET-1 (big ET-1) by endothelin-converting enzymes (for
review, see Shiffrin & Touyz 1998). ET-1 belongs to the endothelin family of peptides with ET-2 and ET-3, the separate genes of which have also been cloned (Inoue et al. 1989). Production of ET-1 in the endothelium is regulated by different stimuli, including shear stress, hypoxia and various chemicals. ECs mainly secrete ET-1 abuminally and ET-1 acts in a autocrine or paracrine fashion on cells in its immediate vicinity. Circulating ET-1 is considered to be a result of peptide overflow from the vascular endothelium. (for review, see Shiffrin & Touyz 1998, Miyauchi & Masaki 1999)

Two receptor subtypes for endothelins have been cloned, namely ET_A receptor (Arai et al. 1990) that preferentially binds ET-1 and ET_B receptor (Sakurai et al. 1990) that is a non-isopeptide-selective receptor. Studies in several vascular preparations, e.g. porcine coronary artery, indicate that ET-1 is the most potent vasoconstrictive substance known so far (Yanagisawa et al. 1988). Consistently, intravenous infusion of ET-1 results in increased mean arterial pressure, which is accompanied with a decrease in HR and CO and dramatic falls in RBF and GFR in dogs (Goetz et al. 1988, Miller et al. 1989). The renal and cardiac effects were preserved when ET-1 was administered at lower, pathophysiological concentrations, although there was no increase in blood pressure (Lerman et al. 1991). ET-1 usually induces vasoconstriction by stimulating ET_A receptor subtype on the VSMCs (Takuwa et al. 1990), but involvement of the ET_B receptor has also been demonstrated under certain conditions (Clozel et al. 1992, Moreland et al. 1992, McMurdo et al. 1993, Seo et al. 1994). The vasopressor effect of ET-1 has been shown to include accumulation of DAG and IP_3 likely following activation of PLC, mobilization of Ca^{2+} from the intracellular calcium stores and activation of voltage-operated calcium channels of VSMCs (Resink et al. 1988, Marsden et al. 1989, Goto et al. 1989, Kasuya et al. 1989, Lang et al. 1997). Early reports showed that ET-1 is also able to release endothelium-derived relaxing factors, prostacyclin and NO, which in turn attenuate the vasoconstrictor response to ET-1 (de Nucci et al. 1988, Lüscher et al. 1990). Hirata et al. showed that this is a result following the activation of the ET_B receptor on ECs, including increases in cellular IP_3 production and [Ca^{2+}], (Hirata et al. 1993). Similarly, stimulation of ET_B receptors releases vasodilator AM from ECs (Jougasaki et al. 1998).

In the heart ET-1 is produced specifically by microvascular (Nishida et al. 1993) and endocardial (Mebazaa et al. 1993) ECs, but also by cardiac myocytes (Suzuki et al. 1993). Although the levels of ET-1 in plasma are low, high concentrations are found in the pericardial fluid (Horkay et al. 1995, Szokodi et al. 1998). Recently, ET-1 concentration in the interstitial transudate of perfused hearts was found to be higher than the concentration in the coronary effluent (Brunner 1997). These findings suggest that ET-1 released by myocardial cells may be an important autocrine/paracrine factor in the regulation of myocardial functions. In support of this, the superfusate of cultured endocardial ECs induces a positive inotropic action in rat ventricular myocytes, which can be blocked by adding ET-1-specific antiserum (Mebazaa et al. 1993). An ET_A receptor antagonist was also shown to reduce the contractility of isolated rat ventricular trabeculae (McClellan et al. 1995). Consistently, exogenous ET-1 has a potent positive inotropic action in myocardial preparations and isolated myocytes (Ishikawa et al. 1988, Hu et al. 1988, Moravec et al. 1989, Kelly et al. 1990, Kramer et al. 1991). However, many studies in isolated hearts (Karwatowska-Prokopczuk & Wennmalm 1990, Neubauer et al. 1990) or in vivo (Domenech et al. 1991, Yang et al. 1991) have failed to show that action or showed negative inotropic action. This may be due to the strong coronary vasoconstrictor
response to ET-1 since by preventing the pressor response with a vasodilator the inotropic effect is unmasked (Beyer et al. 1999).

The inotropic effect of ET-1 has been shown to be associated with degradation of phospholipids in cardiac cells (Vigne et al. 1989). According to the present knowledge, the inotropic response to ET-1 is due to intracellular alkalosis and increased sensitivity of myofilaments to calcium, which is caused by stimulation of PLC, subsequent activation of PKC and plasma membrane Na+-H+ -antiporter (Kelly et al. 1990, Wang et al. 1991, Kramer et al. 1991, Khandoudi et al. 1994, Pi et al. 1997). There are, however, also reports showing that ET-1 is able to raise the [Ca^{2+}] of cardiac myocytes (Wang et al. 1991, Qiu et al. 1992, Lauer et al. 1992, Katoh et al. 1998). A recent report suggested that the inotropic effect of low doses of ET-1 increases the Ca^{2+} transient whereas the responses to higher concentrations may involve a rise in myofilament sensitivity to Ca^{2+} (Yang et al. 1999).

Plasma levels of ET-1 are increased under several pathophysiological conditions (for review, see Miyauchi & Masaki 1999, Shiffrin & Touyz 1998), such as myocardial infarction (Miyauchi et al. 1989) and CHF (McMurray et al. 1992). In experimental heart failure, ET-1 is also increased in the ventricular tissue (Sakai et al. 1996a). In the early phase of CHF, ET-1 may have a beneficial role in the maintenance of myocardial contractility (Sakai et al. 1996a), but in long term this role may be overcome by the adverse effects, such as stimulation of myocyte hypertrophy (Shubeita et al. 1990), since the blockade of ET receptors increases the survival of rats (Sakai et al. 1996b) and improves the left ventricular performance of dogs (Shimoyama et al. 1996) and rabbits (Spinale et al. 1997) with CHF. Interestingly, ET-1 has been shown to increase the contractility of normal cardiomyocytes, but to exert a negative inotropic effect on cells isolated from failing ventricular myocardium (Thomas et al. 1996). Very recently, MacCarthy et al. showed that inhibition of ET_{A} receptors significantly impairs the contractile function of normal human left ventricle, but has no effect in patients with dilated cardiomyopathy (MacCarthy et al. 2000). These findings may partially explain the advantageous effects of the blockade of endogenous ET-1 in CHF.

### 2.2.5.2. NO

Furchgott and Zawadzki demonstrated that vascular endothelium releases a factor, called endothelium-derived relaxing factor, that is essential for the vasodilator effect of acetylcholine (Furchgott & Zawadzki 1980). Later, NO was shown to possess the characteristics of this factor (Palmer et al. 1987). NO is a free radical gas that is synthesized by NOS enzymes that catalyze its formation from L-arginine. Three distinct NOS isoenzymes have been discovered, namely endothelial constitutively expressed NOS (NOS3), inducible NOS (NOS2) and neuronal NOS (NOS1) (for review, see Nathan & Xie 1994, Griffith & Stuehr 1995). NOS1 mainly functions in the central and peripheral nerves. NOS2 is largely undetectable in normal conditions but induced by various stimuli, especially cytokines (Griffith & Stuehr 1995). NOS3 is a calcium dependent enzyme that constitutively produces NO in ECs. The gene expression and activity of vascular NOS3 is
regulated by different factors (for review, see Fleming & Busse 1999), but the fluid shear stress exerted on the endothelium by the streaming blood represents the major stimulus for a continuous production of NO in vivo (Lamontagne et al. 1992). In ECs, NOS3 is located in caveolae, small invaginations of plasma membrane, forming there a heteromeric complex with caveolin, that is a protein attaching NOS3 to the plasma membrane. A transient increase in [Ca^{2+}], by a receptor agonist initiates the activation of NOS3 via Ca^{2+}/calmodulin complex that disrupts the complex of NOS3 and caveolin. (Feron et al. 1998a) Very recently, however, NOS3 has been shown to be activated also by a Ca^{2+}-independent pathway, phosphorylation by cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) (Butt et al. 2000). Consequently, NO released from ECs activates the soluble GC (sGC) of VSMCs leading to increased cellular content of cGMP and vasorelaxation (Fleming & Busse 1999).

NO synthesis also takes place in the heart (for review, see Kelly et al. 1996, Hare & Stamler 1999). NOS3 has been detected in endocardial (Schulz et al. 1991) and in microvascular (Pollock et al. 1993) endothelium but also in cardiac myocytes (Balligand et al. 1993a, Balligand et al. 1995a, Seki et al. 1996), in which it is also localized to the caveolae (Feron et al. 1998b). NOS2 is not detectable in physiological conditions but stimulation with cytokines induces the gene expression in 6 hours in cardiac myocytes (Balligand et al. 1994) and microvascular endothelial cells (Balligand et al. 1995b). Recently, the NOS1 isoenzyme has been reported to be detectable in the heart and localized in the sarcoplasmic reticulum of cardiac myocytes (Xu et al. 1999).

NO has been shown to regulate cardiac contractility (for review, see Kelly et al. 1996, Kojda & Kottenberg 1999). Brady et al. demonstrated that sodium nitroprusside, an NO donor drug, decreased the contraction amplitude by 20 % when added directly to isolated cardiac myocytes (Brady et al. 1993). Later it was shown that physiological concentrations of NO did not elicit any effect on contractility of cat papillary muscle although the same concentrations dose-dependently relaxed cat coronary artery rings (Weyrich et al. 1994). However, there is evidence in the literature that if NO is administered exogenously by NO donors, the effect on contractility is biphasic. Low concentrations that produce a small rise in intracellular cGMP evoke an increase in cell shortening, whereas higher doses strongly increasing cGMP depress myocyte contractility. (Kojda et al. 1996, Mohan et al. 1996, Shirayama & Pappano 1996, Preckel et al. 1997) This is in agreement with the findings showing that low doses of NO donor or cGMP stimulate, but higher concentrations strongly reduce L-type calcium current in ventricular myocytes in cGMP-mediated way (Mery et al. 1993, Kirstein et al. 1995, Shirayama & Pappano 1996).

Cyclic GMP may initiate several intracellular events through binding to its effector proteins such as PKG and cGMP-inhibited cAMP-phosphodiesterase (for review, see Lohmann et al. 1991, Kojda & Kottenberg 1999). PKG may directly inhibit the Ca^{2+} current of cardiac myocytes at relatively high concentrations (Mery et al. 1991). It has also been shown that cGMP analog reduces the sensitivity of myofilaments to Ca^{2+} in cardiac myocytes, which is prevented by PKG inhibitor (Shah et al. 1994). Kojda et al. showed that the positive inotropic response to low doses of NO donors was associated with an increase in intracellular cAMP and abolished by PKA inhibitor (Kojda et al. 1996). This may result from binding of cGMP to the cGMP-inhibited cAMP-phosphodiesterase, which has been shown to lead to an increase in the Ca^{2+} current (Mery et al. 1993), but recent data suggest that the rise in cAMP can also be due to a direct stimulation of adenylate cyclase by NO
(Vila-Petroff et al. 1999). The NO-cGMP-induced decrease in the Ca\textsuperscript{2+} current may also involve the activation of cGMP-stimulated cAMP phosphodiesterase (Mery et al. 1993). According to the present knowledge, NO is also able to regulate cardiac contractility through cGMP-independent pathways. It was suggested that nitrosylation of the L-type calcium channel by extracellular NO stimulates the myocyte Ca\textsuperscript{2+} current (Campbell et al. 1996). The activity of RyR on SR was also shown to be increased by direct nitrosylation by reactive NO (Xu et al. 1998). Finally, SR was demonstrated to possess functional NOS, which was shown to inhibit Ca\textsuperscript{2+}-ATPase activity of SR (Xu et al. 1999).

It was shown by Balligand et al. that rat cardiac myocytes respond functionally to products of an endogenous NOS, since the negative chronotropic response to muscarinergic agonist was abolished and the positive inotropic response to β-adrenergic agonist was enhanced by a NOS inhibitor (Balligand et al. 1993a). NOS3 was confirmed to be the isoenzyme responsible for these actions and moreover, for the decrease in Ca\textsuperscript{2+} current in response to muscarinergic agonists (Balligand et al. 1995a). NO release of rat isolated cardiac myocytes in response to β-agonists was demonstrated by a porphyrinic microsensor (Kanai et al. 1997). Cardiac NO synthesis is also upregulated by ET-1 (Ebihara et al. 1996), but there is no evidence of whether this could affect the inotropic action of ET-1. The control of β-adrenergic inotropic response by NOS3 has also been observed in open chest dogs (Keaney et al. 1996). Also the attenuation of β-adrenergic inotropic effect by vagal nerve stimulation is, at least partially, due to endogenous myocardial NO production (Hare et al. 1995a). Consistently, the muscarinergic receptors seem to be functionally coupled to the NOS-caveolin protein complex (Feron et al. 1998b). Surprisingly, however, the mice deficient in NOS3 gene appeared to have unchanged responses to both muscarinergic and β-adrenergic agents when studied in the papillary muscle preparation (Vandecasteele et al. 1999), although two earlier studies reported that cardiac myocytes isolated from the same animals did not respond normally to muscarinergic agonists (Han et al. 1998, Feron et al. 1998b, see also Hare & Stamler 1999). Of importance is the finding that treatment with cAMP-elevating agents decreases the expression of NOS3 in cardiac myocytes and augments the contractile response to β-agonists, while the negative inotropic effect of muscarinic cholinergic agonists is attenuated (Belhassen et al. 1996).

Kojda et al. reported that NOS inhibitors have a negative inotropic effect in constant flow perfused rat heart, suggesting that endogenous NO maintains the contractility of the heart (Kojda et al. 1997). A result along the same line is that NOS inhibitors attenuate the Frank-Starling response of isolated ejecting guinea-pig hearts, the mechanism of which is thought to probably involve direct effects on myocardial diastolic and/or systolic function (Prendergast et al. 1997). In support of this, NO has been shown to improve the relaxation parameters of the heart (Grocott-Mason et al. 1994, Paulus et al. 1994).

The NOS2 isoenzyme may be involved in some paracrine effects of NO under pathophysiological conditions. Depressed contractility of cardiac myocytes of endotoxin-treated guinea-pigs was reversed by NOS inhibition (Brady et al. 1992). Similarly, exposure of cardiac myocytes to culture medium from macrophages treated with endotoxin resulted in decreased inotropic responses to β-adrenergic agonists, which was prevented with NOS inhibitor (Balligand et al. 1993b). Coculture of cardiac myocytes with IL-1β-treated cardiac microvascular ECs produced a similar NOS inhibitor-sensitive suppression
of β-agonist effect (Ungureanu-Longrois et al. 1995). In dogs with rapid-pacing induced heart failure, the NO production was increased in the myocardium. N⁶-nitro-L-arginine methyl ester (L-NAME) enhanced the inotropic response to isoprenaline in cardiac myocytes isolated from those hearts. (Yamamoto et al. 1997) Poor responsiveness of failing human myocardium to β-adrenergic agonists was shown to be explained by NO production, since NO inhibitor could augment the responses in patients with left ventricular dysfunction (Hare et al. 1995b, Hare et al. 1998) but not in control subjects with normal left ventricular performance (Hare et al. 1998). It was suggested that this may be due to the increased production of NO by NOS2 isoenzyme, because Ca²⁺-independent NOS activity seems to be dominant in the right ventricular endomyocardial biopsies in patients with dilated cardiomyopathy (de Belder et al. 1993). However, experiments in dogs with pacing-induced heart failure show that after an early rise observed at two weeks (O’Murchu et al. 1994), cardiac NO production diminishes after the transition to decompensated cardiac failure (Recchia et al. 1998). Recently, Heymes et al. reported a positive correlation between NOS2 and NOS3 gene expression and indices of left ventricular performance in failing human heart. Stimulation of NO release by substance P was shown to improve left ventricular dysfunction, suggesting that NO may exert beneficial hemodynamic effects through maintenance of the Frank-Starling response in failing heart. (Heymes et al. 1999)

2.2.5.3. Others

All members of the renin-angiotensin system are found in the heart, and angiotensin II is produced in the myocardium (for review, see Dostal & Baker 1999). Angiotensin II is known to have a positive inotropic action that may involve phosphoinositide hydrolysis (Ishihata & Endoh 1993), suggesting that it may be one of the autocrine/paracrine regulators of myocardial contractility. Endogenous bradykinin has been shown to have a role in left ventricular relaxation, probably through a release of NO (Anning et al. 1995). Natriuretic peptides may also have minor effects on contractility, particularly the relaxation parameters, but the results of the role of myocardium-derived prostaglandins are very inconsistent (Shah 1996).

Ramaciotti et al. used the coronary effluent of differently perfused rat hearts as bathing medium for isolated ventricular trabeculae. It was concluded that the effluent contains both contractility up- and down-regulating factors that are released in response to changes in perfusate oxygen saturation or coronary flow, but these were deduced not to be ET-1 or NO, however (Ramaciotti et al. 1993). Therefore, it is possible that new local regulators of myocardial contraction will be discovered in the future.
2.3. Coronary vascular tone

Regulation of coronary artery tone is an important topic of research since it partially determines the adequate blood supply of the myocardium. The pathogenesis of acute coronary artery syndromes, a leading cause of morbidity and mortality in western countries, involves dysfunction of vascular endothelium. It is characterized by increased synthesis of the local endothelial vasoconstrictor factors, such as ET-1 (Zeiher et al. 1994), and decreased synthesis of endothelial vasodilator factors, such as NO. (for review, see Fuster et al. 1992, Lüscher et al. 1995) NO is the main mediator of the endothelium-mediated vasodilation (see Section 2.2.5.2.). The major stimulus for NO release also in coronary artery endothelium is shear stress (Lamontagne et al. 1992). However, in addition to adapting to the changes in the coronary blood flow, NO synthesis continuously counterbalances the vasoconstrictor effect of ET-1, which is the main endothelium-derived vasoconstrictor. An electron microscopic autoradiographic study showed that in human coronary artery ETA receptor is the predominant subtype and ECs possess virtually no ET receptors (Russell et al. 1997). In porcine coronary artery, vasoconstriction induced by low doses of ET-1 was not inhibited by ET_A receptor antagonist. On the other hand, a selective ET_B receptor agonist, sarafotoxin S6c, induced a pressor response under conditions of ET_A blockade, suggesting that in porcine coronary arteries the ET_B receptor, as well as the ET_A receptor, mediates vasoconstriction. (Seo et al. 1994) However, it has also been demonstrated in the coronary vasculature that the pressor effect of ET-1 is enhanced by inhibition of NOS (Wang et al. 1994, Garcia et al. 1996), which indicates that coronary artery ECs express functional ET receptors capable of NO release. Recently, a new mechanism to endogenously oppose the pressor effect of ET-1 was suggested by Jougasaki et al. by demonstrating that stimulation of ET_B receptor on ECs results in endothelial release of AM (Jougasaki et al. 1998). In coronary arteries of perfused rat hearts (Entzeroth et al. 1995), open-chest dogs (Sabates et al. 1997) and in isolated porcine (Yoshimoto et al. 1998) and rat (Sheykhzade & Nyborg 1998) coronary artery rings and porcine coronary artery strips (Kureishi et al. 1995), AM has been shown to exert a vasodilator effect. In porcine coronary artery rings the effect of AM was shown to be independent of NO production (Yoshimoto et al. 1998), but this has not been confirmed in other coronary artery preparations.

2.4. Natriuretic peptide system

The heart was demonstrated to take part in the hormonal regulation of body fluid and electrolyte balance as extracts of atrial myocardium were observed to result in potent diuretic and natriuretic effect when infused into rat circulation (de Bold et al. 1981). This observation led to the discovery of members of the natriuretic peptide family, ANP (Flynn et al. 1983, Atlas et al. 1984), BNP (Sudoh et al. 1988) and C-type natriuretic peptide (CNP) (Sudoh et al. 1990). Structurally, natriuretic peptides share a common 17-residue ring motif formed by an intramolecular disulfide linkage between two cysteine residues. The amino acid sequence of ANP and CNP is highly homologous between species, but the
sequence of BNP is more variable (for review, see Ruskoaho 1992). There are three receptors for natriuretic peptides, namely NPRα, NPRβ and NPRC receptors, that are widely distributed in different tissues and cell types. NPRα and NPRβ are coupled to guanylate cyclase, while NPRC is regarded as a clearance receptor. (for review, see Nakao et al. 1992b) By activating these receptors ANP and BNP secreted by the heart initiate a number of important biological effects, most importantly including diuresis and natriuresis, vasodilator and hypotensive action, and inhibition of renin, aldosterone, vasopressin and ET-1 release (for review, see Wilkins et al. 1997, Levin et al. 1998).

Mature ANP, which is the biologically active peptide secreted to the circulation, is a 28-amino acid peptide that is stored in atrial secretory granules in its 127-amino acid proANP form (for review, see Ruskoaho 1992). Atrial tissue is very rich in ANP mRNA (Seidman et al. 1984), but the synthesis of ANP is also present in the ventricles, although to a clearly lesser degree (Zivin et al. 1984). BNP consists of 32 amino acids in humans and 45 amino acids in rats (see e.g. Nakao et al. 1992a). It is produced in both ventricles and atria, but because of the larger mass of the ventricular muscle, circulating BNP is considered to be mainly of ventricular origin (Ogawa et al. 1991). CNP was originally found in the brain (Sudoh et al. 1990), but there is some evidence suggesting that CNP could be produced by vascular ECs and act as a local regulator of vascular tone (Suga et al. 1992).

Wall stretch is the major stimulus for the release of ANP (Lang et al. 1985, Ruskoaho et al. 1986, Mäntymaa et al. 1993) and BNP (Mäntymaa et al. 1993) in the atria. This is a consequence of the fact that BNP is largely colocalized with ANP in the same atrial granules (Nakamura et al. 1991, Hasegawa et al. 1991). Additionally, many hormones and chemicals affect the ANP exocytosis (for review, see Ruskoaho 1992). For example, ET-1 has been shown to increase the secretion of ANP in vivo (Goetz et al. 1988, Miller et al. 1989) and also in experimental models in vitro, such as cultured isolated atrial myocytes (Fukuda et al. 1988) and isolated perfused rat heart (Mäntymaa et al. 1990). There is also evidence that NO production could affect ANP release. In isolated rat atria, removal of endothelium by saponin or administration of NO inhibitors increases the release of ANP (Sanchez-Ferrer et al. 1990). Furthermore, intravenous infusion of L-NAME dose-dependently stimulates the plasma ANP of rats, and the increased ANP does not correlate with changes in right atrial pressure (Leskinen et al. 1995).

In the ventricles, a small number of secretory granules exist, and ANP and BNP are preferably secreted via a constitutive pathway, which indicates that the changes in their gene expression are a crucial factor in determining the ventricular secretory rate of ANP and BNP (see e.g. Ruskoaho 1992). Ventricular gene expression of BNP is increased in response to various stimuli (for review, see Ruskoaho 1992), but most characteristically to ventricular wall stretching (Kinnunen et al. 1993). It is also well established that ET-1 stimulates BNP gene expression in cultured ventricular myocytes (Nakagawa et al. 1995) as well as in ventricles of perfused rat heart, where the induction is greater in the right than in the left ventricle (Magga et al. 1998).
3. Aims of the research

The aim of the present study was to investigate the effects of AM on myocardial contractile and endocrine function and coronary artery tone. Specifically the aims were:

1. To determine the effects of AM on myocardial contractility and heart rate.

2. To study the intracellular mechanisms of the cardiac actions of AM. Because AM is known to stimulate cAMP in many cell types, the hypothesis that the cardiac actions of AM are also mediated by cAMP was tested first.

3. To investigate the interaction of AM, ET-1 and NO at the level of myocardial contractility.

4. To find out whether exogenous AM can attenuate the pressor effect of ET-1 and whether or not the vasodilatory action of AM in the rat coronary vasculature is NO-dependent.

5. To study the influence of AM on the synthesis and release of natriuretic peptides.
4. Materials and methods

4.1. Materials

Synthetic rat peptides AM, PAMP, CGRP, CGRP_8-37 and ET-1 were purchased from Peninsula Laboratories Europe or Phoenix Pharmaceuticals, Inc. Other drugs used were N-[2-((p-bromo-cinnamyl)amino)ethyl]-5-isoquinoline-sulfonamide (H-89) (Seiaku Corp); ryanodine and thapsigargin (Calbiochem); staurosporine and isoprenaline hydrochloride (Sigma Chemical Co); diltiazem hydrochloride (Orion Pharmaceuticals Ltd); L-NAME (Sigma Chemical Co) and heparin (Leiras). Bosentan was generously supplied by Dr. Martine Clozel from F. Hoffmann-La Roche Ltd. Peptides, ryanodine, isoproterenol, diltiazem and L-NAME were dissolved in 0.9% saline, other compounds in DMSO. The final concentration of both solvents was <0.03%.

4.2. Animals

Male 1.5 - 2 month-old Sprague-Dawley (SD) rats from the colony of the Center of Experimental Animals at the University of Oulu, Finland, were used. The rats were housed in plastic cages in a room with a controlled humidity of 40% and a temperature of 22 °C. A 12 h light and 12 h dark environmental light cycle was maintained. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu. The investigation conforms with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes.

4.3. Isolated perfused rat heart preparation (I-V)

The isolated, perfused rat heart preparation used in this study was a modification of that previously described (Ruskoaho et al. 1986, Magga et al. 1997). Briefly, rats were injected with heparin (500 IU/kg body weight i.p.) and decapitated 20 minutes later. The
abdominal cavity was immediately opened, the diaphragm transected, lateral incisions made along both sides of the rib cage, and the heart cooled with perfusion fluid (4-10 °C). The aorta was cannulated above the aortic valve and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH 7.40, equilibrated with 95 % O₂ - 5 % CO₂ at 37 °C. The composition of the buffer was (mmol/L): NaCl 113.8, NaHCO₃ 22.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.1, CaCl₂ 2.5 and glucose 11.0.

Variations in the perfusion pressure, arising from changes in coronary vascular resistance, were recorded on a Grass polygraph (model 7DA, Grass Instruments) with a pressure transducer (model MP-15, Micron Instruments) situated on a side-arm of the aortic cannula. Isometric force of contraction (apicobasal displacement) was recorded by a force-displacement transducer (model FT03, Grass Instruments) connected to the Grass polygraph. The heart rate was counted from contractions by a Grass tachograph. The hearts were set under resting tension (RT) of 1.0, 2.0 or 3.0 g and stimulated via atrial electrodes (spontaneously beating hearts were also used) (11 V, 0.5 ms) with a Grass stimulator (model S88, Grass Instruments) to increase heart rate to the level of 300 beats/min or 15-20 % above the spontaneous rhythm. Each experiment was started by perfusing the heart for 50 or 60 min (equilibration period) using a flow rate of 7 or 5 ml/min with a peristaltic pump (Minipuls 3, model 312, Gilson). During the experimental period that followed, the hearts were perfused at a constant flow rate of 5 ml/min. Drugs were infused via an infusion pump (Secan PSA 55, Skyelectronics S.A.) into the aortic perfusion cannula at a rate of 0.5 ml/min. In a subset of experiments, the pulmonary veins and the superior cava vein were ligated and a cannula was inserted to the pulmonary artery in order to adjust the right atrial pressure by means of changing the vertical position of the distal tip of the cannula (Magga et al. 1997). In those experiments, 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).

4.4. Experimental design

4.4.1. Effect of AM on spontaneously beating perfused rat heart (I)

In order to characterize first the effects of AM on inotropic and chronotropic state of the isolated perfused rat heart, the spontaneously beating preparation was used. After the equilibration period and a 10 min control period, vehicle, AM (0.01 to 10 nmol/l) and AM combined with the CGRP receptor antagonist CGRP8-37 (360 nmol/l) were infused to the coronary circulation for 30 min. (I)
4.4.2. Intracellular mechanisms underlying the inotropic effect of AM (II)

Since we found that AM had a positive inotropic effect but no effect on chronotropism, we performed the next set of experiments in a similar preparation but with atrial pacing to study the intracellular signaling mechanisms underlying the inotropic action. The initial RT was set at 2 g. First, vehicle, AM (0.1 to 1 nmol/l), PAMP (10 to 100 nmol/l) or CGRP (1 nmol/l) were infused to the coronary circulation for 30 min to clarify their responses in paced heart preparation. CGRP<sub>8-37</sub> (100 nmol/l) was infused in combination with AM (1 nmol/l) to study the involvement of CGRP receptors. In signal transduction studies, AM was infused at a concentration of 1 nmol/l. Vehicle, AM alone or in combination with different drugs (H-89, 100 nmol/l; ryanodine, 3 nmol/l; thapsigargin, 30 nmol/l; staurosporine, 10 nmol/l and diltiazem, 1 µmol/l) were added into the perfusion fluid for 30 min. Thapsigargin was also administered as a pretreatment (10 min, 30 nmol/l), following a 10 min control period and subsequent AM infusion. The efficiency of H-89 as PKA inhibitor was tested separately by means of inhibiting the actions of isoprenaline (1 µmol/l). Coronary venous effluent was collected at 2-minute intervals during the control period and the 30 min experimental period for the ANP radioimmunoassay. For measurement of ventricular cAMP, the hearts were perfused with vehicle, AM (1 nmol/l) or isoprenaline (1 µmol/l) for 2, 5 or 30 minutes, after which the ventricular tissue was immediately frozen (-70°C) until assayed.

4.4.3. Interaction of AM, ET-1 and NO (III)

To study the role of endogenous myocardial NO as modulator of inotropic responses to AM (0.03 and 1 nmol/l) and ET-1 (0.08 and 1 nmol/l), vehicle and peptides were added alone or in combination with L-NAME (300 µmol/l) or bosentan (1 µmol/L, a mixed ET<sub>A/B</sub> receptor antagonist) to the aortic perfusion cannula of perfused, paced rat heart as a continuous infusion for 30 minutes. Preliminary experiments were performed to determine the maximal responses to AM and ET-1 and also concentrations of the peptides resulting in clearly submaximal but equal responses. The contractile response to the combined infusion of AM and ET-1 was also studied.

4.4.4. Effect of AM on coronary artery tone (IV)

To characterize the coronary vasodilator action of AM, the paced perfused rat heart was employed at constant coronary flow of 5 ml/min. AM (0.03 and 1 nmol/l) was infused to the coronary circulation for 30 min in the absence or presence of L-NAME (300 µmol/l). Since ET-1 has been shown to release AM from ECs (Jougasaki et al. 1998) and suggested to counterbalance the vasoconstrictor effect of ET-1, we determined the effect of AM on ET-1 (0.08 and 1 nmol/l)-induced vasoconstriction.
4.4.5. Effect of AM on the natriuretic peptide system (V)

Effect of AM on atrial ANP release was studied by infusing AM (0.03 and 1 nmol/l) into the coronary perfusion fluid of perfused, paced rat hearts for 30 min in the absence or presence of L-NAME. Since atrial wall stretch is the main stimulus for ANP release (Lang et al. 1985), in part of the experiments the right atrial pressure was elevated by 5 mmHg, as described previously (Magga et al. 1977), for 10 min after a 20-min pretreatment with AM and/or L-NAME. Coronary venous effluent was collected at 2-minute intervals for the ANP radioimmunoassay. In normal Langendorff preparation, the effect of AM on ventricular BNP gene expression was studied by infusing vehicle or AM (0.03 nmol/l) for 2 hours under basal or ET-1 (1 nmol/l)-stimulated (Magga et al. 1998) conditions. Immediately after the experiment the left and right ventricular tissue was frozen (-70°C) separately for assays of BNP mRNA and ir-BNP. Coronary effluent samples were collected for measurement of ir-ANP and ir-BNP.

4.5. Atrial superfusion and electrophysiological recordings (II)

To study the effect of AM on myocyte action potentials, the isolated atrial preparation was employed. The left atrial appendage was prepared as described previously (Laine et al. 1994). Atrium, placed in a constant-temperature (37 °C) organ bath, was superfused with a modified Krebs-Henseleit bicarbonate buffer (composition described above) at a flow rate of 3 ml/min with a peristaltic pump (Cole-Parmer Instrument, model 7553-85). Glass microelectrodes filled with a solution containing 2 mol/l K-acetate and 5 mmol/l KCl, pH 7.0, and having input resistance of 70 to 120 MΩ were used for membrane potential recordings. The atrial appendage was quiescent unless stimulated electrically through bipolar Ag/AgCl electrodes placed in contact with the auricle. Electrical stimulation (steps of 1 ms duration, 50 % over treshold voltage) was provided by a stimulator (Grass Instruments, model S44). All electrical signals were amplified with an intracellular amplifier (Dagan model 8100-1) and stored by a DAT recorder (Biologic DTR-1800). Data analysis was done with DT VEE (Data Translation Inc) and MATLAB (The Math Inc Natick) software programs. Sampling frequency was 3 kHz in all recordings. AM (1 and 10 nmol/l) was added to the atrial superfusion buffer if a stable impalement, resting potential of at least -70 mV and an overshoot of action potential of at least 10 mV were reached.

4.6. Isolation and analysis of cytoplasmic RNA (V)

RNA was isolated from right and left ventricle by the guanidine thiocyanate-CsCl method (Chirgwin et al. 1979). Northern hybridization, in which the size and amount of specific mRNA molecules in preparations of total RNA are determined (Alwine et al. 1977), was done after isolation of RNA. For the RNA Northern blot analysis, 22 µg samples from ventricles was transferred to the Amersham Hybond N+ nylon membranes. A 390 bp
fragment of rat BNP cDNA probe (Ogawa et al. 1991) (a generous gift from Dr. Kazuwa Nakao, Kyoto University School of Medicine, Kyoto, Japan) and oligonucleotide probe complementary to rat 18 S ribosomal RNA (Magga et al. 1997) were labeled with [\(^{32}\)P]-dCTP with T7 Quick Prime Kit (Pharmacia LKB Biotechnology). The membranes were hybridized overnight at 42 °C in 5 x SSC (saline sodium citrate, 1 x SSC = 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate, pH 7), 0.5 % sodium dodecyl sulfate (SDS), 5 x Denhardt's solution, 50 % formamide and 100 µg/mL sheared herring sperm DNA. After hybridization, the membrane was washed in 0.1 x SSC, 0.1 % SDS three times for 20 min at 50 °C and exposed to Phosphor Screen (Molecular Dynamics) at room temperature. Phosphor Screens were scanned with a Phosphor Imager.

4.7. Radioimmunoassays of ANP and BNP (II,V)

For coronary effluent ANP radioimmunoassay, samples were not extracted. For the BNP radioimmunoassay, the 5 ml of perfusate sample was extracted by Sep-Pak C\(_{18}\) cartridges, lyophilized and redissolved to 500 µl of RIA buffer. 150 µl of ventricular guanidine thiocyanate extract was diluted to 700 µl of RIA buffer. The tissue extracts and unextracted perfusate samples in duplicates of 100 µL were incubated with 100 µL of the specific rabbit BNP (Ogawa et al. 1991) or ANP antiserum (Vuolteenaho et al. 1985). Synthetic rat BNP\(_{51-95}\) (BNP-45) and synthetic rat ANP\(_{99-126}\), ranging from 0 to 125 fmol and 0 to 500 pg per tube, respectively, were incubated as standards. The BNP and ANP tracers were prepared by chloramine-T iodination of synthetic rat [Tyr\(_0\)]-BNP\(_{51-95}\) and rat ANP\(_{99-126}\), followed by reverse phase high performance liquid chromatography purification. After incubation for 48 hours at 4 °C the immunocomplexes were precipitated with sheep antiserum directed against rabbit gammaglobulin in the presence of 500 µL of 8 % Polyethylene Glycol 6000, pH 7, followed by centrifugation at 3000 g for 30 min. The sensitivities of the BNP and ANP assays were 2 fmol/tube and 1 fmol/tube, respectively. Fifty percent displacements of the respective standard curves occurred at 16 and 25 fmol/tube. The intra- and inter-assay variations were less than 10 % and 15 %, respectively. Serial dilutions of perfusate and tissue extracts showed parallelism with the standards. The ANP antiserum recognized ANP and proANP with equal avidity but did not cross-react with BNP or CNP (<0.01 %). The BNP antiserum did not recognize ANP or CNP (<0.01 %).

4.8. Statistical analysis

The results are expressed as mean±SEM. For the comparison of statistical significance between two groups, Student's t-test was used. The hemodynamic variables were analyzed with one-way ANOVA followed by Bonferroni’s post hoc test. Repeated measures ANOVA for repeated measurements was used for multivariate analysis. Differences with P<0.05 were considered statistically significant.
5. Results

5.1. Effect of AM on chronotropy and inotropy of isolated rat heart (I, II, III)

The basal HR and developed tension (DT) at 2 g initial RT of spontaneously beating perfused rat heart were 242±10 beats/min and 3.2±0.3 g, respectively. When AM (0.01 to 10 nmol/l) was infused into the coronary circulation, HR did not change significantly (to 257±7 beats/min, AM 1 nmol/l, P=NS). However, AM caused a dose-dependent elevation in DT with an EC50 value of 7×10^{-11} nmol/l (Fig. 4A).

Fig. 4. Inotropic effect of AM in isolated, perfused rat heart. A, The dose-response curve in spontaneously beating hearts. B, Time course of the inotropic response to AM compared to that of ET-1 at doses producing maximal and submaximal effects. The infusion of different substances was started at 0 min. The values are expressed as mean±SEM. *P<0.001 AM vs. vehicle; †P<0.001 ET vs. vehicle; ‡P<0.001 vs. AM 0.03 nmol/l; §P<0.001 vs. ET-1 0.08 nmol/l.
50

The inotropic effect of AM was dependent on the initial RT; the lower the initial RT, the higher the rise in DT (RT 1 g: \(\Delta DT 62\%\), RT 2 g: \(\Delta DT 45\%\), RT 3 g: \(\Delta DT 35\%\), AM 0.1 nmol/l, \(P<0.05\)). In paced isolated rat heart, AM induced an inotropic action similar to the response in the spontaneously beating heart (Fig. 4B). PAMP (10 to 100 nmol/l), a peptide derived from the AM gene (Kitamura et al. 1994b) and CGRP (1 nmol/l), an AM related peptide, did not elicit any effect on the contractility of perfused rat heart. The inotropic action of AM was compared to the effects of isoprenaline and ET-1 in paced rat heart preparation. The maximal elevation in DT induced by AM (1 nmol/l) was 90\% of that produced by isoprenaline (1 \(\mu\)mol/l, 44 vs. 49\%, \(P=NS\)). Of note is, however, the difference in the time course of these responses. The maximal increase in DT in response to isoproterenol developed very radiply, in a few seconds as previously described (Kelly et al. 1990), whereas the mean time to maximal response to AM was 25.4±1.3 min. The maximal responses to AM and ET-1 at the concentration of 1 nmol/l were equal (Fig. 4B.). Moreover, AM at 0.03 nmol/l elicited an equivalent inotropic effect to ET-1 at 0.08 nmol/l. In agreement with previous results (Kelly et al. 1990), ET-1 also induced a slowly-developing inotropic response which was, however, significantly faster than the response to AM (Fig. 4B). The time to reach the maximum increase in DT was 15.7±2.7 minutes with ET-1 (ET-1 1 nmol/l vs. AM 1 nmol/l, \(P<0.01\)). The inotropic effect of AM was not affected by bosentan (1 \(\mu\)mol/l), whereas the effect of ET-1 was abolished. Thus, the inotropic action of AM was not apparently dependent upon stimulation of endogenous ET-1 or its receptors.

In spontaneously beating heart, the inotropic effect of AM (0.1 nmol/l) was attenuated by CGRP\(_{8-37}\) (360 nmol/l, \(\Delta DT AM 45\%\) vs. AM + CGRP\(_{8-37}\) 21\%, \(P<0.05\)). However, the maximal inotropic response to AM (1 nmol/l) in paced heart was not affected by CGRP\(_{8-37}\) at a dose (100 nmol/l) that has been shown to inhibit AM-induced potentiation of NO synthesis in cytokine-activated cardiac myocytes (Ikeda et al. 1996).

5.2. Intracellular signaling pathways underlying the inotropic effect of AM (II)

5.2.1. cAMP

AM is known to elevate the intracellular cAMP content of many cell types, including cardiac myocytes (see Section 2.1.4.). Since the cAMP-PKA pathway is a well-established pathway mediating positive inotropic actions, it was hypothesized to have a role also in the inotropic response to AM.

H-89 (100 nmol/l), which has been shown to be a potent inhibitor of PKA at the dose used, had no effect on either the basal contractile force or the AM (1 nmol/l)-induced inotropic action (Fig. 5A). The efficiency of H-89 to inhibit cAMP-PKA -mediated responses was demonstrated by the finding that H-89 decreased the isoprenaline-induced increase in resting tension (ART: isoproterenol 1.7 g vs isoproterenol+H-89 0.6 g, \(P<0.001\)). Furthermore, AM (1 nmol/l) failed to elevate the myocardial cAMP concentration whereas isoprenaline (1 \(\mu\)mol/l) induced a significant rise (Fig 5B).
5.2.2 Intracellular Ca^{2+} stores

Intracellular calcium stores of SR play an essential role in cardiac E-C coupling (see Fig. 3.). Therefore two important molecules in SR calcium release and reuptake, RyR, which is responsible for the CIRC, and Ca^{2+}-ATPase, which pumps the released calcium back to the SR stores, were studied under stimulation with AM (1 nmol/l). Ryanodine (3 nmol/l), an opener of RyR, had only a small negative effect on basal contractility but significantly attenuated the AM-induced elevation in DT maximally by 72 % at the end of the infusion period (P<0.001, Fig. 6). Thapsigargin (30 nmol/l), a selective inhibitor of SR Ca^{2+}-ATPase, alone had no effect on contractility for the first 16 min of infusion, but significantly enhanced the AM-induced inotropic effect during that phase (P<0.001, Fig. 6.). However, pretreatment with thapsigargin (10 min, 30 nmol/l), which was assumed to lead to depletion of the SR calcium stores since thapsigargin irreversibly inhibits Ca^{2+}-ATPase (Lytton et al. 1991), significantly attenuated the early phase of AM-induced inotropic effect (maximally by 40 % at 14 min, P<0.01).

5.2.3. PKC

PKC plays a role in the cAMP-independent inotropic effects of some substances, e.g. ET-1 (Krämer et al. 1991). A PKC inhibitor, staurosporine (10 nmol/l), alone had no effect on the contractile force of isolated rat heart. However, it remarkably attenuated the plateau phase of the AM-induced rise in DT (P<0.001). Maximally, staurosporine reversed the action of AM at the end of the infusion period by 63 %. The concentration of
staurosporine used has been shown to abolish the PKC-mediated TPA phorbol ester (12-0-tetradecanoyl-phorbol-13-acetate)-stimulated ANP release and coronary vasoconstriction under these experimental conditions (Pitkänen et al. 1991, Kinnunen et al. 1993).

Fig. 6. Effect of ryanodine (A) and thapsigargin (B) on AM-induced inotropic effect. The infusion of different substances was started at 0 min. The values are expressed as mean±SEM.*P<0.05, †P<0.01 and ‡P<0.001 vs. AM.

5.2.4. L-type Ca\textsuperscript{2+} channels

L-type Ca\textsuperscript{2+} channels are voltage-dependent channels that are opened in response to the depolarization of the sarcolemma to cause an inward Ca\textsuperscript{2+} current that triggers CIRC. Several intracellular pathways can modulate the L-type calcium current (see Fig. 3). Diltiazem (1 µmol/l), an inhibitor of L-type calcium channels (Chaffman & Brogden 1985), was used to determine the role of Ca\textsuperscript{2+} influx in the AM-induced inotropic effect. Diltiazem alone had an insignificant negative effect on DT, but statistically significantly attenuated the late phase of AM-induced elevation in DT (P<0.01, Fig. 7A).

In isolated rat atria, myocyte action potentials were recorded under the stimulation with AM (1 nmol/l). AM lengthened the duration parameters at 15%, 30%, 60% and 90% repolarization levels (Fig. 7B). The largest increase was observed at 60% repolarization level (by 44%, P<0.001). A similar result, with a somewhat more prominent increase in the duration of action potential, was achieved by a higher concentration of AM (10 nmol/l). These findings support the concept that L-type Ca\textsuperscript{2+} channels are activated by stimulation with AM.
Fig. 7. A, Effect of diltiazem on AM-induced inotropic action. The infusion of substances was started at 0 min. The values are expressed as mean±SEM. B, Effect of AM on action potentials of rat atrium. Ten action potentials from each cell in the control group (n=17) and AM-group (n=6) were averaged. *P<0.05 vs. AM.

5.3. AM-induced inotropic response and endothelium-derived factors (III)

5.3.1. Modulation of inotropic responses by endogenous NO

It was studied here whether the inotropic effects of AM and ET-1 are subjects of secondary modulation by endogenous NO. When L-NAME (300 µmol/l), an inhibitor of NO synthase, was infused alone into the coronary circulation the contractile parameters remained constant, indicating that the impact of NO on basal contractility is minimal under these experimental conditions. However, the early phase of the inotropic response to 1 nmol/l ET-1 was markedly augmented by L-NAME (P<0.05). The inotropic effect of AM at 1 nmol/l was unchanged by inhibition of NOS, although there appeared to be a trend toward an enhanced effect with the lower dose (0.03 nmol/l) of AM (P=NS).

5.3.2. Combined effect of AM and ET-1 on cardiac contractility

Both AM and ET-1 are peptides produced by the myocardium (Suzuki et al. 1993, Horio et al. 1998) and may therefore have an impact on the regulation of myocardial contractility. Ventricular synthesis of both peptides is increased in response to cardiac overload (Jougasaki et al. 1995a, Sakai et al. 1996a). Based on this background, the interaction of these peptides at the level of cardiac contractility was studied. Equipotent
doses of AM and ET-1 (0.03 nmol/l and 0.08 nmol/l, respectively) which induced submaximal inotropic responses (Fig. 4) were chosen in order to permit identification of their potential additive effect. Unexpectedly, combined administration of AM and ET-1 induced a significantly smaller increase in DT than either peptide alone (19 % vs. AM 30 %, P<0.001; vs. ET-1 29 %, P<0.05). Although the individual responses to the peptides at these doses were not increased significantly by L-NAME treatment (See 5.3.1.), it was tested whether NO was responsible for the observed attenuated effect induced by simultaneous infusion of AM and ET-1. Indeed, concomitant administration of L-NAME more than reversed the depressed inotropic response to the two peptides (43 % vs 19 %, P<0.001), showing that the degree of suppression elicited by NO was potentiated.

5.4. Regulation of coronary vascular resistance by AM (IV)

Since AM is a potent vasodilator that is released from ECs e.g. in response to ET B receptor stimulation (Jougasaki et al. 1998), it may also be an important regulator of coronary vascular tone. In this study, basal perfusion pressure was 32±1 mmHg and there were no significant differences in the baseline values between different experimental groups (Fig. 8). AM (0.03 and 1 nmol/l) had a dose-dependent coronary vasodilator action (6 % and 13 % decrease in perfusion pressure, respectively, P<0.001 vs. vehicle). ET-1 failed to increase the perfusion pressure at a concentration of 0.08 nmol/l, but at 1 nmol/l there was a significant vasoconstrictor effect (19 %, P<0.05). L-NAME, which alone did not influence the coronary perfusion pressure, markedly enhanced the pressor response to ET-1 (to 62 mmHg, P<0.01). The AM-induced vasodilation was not affected by the L-NAME treatment. Furthermore, AM was able to attenuate remarkably the pressor response to ET-1 under the conditions of NOS inhibition (by 67 %, P<0.001, Fig. 8B).
Fig. 8. Effects of AM and ET-1 on perfusion pressure of isolated rat heart under normal conditions (A) and under inhibition of myocardial NOS (B). The infusion of different substances was started at 0 min. The values are expressed as mean±SEM. *P<0.001 vs. vehicle (or L-NAME alone); †P<0.001 vs. ET-1 + L-NAME.

5.5. Interaction of AM with natriuretic peptide system (V)

5.5.1. Atrial ANP release

ANP is a predominantly atrial hormone that is mainly released from the secretory granules in response to atrial wall stretch (Lang et al. 1985). The existence of AM in the atrial tissue (Ichiki et al. 1994) may indicate that it regulates atrial functions, e.g. ANP secretion. AM was infused to coronary circulation at low concentrations that produce significant positive inotropic and coronary vasodilator actions. The basal ANP release after the equilibration period was 303±39 pg/ml. A small decrease (37 %) of perfusate ir-ANP was noted during the 30-min experimental period with vehicle infusion, as reported previously (Taskinen & Ruskoaho 1996). AM (0.03 and 1 nmol/l) as well as L-NAME (300 µmol/l) had no effect on the basal release of ANP. In presence of L-NAME, AM at a dose of 0.03 nmol/l slightly increased the release of ANP during the last 5 minutes of the experimental period (by 56 % vs. vehicle, P<0.05; by 28% vs. L-NAME alone, P=NS). AM at the concentration of 1 nmol/l had no effect on ANP release in the absence (II,V) or presence (V) of L-NAME.

Right atrial stretch (by 5 mmHg) induced a large increase in the ANP release of isolated rat heart (maximally 165±29 % increase after 4 min stretch, P<0.001 vs. control). The stretch-induced release of ANP was augmented by pretreatment with AM at 0.03 nmol/l in the absence and presence of L-NAME (by 71 and 70 %, respectively, Fig.9.). Overall, when the data of the groups with and without L-NAME were combined, AM significantly increased the release of ANP (P<0.01). Pretreatment with L-NAME alone or
AM at the dose of 1 nmol/l did not significantly modulate the stretch-induced ANP release.

Fig. 9. The relation between the change in perfusate ir-ANP concentration and right atrial pressure (ΔRAP) in response to treatment with AM in the absence and presence of L-NAME. The change in ir-ANP is expressed as the difference in the area under secretion curve between control and stretch hearts. For comparison, the ANP secretion values were calculated at ΔRAP of 4 mmHg. The statistical significance was determined by Student’s t-test.

5.5.2. Ventricular BNP synthesis and release

The influence of AM on ventricular gene expression of BNP was studied by infusing AM at a concentration of 0.03 nmol/l to the perfusion fluid of isolated rat hearts for 2 h under basal and ET-1 (1 nmol/l)-stimulated conditions. AM had a positive inotropic effect (50 % at 2 h, P<0.001) at the dose used but had no effect on the basal level of BNP mRNA. ET-1 induced a 1.4-fold induction in the right ventricular BNP mRNA (P<0.05 vs. vehicle). The induction was not affected by AM (1.45-fold change, P=NS).
6. Discussion

6.1. Positive inotropic effect of AM

In the present study, isolated perfused rat heart was employed to investigate the effects of AM, a novel vasoactive peptide (Eto et al. 1999, Samson 1999), on myocardial contractile function. This approach revealed that pico-nanomolar concentrations of AM have a dose-dependent positive inotropic effect as administered into the perfusion buffer of either spontaneously beating or paced rat hearts.

The direct inotropic action of AM is in agreement with a number of reports describing the effects of intravenously administered AM on conscious animals. In those experiments, AM regularly increased HR, CO (Ishiyama et al. 1993, Parkes 1995, Fukuhara et al. 1995, Parkes & May 1997, Charles et al. 1997, Lainchbury et al. 2000) and the indices of left ventricular contractility (Parkes 1995, Parkes & May 1997) in addition to the decrease in mean arterial pressure. In part of the studies it was observed that the hypotensive action of AM is associated with a rise in sympathetic nerve activity (Takahashi et al. 1994, Fukuhara et al. 1995, Saita et al. 1998a). It is thus possible that the decrease in blood pressure initiates the baroreceptor reflex leading to a sympathetic discharge which, in turn, causes a rise in CO by elevating the frequency and contractility of the heart. Since AM had no effect on the HR of isolated rat heart, the increase in HR is likely to be explained by reflex mechanisms. In the experiments of Parkes & May the blockade of autonomic ganglia by hexamethonium prevented the indirect chronotropic response to intravenous AM, but it could not totally block the increase in CO and left ventricular stroke volume (Parkes & May 1997). Accordingly, these findings suggest that AM may have a direct inotropic effect on myocardium also in vivo. The perfused rat heart, which responded to AM by a positive inotropic action in this study, as well as the papillary muscle preparation used by others (Ihara et al. 2000), are isolated from the signals of autonomic nervous system, confirming that AM has a direct effect on myocardial contractility. It could, however, be assumed that the accompanying coronary vasodilatory action of AM could act to facilitate the myocardial performance and thus enhance the inotropic action. In this study, a constant flow perfusion model was used, which means that changes in the coronary vascular tone do not alter the perfusate supply of the myocardium. On the other hand, the well-established Gregg phenomenon states that the contraction force of constant
flow-perfused heart rises in response to increases in coronary perfusion pressure, not to decreases (Dijkman et al. 1998).

Although the direct inotropic effect of AM in perfused rat heart is in agreement with the results obtained from in vivo studies, there are conflicting results achieved by means of cultured rabbit cardiac myocytes (Ikenouchi et al. 1997), porcine atrial and ventricular trabeculae (Saetrum et al. 1999) and isolated rat heart (Perret et al. 1993) preparations. In isolated adult myocytes, AM induced a dose-dependent negative inotropic effect and decreases in amplitude of [Ca\(^{2+}\)], and Ca\(^{2+}\) current. These actions could be abolished by NO inhibitors, suggesting that AM has a negative inotropic effect mediated via NO-cGMP pathway. (Ikenouchi et al. 1997) In the isolated rat heart preparation used in the present study, a NOS inhibitor did not significantly influence the inotropic effect of AM, reflecting the fact that under these experimental conditions AM may not release NO. Another possible explanation for the difference between the results of Ikenouchi et al. and the present study may be the fact that the function of the myocytes depends on the intercellular crosstalk with the adjacent endothelial cells and cardiac fibroblasts (Brutsaert et al. 1988, Shah 1996, Winegrad 1997). Therefore, although there were also cells other than cardiac myocytes present in the incubate of Ikenouchi et al., it is possible that the myocytes are "switched" to respond to AM differently, when isolated from their normal spatial circumstances in intact heart. In addition to the methodological reasons, these conflicting responses could be explained by the differences in the species characteristics of the myocardial E-C coupling. Similarly, unresponsiveness of porcine atrial and ventricular trabeculae to AM may be related to the latter category. In isolated working rat heart preparation, a mild decrease in peak systolic pressure was observed after administration of AM (Perret et al. 1993). In that study, bolus injections were performed to administrate AM into coronary circulation. Taking into account the slow development of the inotropic effect of AM, it is likely to be unrecognized by a bolus administration. Furthermore, a relatively high dose of AM (approximately 10 nmol) was used. In the preparation used in the present study, concentrations above 1 nmol/l have been observed to produce smaller inotropic effects (10 nmol/l, 15% increase in DT) than the concentration of 1 nmol/l, which induced the maximal inotropic effect. Alternatively, these varying observations could be explained by different expression of AM receptors in the cardiac cells or tissue preparations under different experimental conditions. To date, there is only a limited understanding of the receptors mediating different responses to AM (see section 2.1.4.). However, it was suggested that depending on the RAMP subtype expressed by the cell, CRLR receptor is either AM or CGRP receptor (McLatchie et al. 1998). CRLR is abundant in the heart (Aulietlano 1998), suggesting that the RAMP-hypothesis may also be applied to the inotropic effect of AM. Since the regulation of RAMP expression is largely unknown, and the relation between RAMP1 and RAMP2 expression is not known in the experimental preparations described above, the RAMP hypothesis may potentially contribute to their variable responsiveness to AM.

The fact that AM was an equally potent inotropic agent as ET-1 in our preparation indicates that AM may be one of the most potent endogenous inotropic substances identified so far. Until now, ET-1 has been stated to be the most potent inotropic substance on a molar basis (Ishikawa et al. 1988, Kelly et al. 1990). These strong responses of ET-1 are obtained from in vitro studies. In vivo, the potent coronary vasoconstrictor effect of ET-1 masks the positive effect on myocardial contractility by reducing the blood supply of
the myocardium (Beyer et al. 1999). In the present study, a constant coronary flow was used, and therefore, a potent inotropic response to ET-1 was observed.

6.2. Intracellular signaling pathways underlying the inotropic effect of AM

Although previous investigations have suggested an intimate relation between AM-induced biological responses and the increased cAMP levels in several cell types (see Section 2.1.4.), the inotropic effect seems not to be mediated via this second messenger. In isolated cardiac myocytes, the augmentation of cytokine-stimulated NO synthesis (Ikeda et al. 1996) and attenuation of ANP expression (Sato et al. 1997) by AM are reported to be due to the elevated intracellular cAMP. Nevertheless, the inotropic effect of AM was not modulated by a PKA inhibitor or a phosphodiesterase inhibitor (isobutyl methylxanthine, data not shown). Furthermore, AM was not able to elevate myocardial cAMP concentration at the doses that induced an inotropic effect. Finally, the development of the inotropic effect of AM appeared to be very slow compared with the observed rapid response produced by isoproterenol which is known to act through a cAMP-PKA-dependent pathway (Steinberg 1999). The discrepancy between this study and those of Sato et al. and Ikeda et al. could be explained by the fact that the receptor responsible for the cAMP-independent inotropic effect may be distinct from that elevating cAMP. Accordingly, the threshold concentration to activate the cAMP production seems to be higher, not reached by the concentration of AM (1 nmol/l) that exerts the maximal effect on myocardial contractility under these conditions. Indeed, the increase in cardiac myocyte cAMP induced by AM is observed at concentrations of 5 nmol/l and above (Ikeda et al. 1996, Sato et al. 1997). Similarly, the small cAMP-dependent inotropic effect of AM observed in rat papillary muscle was reached by concentrations higher than 10 nmol/l (Ihara et al. 2000). It is also possible that AM at higher concentrations interacts with cardiac receptors that preferentially bind CGRP and are coupled to AC, because the cAMP elevating property of AM in cardiac myocytes (Ikeda et al. 1996) can be inhibited by a CGRP receptor antagonist, CGRP8-37. The inotropic effect of AM appears to be specific to AM since it was only partially attenuated by a high concentration of CGRP8-37, and CGRP itself had no effect on cardiac contractility in our preparation.

Because cAMP seems not to be involved in the inotropic action of AM, other signaling mechanisms involved in the regulation of myocyte E-C coupling may be responsible for the enhanced contraction. Intracellular calcium handling, release from SR by Ca\(^{2+}\) influx or IP\(_3\), and reuptake by Ca\(^{2+}\)-ATPase are basic mechanisms that take part in each contraction cycle (Clapham 1995). It could be demonstrated in the present study that ryanodine and thapsigargin, modulators of SR function, influenced the inotropic effect of AM, although they alone had no effect on the basal contractility. These data suggest that stimulation of AM receptors may release Ca\(^{2+}\) from the intracellular thapsigargin- and ryanodine-sensitive Ca\(^{2+}\) pools, which leads to an enhanced contraction force. Similarly, in bovine aortic ECs, AM released Ca\(^{2+}\) from the endoplasmic reticulum via stimulating PLC and elevating IP\(_3\) level (Shimekake et al. 1995). PLC also generates another signaling molecule, DAG, which activates PKC to produce a positive inotropic effect (Pi et al.
6.3. Contractile interaction of AM and ET-1 with endogenous NO

In the studies of the last decade, myocardial endogenous NO synthesis has appeared as a very important modulator of inotropic responses (Kelly et al. 1996, Hare & Stamler 1999). Most importantly, NO is released in response to β-adrenergic agonists, the positive inotropic responses of which are clearly enhanced by inhibition of NOS (Balligand et al. 1993a). In the present study it was observed that this property of myocardial NOS is not restricted to β-adrenergic receptors, since the inotropic response to ET-1 was markedly augmented by L-NAME. Previously, the inhibition of NO synthesis on ET-1 -induced cardiac responses has been studied in the perfused rat heart preparation. In that study, ET-1 was able to increase the NO production and the ET-1-induced increase in left ventricular end-systolic pressure was augmented by NOS inhibition (Ebihara et al. 1996). Probably because of a strong coronary vasoconstriction, ET-1 had only a small positive inotropic effect, which was not modulated by inhibition of NOS. Under physiological conditions, there are two types of NOS enzymes expressed in the myocardium, NOS1 and NOS3 (Kelly et al. 1996). The detectability of NOS2 gene expression has been shown to demand at least 6 hours of stimulation by cytokines both in cardiac myocytes (Balligand et al. 1994) and in microvascular endothelial cells (Balligand et al. 1995b). NOS1 has for a long time been undetectable in the heart, until a recent report suggested a NOS1-like enzyme in the SR of cardiac myocytes (Xu et al. 1999). Therefore, since L-NAME was able to modulate the response in a few minutes, it is likely that NOS3 is the subtype responsible for the observed suppression of the ET-1-induced inotropic effect, although the other
isoenzymes cannot be entirely excluded in an intact heart preparation. ET-1 has been shown to induce an increase in intracellular Ca\(^{2+}\) that in complex with calmodulin leads to the activation of NO synthesis in ECs (Hirata et al. 1993). ET-1 may potentially also release NO in cardiac myocytes, since it is able to induce a rise in myocyte [Ca\(^{2+}\)] (Wang et al. 1991, Qiu et al. 1992, Lauer et al. 1992, Katoh et al. 1998). In several vascular preparations (see e.g. Hayakawa et al. 1999), the AM-induced vasodilation has been reported, at least in part, to be mediated through endothelial NO release. In ECs, Ca\(^{2+}\) accumulation by AM leads to NO release, deduced from increased cellular content of cGMP, which is inhibited by NOS inhibitors (Shimekake et al. 1995). It was also reported that AM may activate NOS3 in rabbit isolated cardiac myocytes (Ikenouchi et al. 1997) and potentiate the induction of NOS2 by cytokines in rat cardiac myocytes (Ikeda et al. 1996). In this study, the inotropic effect of AM was not modulated significantly by L-NAME. This finding suggest that either AM does not influence the NO release at the concentrations producing an inotropic response, or the NO potentially released does not affect the inotropic effect of AM. The latter is probably not true, since the NO-cGMP system has been shown to interact with the critical sites for E-C coupling (see Fig. 3.), which are also, according to the data of this study, influenced by AM. Therefore, further studies are needed to examine whether AM is able to influence the myocardial NOS activity.

Of particular interest was our finding that equally potent submaximal concentrations of ET-1 and AM together induced a lesser inotropic response than either peptide alone. It was further shown that this depressed effect of combined administration was more than overcome by concomitant infusion of L-NAME, suggesting that the attenuation was due to enhanced synthesis of NO, whereas the individual responses to the peptides at these doses were not modulated by NO. Because the Ca\(^{2+}\)/calmodulin pathway is known to activate NOS3, and both AM and ET-1 are known to influence intracellular Ca\(^{2+}\) metabolism (Hirata et al. 1993, Shimekake et al. 1995) it can be presumed that their simultaneous administration potentiates NO production via manipulation of cardiac myocyte or endothelial cell Ca\(^{2+}\) transients.

### 6.4. AM as a regulator of coronary vascular tone

The present results show that in rat coronary vasculature AM is a potent vasodilator. Previously, AM has been shown to have a coronary vasodilator action in the coronary arteries of perfused rat hearts (Entzeroth et al. 1995), open-chest dogs (Sabates et al. 1997) and in isolated rat (Sheykhzade & Nyborg 1998) and porcine (Yoshimoto et al. 1998) coronary artery rings and porcine coronary artery strips (Kureishi et al. 1995). In porcine coronary artery rings the effect of AM was shown to be independent of NO production (Yoshimoto et al. 1998). The results of the present study confirm the coronary vasodilatory action of AM, but furthermore, they show that AM is capable of inducing a relaxation of smooth muscle independently of coronary endothelial NO production. L-NAME was able to enhance significantly the vasoconstrictor effect of ET-1, but it had no effect on AM-induced vasodilation. The coronary vasoconstriction induced by ET-1 observed here is consistent with the original finding (Yanagisawa et al. 1988) that ET-1
dose-dependently constricts the porcine coronary artery strips. In rat, it was suggested that the constriction is due to activation of the ET<sub>A</sub> receptor, but that simultaneous activation of ET<sub>B</sub> receptor may counteract the response by increasing NO release (Wang et al. 1994).

Of special interest was the finding that AM attenuated the pressor response to ET-1. In VMSCs, ET-1 causes an increase in the intracellular Ca<sup>2+</sup> level, probably via activation of PLC (Takuwa et al. 1990), whereas AM has been shown to decrease the Ca<sup>2+</sup> concentration associated with an increased intracellular cAMP content (Yoshimoto et al. 1998). Since AM attenuated the effect of ET-1 in an intact coronary circulation in the present study, it is predictable that the quantitative balance between AM and ET-1 regulates the intracellular Ca<sup>2+</sup> and the contractile state of the smooth muscle also in vivo. It has previously been shown that ET<sub>B</sub> receptor activation increases AM release from cultured ECs (Jougasaki et al. 1998). Moreover, a recent study shows that ET-1 stimulates the release of AM to the coronary effluent also under the conditions of this study (Piuhola et al. 2000, unpublished observation). Thus, it is possible that AM is acting as an endogenously released substance that counterbalances the vasoconstrictor effect of ET-1. This endogenous regulatory loop should be confirmed as soon as a selective antagonist for AM receptor is developed. In our experimental setup, AM and CGRP receptor antagonists AM26-52 and CGRP<sub>8-37</sub>, respectively, failed to antagonize the coronary vasodilatory effect of AM (data not shown), and can not thus be used in characterization of the counteracting role of AM in the ET-1-induced vasoconstriction.

Atherosclerotic coronary arteries are characterized by an increased synthesis of ET-1 and a decreased synthesis of NO (Fuster et al. 1992). Based on the results of this study, it would be of interest to study the ability of damaged vessels to produce AM in order to gain more precise knowledge of the pathophysiology underlying the coronary artery disease. It has been shown that in addition to ECs (Jougasaki et al. 1998), ET-1 liberates AM also from VSMCs (Sugo et al. 1995a). Taking into account that the dysfunction of endothelium in coronary artery disease is usually accompanied by a migration of VSMCs of arterial media to the intima layer (Ross 1986), AM may therefore serve as an important compensatory mechanism against the exaggerated vasoconstriction under those conditions. The results of the present study show that exogenous AM is able to remarkably attenuate the vasoconstriction due to ET-1 under conditions of low NO production typical of the coronary artery disease.

### 6.5. AM and natriuretic peptides

In previous reports, AM has been shown to inhibit ANP expression and release in cultured cardiac myocytes (Sato et al. 1997) and attenuate its release in response to elevated atrial pressure (Kaufman & Deng 1998). The present study produced opposing results, since AM at a low concentration enhanced the stretch-induced release of ANP in the perfused rat heart preparation. The concentrations of AM used in this study were in the pico-nanomolar range, and the activity of the peptide at these concentrations was verified by a simultaneous positive inotropic effect. Inhibitory effect on ANP synthesis and release by higher than 5 nmol/l AM was associated by an increased cAMP content of cardiac
myocytes (Sato et al. 1997), while the positive inotropic effect of AM did not involve the activation of AC (II). Therefore, it is reasonable to assume that there may be at least two receptors that bind AM in the myocardium. Accordingly, the saturation of the putative AM receptor(s) that mediates the increased contractility and enhanced ANP release seems to be achieved by lower concentrations than that of the cAMP-linked receptor that possibly mediates the ANP-suppressive action of AM (Sato et al. 1997). In support of this hypothesis it has been shown that forskolin, a cAMP elevating substance, attenuates the stretch-induced secretion of ANP under our experimental conditions (Ruskoaho et al. 1990). The failure of AM at a concentration of 1 nmol/L to modify ANP secretion is in agreement with this proposed biphasic effect of AM. Since the inhibitory effect of AM on stretch-induced ANP release was previously observed at approximately 175 nmol/l (Kaufman & Deng 1998), it could also be related to the cAMP-mediated mechanisms.

There is evidence that myocardial NO production could be a factor controlling ANP release. In isolated rat atria, removal of endothelium by saponin or administration of NO inhibitors increase the release of ANP (Sanchez-Ferrer et al. 1990). Furthermore, intravenous infusion of L-NAME in conscious rats dose-dependently stimulates the plasma ANP levels (Leskinen et al. 1995). In the present study, however, L-NAME alone did not significantly augment ANP release, suggesting that the role of basal NO production is minimal in the regulation of the ANP release under our experimental conditions. Since in rabbit ventricular myocytes AM has been previously shown to augment NO synthesis (Ikenouchi et al. 1997), we assumed that the blockade of NO synthesis could modify the effect of AM on ANP secretion. In normal Langendorff preparation the release of ANP in response to AM was somewhat increased by concomitant infusion of L-NAME, but the stretch-induced ANP release was augmented by AM to a similar degree in the absence and in the presence of NO inhibition. Accordingly, the stimulatory effect of AM on ANP secretion seems not to be essentially controlled by endogenous NO system. Overall, these data indicate that differences between species and experimental models as well as atrial and ventricular myocardium may account for the varying importance of NO as a modulator of AM effects.

The biological significance of the ANP-stimulatory role of AM in vivo remains to be studied. AM increased ANP secretion in response to stretch by approximately 70 %, which is comparable to the effect of ET-1 that is considered a potent secretagogue of ANP (Mäntymaa et al. 1990). This may be important, because stretch is the main stimulus for ANP release (Lang et al. 1985, Ruskoaho 1992). Since atria are one of the major sites of AM synthesis (Ichiki et al. 1994, Jougasaki et al. 1995a, Jougasaki et al. 1995b, Jougasaki et al. 1997, Asada et al. 1999), it can be assumed that AM may play a role in the regulation of ANP release. This is supported by an in vivo study in conscious sheep, showing that intravenous AM caused a trend for plasma ANP to rise, despite that there was a marked reduction in the right atrial pressure (Charles et al. 1997).

Long (2 h) infusion of ET-1 into the perfusion fluid produced an increase in right ventricular BNP mRNA, as described previously (Magga et al. 1998). This is in agreement with the property of ET-1 to induce hypertrophic changes in the myocardium, including the expression of the early-inducible genes like BNP (Nakagawa et al. 1995). AM has been shown to attenuate the hypertrophic changes induced by angiotensin II or fetal bovine serum in cardiac myocytes (Tsuruda et al. 1998). In this study, a concentration of AM that produced a significant inotropic effect failed to affect either basal or ET-1-induced BNP
expression. It is known that the plasma level of BNP is far lower than that of ANP under normal conditions, but following a prolonged ventricular overload, the levels of BNP may surpass the levels of ANP in severe cases of left ventricular dysfunction (Mukoyama et al. 1991). Therefore, although AM seems not to affect the synthesis of BNP in the perfused normal rat heart, it may be important to study the effect of AM on the diseased heart that possess a high baseline of BNP synthesis. Preliminary results suggest, that when infused intravenously to patients with left ventricular dysfunction, AM does not interfere with the BNP levels, at least in short term (Nagaya et al. 2000a). This may be of importance, because both AM (Nagaya et al. 2000a) and BNP (Yoshimura et al. 1991) exert beneficial effects on the cardiorenal parameters of patients with congestive heart failure, serving in that case potentially two independent mechanisms to be utilized by medical interventions in the future.

![Hypothetical signaling pathways mediating the inotropic effect of AM.](image)

Fig. 10. Hypothetical signaling pathways mediating the inotropic effect of AM.
7. Summary and conclusions

Overall, AM exerts regulatory actions on the heart by increasing cardiac contractility, dilating coronary arteries and modulating stretch-induced ANP release. In detail, the findings of the present study are summarized as follows:

1. This study showed that AM has a potent positive inotropic effect in perfused rat heart. Since AM is present in circulation in low picomolar range and is abundant in myocardial tissue, it is assumed that AM may play a role in the regulation of myocardial contractile function in endocrine and/or paracrine way.

2. The intracellular signaling mechanism underlying the inotropic action of AM seemed to involve activation of PKC, stimulation of L-type Ca\(^{2+}\) current and release of Ca\(^{2+}\) from SR calcium stores. Hypothetical signaling pathways are presented in Fig. 10.

3. The inotropic effect of AM was not a subject of secondary modulation by endogenous NO synthesis, whereas the inotropic response of ET-1 was suppressed by NO release. AM and ET-1 did not produce an additive inotropic effect, but in contrast, the response to combined administration of the peptides resulted in a smaller inotropic effect than that induced by the peptides alone. This appeared to be due to an enhanced impact of endogenous NO synthesis by two simultaneous inotropic stimuli. This potentiation may represent a new phenomenon in the regulation of cardiac contractility by NO.

4. AM had a dose-dependent coronary vasodilator action in the perfused rat heart preparation. AM attenuated the pressor response of ET-1. Since the vasodilator effect of AM was shown to be independent of endothelial NO production, this property may be of importance in the endothelial dysfunction states, where the production of constrictors like ET-1 have been proposed to overcome the production of dilators such as NO.

5. AM enhanced the stretch-induced ANP release from right atrium, but it had no effect on the ET-1-induced BNP expression in the right ventricle.
8. References


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