# Co-expression of natriuretic peptide receptors and its implications on receptor regulation in smooth muscle cells of the aorta

Inaugural Dissertation

submitted to the

Faculty of Medicine

In partial fulfillments of the requirements

for the Doctor medincinae Degree

of the Faculty of Medicine

of the Justus-Liebig University Giessen

by

Guy Waisbrod

of

Tiberias, Israel

Giessen 2011

From the Institut für Anatomie und Zellbiologie

Chairman: Prof. Dr. Wolfgang Kummer

of the Faculty of Medicine of the Justus-Liebig University Giessen

First Supervisor: Prof. Dr. Ralf Middendorff

Second Supervisor: Prof. Dr. Heinrich Sauer

Date of Doctoral Defense: 20. 03. 2012

# Table of Contents

# 1 Introduction

## 1.1 Natriuretic Peptides

A long predicted humoral link between the heart and the kidney was found by de Bold, by isolating a substance from atrial cardiac tissue capable of eliciting a rapid decrease of blood pressure, accompanied by increased diurese (de Bold, Borenstein et al. 1981). Soon, two related peptides, possessing a common disulfide linked ring structure, were purified.

The family of these natriuretic peptides consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Structurally related but genetically distinct, these hormones (ANP, BNP) or paracrine factors (CNP) regulate blood pressure and blood volume and play a crucial role in modulating ventricular hypertrophy, fat metabolism, bone growth and pulmonary hypertension among others (Maack, Suzuki et al. 1987; Stein and Levin 1998; Kuhn 2004; Potter, Abbey-Hosch et al. 2006). Both ANP and BNP are synthesized in atrial cardiomyocytes and secreted into circulation in response to cardiac wall stretch. Thus they exert important cardiovascular functions by acting as true endocrinal factors (Brenner, Ballermann et al. 1990; Ruskoaho 1992). By contrast, the third member, CNP, is mainly produced in vascular endothelial cells and chondrocytes while also found in high concentrations in brain tissue. It exhibits various local and paracrine functions in the cardiovascular system as well as outside of it (Sudoh, Minamino et al. 1990; Suga, Nakao et al. 1992; Hagiwara, Sakaguchi et al. 1994).

For the sake of completeness another peptide of this family, urodilantin, should be mentioned. This peptide represents an elongated form of ANP, produced exclusively in the kidney (Schulz-Knappe, Forssmann et al. 1988).

Evolutionary analyses uncovered CNP as the most ancient family member of natriuretic peptides, suggesting that both ANP and BNP evolved from CNP gene duplications (Inoue, Naruse et al. 2003).

### 1.1.1 Biosynthesis and structure of natriuretic peptides

All members of the natriuretic peptides family are synthesized as so called preprohormones. The length of human preproANP is 151 amino acids and cleavage of the N-terminal signal sequence results in a precursor protein, proANP, of 126 amino acids. This molecule is the predominant form to be stored in granules. Upon secretion, biologically active ANP emerges after a second cleavage, catalyzed by a protease called corin, as a 28-amino acid carboxyl-terminal fragment (Yan, Wu et al. 2000).

The signal sequence of human preproBNP is cleaved likewise to form a proBNP containing 108 amino acids. Further proteolytical process leads to a mature 32-amino acid BNP. The protease responsible for this process is not identified yet.

Mature CNP is known to exist in two variants, a 22- and a 53-amino acid form, each derived from the 103-amino acids proCNP. While the enzyme responsible for the 53-amino acid version is identified as forin, the protease for processing the 22-amino acid CNP is unknown. Although similar if not identical in function, tissue expression of both CNP forms differs (Potter, Abbey-Hosch et al. 2006): CNP-22 is found mainly in human plasma and cerebral spinal fluid, CNP-53 predominates in brain tissue, vascular endothelial cells and the heart (Minamino, Makino et al. 1991; Stingo, Clavell et al. 1992; Togashi, Kameya et al. 1992; Totsune, Takahashi et al. 1994).

The three mature natriuretic peptides contain a common 17-residue disulfide-linked ring structure (Yandle 1994; Levin, Gardner et al. 1998; Stein and Levin 1998). This ring along with parts of the N- and C-terminal extensions are essential for the natriuretic peptides to exert their biological activity (Chen and Burnett 1998).

## 1.2 Natriuretic Peptide Receptors

There are three known transmembrane receptors binding natriuretic peptides: NPR-A, NPR-B and NPR-C, “NPR” standing for natriuretic peptide receptor. They are also referred to as guanylyl cyclase A (GC-A), guanylyl cyclase B (GC-B) and the

clearance receptor, stressing the fact that GC-A and GC-B are guanylyl cyclases (Potter 2005), while the latter lacks any guanylyl cyclase activity and is involved in metabolic clearance of the peptides (Fuller, Porter et al. 1988). Hence, GC-A and GC-B convert guanosine triphosphate into cyclic 3',5'-guanosine monophosphate (cGMP). Thus, cGMP is the second messenger in the signaling pathways of natriuretic peptides (Lucas, Pitari et al. 2000). The cGMP-generating receptors resemble growth factor receptor topology, containing an extracellular ligand-binding site (approximately 450 amino acids), a single hydrophobic membrane-spanning domain and an intracellular region of about 570 amino acids (Potter 2005).

GC-A mRNA is expressed in many tissues and cell types. It is predominantly found in kidney, adrenal, vascular, pituitary and fat tissue (table 1). GC-B is observed in above mentioned tissues, though in a more restricted manner. Additional expression sites such as chondrocytes are reported (Lucas, Pitari et al. 2000; Potter, Abbey-Hosch et al. 2006). The clearance receptor was localized to atrial, kidney and venous tissue as well as to smooth muscle cells and endothelial cells of the aorta (Fuller, Porter et al. 1988; Porter, Arfsten et al. 1990; Nagase, Katafuchi et al. 1997).

Table 1: Natriuretic peptide receptors, their ligand preferences, sites of expression and physiological functions

| NATRIURETIC PEPTIDE RECEPTOR | LIGAND PREFERENCES | SITES OF EXPRESSION | MAIN FUNCTIONS |
|---|---|---|---|
| GC-A (NPR-A) | ANP>BNP>>CNP | Kidney, vasculature, heart, brain, lung, pituitary, testis, smooth muscle cells (additional sites are reported) | Vasorelaxation, natriuresis, diuresis, inhibition of renin and aldosterone secretion, regulation of cell proliferation |
| GC-B (NPR-B) | CNP>>ANP>BNP | Kidney, vasculature, heart, brain, lung, pituitary, pineal gland, penis, endothelium, ovary, uterus, chondrocytes (additional sites are reported) | Vasorelaxation, regulation of bone growth, control of neuronal differentiation, regulation of hormone secretion |
| Clearance receptor (NPR-C) | ANP, BNP, CNP | Kidney, vasculature, testis, lung, placenta, mesentery (additional sites are reported) | Regulation of local concentrations of natriuretic peptides, inhibition of adenylyl cyclase |

The three members of the natriuretic peptide family bind differentially to these receptors.

The rank order binding affinity for GC-A is ANP greater than BNP and much greater than CNP. The affinity rank order for GC-B is almost completely inversed, with CNP as the most potent activator and BNP with the least potency (Koller, Lowe et al.

1991; Suga, Nakao et al. 1992; Lopez, Garbers et al. 1997). These findings suggest that GC-A is the main binding protein for ANP and BNP, whereas GC-B represents the endogenous receptor for CNP. In contrast the clearance receptor, binds all three receptors with similar affinity (Bennett, Bennett et al. 1991; Suga, Nakao et al. 1992; Kuhn 2004; Potter, Abbey-Hosch et al. 2006). The physiological role of this third natriuretic peptide receptor is not fully understood. It has been shown that the clearance receptor keeps natriuretic peptides from circulation via internalization, thus modulating their circulating and local concentrations (Maack, Almeida et al. 1988; Jaubert, Jaubert et al. 1999; Matsukawa, Grzesik et al. 1999; van den Akker 2001). Furthermore, some reports postulate that NPR-C reduces adenylyl cyclase activity in membranes or decrease cAMP concentrations in whole cells (Anand-Srivastava, Sairam et al. 1990). The clearance receptor has also been shown to stimulate phospholipase C (Berl, Mansour et al. 1991; Murthy, Teng et al. 2000; Pagano and Anand-Srivastava 2001). In addition it should be mentioned, that unlike GC-A and GC-B, the clearance receptor binds the synthetic ANP analog called cANF. Functions stimulated by this substance, lacking the carboxyl-terminal tail and a part of the disulfide ring structure, can be attributed to NPR-C (Maack, Suzuki et al. 1987; Potter, Abbey-Hosch et al. 2006).

### 1.2.1 Structure of guanylyl cyclase-linked natriuretic peptide receptors

Basically, membrane guanylyl cyclases share a common topology containing an extracellular ligand binding domain at the N-terminus, a short hydrophobic transmembrane part and an intracellular site with the catalytic component at its C-terminal end. Hence, the basic structures of GC-A and GC-B are quite similar. As described above, their extracellular domain consists of approximately 450 amino acids whereas the single hydrophobic transmembrane region is about 20-25 residues. The intracellular domain consists of three further subdivisions: a juxtamembranous protein kinase-homology domain, an amphipathic hinge region of 41 amino acids as well as a roughly 250-amino acid C-terminal cyclase-homology catalytic domain (Potter and Hunter 2001; Kuhn 2003). In the absence of their ligand,

GC-A and GC-B exist as homodimers. The hinge region mediates the oligomerization of the receptors (Forte and Currie 1995). Deletion of this region has proved to result in monomeric inactive entities. Therefore dimerization is essential for the activation of the catalytic domain (Kuhn 2003).

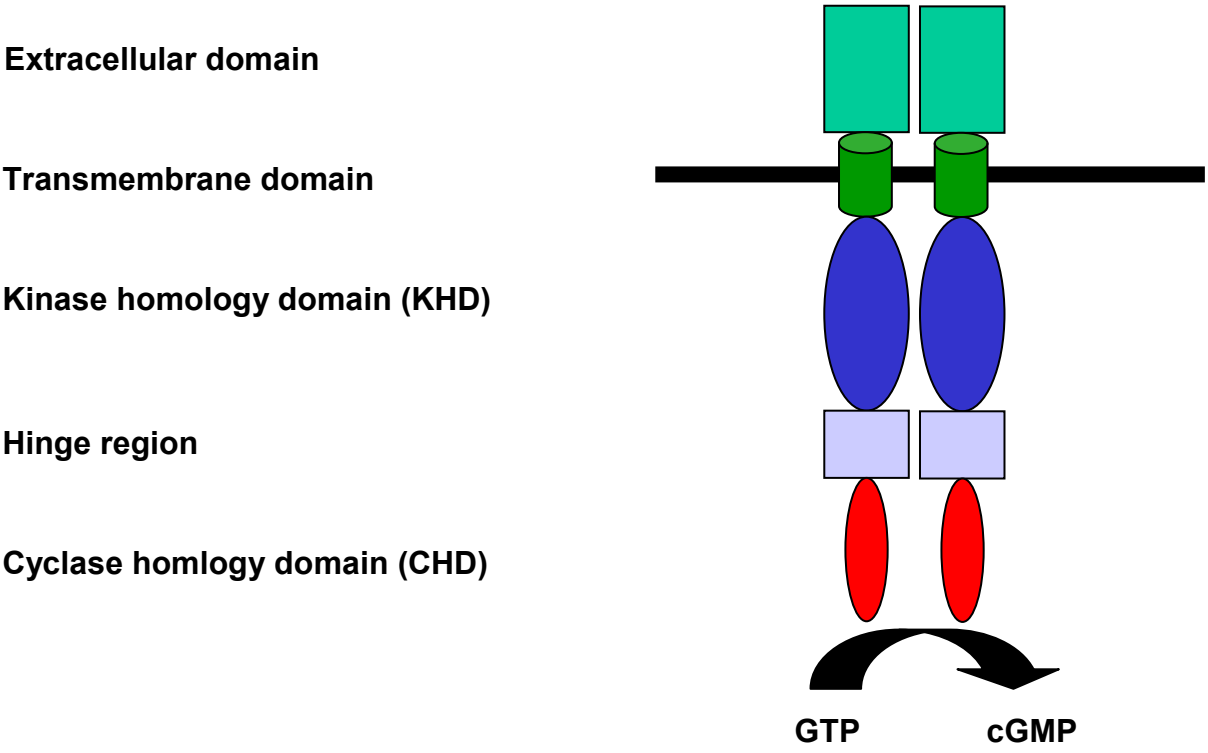


**Figure 1: Common structure of the cGMP-generating natriuretic peptide receptors**

The basic topology of GC-A and GC-B consists of a ligand binding extracellular domain, a single hydrophobic transmembrane domain and an intracellular domain. The latter can be further subdivided into a kinase homolgy domain and a catalytic domain, bisected by a hinge region. As the figure indicates, the receptors exist as homodimers (Kuhn 2003).

#### 1.2.1.1 Extracellular domain

The extracellular region of the receptors represents the site of binding the natriuretic peptide. Though similar in topology, the difference in primary structure provide the specifity for ligand binding, thus it is the most dissimilar region between the two proteins (Potthast and Potter 2005). The mechanism of ligand binding is not completely resolved and studies are discordant about ligand-receptor stoichiometry, ranging between 1:1 (Shyjan, de Sauvage et al. 1992; Lowe, Dizhoor et al. 1995), 2:2 (Schulz, Lopez et al. 1997; Yang, Robinson et al. 1999) and 1:2 (Fulle, Vassar et al. 1995). Latter is underpinned by novel reports that propose an A-shaped head-to-head dimerization with a stoichiometry of one molecule of ligand to two molecules of receptor (Ogawa, Qiu et al. 2004; Potter, Abbey-Hosch et al. 2006).

#### 1.2.1.2 Transmembrane domain

The single transmembrane span represents the part which divides the receptor molecule into an extracellular and an intracellular domain while its hydrophobic configuration allows it to be integrated in the hydrophobic cell membrane. Besides its localization further functions are still unraveled.

#### 1.2.1.3 Kinase homology domain (KHD)

This receptor domain, consisting of an approximately 250-amino acid chain and located between the transmembrane domain and the catalytic domain, exposes a structure conserved in many protein kinases, though no kinase activity has been identified. Its function is remained to be fully characterized, however, studies indicate that it represses the enzyme activity of the catalytic domain but is also essential for the latter's activation. Furthermore it probably binds ATP (Garbers 1991; Koller, de Sauvage et al. 1992; Potter and Hunter 2001; Kuhn 2003). Phosphorylation sites within the KHD, mainly serine and threonine, are predicted to be involved in the activity state of both GCs (Potter and Garbers 1992; Potter and Hunter 2001; Potthast and Potter 2005). The role of this domain in receptor regulation will be discussed below.

#### 1.2.1.4 Hinge region

The coiled coil proline-rich hinge region, composed of 41 amino-acids, is proposed to be involved in the receptor dimerization process, required for its catalytic activation.(Thompson and Garbers 1995; Wilson and Chinkers 1995). As already mentioned, deletion of this region will lead to monomeric and inactive catalytic domains.

#### 1.2.1.5 Cyclase homology domain (CHD)

The catalytic subunit at the intracellular tail is considered to resemble the catalytic domain of adenylyl cyclases in structure. A crystal structure of the GC domain is still unexistent. It appears that the catalytic domain contains two active sites per homodimer (Potter and Hunter 2001). Finally, the active catalytic domain converts guanosine triphosphate (GTP) into cGMP and pyrophosphates.

## 1.3 Activation and Regulation of Guanylyl Cyclase-linked Receptors

### 1.3.1 Activation of GC-A and GC-B

Hypothetically, there exist three states of receptor activity: basal, highly active, and desensitized (Potter, Abbey-Hosch et al. 2006). In the basal state the enzymatic activity is strictly repressed. Upon ligand binding, a conformational change is induced, bringing together the juxtamembrane regions of the receptor's extracellular domain (Labrecque, Mc Nicoll et al. 1999). Via an unknown mechanism, this signal is transduced across the membrane and relieves the repression of the KHD on the catalytic domain. Thus the latter is allowed to dimerize, exposing two active sites on each dimer (Potter, Abbey-Hosch et al. 2006). Ligand binding does not lead to a change in the oligomeric state of the receptor (Currie, Fok et al. 1992; Hasegawa, Hidaka et al. 1999; Potthast and Potter 2005).

Several groups suggested that ATP has a crucial role in this activation process, though its exact function remains controversial. Originally seen rather as an inhibitor of guanylyl cyclase activity, other studies reported that ATP was essential for both GC-A and GC-B activation (Waldman, Rapoport et al. 1984; Winquist, Faison et al. 1984; Kurose, Inagami et al. 1987; Chinkers, Singh et al. 1991; Marala, Sitaramayya et al. 1991; Wong, Ma et al. 1995; Potter and Hunter 1998) .Based on recent studies, ATP rather stabilizes both receptors, but does not activate them (Potter, Abbey-Hosch et al. 2006).

## 1.3.2 Regulation of GC-A and GC-B

Cells possess a whole range of mechanisms to regulate their receptors or ligand-dependant activity. First, the abundance of receptors at the cell surface can be controlled genetically, by receptor degradation or by receptor internalization. Another mechanism is to control local ligand availability by sequestrating the hormone via a clearance receptor or by ligand degradation. Yet, none of these mechanisms would affect the responsiveness of each receptor to its ligand, but the responsiveness of the cell itself (or at least of a cell's microdomain). A further mechanism used by cells to regulate their receptors is the modulation of the receptor's responsiveness itself.

The states of the hormone-responsiveness of both GC-A and GC-B are regulated primarily by phosphorylation / dephosphorylation (Potter and Garbers 1992; Potter and Hunter 1998; Kuhn 2003; Potthast and Potter 2005). Moreover, these receptors are apparently not down-regulated by internalization (Fan, Bryan et al. 2005).

### 1.3.2.1 Receptor phosphorylation

Phosphorylation and dephosphorylation is seen as a general mechanism within cells to regulate cellular protein activities. The tools of this control mechanism are kinases and phosphatases which provide the cell the ability to adapt to different physiological changes (Mumby and Walter 1993).

Accordingly, receptors can alter their activation states depending on the degree of phosphorylation. As well known, G-protein coupled receptors are active in a

dephosphorylated state, whereas ligand binding induces desensitization through G-protein receptor kinases (Lefkowitz and Caron 1986). The studies of several groups revealed that natriuretic peptide receptors are regulated by phosphorylation as well. GC-A and GC-B, expressed in HEK293 cells, show a decreasing guanylyl cyclase activity during prolonged incubation with ANP or CNP, respectively. This effect correlates with and is explained by dephosphorylation (Potter and Garbers 1992; Koller, Lipari et al. 1993; Joubert, Labrecque et al. 2001). Thus, natriuretic peptide receptors are regulated in a different manner than G-protein coupled receptors. It is thought that GC-A and GC-B are fully phosphorylated in their basal activity state, meaning they are most responsive to ligand-dependent stimulation.

For GC-A, six residues within the KHD were reported to be phosphorylated, four serine and two threonine residues. The identified positions are Ser-497, Thr-500, Ser-502, Ser-506, Ser-510 and Thr-513. The lack of four or more of these phosphor acceptor sites results in a complete unresponsiveness of the receptor to hormone stimulation (Koller, Lipari et al. 1993; Potthast and Potter 2005; Potter, Abbey-Hosch et al. 2006). GC-B possesses five residues which upon phosphorylation enhance receptor activity. The identified sites are Thr-513, Thr-516, Ser-518, Ser-523 and Ser-526, also located within the KHD (Potter and Hunter 1998). Reports indicate that a mutation of any of these residues to alanine reduces receptor phosphorylation and accordingly its enzymatic activity (Potthast and Potter 2005). Unlike GC-A, which is totally unresponsive to ANP in a completely dephosphorylated state, GC-B could be still stimulated in the same state (Potthast and Potter 2005).

### 1.3.3 Homologous desensitization

Despite enhanced production and secretion of ANP under conditions of atrial hypertension and cardiac insufficiency ANP-dependent GC-A effects are markedly attenuated. Thus, understanding the mechanisms involved in down regulation of GC-A (and GC-B) during prolonged exposure to their ligands could have important consequences from a pathophysiological and clinical point of view.

As reported by several groups, prolonged ligand-receptor interactions unleash a signaling cascade resulting in receptor dephosphorylation and desensitization (Foster, Wedel et al. 1999; Kuhn 2003; Potter, Abbey-Hosch et al. 2006).
Desensitization caused by the physiological ligand of a receptor is generally called homologous desensitization. The conformational change triggered by ligand binding leads to the following events: the inhibitory effect of the KHD is abolished, then an increased dissociation rate decreases the extracellular domain's affinity for natriuretic peptides (Jewett, Koller et al. 1993). Finally, a conformational change within the KHD results in the exposure of the phosphorylated residues to a phosphatase. At the end, the receptor is dephosphorylated and less responsive to further ligand stimulation. Although, this model was initially proposed for GC-A it similarly holds for GC-B (Potter 1998).
The model for homologous desensitization is illustrated schematically in Figure 2.

### 1.3.4 Heterologous desensitization

Any mechanism that results in reduction of receptor activity not due to sustained agonist exposure is defined as heterologous desensitization. Signaling by substances such as angiotensin II, endothelin or vasopressin can similarly facilitate a reduction of receptor responsiveness of membrane-linked guanylyl cyclases (Potter and Garbers 1994). The vasoconstricting factors such as angiotensin II, endothelin and vasopressin induce GC-A desensitization via stimulation of protein kinase C (PKC) (Nambi, Whitman et al. 1986; Haneda, Kikkawa et al. 1991; Jaiswal 1992). Inositol triphosphate-calcium pathway contributes to GC-B desensitization, suggesting that arginine vasopressin-dependent elevated calcium concentration within rat aortic smooth muscle cells reduce GC-B activity (Abbey and Potter 2002). PKC-independent desensitization of GC-B can be induced by lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) (Abbey-Hosch, Cody et al. 2004; Potthast, Abbey-Hosch et al. 2004). S1P was shown to play a desensitizing role in transfected NIH3T3 fibroblasts and A10 vascular smooth muscle cells, whereas LPA

reduces GC-B activity in NIH3T3 cells and GC-A activity in MA10 Leydig cells (Muller, Cortes-Dericks et al. 2006).

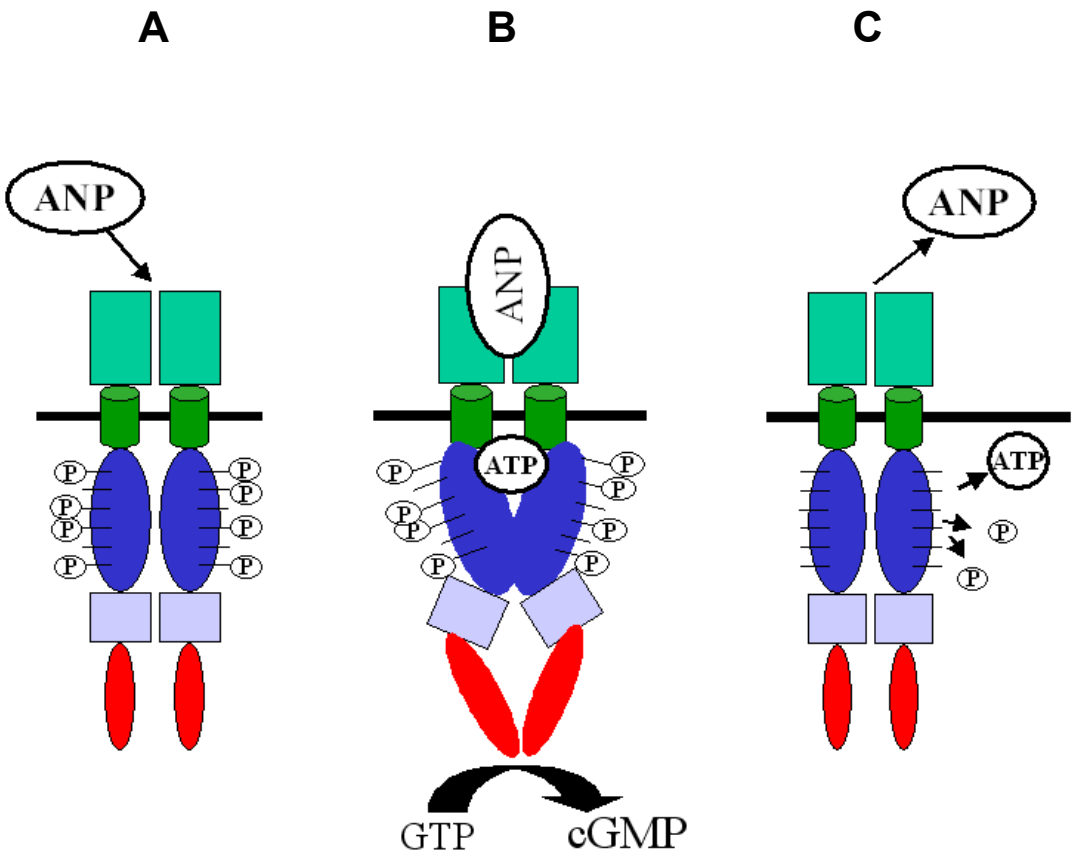


**Figure 2: Hypothetical model of GC-A activation and homologous desensitization**

In the basal state, GC-A is phosphorylated on serine and threonine residues within the kinase homolgy domain (A). ANP binding to the highly phosphorylated but inactive receptor (in the presence of ATP) results in a conformational change that relieves the repression of the KHD on the guanylyl cyclase domain (red). This process permits the dimerization of the cyclase domain resulting in its enhanced cyclase activity (B). Upon sustained exposure to ANP, dephosphorylation is stimulated and ANP dissociates (C). The receptor is now dephosphorylated and thus unresponsive towards further ANP stimulation. This process is called homologous desensitization. Phosphate is indicated by P in *small circles* (Kuhn 2003).

## 1.4 Signaling pathways

The link between ligand-dependent receptor activation and the cellular responses are molecules referred to as second messengers. This term was initially formulated after the discovery of 3',5' cyclic adenosine monophosphate (cAMP) and extended onto further molecules that transform receptor activation into cell response.

Activation of natriuretic peptide receptors GC-A and GC-B results in enhanced synthesis of cGMP. This cyclic nucleotide, first described in 1963 acts as a second messenger in multiple signaling pathways (Lucas, Pitari et al. 2000).

Playing a vital role in processing intracellular information, elevation of intracellular cGMP results in modulation of several target proteins, involved in downstream signaling events. These target proteins are mainly cGMP-regulated phosphodiesterases (PDEs), cGMP-gated ion channels and cGMP-dependent protein kinases, (Corbin and Francis 1999; Francis and Corbin 1999).

Since cGMP is produced by different soluble and membrane-linked guanylyl cyclases, which are co-expressed by many cell types, its production and function is compartmentalized (Kuhn 2004; Piggott, Hassell et al. 2006).

### 1.4.1 cGMP-dependent protein kinase (PKG)

The most important target protein of cGMP seems to be the cGMP-dependent protein kinase, known as PKG. This enzyme exhibits crucial functions in cGMP-dependent cellular signaling pathways (Yuasa, Omori et al. 2000). PKG is a dimer consisting of two identical monomers, each containing two sites: a catalytic domain and a regulatory domain (Francis and Corbin 1999; Pfeifer, Ruth et al. 1999). cGMP-binding transforms the protein into an active enzyme that phosphorlyates other proteins and thus induces physiological effects such as relaxation of vascular smooth muscle cells, bronchodilatation, inhibition of cell proliferation and platelet aggregation (Lohmann, Vaandrager et al. 1997; Francis and Corbin 1999).

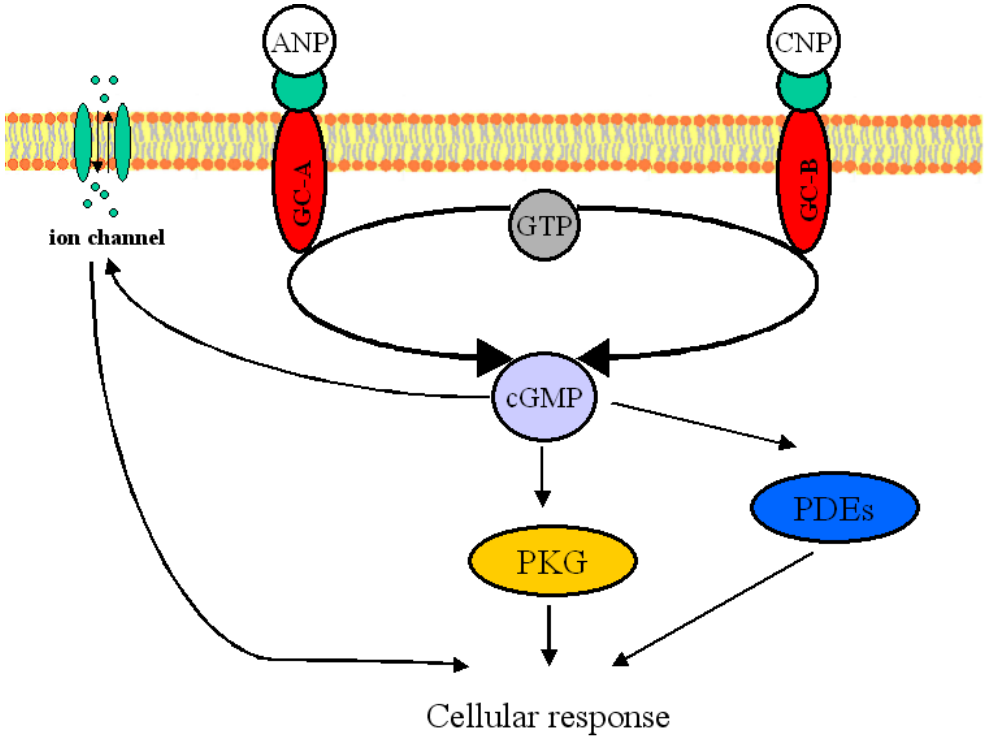


**Figure 3: Cellular target proteins of cGMP**

Both GC-A and GC-B convert GTP into cGMP upon natriuretic peptide activation. Increased cGMP modulates the activity of target proteins such as cGMP-regulated phosphodiesterases (PDEs), cGMP-dependent protein kinases (PKG) and cGMP-controlled ion channels. These target proteins in turn are involved in a variety of physiological processes in the nervous, the cardiovascular and the gastrointestinal systems as well as the kidney, bone and other tissues.

Two types of PKG have been identified, namely PKG I and PKG II. The former is found predominantly in cytoplasm whereas the latter is a membrane-bound protein. PKG I can be further subdivided into two isoforms: PKG I-α and PKG I-β. These isoforms differ only in their N-terminal sequence and exhibit different affinities for cGMP (Wall, Francis et al. 2003). Interestingly, one study indicates that ANP-dependent cGMP elevation stimulates the recruitment of PKG I from the cytoplasm to the plasma membrane. This rebound effect leads to the anchoring of PKG to GC-A

and is suggested to represent a mechanism for compartmentation of cGMP signaling (Airhart, Yang et al. 2003).

### 1.4.2 cGMP-regulated phosphodiesterases (PDEs)

PDEs comprise a large family of structurally related enzymes that hydrolyze cyclic nucleotides into the inactive nucleoside 5'-monophosphates.

The PDE superfamily is classified into 11 families, namely PDE1-PDE11, based on amino acid sequences, substrate specificities, regulatory properties, pharmacological properties and finally tissue distribution (Dousa 1999; Soderling and Beavo 2000). PDEs have different substrate specificities. Some are cAMP-selective (PDE 4, -7 and -8), and others are cGMP-selective (PDE 5, -6 and -9). There are also PDEs that can hydrolyze both cAMP and cGMP (PDE1, -2, -3, -10 and -11). The cAMP-degradation by PDE3 is inhibited by cGMP through competition to the catalytic site. Although PDE2 can hydrolyze both cyclic nucleotides, binding of cGMP to the regulatory domain increases cAMP affinity and hydrolysis. These mechanisms allow for cross-regulation of the cAMP and cGMP pathways (Mehats, Andersen et al. 2002). PDE enzymes are often targets for pharmacological inhibition due to their unique tissue distribution, structural properties, and functional properties (Jeon, Heo et al. 2005). The introduction of sildenafil, a drug designed to inhibit PDE5 and thus to prevent the degradation of intracellular cGMP, is used to treat male erectile dysfunction and pulmonary hypertension. Ultimately, PDEs control the level of second messengers and thus modify their access to target proteins.

### 1.4.3 Cyclic nucleotide-gated ion channels (CNG)

Cyclic nucleotide-gated ion channel is any ion channel that opens in the presence of cyclic nucleotides. Thus, binding of cGMP to its target ion channel may lead to changes in membrane potential. The mammalian visual system contains the most

famous member of this family, located within the outer membrane of retinal photoreceptor cells. The channels are open and allow positively-charged ions to flow into the cell, causing depolarization. CNGs play also a key role in the olfactory system (Fesenko, Kolesnikov et al. 1985; Nakamura and Gold 1987). They have also been detected in many other tissues, including heart, kidney, testis, lung, skeletal muscle, and the colon (Kaupp and Seifert 2002; Brown, Strassmaier et al. 2006). Despite their well described role in sensory tissue, their physiological function in other tissues remains poorly understood. However, this study will mainly focus on the first two described effectors of cGMP, the PKG and the PDEs.

# 1.5 Aims of the study

## 1.5.1 Background

Many studies have provided evidence for regulation of natriuretic peptide receptor activity by homologous (and heterologous) desensitization, and that this desensitization is based on receptor dephosphorylation reactions. However, crucial questions remain to be addressed. For example, the kinase(s) and phosphatase(s) acting at the receptor molecules have not yet been identified. Moreover, the signaling mechanism(s) responsible for re-sensitization/phosphorylation are unknown. In addition, it is still unclear whether desensitization pathways are affected in a cell type-specific manner.

One important further question concerns the receptor specificity of such reactions. To address this item, Cortes-Dericks investigated homologous desensitization of natriuretic peptide receptors in pituitary αT3-1 cells, which endogenously co-express ANP (GC-A) and CNP (GC-B) receptors (Muller, Cortes-Dericks et al. 2006). These studies revealed that ANP pre-treatments induced desensitization of GC-A, while pre-exposure of cells to CNP failed to elicit desensitization of GC-B. Since both receptors produced large amounts of the (common) second messenger cGMP in

response to ligand binding, it was of interest to examine whether agonist-induced increases in cellular cGMP could affect the activity of the other receptor each. Data showed that stimulation of GC-A had no effect on GC-B activity. However, and most surprisingly, CNP-induced stimulation of GC-B resulted in strongly enhanced activity of GC-A. These findings uncovered for the first time a mechanism by which a membrane guanylyl cyclase receptor becomes sensitized rather than desensitized. Further studies revealed that the CNP-induced sensitization of GC-A is blocked by cAMP-dependent protein kinase (PKA) inhibitors, indicating that PKA acts as an essential component in the signaling pathway.

### 1.5.2 Experiments with rat aorta

The observed sensitization of GC-A by CNP/GC-B signaling in pituitary αT3-1 cells raised the significant question whether such a regulation may take place also in other cell types co-expressing GC-A and GC-B. A particularly interesting cell type in this regard is the vascular smooth muscle cell. ANP signaling plays a pivotal role for regulating vascular contraction/relaxation via cGMP-mediated pathways (Pandey 2005), and several reports suggested the expression of both ANP (GC-A) and CNP (GC-B) receptors in smooth muscle cells of the aorta (Nagase, Katafuchi et al. 1997; Steinmetz, Potthast et al. 2004; Potter, Abbey-Hosch et al. 2006). The latter conclusion, however, was primarily based on investigations of receptor gene expression, and convincing data indicating co-expression at the protein level have not yet been provided.

Thus, the first goal of my investigations was to examine the protein expression of GC-A and GC-B in cells of the rat aorta. In the case of evidence for co-expression in vascular smooth cells, the aorta could be used in a proper way by functional approaches (e. g., tension recording, measuring of ligand-induced cGMP production) to address the following key questions:

1. Can CNP pre-exposure enhance the vasorelaxing potency of ANP?
2. Can CNP pre-exposure increase ANP-elicited cGMP production?

In addition, and partially dependent on the results obtained, further studies should clarify the following items:

3. If CNP pre-exposure in fact evokes sensitization of GC-A: Is this reaction (like in αT3-1 cells) mediated by PKA?
4. Considering conflicting data in the literature: Can stimulation of either GC-B (by CNP) or NPR-C (by c-ANF) also induce vasorelaxation?
5. Is the activity of GC-A in vascular smooth muscle cells regulated by homologous desensitization? Which kinase(s) mediate(s) this reaction?
6. Can CNP signaling counteract homologous desensitization of GC-A?

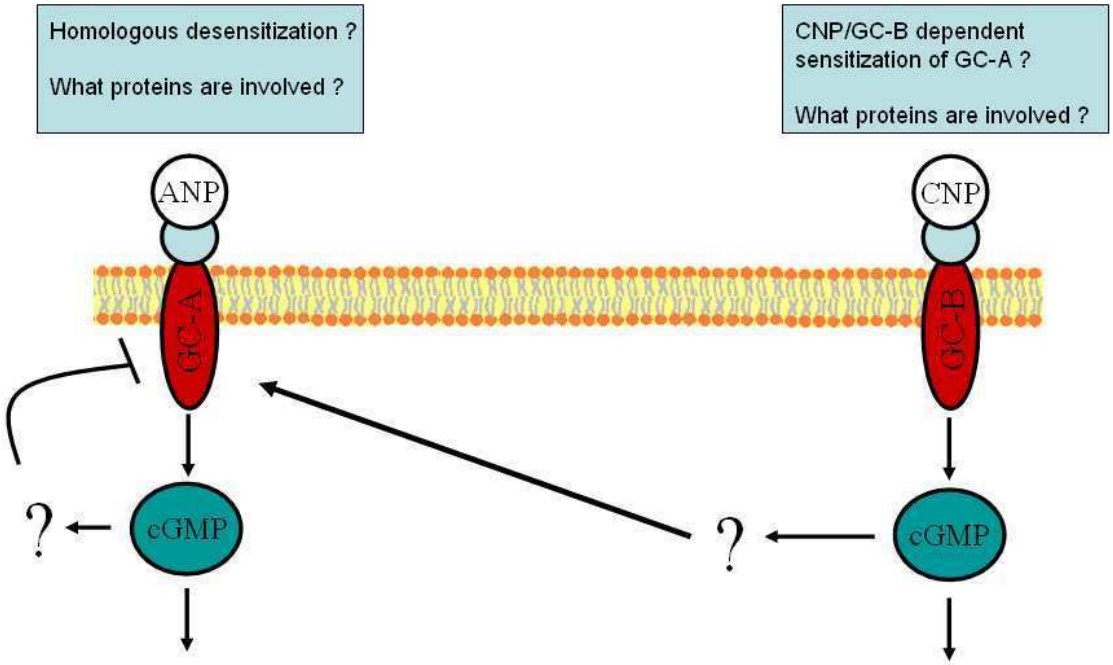


**Figure 4: Overview of underlying questions for experiments with rat aorta**

Studies are designed to examine (a) whether prolonged exposure to ANP results in desensitization of GC-A, (b) whether desensitization can be relieved by a CNP/GC-B-dependent pathway, (c) if specific molecules can be identified as co-actors in this process.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Wistar rats

Thoracic aortae were derived from healthy adult male Wistar rats (~250g), kindly provided by the Institute of Physiology, Justus-Liebig-Universität, Gießen. The animals were housed in cages, were fed a standard diet and had access to tap water ad libitum.

### 2.1.2 Reagents, solutions and substances

Solutions used

| | | |
|---|---|---|
| Bouin's Fixative | Saturated picric acid | 15ml |
| | Formaldehyde | 5ml |
| | Glacial acetic acid | 1ml |
| Minimum Essential Medium (MEM, containing Earle's salts and L-glutamine): | 2mM L-glutamine | |
| | 5.56 mM glucose | |
| | 1.8 mM $CaCl_2$ | |
| | 0.813 mM $MgSO_4$ | |
| | 26.19 mM $NaHCO_3$ | |
| | 117.24 mM NaCl | |
| | 5.33 mM KCl | |
| | 1.09 mM $NaH_2PO_4$ | |

| | |
|---|---|
| Hank's Balanced Salt Solution (HBSS, containing calcium and magnesium): | 5.56 mM glucose |
| | 1.26 mM $CaCl_2$ |
| | 0.493 mM $MgCl_2$ |
| | 0.407 mM $MgSO_4$ |
| | 0.441 mM $KH_2PO_4$ |
| | 4.17 mM $NaHCO_3$ |
| | 5.33 mM KCl |
| | 137.93 mM NaCl |
| | 0.338 mM $Na_2HPO_4$ |
| Homogenizing buffer: | 50mM Tris-HCl, pH 7.5 |
| | 1mM EDTA |
| | 1mM Dithiothreitol (DTT) |
| | 0.1 mM Phenyl-methyl-sulfonyl-fluoride (PMSF) |
| 4x Separating gel buffer: | 0.375 M Tris-HCl, pH 8.8 |
| | 0.1% (w/v) SDS |
| 4x Stacking gel buffer: | 0.5 M Tris-HCl, pH 6.8 |
| | 0.1% (w/v) SDS |
| PAGE buffer: | 0.025 M Tris-HCl, pH 8.4 |
| | 0.192 M glycine |
| | 0.1% (w/v) SDS |
| 3x SDS PAGE sample buffer: | 0.375 M Tris-HCl, pH 6.8 |
| | 0.2 M DTT |
| | 15% (w/v) SDS |
| | 20% (v/v) glycerine |
| | 0.6 mg/ml bromphenol blue |
| Tank blotting transfer buffer: | 100 mM Tris base |
| | 193 mM glycine |

| | |
|---|---|
| 10x TBS buffer: | 200 mM Tris base |
| | 1.37 M NaCl |
| | adjust pH to 7.6 |
| TBST tween buffer: | 1x TBS buffer + 0.05% Tween 20 |
| Blocking buffer: | 1% Amersham blocking reagent<br>0.1 M maleic acid/NaOH |
| | 0.15 M NaCl |
| | adjust pH to 7.5 prior to addition of |
| | 0.005% thimerosal |
| Antibody dilution buffer: | 90% TBST |
| | 10% blocking buffer |
| | 0.005% thimerosal |
| E-PBS buffer: | 0.1 M $Na_2PO_4$ |
| | 0.15 M NaCl |
| | 0.005 M EDTA |
| | 0.2% BSA |
| | 0.01% thimerosal |
| | pH 7.0 |
| Substrate buffer: | 4.8 M Na-acetate |
| | 0.24 M citric acid |
| Wash solution: | 0.02% Tween 20 in $H_2O$ |
| | 0.5% NaCl |

Table 2: Substances and concentrations used

| **Substance** | **Supplier** | **Concentrations used (M)** |
|---|---|---|
| Norepinephrine | Hoechst Marion Roussel | $1x10^{-5}$ |
| Atrial natriuretic peptide, (ANP) | Bachem | $5x10^{-7}$ |
| C-type natriuretic peptide, (CNP) | Bachem | $5x10^{-7}$, $1x10^{-7}$, $5x10^{-8}$ |
| Rp-cAMPS (PKA inhibitor) | BioLog | $3x10^{-5}$ |
| (Cys18)-atrial natriuretic factor (4-23) amide, cANF (NPR-C agonist) | Bachem | $5x10^{-7}$ |
| 3-Isobutyl-1-Methyl-Xanthin (IBMX) | Sigma | $2x10^{-4}$ |
| Rp-8-Br-cGMPS (PKG-inhibitor) | BioLog | $1\text{-}5x10^{-6}$ |
| HS-142 (Inhibitor of both GC-A and GC-B) | Mr. Yuzuru Matsuda, Tokyo Research Laboratories | $10^{-3}$ |
| 8-br-cAMP (8-bromo-cyclic- adenosine monophosphate) | Biolog | $10^{-3}$ |
| $^{125}$I-ANP | Amersham | $1x10^{-9}$ |
| $^{125}$I-CNP | Amersham | $3x10^{-9}$ |

# 2.2 Methods

## 2.2.1 Vessel preparation

Rats were anesthetized with isoflorane (Baxter) and subsequently sacrificed by cervical dislocation. The abdomen and thorax were opened with a midline incision and both the heart and the aorta were carefully excised. Aortae were dissected from the heart and placed immediately in HBSS at 4° C.

Depending on the experiments intended, vessels were either used for immunohistochemistry (2.2.2), protein extraction (2.2.3), cut into rings for organ bath experiments (2.25) or dissected longitudinally for cGMP measurement (2.2.6)

## 2.2.2 Immunohistochemistry (IHC)

Immunohistochemistry visualizes the localization of proteins in cells and tissues. For that purpose tissues are fixed and cut into thin slices prior to incubation with primary antibodies in question. The binding sites of these antibodies become detectable by usage of secondary antibodies that produce visible reaction products.

In this study, both paraffin and cryostat sections were used.

For former sections, aortae were fixed in Bouin's fixative and then dehydrated in ascending ethanol concentrations. Afterwards tissues were embedded with paraffin at 60° C by using a tissue processor (Leica TP 1020, Bensheim, Germany). Sections of 6 μm were sliced with a microtome (Autocut 1140, Reichert-Jung) and mounted onto chrome gelatine-coated slides followed by a drying period at 37° C overnight. Subsequently sections were deparaffinised in descending ethanol concentrations and rehydrated with $H_2O$. In order to block endogenous peroxidase activity, sections were treated with 1.2% $H_2O_2$ in methanol for 30 min and then washed with phosphate buffered saline (PBS) for another 15 min. Sections were blocked with 2% swine non-immune serum. After incubation with primary antibodies (see table below) overnight at 4° C, sections were washed with PBS for 3x10 min. Sections were treated with anti-rabbit IgG-biotin, (swine 1:250 in PBS) for 60 min at room temperature and washed afterwards 2x10 minutes with PBS. A combination of the peroxidase antiperoxidase (PAP) technique with the avidin-biotin-peroxidase complex method was employed, visualizing peroxidase activity by the nickel-glucose oxidase approach (Middendorff, Muller et al. 2002):

For cryosections, rat aortae were embedded in Tissue Tec® (Sakura, Torrance, CA, USA) and frozen in 2-methylbutane (Fluken) over liquid nitrogen. Sections of 10μm were cut with a cryostat (Leica CM 1900, Bensheim, Germany). Slices were mounted onto chrome gelatine-coated slides and fixed by 4% paraformaldehyde in PBS for 20 min, followed by 10 min washing in $H_2O$ and drying for one h at room temperature. After preincubation with 2% normal goat serum in PBS, primary antibodies directed against GC-A or GC-B (see table below) were applied and incubated at 4° C overnight. Sections were washed with PBS for 2x10 min and then incubated with Cy3-conjugated secondary goat-anti-rabbit-IgG-antibody (Dianova) for 1 h at room temperature in darkness. After washing with PBS, sections were analysed.
For negative controls, sections were used, in which primary antibodies were replaced by PBS.
Sections were viewed with a fluorescence microscope (Axioskop2 plus, Zeiss, Germany). Micrographs were taken by a digital camera (Axiocam Mrc, Zeiss) and edited with manufactor's software (Axiovision Release 4.6.3, Zeiss).

Table3: Antibodies used in immunohistochemistry

| **Primary Antibody** | **Host** | **Dilution** | **Manufacturer** |
|---|---|---|---|
| GC-A | Rabbit | 1:50 | FabGennix |
| GC-A | Rabbit | 1:50 | Kuhn, University of Würzburg |
| GC-B | Rabbit | 1:100 | FabGennix |

## 2.2.3 Protein extraction

Proteins were extracted from isolated aortas. The surrounding connective tissue was either removed or not. Frozen in liquid nitrogen, tissues were grinded with a mortar, suspended in homogenizing buffer and then homogenized in a Potter-Elvehjem homogenizer by ~10 strokes. To remove cell debris and nuclei, the resulting

homogenates were centrifuged for 8 min at 3,000 x g at 4° C (Eppendorf 5804 R, Hamburg, Germany). The supernatant fractions containing cytosol and particulate fractions were re-centrifuged at 100,000 x g for 30 min at 4° C using an ultracentrifuge (Ultra Pro 80, Savall, Wilmington, DE, USA). Resulting pellets, containing membranes, were resuspended with 50 mM Tris-buffer, pH 7.5. Protein concentration was determined according to Bradford (Bradford, 1976) using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Munich, Germany) by means of BSA (fraction V) as standard.

## 2.2.4 Western Blot

### 2.2.4.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a method to separate proteins based on their size. Proteins are maintained in a denatured state after treatment with reducing agents to remove secondary and tertiary structure (including disulfide bonds). Sample proteins become covered in anionic detergent SDS and move to the positively charged electrode through the acrylamide mesh. The migrations velocity is dependent on the protein size.

In my studies, SDS-Page was performed according to Laemmli (1970).
Separating gels were pipetted carefully between glass plates avoiding formation of air bubbles. Gel solutions were overlayered with 400 µl butanol and allowed to polymerize at room temperature overnight.
The following day, butanol was removed and combs were inserted between glass plates to enable the formation of wells. Stacking gel solutions were pipetted down on the top of the separating gel. After polymerization (60min) combs were removed and the wells were filled with PAGE buffer using a syringe. Samples were boiled at 100° C for 2 min and subsequently chilled on ice for 20 seconds followed by loading the samples.

Electrophoresis was performed in Hoefer SE 600 units (Hoefer Scientific, Instruments, San Francisco, CA, USA). An initial power of 13 W was applied until samples have reached separating gel. Thereafter the electrophoresis was continued at 17 W for 2 h. Molecular weight standards (Sigma) were used as size references (see table 4).

Table 4: Reference proteins for SDS-PAGE

| **Protein** | **Molecular Weight** |
|---|---|
| Myosin, porcine | 200.000 |
| B-Galactosidase, E. coli | 116.000 |
| Phosphorylase b, rabbit muscle | 97.000 |
| Albumin, bovine | 66.000 |
| Albumin, chicken egg white | 45.000 |
| Carbonic Anhydrase, bovine erythrocytes | 29.000 |

### 2.2.4.2 Protein transfer to membranes (blotting)

Size-separated proteins from gels were transferred to nitrocellulose membranes (Hybond ELL, Amersham Biosciences, Germany) using tank blotting transfer buffer.
Membranes and filter paper were cut according to the size of the gel and equilibrated in transfer buffer. The gel-blot-package, consisting of 2 sheets of 3MM-Whatman® paper (Maidstone, UK), the gel, the membrane and another 2 sheets of Whatman® paper were laid onto a glass plate. By rolling carefully a glass pipette on top of the blots any bubbles that may have been stuck underneath the gel, were removed. The blots were mounted in holder cassettes which were installed in the blot tank filled with transfer buffer.
Electroblotting was carried out at 29 V at 4° C overnight. Blots were stained with Ponceau S for ten min in order to control the efficiency of protein transfer. Membranes were rinsed with $H_2O$ for 2 min, scanned for digitalized documentation

and thereafter blocked in blocking buffer for 2 h in a shaking apparatus (PMR-30, Grant-Bio, Cambridgeshire, UK). Prior to immunostaining, membranes were shaken in TBST for 5 min.

### 2.2.4.3 Immunostaining of membranes

After blocking, primary antibodies (see table 5) diluted with antibody dilution buffer were added and incubated for 1 h at room temperature. Subsequently membranes were washed (3x5 min) in TBST and then incubated with peroxidase-linked secondary antibodies for one h at room temperature. After another washing series in TBST as before, binding of the antibody was visualized using enhanced chemiluminescence (ECL) according to manufacturer's protocol (Amersham, GE Healthcare, Buckinghamshire, UK). Blots were wrapped with a plastic foil, placed into a film cassette (Hypercassette, Amersham, Biotek, UK) and exposed to x-ray films (Fuji Medical X-Ray, 18x24) up to 5 min. Films were developed by incubation in appropriate agents (Agfa, G138i) for 2 min and fixed (Agfa, 334i) for 1 min. Finally the films were rinsed with water and hanged up for drying.

Table 5: Primary antibodies used in immunoblotting

| **Primary Antibody** | **Dilution** |
|---|---|
| GC-A (Kuhn) | 1:500 |
| GC-A (FabGennix) | 1:500 |
| GC-B (FabGennix) | 1:500 |

Table 6: Secondary antibodies used in immunoblotting

| **Secondary Antibody** | **Dilution** | **Manufacturer** |
|---|---|---|
| Peroxidase-conjugated goat-anti-rabbit IgG | 1:2000 | Pierce Biotechnology |

## 2.2.5 Affinity cross-linking

This method enables detection of receptors after cross linking with their radioactively labelled ligands (Muller, Hida et al. 2009).

Aorta membrane preparations were incubated with either $^{125}$I-ANP (1 nM) or $^{125}$I-CNP (3 nM) in total volumes of 40 µl of 20 mM HEPES buffer, pH 7.5, containing 5 mM $MgCl_2$, 125 mM NaCl, and the protease inhibitors parahydroxymercury benzoate (60 µg/ml), bacitracin (1 mg/ml), bestatin (50 µg/ml), phosphoramidon (50 µg/ml), and 1.10-phenanthroline (1 mM) for 15 min at 20°C. Samples were then irradiated in the dark for 10 min with UV light (peak wavelength 302 nm) followed by chilling and immediate addition of 20 µl of 3X SDS-PAGE sample buffer. Prior to analysis by SDS-PAGE under reducing conditions according to Laemmli (1970) in 7% polyacrylamide separation gels, samples were boiled for 3 min. For visualization of molecular weight marker proteins (Sigma, SDS-6H), gels were stained with Coomassie brilliant blue, then dried in a Gel dryer (Bio-Rad, model 583, USA) and exposed for 5 days to X-ray film (Kodak XAR-5) between intensifying screens at -70°C.

To confirm the identity of GC-A and GC-B, reactions were also performed in the presence of an excess (1 µM) of unlabelled ANP or CNP, respectively.

## 2.2.6 Isometric tension studies

Isometric tension studies in organ baths enable sensitive measurements with the intact biological structures ex vivo. Contraction of vessels is detectable by an increase in force transduction, whereas relaxation correlates with decreasing force.

For isometric tension studies in the organ chamber, a ring of approximately 3mm length was excised gently off the thoracic part of the vessel. In order to avoid holes in the inner wall of the ring, segments were cut always between the outlets of the aorta. All connective tissue and periadventitial fat were removed from the segment with care under microscopic view while harming of the endothelium was avoided.

The rings were suspended in a 15 ml organ bath using two stainless-steel hooks placed vertically through the lumen. The lower hook was fixed, while the upper one was connected to isometric force recording transducer (SG4-90, Hugo Sachs, Freiburg, Germany). The output of the transducer was digitized at 1 Hz by using a Metrabyte DAS 1202 interface (Keithley Instruments, Cleveland, OH). Simultaneously, isometric force was recorded analogue on a chart recorder (200 E, Linseis, Selb, Germany).

Each organ bath contained 15 ml MEM, continuously gassed with carbogen (95% $O_2$ and 5% $CO_2$) to provide oxygenation and pH stability.

Prior to drug addition the rings were stretched to a preload tension of 10 mN. It was allowed to equilibrate for 1-2 h and to attain a steady-state resting tension to prevent stretch-induced contractions (Mewe, Bauer et al. 2006).

Data collection was carried out by using a DOS program developed by P. Bassalay (Institute of Physiology, UKE, Hamburg, Germany). Further data processing was performed with Sigma Plot 5.0 (SPSS, Chicago, IL).

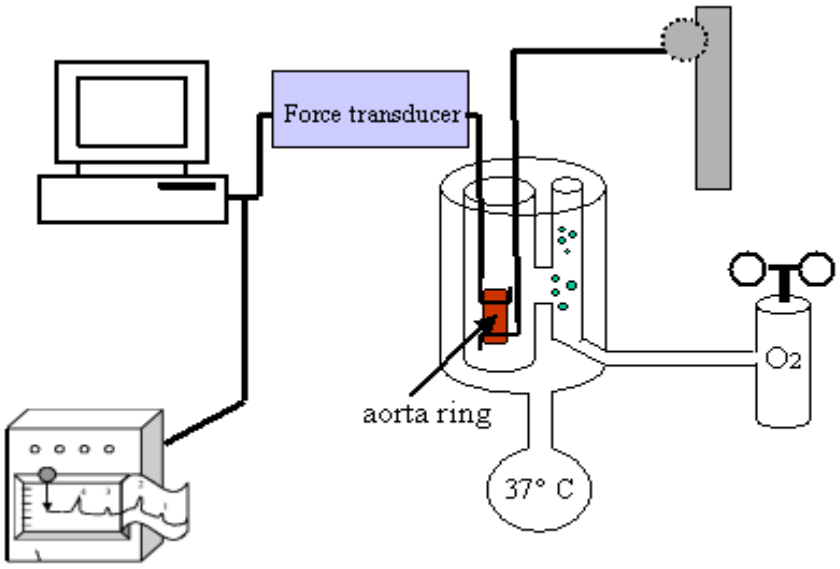


**Figure 5: Setting of isometric tension studies**

Aortic ring was suspended into medium-filled and carbogen-gassed organ bath and mounted between two stainless steel hooks, one fixed, the other connected to a force transducer. Force, exerted by the tension of the rings, was digitized via computer and simultaneously recorded on an analogue chart recorder. The temperature of the organ bath was maintained at 37°C.

After the resting tension of aortic rings reached a steady state, a trial of 3-5 minutes was recorded before experimental treatments. Experiments started with a precontraction induced by $1x10^{-5}$M norepinephrine. Once maximum responses had developed and a plateau of contraction had been reached, relaxation was initiated by adding natriuretic peptides, response curves for ANP and CNP were then recorded.
Relaxation was expressed as percentage of the maximum steady-state tension (100%) after initial contraction.
Between experiments organ chambers were rinsed several times with deionised water to wash out agents from previous experiments and to avoid accumulation of metabolites.

## 2.2.7 Measurements of cGMP production

Aortae were freed from surrounding tissue, and all outlets were cut off directly along the vessel's wall. Purified vessels were divided into 2 stripes of similar width and a moist mass of approximately 2-4 mg. In each case, one of these stripes served as control during following experimentation. The stripes were stored in MEM (4° C) until being used for experiments.

Stripes from 1-4 aortae were used per experiment, each divided into treatment groups A and B.

Both groups were washed again and pre-incubated in MEM for 5 min at 37° C in a heat block. After short (3 sec) centrifugation, medium was removed with a pipette.

To induce cGMP generation, samples were treated with ANP (or CNP) for 20 or 30 min at 37° C in the presence of 0.2 mM 1-methyl-3-isobutyl-xanthine (IBMX, a phosphodiesterase inhibitor used to avoid cGMP degradation).

In some experiments, samples were preincubated once or twice for predetermined periods with different substances (see protocols below). In those cases, medium was removed again after preincubation as described before and the second incubation was carried out accordingly.

Reactions were terminated by chilling. The medium (as well as the vessel stripes) were removed and frozen in liquid nitrogen and stored at -80° C cGMP measurements by means of a commercial ELISA.

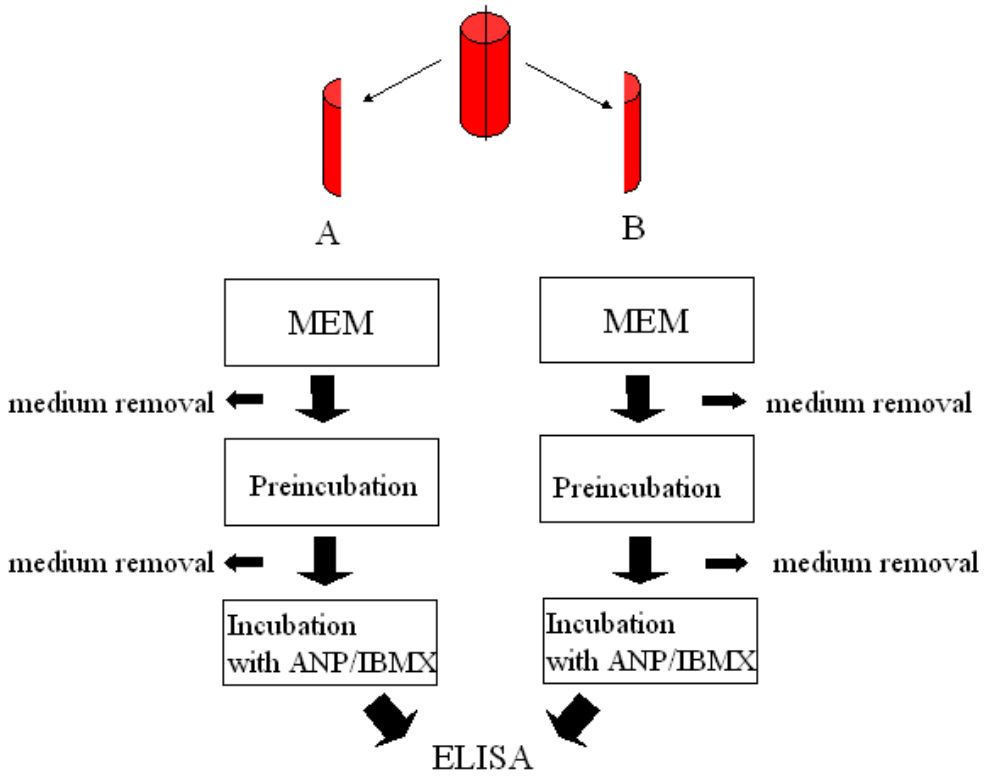


**Figure 6: Experimental design for measurements of ANP-induced cGMP generation by of rat aorta**

Aortae were dissected longitudinally, one half assigned to group A, the other half to group B. After pre-incubation in MEM for 5 min at 37°C, samples were pre-treated with different agents. Finally samples were incubated with ANP to elicit cGMP production. IBMX was added to avoid degradation of cGMP. Reactions were terminated by putting tubes on ice. The amounts of cGMP were determined by ELISA.

### 2.2.7.1 Enzyme-linked-immuno-sorbent-assay (ELISA)

The amounts of cGMP after different treatments were determined by enzyme-linked-immuno-sorbent-assay (ELISA). It is based on a competitive double-antibodies enzyme immuno assay with solid phase technique. The immunologic reaction takes place in goat-anti-rabbit-antibodies-coated wells. The cGMP in the sample competes with a biotin labelled cGMP tracer for binding to the (primary) anti-cGMP-antibody. Thus, both cGMP-antibody and tracer-antibody complexes are simultaneously bound to the surface of the wells via the secondary antibody. In a second step, a streptavidin-coupled horseradish peroxidase (HRP) is added, which binds selectively

to the tracer-antibody complex. The enzyme activity converts the colourless substrate tetramethyl benzidin (TMB) into blue. After adding $H_2SO_4$ blue turns into yellow. The colour change of the chromogen depends on the amount of enzyme linked to the wells and is inversely proportional to the cGMP concentration.

cGMP standards were prepared at the following concentrations: 34.02, 11.34, 3.78, 1.26, 0.42, 0.14, 0.00 pmol/ml assay buffer (EPBS). Before starting the assay, a 96-well plate coated with goat-anti-rabbit-antibodies was prewashed with EPBS at room temperature. 50 µl of standards and 50 µl of samples were pipetted first, followed by 50 µl of cGMP-biotin. Finally 100 µl cGMP antiserum was added. The plate was incubated in a $H_2O$ moistened chamber at 4° C in darkness for 18 h.
After washing 0.2 ml HRP-Streptavidin was substituted and incubated for 30 min at 4° C in darkness. Subsequently, well plates were washed 4 times with wash solution at room temperature. The substrate solution (250µl TMB) was incubated for 40 min at room temperature in darkness.
Reactions were terminated by addition of 50 µl 2M $H_2SO_4$. Plates were measured in an ELISA reader (Dynex revelation) at 450 nm. The minimum detection limit was 0.14 pmol/ml and cross-reactivity with cAMP less than 0.0001%.

### 2.2.8 Data presentation and statistical analysis

Data were analysed and presented using Prism 4.00 (GraphPad software Inc., San Diego, CA, USA) and Excell (Office XP, Microsoft Inc.). The significance of effects was assessed by unpaired *t* test or 1way ANOVA. Significant effects (P values <0.05) are indicated by asterisks. Standard deviations are indicated by vertical bars.

# 3 Results

Earlier studies revealed a co-expression of the two cGMP-generating natriuretic peptide receptors, GC-A and GC-B in αT3-1 cells (Muller, Cortes-Dericks et al. 2006) and provided evidence for a regulatory cross-talk between the two receptor signaling pathways in this cell (see Introduction). Based on these findings, rat aortic tissue was thought to represent an attractive experimental model to investigate whether similar regulatory mechanisms are present in the vascular system.

## 3.1 Characterization of natriuretic peptide receptors in vasculature

Although gene expression of natriuretic peptide receptors in the vasculature has been reported, reliable data on these receptors at the protein level have not yet been published. By means of highly specific antibodies, this study used immunohistochemical and immunoblotting approaches to examine the expression of GC-A and GC-B in rat aorta. Additionally, affinity cross-linking experiments were performed as a further approach for analyzing these proteins and allowed to co-examine the local expression of the third natriuretic peptide receptor, NPR-C.

### 3.1.1 Immunohistochemical analysis of GC-A and GC-B expression in rat aorta

To examine the distribution of natriuretic peptide receptors in vascular tissue, rat aortae were isolated and fixed for immunohistochemical analyses. Both cryosections and paraffin sections of rat aortae were incubated with antibodies against GC-A and GC-B. While visualization of immunoreactivity in paraffin sections was induced by the use of the peroxidase antiperoxidase technique with nickel glucose oxidase amplification (Middendorff, Muller et al. 2002), Cy3-conjugated secondary antibodies

served to visualize immunoreactivity on cryosections. For specifity controls, analogous assays were performed with PBS instead of primary antibodies.

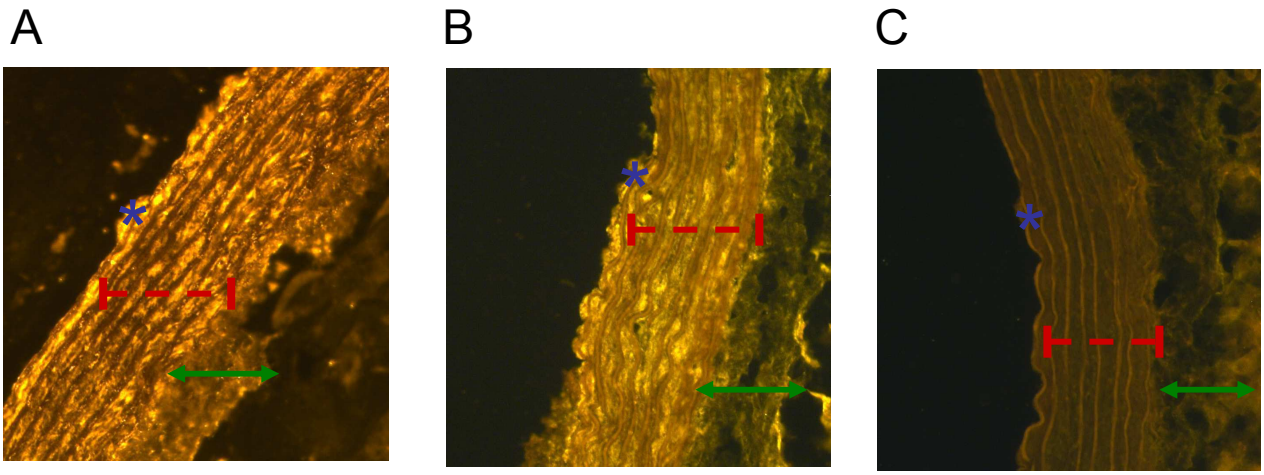


**Figure 3: Immunohistochemical analysis of GC-A and GC-B expression in cryosections of rat aorta**

Rat aorta sections (10µm) were treated with anti-GC-A antibody (A) or anti-GC-B antibodies (B). Control incubations with PBS instead of primary antibodies are shown in C. Cy3-conjugated secondary antibody served to visualize immunoreactivity. Specific staining is evident in endothelial cells (*) and in smooth muscle cells located in the media (I----I). Connective tissue (green arrows) lacks almost completely any immunoreactivity.

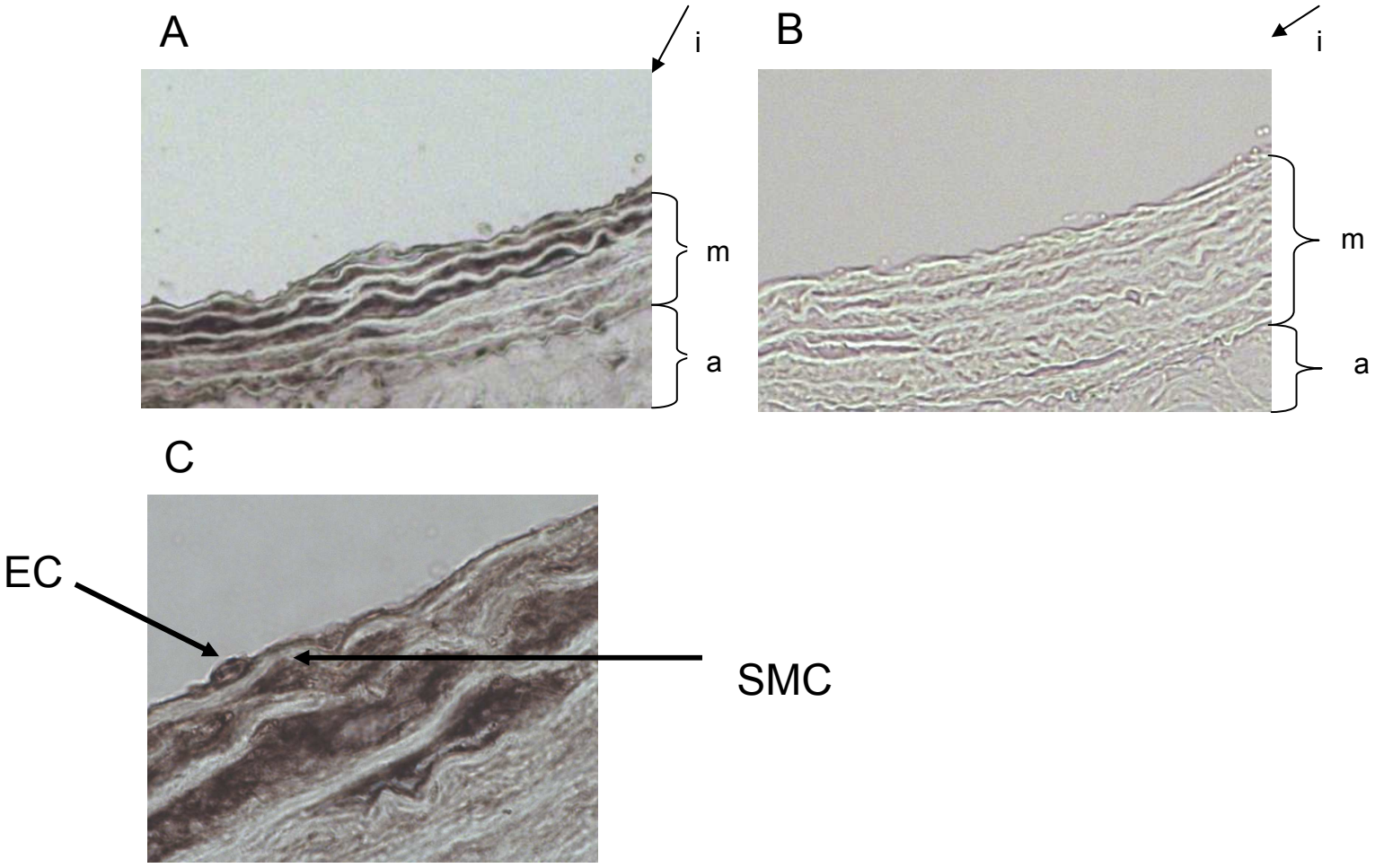


**Figure 4: Immunohistochemical analysis of GC-A and GC-B expression in paraffin sections of rat aorta**

Paraffin sections (6 μm) were incubated in either the presence (A) or absence (B) of anti-GC-A-antibodies. A combination of the peroxidase antiperoxidase (PAP) technique with the avidin-biotin-peroxidase complex method was employed to visualize immunoreactivity. GC-A expression is detectable in the intima (i) and media (m), but not in the adventitial connective tissue (a) of the aorta (A). At higher magnification (C), staining of endothelial cells (EC, arrow) and smooth muscle cells (SMC, arrow) becomes evident.

These studies revealed abundant expression of GC-A (Fig. 3A) and GC-B (Fig. 3B) in the vessel's intima and media, indicating localization in endothelial cells and smooth

muscle cells. On the other hand, both receptors are barely detectable in the connective tissue surrounding the vessel. Control sections (Fig. 3C) proved the specifity of antibody reactions. These findings were confirmed by corresponding analyses of GC-A (Fig. 4) and GC-B (data not shown) on paraffin sections. Thus, these results obtained clarified the distribution of these receptors in aortic tissue and provided strong evidence for co-expressions of GC-A and GC-B in both endothelial and smooth muscle cells.

### 3.1.2 Immunoblot analysis of GC-A and GC-B expression in rat aorta

To further characterize the expression of natriuretic peptide receptors in the vasculature, samples of rat aortae were homogenized and analyzed via immunoblotting. In these experiments vessels with or without surrounding connective tissue were used.

After separation by SDS-PAGE equal amounts of membrane protein were blotted and receptor levels were detected by use of antibodies against GC-A and GC-B (Fig. 5). These studies revealed a strong expression of both GC-A and GC-B in aortic tissue freed of surrounding connective tissue, whereas receptor levels were much lower in samples including connective tissue remained low (Fig. 5). The results confirmed the immunohistochemical data showing that both receptor types are predominantly distributed within the media and intima layers of the vessel and localized to endothelial and smooth muscle cells.

Protein size markers were used to assess the apparent receptor molecular masses. Consistent with recent findings (Muller, Hildebrand et al. 2010), GC-A has a size of 127 kDa in rat aorta. The higher molecular mass of GC-B (130 kDa) is explained by an enhanced extent of N-linked glycolization (Muller, Hildebrand et al. 2010) .

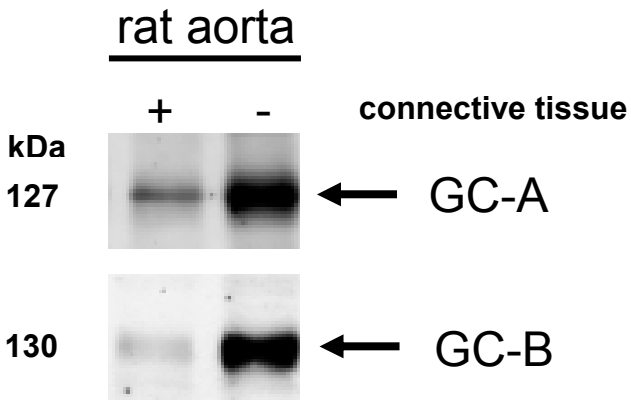


**Figure 5: immunoblot analyses of GC-A and GC-B in isolated rat aortae**

Aortae were isolated from male Wistar rats. Connective tissue was either carefully removed (-) or not (+). Samples derived from at least three animals were homogenized and membrane proteins were separated by SDS-PAGE and subsequently subjected to immunoblot analyses using antibodies directed against GC-A and GC-B respectively. Positions of antigens (arrows) and receptor sizes (in kDa) are indicated. Data is a representative of at least three experiments performed.

### 3.1.3 Affinity labeling of natriuretic peptide receptors, GC-A, GC-B and NPR-C, in rat aorta

To further investigate natriuretic peptide receptor expression in rat vascular tissue photoaffinity labeling were used.

Membranes prepared as described above were incubated with radiolabeled ANP (the natural ligand to GC-A) or CNP (ligand to GC-B), respectively. Again, vessels with connective tissue were compared with those freed of surrounding tissue.

Receptor expression was analyzed by UV light-induced cross linking followed by SDS-PAGE and autoradiography. Several previous studies have proved this method to be a reliable approach for detection of NP receptors in tissues (Muller, Mukhopadhyay et al. 2004; Muller, Hida et al. 2009; Muller, Hildebrand et al. 2010).

Consistent with the immunohistochemical and immunoblotting data, these experiments demonstrated the presence of GC-A (Fig. 6a) and GC-B (Fig. 6b) in

vascular tissue. Membranes prepared from aortae without connective tissue showed much higher receptor concentrations then those where connective tissue was not removed. These findings confirmed the predominant expression of GC-A and GC-B in membranes of endothelial and smooth muscle cells.

Importantly, cross linking approach also revealed detectable amounts of the third natriuretic receptor, NPR-C, which is labeled by both radioligands and migrates at 60 kDa (Fig. 6a, b). In contrast to GC-A and GC-B, levels of NPR-C are much more pronounced in aortae unfreed of surrounding tissue, indicating its predominant expression in the connective tissue and rather than in the media and intima layers of the vessel.

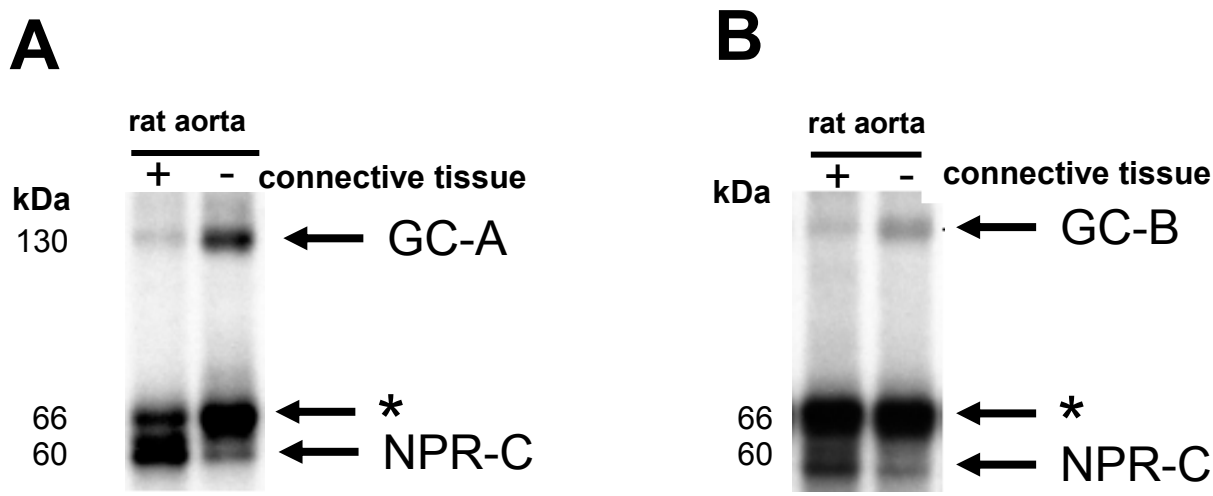


**Figure 6: Analysis of natriuretic peptide receptors expression in isolated rat aorta by photoaffinity labeling**

Equal amounts of membrane protein, containing (+) or derived of (-) surrounding connective tissue extracted from rat aortae were incubated with either $^{125}$I-ANP (A) or $^{125}$I-CNP (B) in the presence of protease inhibitor (see 2.2.4). Receptor/radioligand cross linking was induced by UV irradiation and reaction products were resolved by SDS-PAGE and autoradiography. Radiolabelled GC-A (A, 130kDa) and GC-B (B, 132 kDa) are indicated by arrows. NPR-C, labeled by both radioligands appears at nearly 60 kDa. Bands at 66 kDa marked by asterisks represent unspecifically labeled serum albumin, usually present in $^{125}$I-labeled peptide solutions.

Thus three distinct approaches, namely immunohistochemistry, immunoblotting and affinity cross linking, consistently revealed a co-expression of GC-A and GC-B in aortic tissue. The obtained results further demonstrate that these receptors are predominantly localized in the intima and media layers of the vessel, indicating that mainly endothelial cells and smooth muscle cells are the sites of expressions.
Additionally, data shows a differential distribution of the third natriuretic peptide receptors, NPR-C, which is predominantly expressed in cells of the adventitial connective tissue surrounding the aorta.
These studies revealed for the first time on a protein basis, that both receptors are co-expressed in aortic tissue, predominantly in endothelial cells and smooth muscle cells, while the third natriuretic receptor type, NPR-C, was found mainly in the surrounding tissue.

Considering the identified GC-A regulation by GC-B/CNP signaling in $\alpha$T3-1 cells which co-express both receptors, the above results provided and experimental basis to examine whether such a cross talk is present and active also in cell types of the aorta.

## 3.2 Isometric tension studies with rat aorta

After expression of natriuretic peptide receptors has been characterized in rat aorta, functional experiments with isolated vessels were carried out in order to assess the activities of these receptors by measuring their impact on change in vascular tone. Since all three receptors are expressed in rat aorta, with GC-A and GC-B predominantly in smooth muscle cells, preliminary experiments served to determine which receptor is primarily responsible to mediate vessel relaxation through ligand activation. Moreover, the role of the so-called clearance receptor, NPR-C, in vasorelaxation was investigated.
In general, thoracic aortae, obtained from male Wistar rats, were carefully cleaned of their fascia. To assure the integrity of the wall, rings were cut only between vessel

outlets. Rings were placed in a double-jacketed tissue bath in oxygenated medium at 37° C, mounted on two stainless-steel hooks, the lower hook fixed while the upper one transmitted isometric force via a force transducer.

After rings were stretched to a preload tension of 10 mN, they relaxed to a steady-state resting tension after 1-2 hours. Following a trial of 3-5 minutes of resting tension, experiments started with addition of norepinephrine (NE) to induce vessel contraction. Rings were then treated with vasorelaxant agents once maximum response had developed and a stable contraction tension had been reached.

Degree of relaxation was expressed as the percentage of tension reduction between the maximum norepinephrine-induced tension before application of vasorelaxant agents and the baseline tension prior to NE administration.

### 3.2.1 Vasorelaxant potency of ANP

Following treatment with norepinephrine, aortic rings were exposed in a cumulative manner to increasing concentrations ($10^{-9}$ to $5x10^{-7}$ M) of ANP. Single doses were given at intervals of 2 minutes or after a stable tension was attained (Fig. 7). Significant vasorelaxation was detectable at low ANP concentrations (10% at 10nM) and after the final administration of 500 nM, the NE-induced tension was reduced by 80%.

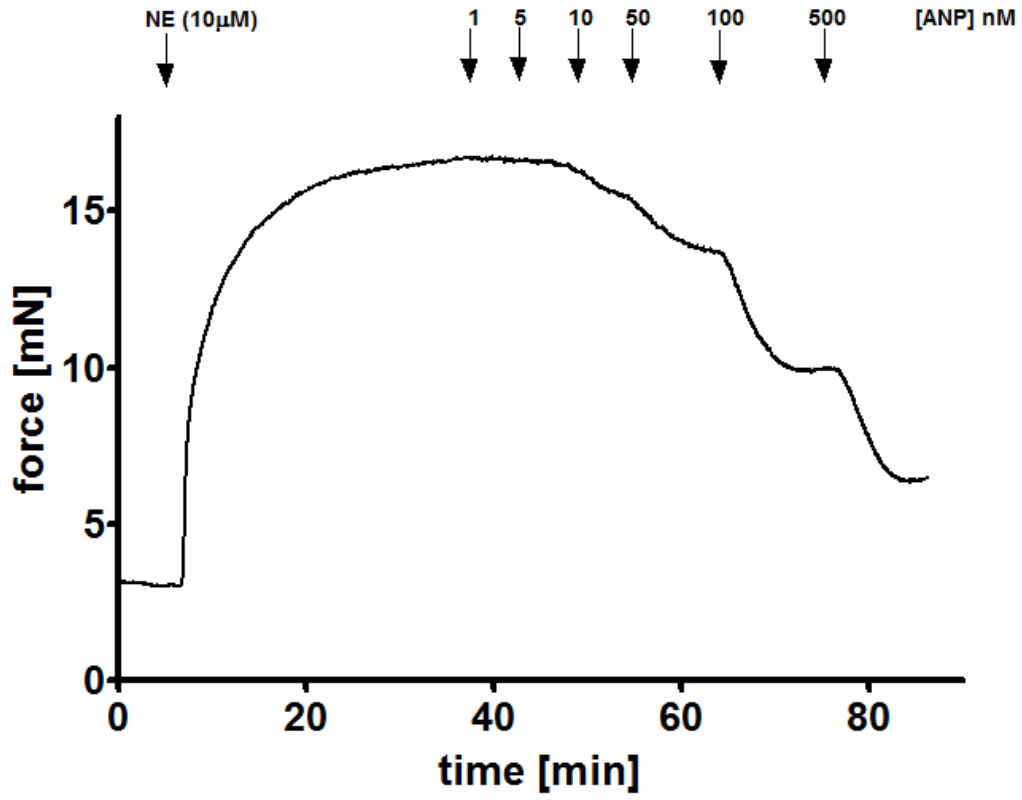


**Figure 7: Concentration dependency of ANP-induced vasorelaxation:**
Relaxation of the aorta by increasing concentration of ANP after precontraction with norepinephrine (NE) was recorded. The times and concentrations of the agents applied are indicated.

## 3.2.2 Vasorelaxant potency of CNP

To investigate vasorelaxant effects induced by the CNP receptor, GC-B, analogous experiments were carried out with CNP instead of ANP. Again, precontracted aortae were treated with increasing peptide concentrations ranging from $10^{-9}$ to $5x10^{-7}$ M (Fig.8).

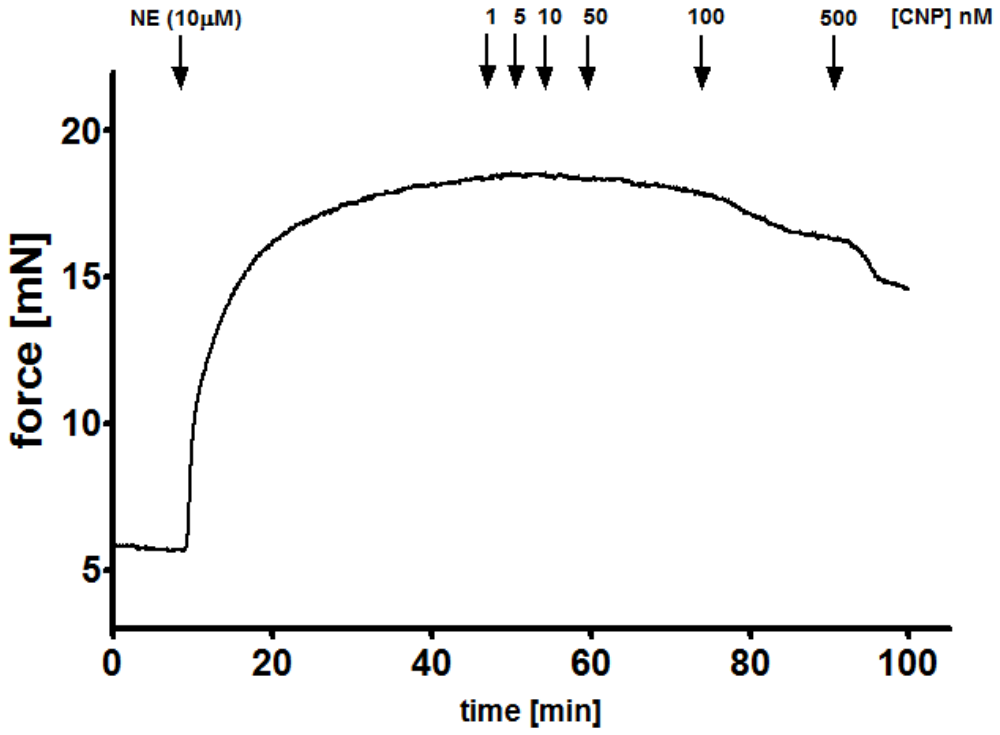


**Figure 8: Concentration dependency of CNP-induced vasorelaxation:**

Relaxation of the aorta by increasing concentration of CNP after precontraction with norepinephrine (NE) was recorded. The times and concentrations of the agents applied are indicated.

As compared to ANP (Fig.7), CNP was less effective in eliciting vasorelaxation. Significant effects were detectable only at relatively high (≥100nM) peptide concentrations and the overall reduction of NE-induced vessel tension remained low (<30%). Since CNP can interact with GC-A at concentrations ≥ 100nM (Muller, Middendorff et al. 2002), these findings raised the possibility that the vasorelaxing effects of CNP were due to cross activation of GC-A.

### 3.2.3 Vasorelaxant potency of the NPR-C ligand cANF

The third member of the natriuretic peptide receptor family is NPR-C, a non-guanylyl cyclase receptor, also referred to as natriuretic peptide clearance receptor. In addition to its clearance function, NPR-C may also have a signaling role by affecting intracellular cAMP levels (Anand-Srivastava, Sairam et al. 1990). To investigate whether NPR-C has effects on vascular tone precontracted aortae were treated with the synthetic ANP analog, cANF (ANP 4-23), which lacks part of the peptide ring structure and represents a selective NPR-C agonist (Muller, Mukhopadhyay et al. 2004).

After inducing contraction by norepinephrine, aortic rings were treated with 500 nM cANF (Fig. 9). In contrast to ANP and CNP, cANF failed to evoke any vasodilatory effects, even at high peptide concentrations.

These experiments showed that relaxation of precontracted aortic rings is essentially induced by ANP/GC-A signaling, whereas effects elicited by the GC-B ligand CNP are low and those by the NPR-C agonist cANF completely undetectable.

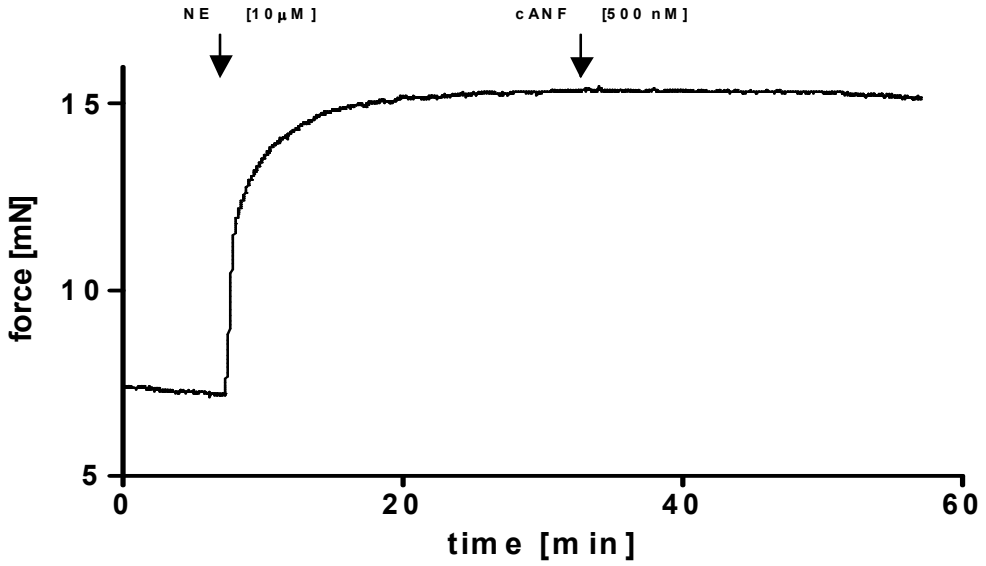


**Figure 9: The NPR-C ligand, cANF, does not induce vasorelaxation**

Precontracted aortae were incubated with high concentrations (500nM) of cANF. These treatments did not induce any detectable effects. Arrows indicate the times of NE and cANF administrations.

## 3.2.4 ANP-induced relaxation

The next experiments served to characterize the ANP induced vasorelaxation under defined conditions (induction by 500nM of the peptide). These studies (Fig. 10 shows a typical trace) revealed that administration of 500nM ANP to precontracted aortae consistently elicits tension reduction in the range of 40-70% (ẋ: 56%, n=13).

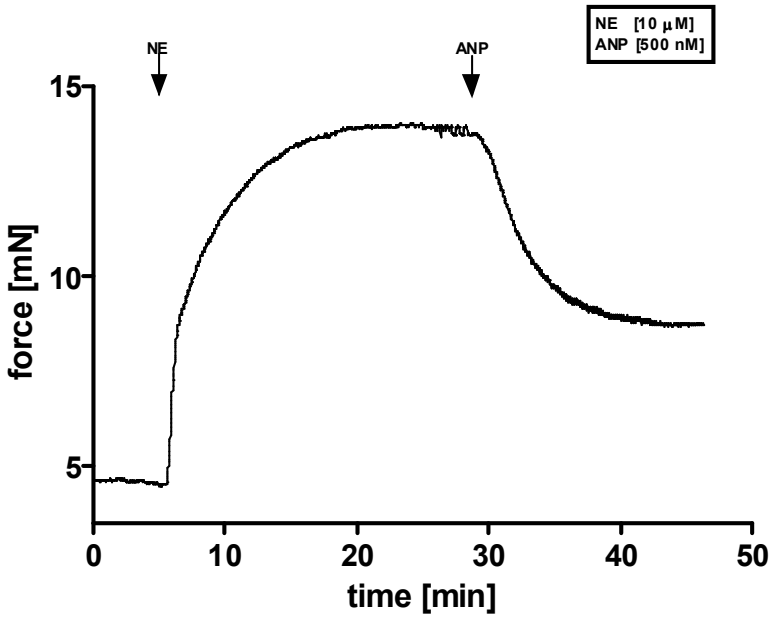


**Figure 10: ANP-induced relaxation of precontracted rat aorta:**

Precontracted aortae were treated with 500 nM ANP after development of a stable baseline tension. Recorded results show an ANP-induced relaxation of 56%. The trace is typical of more than ten experiments performed..

## 3.2.5 ANP-induced relaxation after pretreatment with 100 nM CNP

After characterizing the role of GC-A in mediating vasorelaxation by activation through ANP (or high doses of CNP) and ruling out any involvement of NPR-C, the next experiments were designed to scrutinize a possible role of GC-B as a regulator of its cognate receptor GC-A. To address this issue, precontracted aortic rings were

incubated for 15 minutes with CNP (100nM) prior to the addition of ANP (500nM). Under these conditions, the finally induced relaxation was generally more pronounced (ẋ: 71%) than in control assays without CNP pre-exposure (ẋ: 56%, n=13; Fig. 11).

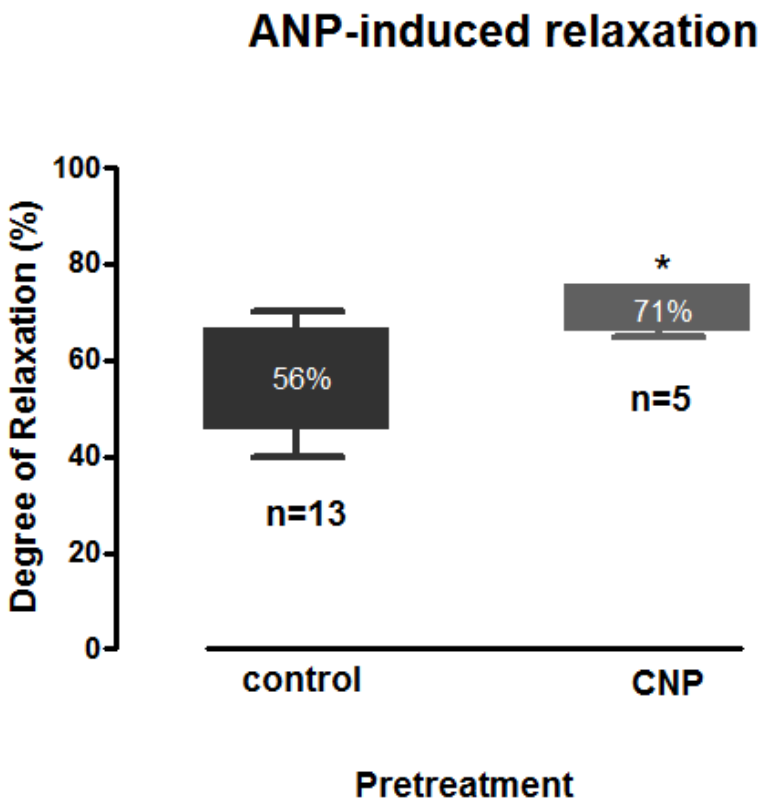


**Figure 11: ANP-induced relaxation increases in response to pre-incubation with CNP:** Precontracted aortic rings were treated with ANP (500nM) in either the absence (control) or presence of CNP (100nM).CNP was added 15 minutes before ANP. CNP treatments resulted in an intensified relaxation (ẋ: 71%). This effect was significant (*P<0.05). Values are mean ± SD from 13 or 5 experiments respectively.

### 3.2.6 ANP-induced relaxation after pretreatment with 50 nM CNP

The above experiments have demonstrated that pretreatment with 100nM CNP significantly enhanced the degree of vasorelaxation elicited by subsequent administration of ANP.

Considering the findings in αT3 cells, where CNP treatment enhances the hormone-sensitivity of GC-A (Introduction), these results would lead to the conclusion, that the ANP receptor GC-A was more active in the presence of CNP. However, since CNP at 100nM per se elicits a vasorelaxation by 10% (Fig.8), such an interpretation remained questionable.

In order to avoid any direct effects of CNP on vascular tone, analogous experiments were carried out with 50 nM CNP, a concentration shown to be ineffective in eliciting tension reduction (Fig. 8). Remarkably, these studies revealed that pretreatments with 50nM evoked even higher increases in vasorelaxation than those with 100nM (Fig.12). The resulting mean relaxation amounted 89% as compared to 71% in the case of 100nM. A typical trace of such experiments is shown in Fig. 13. Thus, the letter findings provided strong evidence that CNP/GC-B signaling enhances the agonist-dependent activity of GC-A not only in αT3 cells but also in smooth muscle cells of the aorta. The striking observation that 100nM CNP was less effective than 50nM may be explained by CNP binding to GC-A at the former concentration, leading to a certain degree of receptor desensitization.

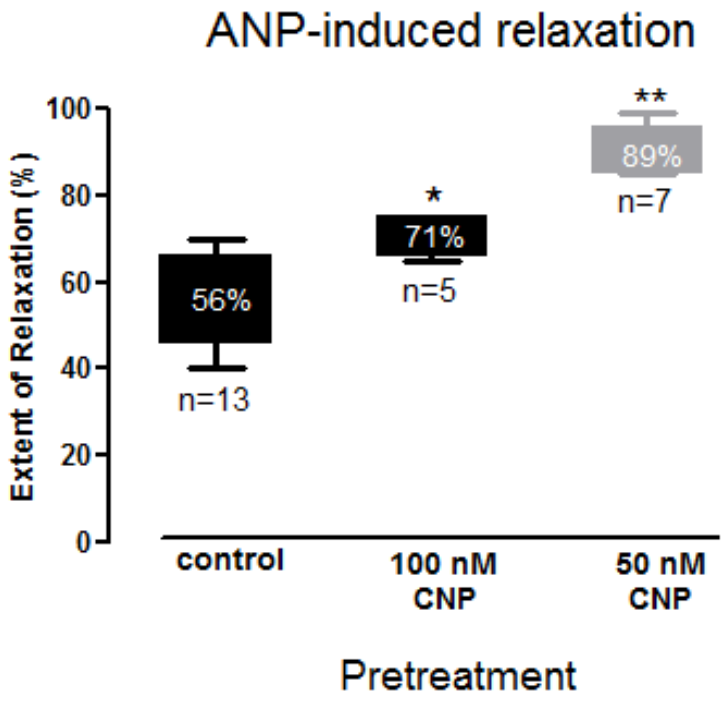


**Figure 12: Dose-dependent effect of CNP on ANP-induced relaxation:**

Aortic rings were preincubated with either 100 nM or 50 nM CNP followed by ANP (500nM) addition 15 minutes later. Controls were performed in the absence of CNP preincubations. Data shown represents means ± SD of 13 control experiments, 5 experiments with 100 nM and 7 experiments with 50 nM CNP, respectively. CNP induces significant (*P<0.01) increases in vasorelaxation.

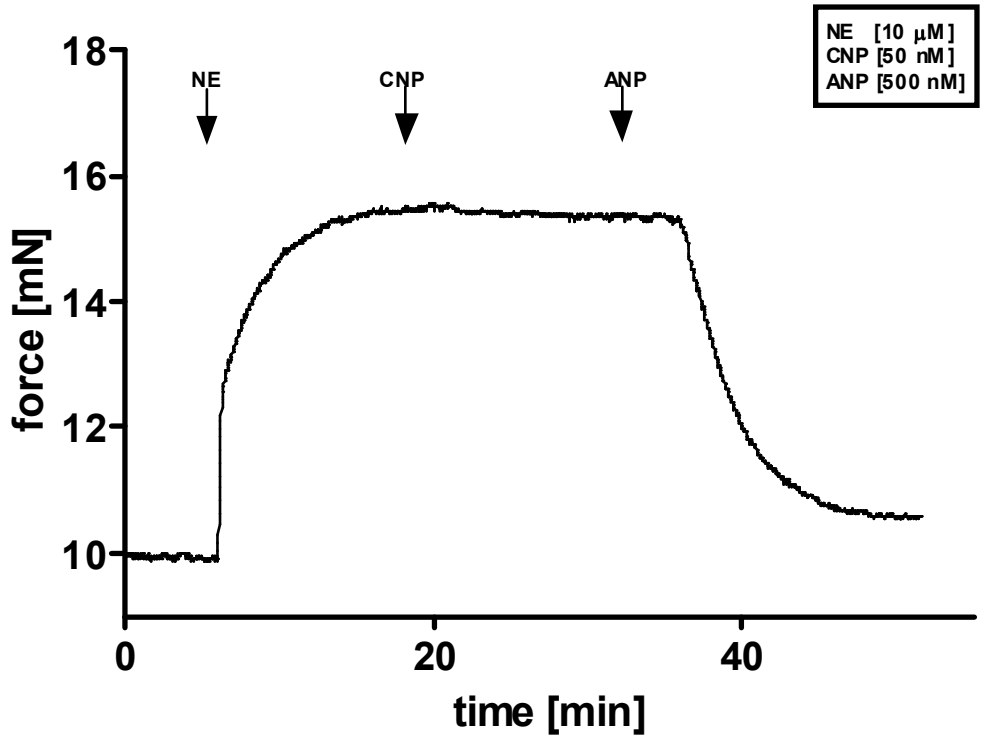


**Figure 13: ANP-induced relaxation of precontracted rat aorta after 50 nM CNP preincubation:**

Precontracted aortae were incubated with CNP (50nM) for 15 minutes prior to the addition of ANP (500nM), as indicated by arrows. While CNP itself did not induce any tension reduction, vasorelaxations by ANP was intensified to a value of 86%. Data shown is a representative of 7 experiments performed

### 3.2.7 ANP-induced relaxation after pretreatment with PKA-inhibitor and 50 nM CNP

The apparent booster effect by CNP on ANP-induced relaxation suggested a CNP-mediated sensitization of GC-A. In this case an intracellular signaling pathway is required to elicit GC-A sensitization in response to GC-B activation by CNP. Since protein kinase A (PKA) was found to be an essential component of these signaling pathways in αT3 cells, the next experiment served to examine whether PKA is also implicated in CNP-induced GC-A sensitization in smooth muscle cells of the aorta.

For this purpose, aortic rings were pretreated with Rp-8-Br-cAMPS, a cell permeable inhibitor of PKA, prior to CNP exposure with50 nM CNP and ANP was added 15 minutes later to elicit vasorelaxation.

These studies (Fig. 14 shows a typical trace) revealed that inhibition of PKA completely abolished the CNP effect demonstrated in prior experiments. Quantitative evaluation of such assays (Fig. 15) confirmed that the CNP-induced increase in ANP-evoked relaxation was completely blocked when aortae were pretreated with PKA inhibitor. Thus, these findings provided evidence that PKA is implicated as mediator of CNP-induced sensitization of GC-A not only in αT3 cells but also in vascular smooth muscle cells.

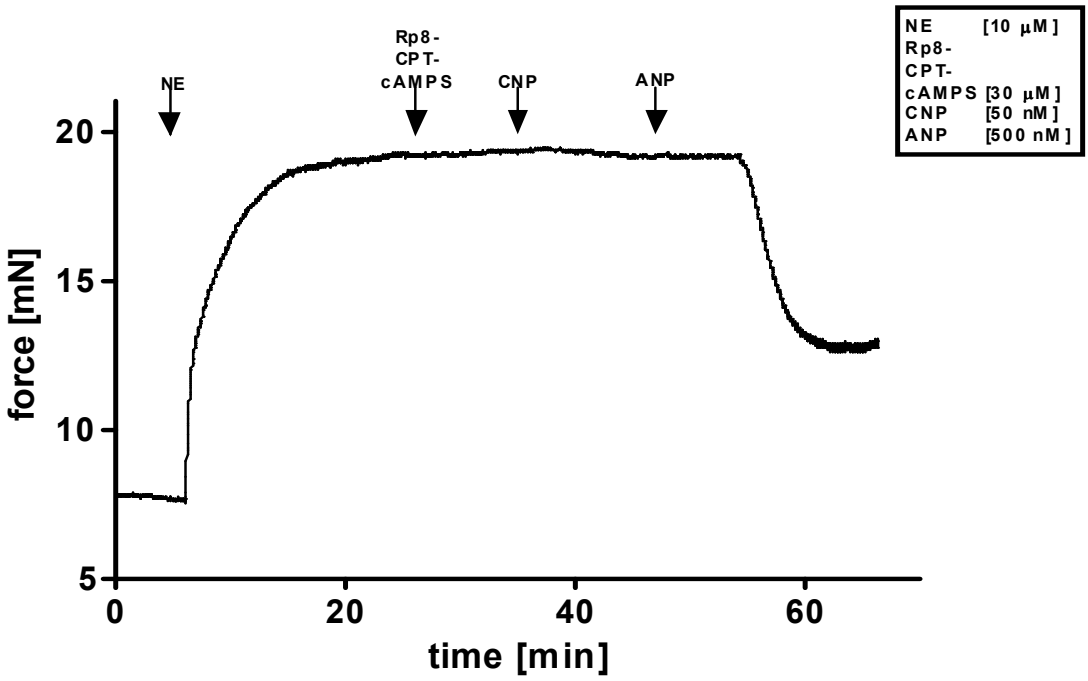


**Figure 14: ANP induced vasorelaxation after preincubation with PKA inhibitor and CNP**

After contraction was induced by NE and vasotone reached steady state levels, Rp-8-Br-cAMPS (30µM), a PKA inhibitor, was pipetted into the organ chamber, followed by addition of CNP ( 50 nM) 10 min later. After additional 15 min, relaxation was induced by 500 nM ANP. Times of agent addition are indicated by arrows. Results shown are representative of 6 experiments performed.

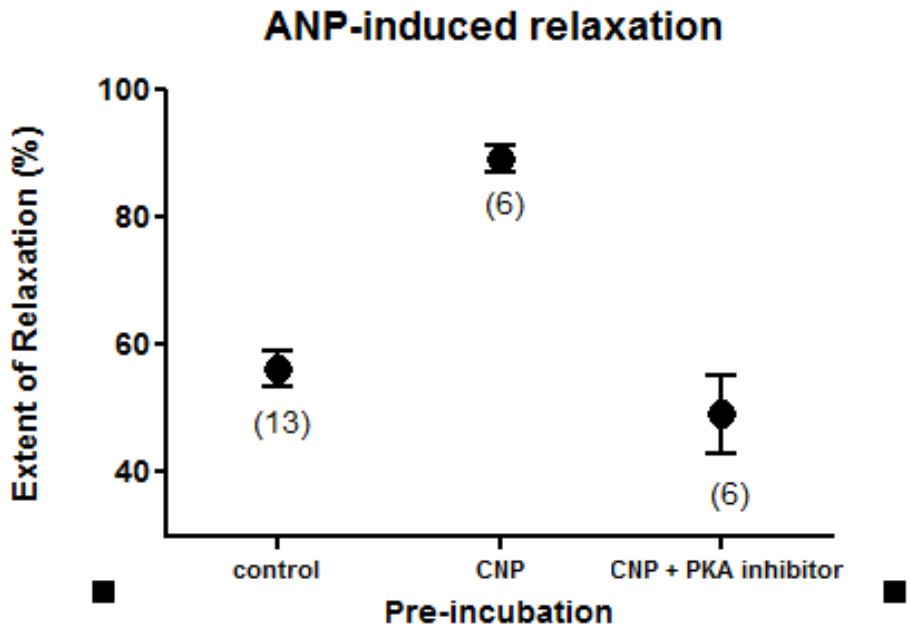


**Figure 15: PKA inhibition abrogates CNP effect:**

NE-contracted aortic rings were pretreated in the absence (control) or presence of CNP (50nM) alone or CNP+PKA inhibitor (Rp-8-Br-cAMPS) (30µM). Data shown represents means ± SD, number of experiments are indicated in parentheses.

## 3.3 Measurement of cGMP production in rat aorta

To further investigate function of GC-A and GC-B, experiments were carried out to assess their enzymatic activities by measuring ligand-dependent cGMP production. These experiments were performed with freshly dissected thoracic aortae after removal of adherent fat and connective tissue. Individual aortae were cut longitudinally into two halves (approximately 18mg each) and these were further processed in parallel to examine treatment effects. To facilitate physiologically relevant reactions, pre-treatments were generally performed in the absence of phosphodiesterase inhibition. To assess receptor activities after pretreatments,

samples were treated with high ligand (ANP or CNP) concentrations (0.5 µM) in the presence of IBMX to inhibit cGMP degradation.

### 3.3.1 ANP is more potent than CNP to stimulate cGMP production in rat aorta

Initial studies served to characterize basal (unstimulated) and natriuretic peptide-induced cGMP production in the rat aorta. Equivalent aorta samples were incubated in either the absence or presence of natriuretic peptides (ANP, CNP, 0.5µM each) for 30 minutes at 37°C under conditions (co-incubation with the phosphodiesterase inhibitor IBMX to prevent cGMP degradation). Reactions were stopped by chilling. After immediate centrifugation (3min, 15.000g), supernatants were removed and used for determination of cGMP by ELISA.

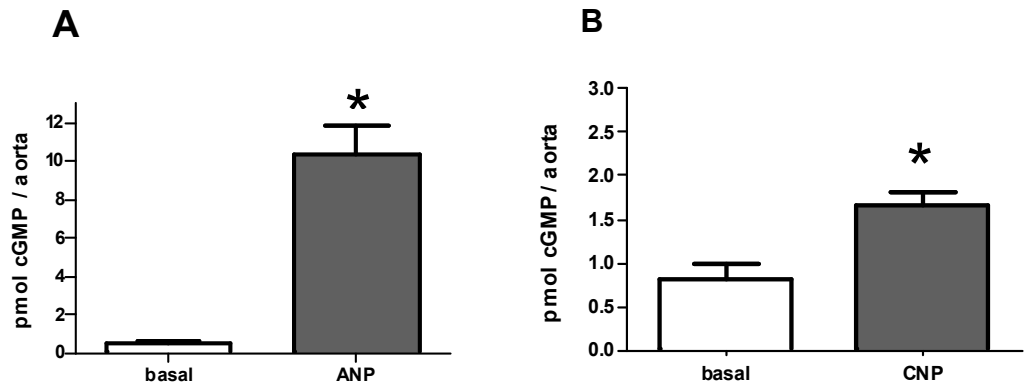


**Figure 16: ANP- (A) and CNP- (B) stimulated cGMP production in rat aorta:**

Aorta samples were incubated in the absence (basal) or presence (0.5µM) of either ANP (A) or CNP (B). Solutions contained IBMX (0.2mM) to inhibit PDE activity. After 30 min at 37°C, reactions were terminated by chilling. Samples were centrifugated, and levels of cGMP in the supernatant fractions were determined by ELISA. Data shown represents means ± SD of 5 experiments with duplicate determinations of cGMP each. Both ANP and CNP induced significant (*P<0.005) increases in cGMP.

As calculated for simple thoracic aortae, these studies (Fig.16) revealed basal values of about 0.8pmol cGMP and significant increases elicited by ANP (Fig. 16A) and CNP (Fig. 16B). Interestingly, ANP was much more effective than CNP to accumulate cGMP, resulting in 18-fold (ANP) versus 2-fold (CNP) increases as compared to basal (Fig. 17).

These findings provided evidence for a markedly higher abundance of enzymatically active ANP (GC-A) than CNP (GC-B) receptors in aortic tissue. Remarkably, this situation is different from that in αT3 cells, where CNP is more effective than ANP in promoting cGMP production (Introduction).

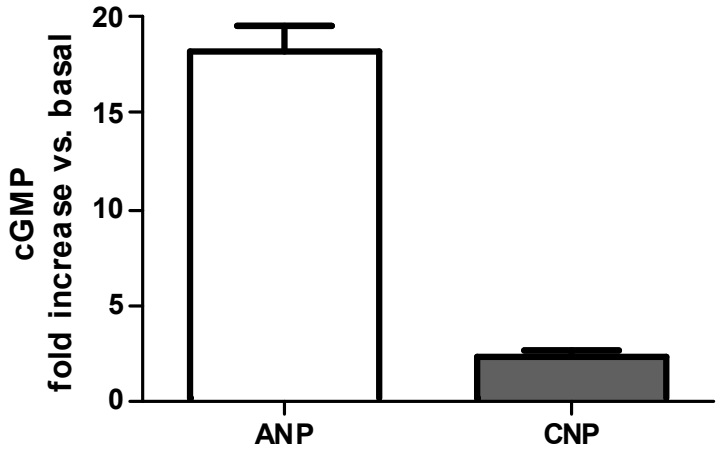


**Figure 18: Fold increase of cGMP induced by ANP and CNP, respectively**

Figure demonstrates the potencies of ANP and CNP to accumulate cGMP in aortic tissue. Values represent the mean ± SD of ANP/CNP elicited fold increase of cGMP compared to those cGMP levels measured in the absence of natriuretic peptides. Data is caculated from experiments shown in Fig. 16.

## 3.3.2 Pre-treatment with CNP enhances ANP-induced cGMP production

Relaxation of smooth muscle by ANP is mediated via GC-A produced cGMP (Potter, Abbey-Hosch et al. 2006). Since pretreatments with CNP enhanced the vasorelaxing activity of ANP in isometric tension studies, the next experiments served to examine whether CNP also enhances the cGMP-generating potential of ANP. To address this

issue, aorta samples were incubated for 15 minutes in either the absence or presence (100nM) of CNP. After medium removal and washing, both samples were exposed to ANP (500nM) plus IBMX, and cGMP production determined. Results show (Fig. 19), that pre-exposure to CNP increases ANP-triggered cGMP accumulation significantly. This outcome is completely consistent with the results of the organ bath experiments, indicating a direct correlation between vasorelaxation and accumulation of cGMP due to activation of GC-A.

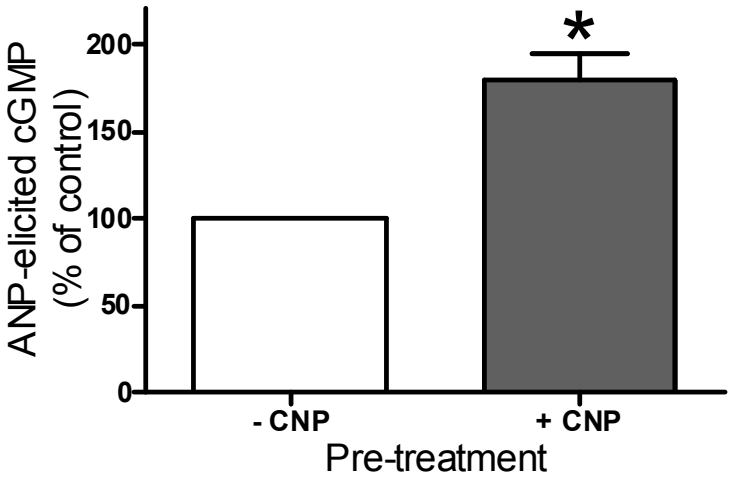


**Figure 19: Pre-treatment with CNP enhances ANP-induced cGMP production**

Aorta samples were pre-incubated for 15 min at 37°C in either the absence (-CNP) or the presence (100nM) of CNP (+CNP). After medium removal and washing, samples were incubated for 20 min in the presence of ANP (500nM) and IBMX (0.2 nM). Amounts of cGMP produced were measured by ELISA. As figure indicates, pre-treatment with CNP enhances ANP-induced cGMP production significantly (*$p<0.001$). Amounts of cGMP measured in the control (-CNP) are indicated as 100%. Data represents means ± SD of 7 assays with duplicate determinations of cGMP each.

### 3.3.3 Pre-treatment with different CNP concentrations

Since organ bath studies revealed a reciprocal dose-dependent effect of CNP (50nM>100nM) on ANP-induced vasorelaxation, analogous experiments for cGMP measurements were carried out. Samples were pre-exposed to CNP prior to ANP-dependent stimulation in the same manner as described above, using two different CNP concentrations (50nM and 100nM). These studies (Fig. 19) showed that CNP is more effective at lower doses, suggesting that higher peptide concentrations attenuate this effect. Thus, the findings corroborated a CNP-induced sensitization of GC-A and supported the view that pre-incubations with CNP at concentrations (=100nM) allowing interaction of this peptide with GC-A diminish maximum effects by inducing GC-A desensitization.

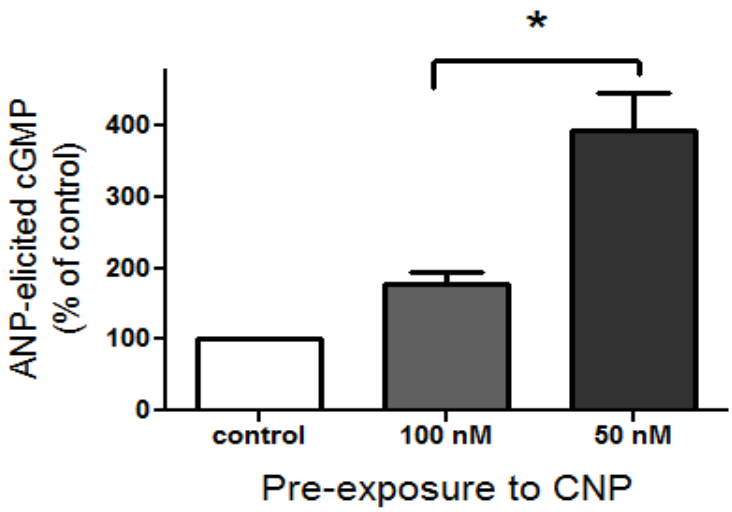


**Figure 20: Comparison between different CNP concentrations used as pre-treatment**

Samples were incubated with either 50 nM or 100 nM CNP for 15 minutes. After medium removal and washing, samples were incubated with 500 nM ANP and IBMX to facilitate ANP-dependent cGMP production. Reactions were terminated by chilling, after which supernatant fractions were analyzed for cGMP by ELISA. Controls were incubated in the absence of CNP. The amounts of cGMP measured in control samples were indicated as 100%. Data represents means ± SD of at least 3 independent experiments. 50nm CNP generates significantly (*$p<0.05$) higher effects than 100nM with duplicate determinations of cGMP each.

Moreover, these findings provided further and convincing evidence for a direct correlation between the enzymatic activity of GC-A in synthesizing cGMP and the cellular response (relaxation) in vascular smooth muscle cells.

### 3.3.4 GC-A sensitization is mediated through PKA

Based on the results from the isometric tension studies with regard to signaling role of PKA, analogous experiments were performed to see whether CNP-induced enhancement of cGMP production is also affected by the PKA inhibitor, Rp-8-Br-cAMPS. Test samples were pre-incubated with Rp-8-Br-cAMPS for 10 minutes to allow for cell entry and inhibition of PKA. Then CNP was added and incubated with aorta samples for 15 minutes. After medium removal and washing, samples were incubated with ANP. After 30 minutes, supernatant fractions were removed and analyzed via ELISA for cGMP accumulation. Controls were carried out in the same manner except for omission of the PKA inhibitor during the first step.

The results (Fig. 21) demonstrated that, similar to the tension studies, Rp-8-Br-cAMPS significantly reduces the CNP effect on GC-A activity. Thus, these results supported an involvement of PKA in the signaling pathway leading to sensitization of GC-A.

If this is true, an activation of PKA should mimic the effect of CNP. To test this hypothesis, samples were incubated with the cell permeable cAMP-analog, 8-bromo-cAMP, for 15 minutes. Again controls were performed 100 nM CNP. After washing and subsequent treatment with ANP, cGMP levels in the 8-bromo-cAMP- and CNP-pretreated samples were in the same range. (Fig. 21) Thus, inhibition of PKA abolishes and activation of PKA mimics the CNP effect on GC-A activity, providing clear cut evidence that PKA serves as an essential mediator of this reaction.

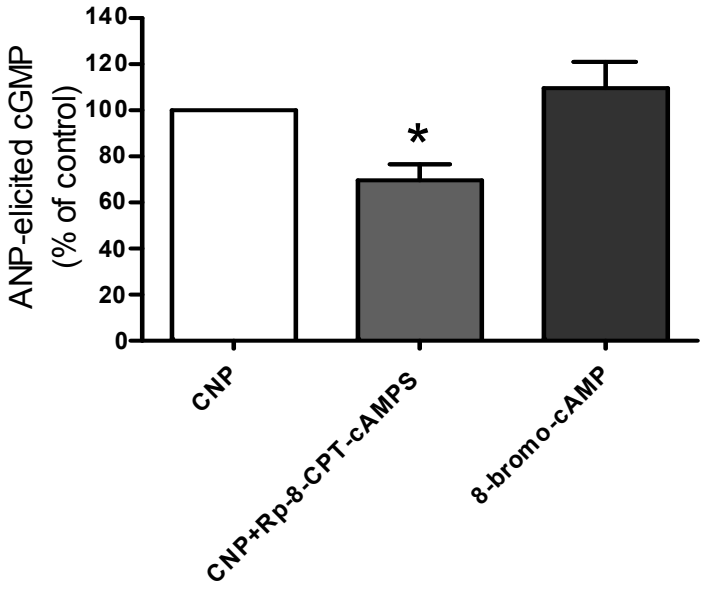


**Figure 21: PKA inhibiton blocks and PKA activation mimics CNP-dependent sensitization of GC-A**

Aorta samples were pre-incubated for 20 min in either presence of CNP (100nM), CNP plus Rp-8-CPT-cAMPS (20µM) or with 8-bromo-cAMP (1mM). After medium removal and washing, all samples were treated with ANP (500nM) and IBMX (0.2 mM) for 30 min to assess GC-A activity. Amounts of cGMP were measured in supernatant fractions after centrifugation. Subsequently medium was removed and and incubation with ANP of 30 minutes followed. Values are means ± SD of 5 assays with duplicate determinations of cGMP each. CGMP levels assayed in controls (CNP) are indicated as 100% (*P<0.01) vs. CNP control.

### 3.3.5 CNP-induced GC-A sensitization is mediated through activation of GC-B

As the previous results established the role of PKA as a mediator of CNP-induced GC-A sensitization, the next studies served to confirm that this signaling pathway is in fact initiated by CNP.

To address this issue, the receptor antagonist (Sano, Imura et al. 1992) HS-142 was used to block receptor-ligand binding. Aorta samples were pre-incubated with CNP alone or CNP plus HA-142 for 15 minutes, then medium was removed and samples

were washed. After addition of ANP and incubation for 30 min supernatant fractions were obtained for determination of cGMP. Results (Fig. 22) demonstrated that co-incubation with HS-142 the CNP effect was completely absent.

Since HS-142 blocks also GC-A, it was important to control whether the ANP-induced cGMP production could have been affected under the experiments conditions. This would be conceivable if HS-142 is resistant to the washing procedure and remains receptor-attached. To check this item samples were treated either with or without HS-142 as above prior to washing and incubation with ANP. These experiments (Fig. 22) did not reveal any effects on ANP-elicited cGMP production, indicating the observed inhibitory activity of HS-142 during CNP-pretreatments was due to blocking CNP/GC-B interaction.

Therefore, these findings showed that the signaling pathway leading to sensitization of GC-A requires CNP-dependent activation of GC-B.

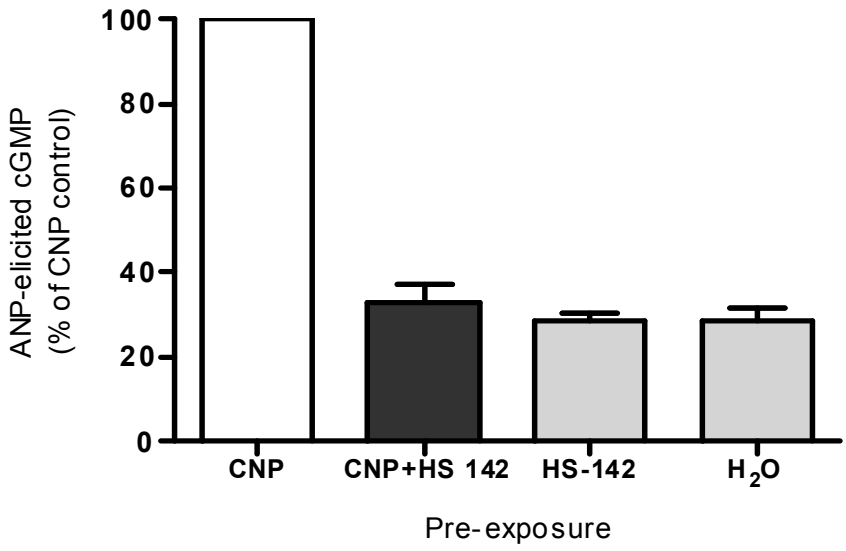


**Figure 22: CNP-induced sensitization is mediated through activation of GC-B**

Aorta samples were pre-incubated with either CNP, CNP plus HS-142, HS-142 or in the absence of these agents ($H_20$) for 15 minutes and then washed and subsequently incubated with ANP for 30 min. Amounts of cGMP were measured via ELISA. Values are means ± SD of 4 assays with duplicate determinations of cGMP each. Data are expressed as percent in relation to the values obtained after CNP treatment (=100).

### 3.3.6 Homologous desensitization of GC-A

After identifying molecular signaling events responsible for sensitization of GC-B, the goal of further studies was to examine homologous (i.g. agonist-induced) desensitization of GC-A in rat aorta. Desensitization of signaling pathways following their activation by ligand binding to plasma membrane receptors represents an important and widely used mechanism to control signal intensity. In the case of GC-A, ANP exposure induces decreases in hormone sensitivity (Muller, Cortes-Dericks et al. 2006; Potter, Abbey-Hosch et al. 2006). Thus, aorta samples were pretreated for 15 minutes in either the absence or presence of ANP. After medium was removed, both samples were re-incubated with ANP for 30 minutes, followed by measurements of cGMP. Results (Fig. 23) show, that pre-exposure to ANP leads to a significantly decreased receptor activity during a following stimulation. These findings demonstrated convincingly that ANP and CNP induce converse changes of GC-A activity in aortic tissue (Fig. 23). Moreover, the detection of ligand-induced desensitization of GC-A confirms the concept that sensitization of GC-A by CNP signaling is diminished at those CNP concentrations (≥100nM) that permit binding to and cross-reaction of GC-A.

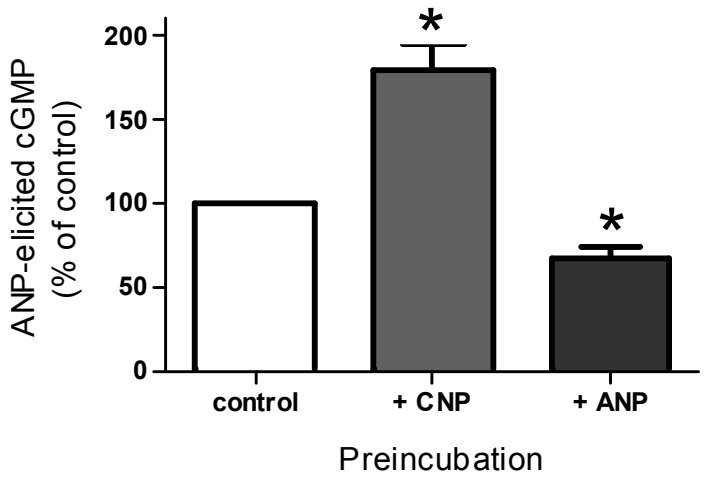


**Figure 23: Homologous desensitization of GC-A in rat aorta**

Samples were pre-incubated for 15 min in either the absence (control) or presence (100nM) of CNP or ANP. After medium removal and washing samples were treated for 20 min with ANP (500nM) in the presence of IBMX. Resulting cGMP levels were determined by ELISA. Amounts of cGMP in control samples were set as 100%. Both CNP and ANP elicited significant effects (*P<0.01)

Values are means ± SD of 7 experiments with duplicate determinations of cGMP each.

## 3.3.7 Homologous desensitization of GC-A is mediated through PKG

Considering that binding of ANP to GC-A initially stimulates the generation of cGMP, it is most likely that cGMP acts as a messenger molecule in the signaling pathway leading to desensitization of GC-A. Sine protein kinase G (PKG) is one of the most important cellular target proteins of cGMP, experiments were designed to examine whether PKG inhibition affects homologous desensitization of GC-A. Pre-incubations of aorta samples with ANP were carried out in either the absence or presence of the cell-permeable PKG inhibitor Rp-8-br-PET cGMPS. After medium exchange and identical treatments with ANP, cGMP levels in the samples pre-treated with ANP plus PKG inhibitor were significantly higher than in the samples exposed to ANP only (Fig. 24). Thus, these findings provided evidence that inhibition of PKG blocks homologous desensitization of GC-A, indicating that PKG activity is implicated in this

reaction. In contrast, analogous experiments performed with PKA-inhibitor (Rp-8-CPT-cAMPS) instead of PKG inhibitors did not show significant effects (Fig. 24). From this data, it can be concluded that PKG but not PKA is implicated in the signaling pathway underlying homologous desensitization of GC-A.

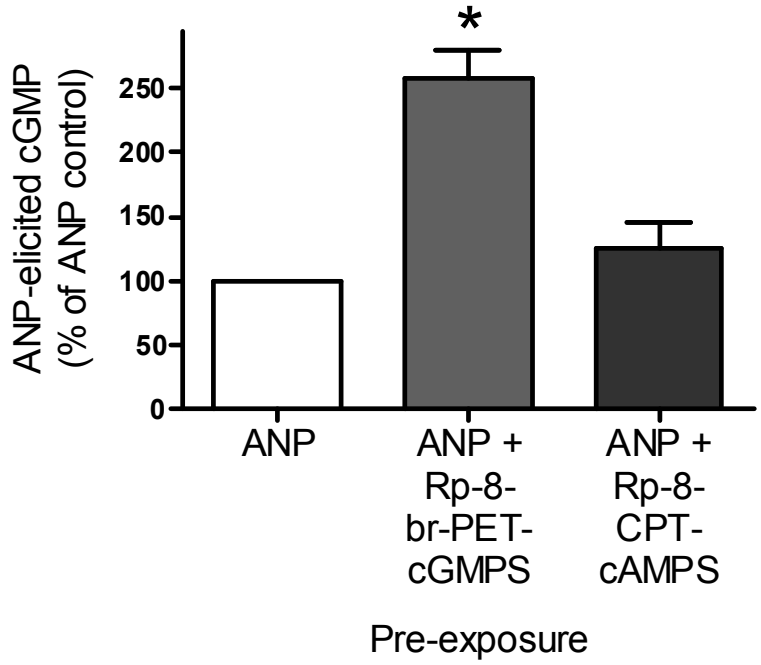


**Figure 24: Effects of PKG or PKA inhibition on ANP-induced homologues desensitization of GC-A**

Aorta samples were pre-incubated for 20 min with either ANP (100nM), ANP plus PKG inhibitor, Rp-8-br-PET-cGMPS (4µM), ANP plus PKA inhibitor, RP-8-CPT-cAMPS ((20µM). After medium removal and washing samples were treated with ANP (500nM) for 20 min. Following centrifugations, supernatant fractions were used for cGMP determination. Data represents means ± SD of 4 experiments, using duplicate determinations of cGMP each. Amounts of cGMP are indicated in relation to those found in ANP-pretreated assays (=100%). *P<0.01 vs. ANP.

### 3.3.8 CNP-elicited sensitization of GC-A under conditions of PKG inhibition

The striking phenomenon that 100 nM CNP was less efficient in evoking GC-A sensitization than 50 nM was explained by opposing effects elicited at the higher peptide concentration: sensitization of GC-A by binding to GC-B and desensitization of GC-A by binding of certain amounts of CNP to GC-A. The above findings that PKG specifically mediates the desensitization of GC-A upon ligand binding raised the possibility to prove this functional interaction experimentally. Inhibition of PKG during pre-treatment with 100 nM CNP should selectively repress the desensitization reaction, leading to enhanced hormone-dependent GC-A activity. In fact, pretreatments of aorta with 100 nM CNP in the presence of the PKG inhibitor Rp-8-br-PET-cGMPS elicited significantly higher (by about 25%) degrees of GC-A sensitization than control assays without the inhibitor (Fig. 25). For comparison, the effects of PKA inhibition and 8-br-cAMP are co-demonstrated.

In conclusion, these findings revealed that GC-A activity in aortic tissue is under concomitant control by both CNP/GC-B and ANP/GC-A signaling. The former leads to PKA-mediated receptor sensitization, the latter to receptor desensitization in a PKG-dependent manner.

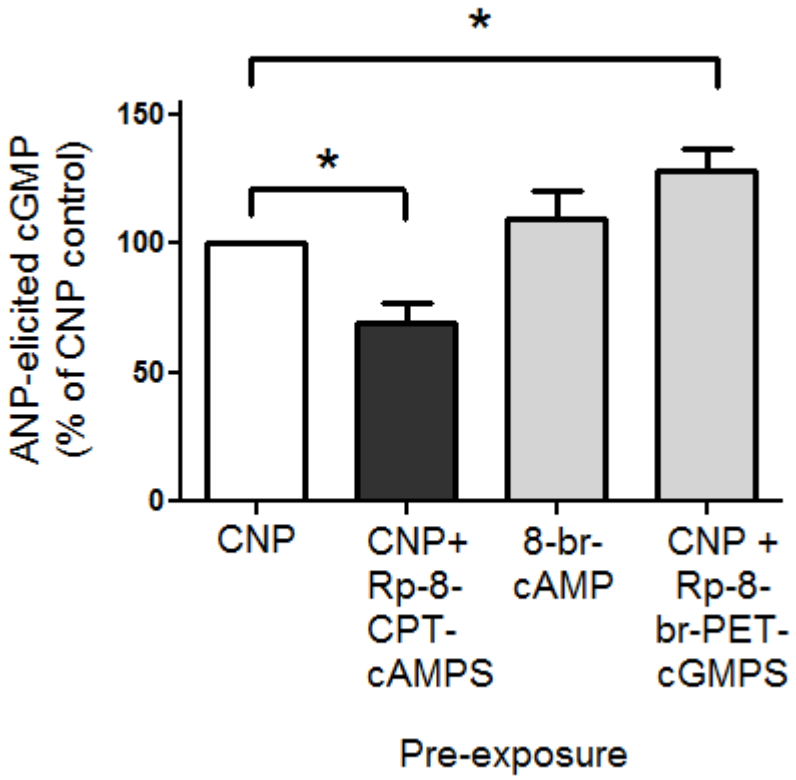


**Figure 25: Effects of PKA or PKG inhibition on CNP-induced sensitization of GC-A**

Aorta samples were pre-exposed to either CNP (100nM), CNP+Rp-8-CPT-cAMPS (20µM), 8-br-cAMP (1mM) or CNP+Rp-8-br-PET-cGMPS (4µM) for 15 min. Pretreatments were followed by medium exchange and addition of ANP (500nM), cGMP amounts, produced after 30 min were determined by ELISA. Values are means ± SD from 5 experiments with duplicate determination of cGMP each, indicated in % in relation to the CNP control (=100). Rp-b-br-PET-cGMPS significantly enhanced (*P<0.05) and Rp-8-CPT-cAMPS reduced (*P<0.005) the CNP effect.

# 4 Discussion

## 4.1 Physiological roles of GC-A and GC-B in mammals

The importance of GC-A in the regulation of blood pressure is well established (Tremblay, Desjardins et al. 2002). Targeted deletion of either the receptor (GC-A) or its ligand (ANP) resulted in chronic arterial hypertension and sudden death (Lopez, Wong et al. 1995; John, Veress et al. 1996; Oliver, Fox et al. 1997; Kuhn 2003). In numbers, the lack of ANP or GC-A in mice, enhanced blood pressure by 20 to 40 mm Hg, whereas blood pressure measured in animals with transgenically induced higher expression of ANP was 20 to 30 mm Hg lower than normal (Steinhelper, Cochrane et al. 1990; Potter, Abbey-Hosch et al. 2006). Though the regulatory functions of the ANP/GC-A system range from effects on intravascular volume to natriuresis and diuresis, the vasorelaxing potency is a key weapon to mediate hypotension (Winquist, Faison et al. 1984; Winquist, Faison et al. 1984; Potter, Abbey-Hosch et al. 2006). Hitherto, the impact of natriuretic peptides on blood pressure has been widely attributed to vasodilatation of peripheral resistance vessels. In contrast, the function of conduit vessels such as the aorta in regulation blood flow has been mainly regarded as a passive mechanism based on the elastic characteristics of its connective tissue compositure.

Further studies observed increases in intracellular cGMP upon ANP-induced vasorelaxation (Waldman and Murad 1987). The experiments of my thesis confirmed the crucial role of ANP as a potent vasodilator. Results show that ANP is capable to relax pre-contracted aortic rings in nanomolar concentrations. The vessel relaxation was pronounced and concomitant cGMP measurements revealed 20-fold increases in response to GC-A activation.

The role of CNP in vasculature is controversial. Other functions, more established, of the CNP/GC-B system include inhibition of smooth muscle proliferation, oxidized low-

density lipoprotein-induced migration of cultured human coronary artery smooth muscle cells, regulation of neointimal formation and suppression of intimal growth caused by artery injuries (Furuya, Aisaka et al. 1993; Shinomiya, Tashiro et al. 1994; Kohno, Yokokawa et al. 1997; Takeuchi, Ohmori et al. 2003; Schachner, Zou et al. 2004). Importantly, CNP dependent GC-B signaling plays a pivotal role in enchondral ossification, and disruption of the murine CNP gene results in severe dwarfism and early death (Chusho, Tamura et al. 2001). Consistently, deletion of the CNP receptor GC-B results in dwarfism (Tamura, Doolittle et al. 2004; Tsuji and Kunieda 2005). In humans, single mutations within GC-B alleles result in statistically shorter patients compared to average persons from respective populations (Bartels, Bukulmez et al. 2004). Moreover, recent findings indicate roles of the CNP/GC-B system in neurogenesis. Perinatal abundance of GC-B in neurons suggests functions for GC-B in proliferation and maturation of neurons (Muller, Hida et al. 2009). Interestingly, some studies identified CNP as the long sought endothelium-derived hyperpolarizing factor (EDHF) (Hobbs, Foster et al. 2004; Scotland, Ahluwalia et al. 2005). However, this activity of CNP was postulated to depend on interaction of the peptide with the clearance receptor (NPR-C) rather than GC-B.

CNP-deficient mice displayed normotensive blood pressure, suggesting that CNP is not important for regulation of blood pressure.

However, vasorelaxing effects were attributed to CNP based on isometric tension studies with precontracted rat aortae (Drewett, Fendly et al. 1995).

This apparent discrepancy could be explained by the possibility that the observed CNP-induced vasorelaxation was elicited by (unphysiological) interaction of CNP with the ANP receptor, GC-A. In fact, there is evidence that CNP can cross-react with GC-A at peptide concentrations ≥ 100 nM (Muller, Middendorff et al. 2002). Experiments of my thesis, where cumulative addition of CNP to precontracted aortic rings generated significant vasorelaxation only at these high peptide concentrations strongly support this view. Therefore, the relevance of CNP signaling in the aorta, linked to GC-B-produced cGMP accumulations, probably applies to other cellular functions. In addition to the well established role of NPR-C as a NP clearance receptor (Matsukawa, Grzesik et al. 1999; Potter 2011), certain reports suggested

also NPR-C-associated vasorelaxing effects (Chauhan, Nilsson et al. 2003; Hobbs, Foster et al. 2004). Two lines of evidence, provided in my thesis, doubt a functional implication of NPR-C in vasodilation. First, NPR-C was localized by affinity labelling experiments predominantly to the vessel-surrounding connective tissue rather than to the media or endothelium. Second, treatments of precontracted aortic rings with the NPR-C-selective agonist cANF failed to generate any changes in vascular tone. Thus, these findings suggest that GC-A (alone) is the principal mediator of NP-induced vasorelaxation in the aorta.

## 4.2 Mechanisms of receptor regulation

Desensitization of receptors following their activation by ligand binding represents an important and ubiquitary mechanism to control signal intensity and cellular responses.

A variety of studies performed with different cell lines showed that prolonged exposure to ANP induces desensitization of GC-A (Koller, Lowe et al. 1991; Joubert, Labrecque et al. 2001; Potter and Hunter 2001; Kuhn 2003; Potter, Abbey-Hosch et al. 2006). Additionally, homologous desensitization of GC-A has been observed and characterized in cultured smooth muscle cells (Cahill, Redmond et al. 1990; Yasunari, Kohno et al. 1992).

It has been well established, that ligand-dependent guanylyl cyclase activity correlates with the degree of receptor phosphorylation and that dephosphorylation represents the molecular mechanism responsible for desensitization (Potter and Garbers 1992; Bryan and Potter 2002; Kuhn 2003; Pandey 2005; Potthast and Potter 2005; Potter, Abbey-Hosch et al. 2006).

Two recent studies, using mass spectrometry for identification of phosphorylated residues, revealed six phosphorylation sites in both rat and human GC-B (Yoder, Stone et al. 2010) and seven sites in GC-A (Schroter, Zahedi et al. 2010; Yoder, Stone et al. 2010). Indicating a more complex regulation than initially thought, the

phosphorylation at one (serine-487) of the sites in GC-A was increased rather than decreased during ANP-induced desensitization (Schroter, Zahedi et al. 2010) .

Homologous desensitization of GC-A is a rapid process, leading to significant decreases in ligand-dependent receptor guanylyl cyclase activity within few minutes. Previous studies performed with cell lines revealed reductions of GC-A activity by 26 % after 10 min (Muller, Cortes-Dericks et al. 2006) and by up to 50 % after 30 min (Potter and Hunter 1999; Muller, Cortes-Dericks et al. 2006). Consistent with and supporting these findings, the data obtained in this thesis with intact aorta showed decreases in GC-A activity by 40 % after 15 min ANP pre-exposure.

The cGMP-generating sGC also undergoes fast (“acute”) desensitization following agonist binding (Bellamy, Wood et al. 2000; Bellamy and Garthwaite 2001; Roy and Garthwaite 2006). Considering that cyclic nucleotide-mediated cellular effects are rapidly elicited (Honda, Adams et al. 2001) using compartmentalized pathways (Piggott, Hassell et al. 2006), acute desensitization may represent a general and essential cellular strategy to protect the cells from second messenger overload and the activation of undesired pathways.

Additional cellular responses can emerge under conditions of chronically elevated hormone levels. As known from other signaling systems, these responses frequently include decreases in cell surface receptor densities. In the case of the GC activity-containing NP receptors (and discriminating these receptors from NPR-C), investigations performed by most (Kato, Lanier-Smith et al. 1991; Koh, Nussenzveig et al. 1992; Vieira, Gao et al. 2001; Fan, Bryan et al. 2005) albeit not all (Pandey, Nguyen et al. 2002) groups argue against the existence of pathways capable of (rapidly) reducing receptor membrane concentrations by internalization/endocytosis. Such reactions may take place, however, after prolonged (>8 h) ligand exposure (Flora and Potter 2010). Indicating the possibility of additional long term effects, there is substantial evidence for agonist-induced downregulation of GC-A (Cao, Chen et al.

1998; Singh, Kuc et al. 2006) and GC-B (Rahmutula and Gardner 2005) at the gene expression level.

Though much attention has been paid to the natriuretic peptide receptor phosphorylation itself, the kinase(s) and phosphatase(s) involved in this process still remain to be identified. Effects induced by the serine/threonine protein phosphatase (PP) inhibitors okaidic acid (Potter and Garbers 1992) and microcystin (Bryan and Potter 2002) suggested an implication of PP1 and/or PP2A in ANP-induced desensitization of GC-A. Further reports documented that heterologous GC-A desensitization in membranes of transfected mouse kidney 293 and NIH3T3 fibroblast cells is mediated through a magnesium-dependent phosphatase, supposedly a member of the PP2C family (Abbey and Potter 2002; Bryan and Potter 2002).

It seems most likely, that cGMP, the second messenger generated by GC-A, is involved in signaling pathways that trigger desensitization of GC-A. In this end, cGMP-dependent protein kinase G (PKG) functions as a key intracellular mediator of cGMP signaling in various cell types (Kotera, Grimes et al. 2003). PKG is already well established as a downstream component of ANP/GC-A signaling. Effects attributed to PKG span from modulation of L-type calcium channels (Tohse, Nakaya et al. 1995) to cross-talks with heterologous receptors, such as G protein-coupled receptors (Pedram, Razandi et al. 2000; Yamamoto, Yan et al. 2001). Another study suggests that ANP-mediated cGMP accumulations regulate PKG-elicited phosphorylation of vasodilator-stimulated phosphoprotein (VASP) Ser-239 in endothelial cells, resulting in reorganization of the actin cytoskeleton and enhancement of angiogenesis (Chen, Levine et al. 2008). This data clearly supports pivotal roles for PKG in ANP signaling. Furthermore, there are reports on upstream targets of PKG, in a manner in which it may be able to regulate GC-A activity. Airhart et al. demonstrated such an upstream pathway in which ANP-activated GC-A recruits PKG to the membrane resulting in increased intrinsic cyclase activity in transfected and untransfected HEK 293 cells

(Airhart, Yang et al. 2003). This is also concordant with data demonstrating that PKG is capable of phosphorylating GC-A *in vitro* (Larose, Rondeau et al. 1992).

As shown in this thesis, the specific PKG inhibitor Rp-8-br-PET-cGMPS blocks homologous desensitization of GC-A in rat aortic tissue. These findings provide evidence that cGMP accumulations in response to GC-A activation not only elicit PKG-mediated smooth muscle relaxation (Weber, Bernhard et al. 2007) but also PKG-mediated decreases in GC-A hormone responsiveness. Whether the latter reaction is based on PKG-induced phosphorylation of GC-A (at serine-487; see (Schroter, Zahedi et al. 2010) or on PKG-stimulated phosphorylation (and activation) of phosphatases that act on other GC-A residues remains to be elucidated. In any case, this data provides a further example for spatial and functional segregation of cGMP signaling pathways within cells (Muller, Greenland et al.; Neves, Tsokas et al. 2008). Desensitization in direct response to elevated cGMP levels has also been observed in the case of sGC (Wykes, Bellamy et al. 2002).

## 4.3 Co-expression of GC-A and GC-B in rat aorta

Expression of GC-A mRNA was described in various tissues such as kidney, adrenal, ileum, lung, adipose and vasculature (Lowe, Chang et al. 1989; Schulz, Singh et al. 1989; Nagase, Katafuchi et al. 1997). Further studies exposed high gene expression of GC-A in specific brain areas including the olfactory bulb, pineal gland, subfornical organ and other circumventricular organs (Langub, Watson et al. 1995; Herman, Dolgas et al. 1996). In contrast, data on GC-A protein levels are rare but identified expression in rodent lung, kidney and testis (Goy, Oliver et al. 2001; Muller, Mukhopadhyay et al. 2004). GC-A was also detected in vascular smooth muscle cells, but these findings were based on GC-A mRNA examinations and on ANP-dependant cGMP accumulations without clear proof for receptor expression at the protein level (Suga, Nakao et al. 1992).

Studies on GC-B expose high expression of mRNA in lung, adrenal, kidney, uterus and certain brain tissues (Schulz, Singh et al. 1989; Wilcox, Augustine et al. 1991;

Chrisman, Schulz et al. 1993; Nagase, Katafuchi et al. 1997). GC-B protein has been detected in fibroblasts (Chrisman and Garbers 1999; Abbey and Potter 2002; Abbey and Potter 2003). Thus, although reports on the tissue expression of both receptors are plenty, clear-cut evidence on their protein levels is scarce and hitherto restricted to few tissues. Since blood pressure regulation is believed to be one of the hallmarks of natriuretic peptide signaling, it was of particular interest (and a major goal of this thesis) to characterize the expression of both receptors at the protein level in rat aorta.

Based on previous findings in αT3-1 cells, where CNP/GC-B signaling was shown to induce sensitization of GC-A (Cortes-Dericks 2005), these studies should also focus on possible sites of cellular co-expression of the two receptors.

Both immunoblotting and affinity crosslinking approaches consistently revealed high abundance of GC-A and GC-B protein in aortic tissue. Immunohistochemical analyses demonstrated that both receptors are localized to and co-expressed in vascular smooth muscle and endothelial cells. Thus, these two cell types are the principal targets for ANP and CNP in aortic tissue and provide a local molecular basis for cellular interactions between GC-A- and GC-B-induced signaling pathways. Moreover, as could be concluded from the immunohistochemical data, the vast majority of both receptors in the aorta are present in smooth muscle cells.

Comparative affinity crosslinking experiments performed with aortic tissue that contained or did not contain the surrounding connective tissue elucidated additional important and novel information. These studies revealed that the natriuretic peptide clearance receptor, NPR-C, is predominantly expressed in the vessel-surrounding connective tissue and of lower abundance than GC-A and GC-B in the media and intima. This particular distribution of NPR-C suggests a most important role for the clearance receptor in controlling natriuretic peptide levels within the connective tissue around blood vessels and further questions an essential direct involvement in blood pressure regulation (Maack, Suzuki et al. 1987).

## 4.4 CNP/GC-B signaling sensitizes GC-A

The above findings proved co-expression of GC-A and GC-B, two enzymes commonly generating the second messenger cGMP, in vascular smooth muscle (and endothelial) cells of the aorta. Therefore, this data allowed examine whether CNP/GC-B signaling in these crucial vascular cell types can also (like in αT3-1 cells) induce GC-A sensitization.

As revealed in this thesis, pre-exposure of CNP to intact aortic tissue elicited an enhanced activity (hormone-responsiveness) of GC-A. This effect was demonstrated at the enzymatic (enhanced ANP-induced cGMP generation) and the functional (enhanced ANP-induced vasorelaxation) level. The latter findings clearly localized the crosstalk between CNP- and ANP-induced signaling, resulting in GC-A sensitization, to vascular smooth muscle cells. Since functional activities of ANP in endothelial cells were not addressed in this study, it remains unclear, whether CNP/GC-B signaling also affects endothelial GC-A.

Strikingly, the potency of CNP to induce GC-A sensitization was greater at 50 nM than at 100 nM. Since CNP can cross-react with GC-A only at the higher (100 nM) concentration, this data indicates that CNP binding to GC-A can induce receptor desensitization and hence diminishes the activation evoked by CNP/GC-B signaling. Thus, these findings provide evidence for a dynamic regulation of GC-A in vascular tissue under the concomitant control of both desensitizing and sensitizing pathways.

Different physiological roles of the two related receptors, GC-A and GC-B, are well established. However, functions exerted by both cognate receptors were examined hitherto almost exclusively with regard to one of them. Moreover, less attention has been paid to compartmentalization and possible distinct pathways of generated cGMP by both GC-A and GC-B in cells where both receptors are active.

Recent studies opened the gate for insight into receptor-specific activities in cells co-expressing GC-A and GC-B. Accordingly, one study revealed that GC-B predominates in neurons of the developing brain whereas GC-A does so in the adult brain. Thus it has been postulated that CNP/GC-B signaling controls perinatal stages of brain development, whereas ANP/GC-A signaling is suggested to be responsible for different functions in the mature brain (Muller, Hida et al. 2009). These findings strongly suggest distinct roles of GC-A and GC-B signaling within the same tissue. Moreover, experiments with αT3 cells (Cortes-Dericks 2005) specifically investigated receptor activities in a cell line co-expressing GC-A and GC-B and uncovered a novel mechanism of intracellular cross talk between these signaling systems.

Enhanced ligand-dependent cGMP generation has been reported previously, when cellular effects of either isoproteonol, a beta adrenergic agonist (Kishimoto, Yoshimasa et al. 1994; Thibault, Lacasse et al. 1996), or NaCl-treatments (Katafuchi, Mizuno et al. 1992) have been investigated. Also, down-regulation of NPR-C levels was found to result in increased GC-A activity (Kishimoto, Yoshimasa et al. 1994). The present findings elucidate for the first time in a functional context (and performed with intact tissue) a mechanism by which the hormone-responsiveness of GC-A is increased.

In conclusion, these findings are of major significance for the following reasons:
First, they strongly suggest a compartmentalized signaling of cGMP in cells co-expressing GC-A and GC-B. It is shown clearly that cGMP generated by GC-A elicits downstream events resulting in vasorelaxation and at the same time it is involved in upstream events leading to enzymatic downregulation of GC-A. Interestingly though, CNP/GC-B dependent cGMP accumulation does not lead to vasorelaxation at physiological CNP levels but induces enhanced activity of GC-A. This provides strong evidence that both receptors cross talk with each other through an intracellular pathway using the same second messenger in a compartmentalized manner. Secondly, this work shows for the first time under *in vivo*-like conditions sensitization rather than desensitization of GC-A, that is triggered via GC-B activation. Hence,

these findings are consistent with a mechanism of two distinct pathways evoking opposite effects on the same receptor. These new insights provide a basis for a dynamic regulation of GC-A. Considering the fact that CNP exerts its function in a paracrine fashion (Potter, Abbey-Hosch et al. 2006), it can be deduced that GC-A activity is controlled by the above mechanism not only rapidly but also locally. Since various cell types have been reported to express both receptors, and the same effect of CNP-induced sensitization has been found in 2 completely different cell types (pituitary αT3-1 and vascular smooth muscle cells), it seems likely that a mechanism of broad significance has been uncovered.

## 4.5 Sensitization of GC-A is mediated via PKA

Remarkably, CNP-induced sensitization of GC-A was found to be completely inhibited by blocking cAMP-dependent protein kinase A (PKA), leading to the conclusion that this kinase is an essential component of the signaling pathway. Involvement of PKA in GC-A regulation has already been reported when inhibition of PKA blocked ANP-induced desensitization of GC-A in MA 10 cells (Muller, Cortes-Dericks et al. 2006). However, these cells do not express PKG, indicating that this PKA effect is a particular and cell type-specific phenomenon.

A possible candidate to deliver the message from the cGMP system to the cAMP system is phosphodiesterase 3 (PDE3). In addition to PKG and cyclic nucleotide gated (CNG) ion channels, phosphodiesterases constitute a further principal target for cGMP. There are at least 11 highly regulated and structurally related PDE gene families (PDE1-11). The proteins control levels of cyclic nucleotides by degrading them upon activation and thus play a pivotal role in regulating cyclic nucleotide signaling. Moreover they are of great significance in maintaining a compartmentalized environment within cells (Conti, Richter et al. 2003). While some of the family members of PDE are strictly confined to degrade either one of the two cyclic nucleotides (cAMP. cGMP), some of them play a double role, providing a basis for cross talks between them. PDE2, which degrades both cGMP and cAMP, has an

allosteric regulatory site with high affinity to cGMP. Binding of cGMP at this site lowers the apparent $K_m$ for cAMP and results in activation of the cAMP-hydrolyzing activity of the enzyme (Martinez, Beavo et al. 2002; Martinez, Wu et al. 2002; Mehats, Andersen et al. 2002). Accordingly, inhibition of aldosterone production due to ANP-dependent GC-A activation in adrenal cells of the zona glomerulosa is a result of cGMP-dependent PDE2 stimulation that evokes attenuated cAMP signaling (MacFarland, Zelus et al. 1991). The significance and potency of this pathway has been recently highlighted by showing that ANP exposure to these cells nearly completely blocks forskolin-induced aldosterone production and PKA activity (Nikolaev, Gambaryan et al. 2005). These findings provide convincing evidence for the existence of rapidly acting and compartmentalized signaling pathways in this cell type, by which ANP evokes effects via a PDE2-mediated cross-talk with cAMP signaling.

A second cAMP-hydrolyzing PDE, considered to be regulated by cGMP *in vivo*, is PDE3 (Shakur, Holst et al. 2001). In this case, cGMP acts as a competitive inhibitor of cAMP hydrolysis. The catalytic site is characterized by a similar high affinity for cGMP and cAMP but the enzyme degrades cAMP much faster (up to 10-fold) than cGMP (Rybalkin, Yan et al. 2003) suggesting that PDE3 *in vivo* primarily functions as a cAMP-degrading enzyme. Several cGMP-mediated responses in cardiac cells, such as potentiation of Ca(2+) currents and a attenuation of the responsiveness to beta-adrenergic receptor agonists, have been shown to result from effects of cGMP on cAMP hydrolysis (Zaccolo and Movsesian 2007). Thus, local accumulations of cGMP are able to evoke increasing cAMP levels through competitive inhibition of PDE3. Evidence for the existence of corresponding signaling pathways has been reported. As demonstrated concordantly using isolated perfused rat kidneys (Kurtz, Gotz et al. 1998) or whole animals (Beierwaltes 2006), stimulation of renin secretion by nitric oxide is mediated via a cGMP-elicited inhibition of cAMP degradation, and PDE3 has been defined as the signaling component implicated in this cross-talk. Other cellular reactions, induced by nitric oxide and suggested to be (at least in part) mediated by cGMP-dependent breakdown of the cAMP-degrading activity of PDE3,

include inhibition of platelet aggregation (Maurice and Haslam 1990), stimulation of calcium currents in cardiac myocytes (Kirstein, Rivet-Bastide et al. 1995), control of renal vascular resistance (Sandner, Kornfeld et al. 1999), and an impairment of TNFα-induced transcriptional activity of NFκB in vascular smooth muscle cells with proposed impact on inflammation and atherosclerosis-promoting processes (Aizawa, Wei et al. 2003).

There is evidence that this mechanism is not confined to nitric oxide induced cGMP, generated by sGC, but also active in pathways originating from membrane guanylyl cyclases. CNP, acting via GC-B, significantly enhanced the heart rate response to vagal nerve stimulation due to a cGMP-PDE3-dependent pathway that increases cAMP-PKA-dependent phosphorylation of presynaptic N-type calcium channels (Herring, Zaman et al. 2001). Similarly, CNP-elicited decreases in atrial stroke volume were reported to be based on this mechanism (Wen, Cui et al. 2004). ANP-induced cGMP accumulations as well as direct cGMP applications with subsequent inhibition of PDE3 and activation of PKA potentiate rectifier potassium channel currents in sinu-atrial node cells (Shimizu, Shintani et al. 2002).

The requirement of PKA activity for sensitization of GC-A by CNP in αT3-1 and aorta smooth muscle cells suggest an involvement of PDE3 in this signaling pathway too. Inhibition of cAMP degradation by PDE3 in response to CNP/GC-B-produced cGMP accumulations represents a well conceivable mechanism by which local increases in cAMP (and subsequent activation of PKA) act as mediators of the reaction. This was confirmed by studies showing that treatment with 8-bromo-cAMP mimics the CNP-induced sensitization of GC-A. More importantly, recent experiments with PDE 3-specific inhibitors (D. Müller, unpublished results) corroborated the role of this phosphodiesterase in the sensitization pathway.

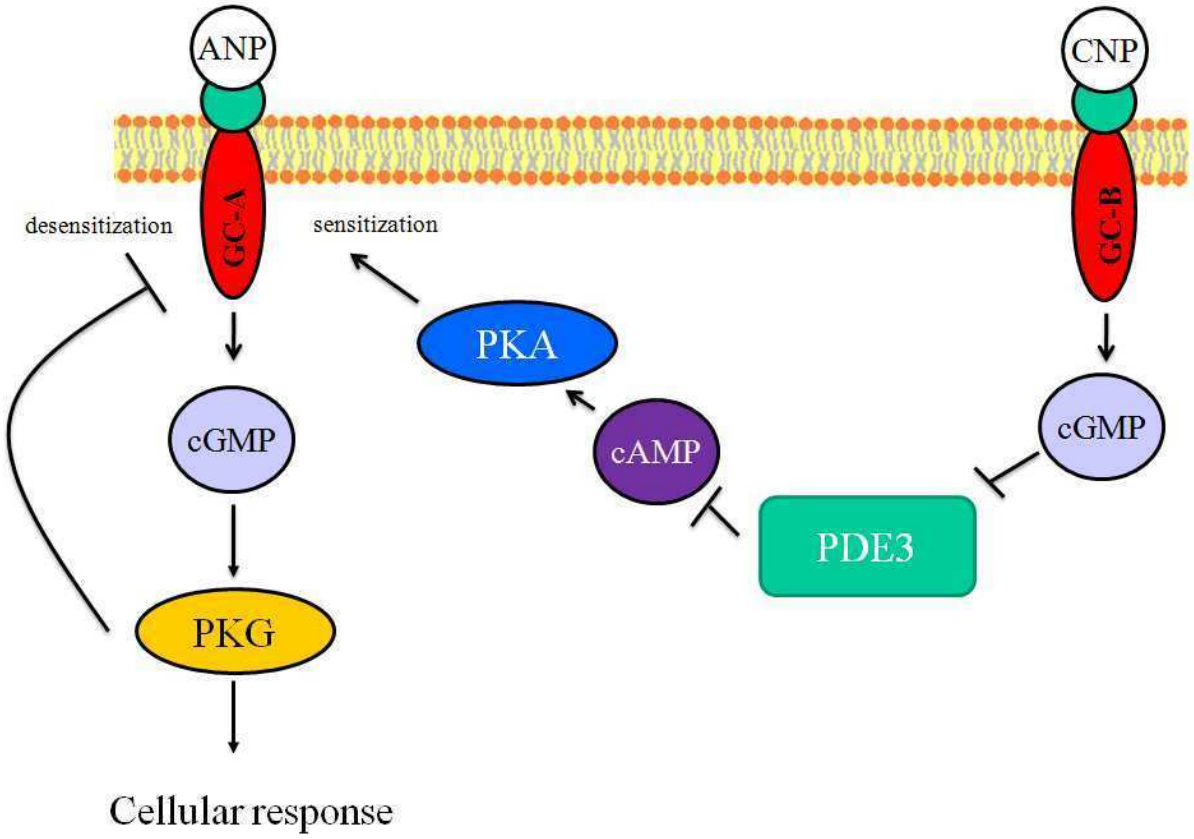


**Figure 26: Signaling pathways of GC-A and GC-B in vascular smooth muscle cells**

ANP-elicited stimulation of GC-A creates local accumulation of cGMP. PKG activation by cGMP evokes major cellular responses (relaxation) and desensitization of GC-A. On the other hand, co-expressed GC-B is stimulated by CNP to produce cGMP. The latter inhibits PDE3 resulting in increased cAMP levels and activation of PKA, which in turn mediates sensitization of GC-A.

## 4.6 The aorta: a crucial independent target organ for natriuretic peptides?

In many studies, including those of my thesis, the aorta was used as a representative for vasculature in general. Results obtained by such experiments generally served to apply them on peripheral vessel. Consequently, most studies about hormone-dependent regulation of vascular tone paid attention to its impact on blood pressure in peripheral resistance vessels, and specific contributions by conduit vessels proximate to the heart such as the aorta were not addressed. In particular, the so called Windkessel effect created by the aorta has been disregarded in the context of hormonal regulation.

It is thought that structural components dominate the biomechanical properties of the aorta. An elastic matrix forms lamella consisting of elastic fibers, collagens (predominantly type 3), proteoglycans, and glycosaminoglycans. When the left ventricle contracts to force blood into the aorta, the aorta expands. This stretching gives the potential energy that will help maintain blood pressure during diastole, as during this time the aorta contracts passively. This Windkessel effect of the great elastic arteries has important biomechanical implications. The elastic recoil helps conserve the energy from the pumping heart and smooth out the pulsatile nature created by the heart. Aortic pressure is highest at the aorta and becomes less pulsatile and lower pressure as blood vessels divide into arteries, arterioles, and capillaries such that flow is slow and smooth for gases and nutrient exchange (Belz 1995) .

Apart from peripheral resistance pressure maintained by small arterioles, the compliance of the aorta as the main conduit artery is a key component in regulation of arterial blood pressure and blood flow velocity, as 80% total compliance of the systemic arterial tree is located in the aorta (Saouti, Westerhof et al. 2010). Accordingly, reduced compliance, referred to as aortic stiffness, is associated with diseases such as cardio-vascular events (Vlachopoulos, Aznaouridis et al. 2010) and kidney insufficiency (Bahous, Blacher et al. 2009). Aortic stiffness is reported to be a

valuable biomarker to predict cardio-vascular events (Mitchell, Hwang et al. 2010). As the Windkessel function is regarded to be completely relying on the architectural composition of aortic tissue, the effects are considered to be mainly passively regulated.

Interestingly, recent findings in our laboratories (D. Müller, unpublished results) showed that the expression density of GC-A and GC-B is much higher in aorta as compared to branching arteries or other established natriuretic peptide target tissues such as kidney and adrenal gland. Similar observations were made by analyses of human tissue samples. Moreover, as assessed by organ bath studies, ANP was found to be much more potent in eliciting vasodilation in aorta as compared to smaller peripheral arteries.

These findings suggest a crucial active role for natriuretic peptides in regulation of the Windkessel effect of the aorta. Thus, the cross talk between GC-A and GC-B identified in this thesis may not only represent a novel mechanism of activity regulation of GC-A in cells co-expressing both receptors but may have particular physiological significance for enabling and maintaining a proper Windkessel function of the aorta.

# Summary

Regulation of hormone activity at the receptor level has crucial physiological impact. In the case of the cGMP-generating receptors (GC-A and GC-B) for the natriuretic peptides ANP, BNP and CNP, such a regulation occurs primarily by changes in the degree of receptor phosphorylation. Prolonged ligand exposure induces dephosphorylation resulting in reduced receptor hormone responsiveness, a process called homologous desensitization.

Previous studies with pituitary cells that co-express GC-A and GC-B revealed an unexpected cross talk between ANP- and CNP-induced signaling pathways. Strikingly, CNP pre-exposure to the cells elicited increases in GC-A activity, and this reaction was apparently mediated by cAMP-dependent protein kinase (PKA).

Goal of the present investigations was to examine whether such a cross talk may exist also in the vasculature, a most important natriuretic peptide target organ. Both GC-A and GC-B were found to be co-expressed at high levels in vascular smooth muscle cells of the rat aorta. Isometric tension studies revealed that CNP pre-exposure enhances the vasorelaxing effect of ANP on vascular tone. Further experiments showed that this regulatory effect of CNP is initiated by activating GC-B and mediated through PKA. These findings were supported by assays carried out to show intracellular cGMP levels as indication for receptor enzymatic activity. Consistently, results demonstrated that pre-incubation with CNP evoked significantly higher amounts of cGMP production by GC-A. Moreover, vessels, pre-incubated with ANP, showed reduced GC-A activity, indicating that smooth muscle GC-A is also sensitive to desensitization mediated through protein kinase G (PKG).

Thus, these findings corroborated the existence of a signaling pathway, by which CNP can increase the hormone-sensitivity of GC-A. This mechanism is apparently present and active in the vasculature to regulate the vasodilatory potency of ANP. Desensitization of GC-A, expected to take place under conditions of elevated ANP plasma levels, can be relieved by CNP/GC-B signaling. Since attenuation of the biological effects of ANP due to GC-A desensitization in various diseases associated

with enhanced ANP levels is a major clinical problem, the data indicates a considerable therapeutic potential of GC-B agonists.

# Zusammenfassung

Die Regulierung der Hormonaktivität auf Rezeptorebene hat entscheidende physiologische Auswirkungen. Im Falle der cGMP-generierenden Rezeptoren (GC-A und GC-B) für die natriuretischen Peptide ANP, BNP und CNP vollzieht sich diese Regulation vorwiegend in der Änderung des Phosphorylierungsgrades des Rezeptors. Andauernde Bindung des Liganden führt zu einer Dephosphorylierung, die eine verminderte Empfindlichkeit des Rezeptors zum Liganden zur Folge hat. Diesen Prozess bezeichnet man als homologe Desensitisierung.

Bisherige Studien mit Hypophysenzellen, die GC-A und GC-B coexprimieren, enthüllten einen unerwarteten „Cross-talk“ zwischen ANP- und CNP-induzierten Signalwegen. Auffallenderweise löst eine Präexposition der Zellen zu CNP eine erhöhte GC-A Aktivität aus. Diese Reaktion wird offensichtlich durch eine cAMP-abhängige Proteinkinase (PKA) vermittelt.

Das Ziel der vorliegenden Untersuchungen war herauszufinden, ob dieses Phänomen auch in Gefäßen vorzufinden sei, die schließlich ein wichtiges Zielorgan für natriuretische Peptide darstellen. Sowohl GC-A, als auch GC-B werden in glatten Muskelzellen der Rattenaorta reichlich coexprimiert. Organbadstudien zeigten, daß Präinkubation mit CNP die vasorelaxierende Wirkung von ANP versärkt. Weitere Experimente verdeutlichten, daß dieser Effekt durch die Aktivierung von GC-B durch CNP ausgelöst und von PKA vermittelt wird. Diese Ergebnisse werden durch Untersuchungen der intrazellulären cGMP-Konzentrationen als Indikator für die Rezeptoraktivität untermauert. Dementsprechend zeigte sich, daß die Präinkubation mit CNP zu einem signifikanten Anstieg der cGMP-Synthese durch GC-A führt. Darüberhinaus konnte demonstriert werden, daß Gefäße, die einer Präinkubation mit ANP ausgesetzt waren, eine verminderte GC-A-Aktivität zu verzeichnen hatten. Dies

weist eindeutig darauf hin, daß auch GC-A in glatter Muskulatur auf Desensitisierung reagiert, welche hingegen durch Proteinkinase G (PKG) vermittelt wird.

Demnach bestätigen diese Ergebnisse einen vorhandenen Signalweg, mithilfe dessen sich die Hormonsensitivität von GC-A durch CNP steigern läßt. Dieser Mechanismus ist offensichtlich in der Vaskulatur präsent und aktiv und reguliert die vasorelaxierende Potenz von ANP. Die Desensitisierung von GC-A, im Falle von erhöhten Plasmakonzentrationen von ANP zu erwarten, kann vom CNP/GC-B System wieder aufgehoben werden. Da die Abschwächung biologischer Eigenschaften von ANP augrund einer Rezeptordesensitisierung in verschiedenen Erkrankungen mit einer erhöhten Konzentration von ANP einhergeht und somit ein relevantes klinisches Problem darstellt, weist diese Arbeit auf die besondere Bedeutung von möglichen CNP/GC-B-Agonisten und ihr therapeutisches Potenzial hin.

# Abbreviations

| | |
|---|---|
| ANP | atrial natriuretic peptide |
| ATP | adenosine triphosphate |
| BNP | brain natriuretic peptide |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine 3', 5' monophosphate |
| cANF | atrial natriuretic factor |
| cGMP | cyclic guanosine 3', 5' monophosphate |
| CHD | cyclase homology domain |
| CNG | cyclic nucleotide gated |
| CNP | C-type natriuretic peptide |
| ECL | enhanced chemiluminescence |
| ELISA | enzyme linked immuno sorbent assay |
| ERK | extracellular-regulated protein kinase |
| GC | guanylyl cyclase |
| GC-A | guanylyl cyclase-A |
| GC-B | guanylyl cyclase-B |
| GPCR | G-protein coupled receptor |
| HEK | human embryonic kidney cell line |
| HBSS | Hank's balanced salt solution |
| HRP | horseradish peroxidase |
| IBMX | 3-isobutyl-1-methylxanthine |
| KHD | kinase homology domain |
| LPA | lysophosphatidic acid |
| NE | norepinephrine |

| | |
|---|---|
| NIH3T3 | mouse embryonic fibroblast cell line |
| NP | natriuretic peptide |
| NPR-A | natriuretic peptide receptor-A (alternative name of GC-A) |
| NPR-B | natriuretic peptide receptor-B (alternative name of GC-B) |
| NPR-C | natriuretic peptide receptor-C |
| OA | okadaic acid |
| PAP | peroxidase antiperoxidase |
| PBS | phosphate-buffered saline |
| PDE | phosphodiesterase |
| PCR | polymerase chain reaction |
| pGC | particulate guanylyl cyclase |
| PKA | cAMP-dependent protein kinase |
| PKC | phospholipid-dependent protein kinase |
| PKG | cGMP-dependent protein kinase |
| PLC | phospholipase C |
| PP | protein phophatases |
| S1P | sphingosine-1-phosphate |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| sGC | soluble guanylyl cyclase |
| SMC | smooth muscle cell |
| SNP | sodium nitroprusside |
| TBS | Tris-buffered saline |
| TBST | Tris buffered saline-Tween 20 |
| TMB | tetramethyl benzidin |

| | |
|---|---|
| Tris | Tris(hydroxymethyl)aminoethane |
| UV | ultraviolet |
| VASP | vasodilator-stimulated phosphoprotein |
| VSMC | vascular smooth muscle cell |

# References

Abbey-Hosch, S. E., A. N. Cody, et al. (2004). "Sphingosine-1-phosphate inhibits C-type natriuretic peptide activation of guanylyl cyclase B (GC-B/NPR-B)." Hypertension **43**(5): 1103-9.

Abbey, S. E. and L. R. Potter (2002). "Vasopressin-dependent inhibition of the C-type natriuretic peptide receptor, NPR-B/GC-B, requires elevated intracellular calcium concentrations." J Biol Chem **277**(45): 42423-30.

Abbey, S. E. and L. R. Potter (2003). "Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B." Endocrinology **144**(1): 240-6.

Airhart, N., Y. F. Yang, et al. (2003). "Atrial natriuretic peptide induces natriuretic peptide receptor-cGMP-dependent protein kinase interaction." J Biol Chem **278**(40): 38693-8.

Aizawa, T., H. Wei, et al. (2003). "Role of phosphodiesterase 3 in NO/cGMP-mediated antiinflammatory effects in vascular smooth muscle cells." Circ Res **93**(5): 406-13.

Anand-Srivastava, M. B., M. R. Sairam, et al. (1990). "Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system." J Biol Chem **265**(15): 8566-72.

Bahous, S. A., J. Blacher, et al. (2009). "Aortic stiffness, kidney disease, and renal transplantation." Curr Hypertens Rep **11**(2): 98-103.

Bartels, C. F., H. Bukulmez, et al. (2004). "Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux." Am J Hum Genet **75**(1): 27-34.

Beierwaltes, W. H. (2006). "cGMP stimulates renin secretion in vivo by inhibiting phosphodiesterase-3." Am J Physiol Renal Physiol **290**(6): F1376-81.

Bellamy, T. C. and J. Garthwaite (2001). "Sub-second kinetics of the nitric oxide receptor, soluble guanylyl cyclase, in intact cerebellar cells." J Biol Chem **276**(6): 4287-92.

Bellamy, T. C., J. Wood, et al. (2000). "Rapid desensitization of the nitric oxide receptor, soluble guanylyl cyclase, underlies diversity of cellular cGMP responses." Proc Natl Acad Sci U S A **97**(6): 2928-33.

Belz, G. G. (1995). "Elastic properties and Windkessel function of the human aorta." Cardiovasc Drugs Ther **9**(1): 73-83.

Bennett, B. D., G. L. Bennett, et al. (1991). "Extracellular domain-IgG fusion proteins for three human natriuretic peptide receptors. Hormone pharmacology and application to solid phase screening of synthetic peptide antisera." J Biol Chem **266**(34): 23060-7.

Berl, T., J. Mansour, et al. (1991). "ANP stimulates phospholipase C in cultured RIMCT cells: roles of protein kinases and G protein." Am J Physiol **260**(4 Pt 2): F590-5.

Brenner, B. M., B. J. Ballermann, et al. (1990). "Diverse biological actions of atrial natriuretic peptide." Physiol Rev **70**(3): 665-99.

Brown, R. L., T. Strassmaier, et al. (2006). "The pharmacology of cyclic nucleotide-gated channels: emerging from the darkness." Curr Pharm Des **12**(28): 3597-613.

Bryan, P. M. and L. R. Potter (2002). "The atrial natriuretic peptide receptor (NPR-A/GC-A) is dephosphorylated by distinct microcystin-sensitive and magnesium-dependent protein phosphatases." J Biol Chem **277**(18): 16041-7.

Cahill, P. A., E. M. Redmond, et al. (1990). "Vascular atrial natriuretic factor receptor subtypes are not independently regulated by atrial peptides." J Biol Chem **265**(35): 21896-906.

Cao, L., S. C. Chen, et al. (1998). "Ligand-dependent regulation of NPR-A gene expression in inner medullary collecting duct cells." Am J Physiol **275**(1 Pt 2): F119-25.

Chauhan, S. D., H. Nilsson, et al. (2003). "Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor." Proc Natl Acad Sci U S A **100**(3): 1426-31.

Chen, H., Y. C. Levine, et al. (2008). "Atrial natriuretic peptide-initiated cGMP pathways regulate vasodilator-stimulated phosphoprotein phosphorylation and angiogenesis in vascular endothelium." J Biol Chem **283**(7): 4439-47.

Chen, H. H. and J. C. Burnett, Jr. (1998). "C-type natriuretic peptide: the endothelial component of the natriuretic peptide system." J Cardiovasc Pharmacol **32 Suppl 3**: S22-8.

Chinkers, M., S. Singh, et al. (1991). "Adenine nucleotides are required for activation of rat atrial natriuretic peptide receptor/guanylyl cyclase expressed in a baculovirus system." J Biol Chem **266**(7): 4088-93.

Chrisman, T. D. and D. L. Garbers (1999). "Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts." J Biol Chem **274**(7): 4293-9.

Chrisman, T. D., S. Schulz, et al. (1993). "Seminal plasma factors that cause large elevations in cellular cyclic GMP are C-type natriuretic peptides." J Biol Chem **268**(5): 3698-703.

Chusho, H., N. Tamura, et al. (2001). "Dwarfism and early death in mice lacking C-type natriuretic peptide." Proc Natl Acad Sci U S A **98**(7): 4016-21.

Conti, M., W. Richter, et al. (2003). "Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling." J Biol Chem **278**(8): 5493-6.

Corbin, J. D. and S. H. Francis (1999). "Cyclic GMP phosphodiesterase-5: target of sildenafil." J Biol Chem **274**(20): 13729-32.

Cortes-Dericks, L. (2005). "Cellular Mechanisms Regulating the Enzymatic Activities of cGMP-Producing Natriuretic Peptide Receptors: Studies in Mus musculus Cell Lines."

Currie, M. G., K. F. Fok, et al. (1992). "Guanylin: an endogenous activator of intestinal guanylate cyclase." Proc Natl Acad Sci U S A **89**(3): 947-51.

de Bold, A. J., H. B. Borenstein, et al. (1981). "A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats." Life Sci **28**(1): 89-94.

Dousa, T. P. (1999). "Cyclic-3',5'-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney." Kidney Int **55**(1): 29-62.

Drewett, J. G., B. M. Fendly, et al. (1995). "Natriuretic peptide receptor-B (guanylyl cyclase-B) mediates C-type natriuretic peptide relaxation of precontracted rat aorta." J Biol Chem **270**(9): 4668-74.

Fan, D., P. M. Bryan, et al. (2005). "Down-regulation does not mediate natriuretic peptide-dependent desensitization of natriuretic peptide receptor (NPR)-A or NPR-B: guanylyl cyclase-linked natriuretic peptide receptors do not internalize." Mol Pharmacol **67**(1): 174-83.

Fesenko, E. E., S. S. Kolesnikov, et al. (1985). "Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment." Nature **313**(6000): 310-3.

Flora, D. R. and L. R. Potter (2010). "Prolonged atrial natriuretic peptide exposure stimulates guanylyl cyclase-a degradation." Endocrinology **151**(6): 2769-76.

Forte, L. R. and M. G. Currie (1995). "Guanylin: a peptide regulator of epithelial transport." FASEB J **9**(8): 643-50.

Foster, D. C., B. J. Wedel, et al. (1999). "Mechanisms of regulation and functions of guanylyl cyclases." Rev Physiol Biochem Pharmacol **135**: 1-39.

Francis, S. H. and J. D. Corbin (1999). "Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action." Crit Rev Clin Lab Sci **36**(4): 275-328.

Fulle, H. J., R. Vassar, et al. (1995). "A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons." Proc Natl Acad Sci U S A **92**(8): 3571-5.

Fuller, F., J. G. Porter, et al. (1988). "Atrial natriuretic peptide clearance receptor. Complete sequence and functional expression of cDNA clones." J Biol Chem **263**(19): 9395-401.

Furuya, M., K. Aisaka, et al. (1993). "C-type natriuretic peptide inhibits intimal thickening after vascular injury." Biochem Biophys Res Commun **193**(1): 248-53.

Garbers, D. L. (1991). "Guanylyl cyclase-linked receptors." Pharmacol Ther **50**(3): 337-45.

Goy, M. F., P. M. Oliver, et al. (2001). "Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide." Biochem J **358**(Pt 2): 379-87.

Hagiwara, H., H. Sakaguchi, et al. (1994). "Subtype switching of natriuretic peptide receptors in rat chondrocytes during in vitro culture." J Biochem **116**(3): 606-9.

Haneda, M., R. Kikkawa, et al. (1991). "Dual mechanism of angiotensin II inhibits ANP-induced mesangial cGMP accumulation." Kidney Int **40**(2): 188-94.

Hasegawa, M., Y. Hidaka, et al. (1999). "The relevance of N-linked glycosylation to the binding of a ligand to guanylate cyclase C." Eur J Biochem **263**(2): 338-46.

Herman, J. P., C. M. Dolgas, et al. (1996). "Localization of natriuretic peptide-activated guanylate cyclase mRNAs in the rat brain." J Comp Neurol **369**(2): 165-87.

Herring, N., J. A. Zaman, et al. (2001). "Natriuretic peptides like NO facilitate cardiac vagal neurotransmission and bradycardia via a cGMP pathway." Am J Physiol Heart Circ Physiol **281**(6): H2318-27.

Hobbs, A., P. Foster, et al. (2004). "Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide." Circulation **110**(10): 1231-5.

Honda, A., S. R. Adams, et al. (2001). "Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator." Proc Natl Acad Sci U S A **98**(5): 2437-42.

Inoue, K., K. Naruse, et al. (2003). "Four functionally distinct C-type natriuretic peptides found in fish reveal evolutionary history of the natriuretic peptide system." Proc Natl Acad Sci U S A **100**(17): 10079-84.

Jaiswal, R. K. (1992). "Endothelin inhibits the atrial natriuretic factor stimulated cGMP production by activating the protein kinase C in rat aortic smooth muscle cells." Biochem Biophys Res Commun **182**(1): 395-402.

Jaubert, J., F. Jaubert, et al. (1999). "Three new allelic mouse mutations that cause skeletal overgrowth involve the natriuretic peptide receptor C gene (Npr3)." Proc Natl Acad Sci U S A **96**(18): 10278-83.

Jeon, Y. H., Y. S. Heo, et al. (2005). "Phosphodiesterase: overview of protein structures, potential therapeutic applications and recent progress in drug development." Cell Mol Life Sci **62**(11): 1198-220.

Jewett, J. R., K. J. Koller, et al. (1993). "Hormonal induction of low affinity receptor guanylyl cyclase." EMBO J **12**(2): 769-77.

John, S. W., A. T. Veress, et al. (1996). "Blood pressure and fluid-electrolyte balance in mice with reduced or absent ANP." Am J Physiol **271**(1 Pt 2): R109-14.

Joubert, S., J. Labrecque, et al. (2001). "Reduced activity of the NPR-A kinase triggers dephosphorylation and homologous desensitization of the receptor." Biochemistry **40**(37): 11096-105.

Katafuchi, T., T. Mizuno, et al. (1992). "Modulation by NaCl of atrial natriuretic peptide receptor levels and cyclic GMP responsiveness to atrial natriuretic peptide of cultured vascular endothelial cells." J Biol Chem **267**(11): 7624-9.

Kato, J., K. L. Lanier-Smith, et al. (1991). "Cyclic GMP down-regulates atrial natriuretic peptide receptors on cultured vascular endothelial cells." J Biol Chem **266**(22): 14681-5.

Kaupp, U. B. and R. Seifert (2002). "Cyclic nucleotide-gated ion channels." Physiol Rev **82**(3): 769-824.

Kirstein, M., M. Rivet-Bastide, et al. (1995). "Nitric oxide regulates the calcium current in isolated human atrial myocytes." J Clin Invest **95**(2): 794-802.

Kishimoto, I., T. Yoshimasa, et al. (1994). "Natriuretic peptide clearance receptor is transcriptionally down-regulated by beta 2-adrenergic stimulation in vascular smooth muscle cells." J Biol Chem **269**(45): 28300-8.

Koh, G. Y., D. R. Nussenzveig, et al. (1992). "Dynamics of atrial natriuretic factor-guanylate cyclase receptors and receptor-ligand complexes in cultured glomerular mesangial and renomedullary interstitial cells." J Biol Chem **267**(17): 11987-94.

Kohno, M., K. Yokokawa, et al. (1997). "Effect of natriuretic peptide family on the oxidized LDL-induced migration of human coronary artery smooth muscle cells." Circ Res **81**(4): 585-90.

Koller, K. J., F. J. de Sauvage, et al. (1992). "Conservation of the kinaselike regulatory domain is essential for activation of the natriuretic peptide receptor guanylyl cyclases." Mol Cell Biol **12**(6): 2581-90.

Koller, K. J., M. T. Lipari, et al. (1993). "Proper glycosylation and phosphorylation of the type A natriuretic peptide receptor are required for hormone-stimulated guanylyl cyclase activity." J Biol Chem **268**(8): 5997-6003.

Koller, K. J., D. G. Lowe, et al. (1991). "Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP)." Science **252**(5002): 120-3.

Kotera, J., K. A. Grimes, et al. (2003). "cGMP-dependent protein kinase protects cGMP from hydrolysis by phosphodiesterase-5." Biochem J **372**(Pt 2): 419-26.

Kuhn, M. (2003). "Structure, regulation, and function of mammalian membrane guanylyl cyclase receptors, with a focus on guanylyl cyclase-A." Circ Res **93**(8): 700-9.

Kuhn, M. (2004). "Molecular physiology of natriuretic peptide signalling." Basic Res Cardiol **99**(2): 76-82.

Kurose, H., T. Inagami, et al. (1987). "Participation of adenosine 5'-triphosphate in the activation of membrane-bound guanylate cyclase by the atrial natriuretic factor." FEBS Lett **219**(2): 375-9.

Kurtz, A., K. H. Gotz, et al. (1998). "Stimulation of renin secretion by nitric oxide is mediated by phosphodiesterase 3." Proc Natl Acad Sci U S A **95**(8): 4743-7.

Labrecque, J., N. Mc Nicoll, et al. (1999). "A disulfide-bridged mutant of natriuretic peptide receptor-A displays constitutive activity. Role of receptor dimerization in signal transduction." J Biol Chem **274**(14): 9752-9.

Langub, M. C., Jr., R. E. Watson, Jr., et al. (1995). "Distribution of natriuretic peptide precursor mRNAs in the rat brain." J Comp Neurol **356**(2): 183-99.

Larose, L., J. J. Rondeau, et al. (1992). "Phosphorylation of atrial natriuretic factor R1 receptor by serine/threonine protein kinases: evidences for receptor regulation." Mol Cell Biochem **115**(2): 203-11.

Lefkowitz, R. J. and M. G. Caron (1986). "Regulation of adrenergic receptor function by phosphorylation." Curr Top Cell Regul **28**: 209-31.

Levin, E. R., D. G. Gardner, et al. (1998). "Natriuretic peptides." N Engl J Med **339**(5): 321-8.

Lohmann, S. M., A. B. Vaandrager, et al. (1997). "Distinct and specific functions of cGMP-dependent protein kinases." Trends Biochem Sci **22**(8): 307-12.

Lopez, M. J., D. L. Garbers, et al. (1997). "The guanylyl cyclase-deficient mouse defines differential pathways of natriuretic peptide signaling." J Biol Chem **272**(37): 23064-8.

Lopez, M. J., S. K. Wong, et al. (1995). "Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide." Nature **378**(6552): 65-8.

Lowe, D. G., M. S. Chang, et al. (1989). "Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction." EMBO J **8**(5): 1377-84.

Lowe, D. G., A. M. Dizhoor, et al. (1995). "Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2." Proc Natl Acad Sci U S A **92**(12): 5535-9.

Lucas, K. A., G. M. Pitari, et al. (2000). "Guanylyl cyclases and signaling by cyclic GMP." Pharmacol Rev **52**(3): 375-414.

Maack, T., F. A. Almeida, et al. (1988). "Clearance receptors of atrial natriuretic factor." Contrib Nephrol **68**: 58-65.

Maack, T., M. Suzuki, et al. (1987). "Physiological role of silent receptors of atrial natriuretic factor." Science **238**(4827): 675-8.

MacFarland, R. T., B. D. Zelus, et al. (1991). "High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells." J Biol Chem **266**(1): 136-42.

Marala, R. B., A. Sitaramayya, et al. (1991). "Dual regulation of atrial natriuretic factor-dependent guanylate cyclase activity by ATP." FEBS Lett **281**(1-2): 73-6.

Martinez, S. E., J. A. Beavo, et al. (2002). "GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides." Mol Interv **2**(5): 317-23.

Martinez, S. E., A. Y. Wu, et al. (2002). "The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding." Proc Natl Acad Sci U S A **99**(20): 13260-5.

Matsukawa, N., W. J. Grzesik, et al. (1999). "The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system." Proc Natl Acad Sci U S A **96**(13): 7403-8.

Maurice, D. H. and R. J. Haslam (1990). "Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP." Mol Pharmacol **37**(5): 671-81.

Mehats, C., C. B. Andersen, et al. (2002). "Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling." Trends Endocrinol Metab **13**(1): 29-35.

Mewe, M., C. K. Bauer, et al. (2006). "Regulation of spontaneous contractile activity in the bovine epididymal duct by cyclic guanosine 5'-monophosphate-dependent pathways." Endocrinology **147**(4): 2051-62.

Middendorff, R., D. Muller, et al. (2002). "The tunica albuginea of the human testis is characterized by complex contraction and relaxation activities regulated by cyclic GMP." J Clin Endocrinol Metab **87**(7): 3486-99.

Minamino, N., Y. Makino, et al. (1991). "Characterization of immunoreactive human C-type natriuretic peptide in brain and heart." Biochem Biophys Res Commun **179**(1): 535-42.

Mitchell, G. F., S. J. Hwang, et al. (2010). "Arterial stiffness and cardiovascular events: the Framingham Heart Study." Circulation **121**(4): 505-11.

Muller, D., L. Cortes-Dericks, et al. (2006). "Homologous and lysophosphatidic acid-induced desensitization of the atrial natriuretic peptide receptor, guanylyl cyclase-A, in MA-10 leydig cells." Endocrinology **147**(6): 2974-85.

Muller, D., K. J. Greenland, et al. "Neuronal differentiation of NG108-15 cells has impact on nitric oxide- and membrane (natriuretic peptide receptor-A) cyclic GMP-generating proteins." Mol Cell Endocrinol **320**(1-2): 118-27.

Muller, D., B. Hida, et al. (2009). "Expression of guanylyl cyclase (GC)-A and GC-B during brain development: evidence for a role of GC-B in perinatal neurogenesis." Endocrinology **150**(12): 5520-9.

Muller, D., M. Hildebrand, et al. (2010). "The membrane receptors guanylyl cyclase-A and -B undergo distinctive changes in post-translational modification during brain development." J Neurochem **115**(4): 1024-34.

Muller, D., R. Middendorff, et al. (2002). "Central nervous system-specific glycosylation of the type A natriuretic peptide receptor." Endocrinology **143**(1): 23-9.

Muller, D., A. K. Mukhopadhyay, et al. (2004). "Spatiotemporal regulation of the two atrial natriuretic peptide receptors in testis." Endocrinology **145**(3): 1392-401.

Mumby, M. C. and G. Walter (1993). "Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth." Physiol Rev **73**(4): 673-99.

Murthy, K. S., B. Q. Teng, et al. (2000). "G(i-1)/G(i-2)-dependent signaling by single-transmembrane natriuretic peptide clearance receptor." Am J Physiol Gastrointest Liver Physiol **278**(6): G974-80.

Nagase, M., T. Katafuchi, et al. (1997). "Tissue distribution and localization of natriuretic peptide receptor subtypes in stroke-prone spontaneously hypertensive rats." J Hypertens **15**(11): 1235-43.

Nakamura, T. and G. H. Gold (1987). "A cyclic nucleotide-gated conductance in olfactory receptor cilia." Nature **325**(6103): 442-4.

Nambi, P., M. Whitman, et al. (1986). "Vasopressin-mediated inhibition of atrial natriuretic factor-stimulated cGMP accumulation in an established smooth muscle cell line." Proc Natl Acad Sci U S A **83**(22): 8492-5.

Neves, S. R., P. Tsokas, et al. (2008). "Cell shape and negative links in regulatory motifs together control spatial information flow in signaling networks." Cell **133**(4): 666-80.

Nikolaev, V. O., S. Gambaryan, et al. (2005). "Real-time monitoring of the PDE2 activity of live cells: hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis." J Biol Chem **280**(3): 1716-9.

Ogawa, H., Y. Qiu, et al. (2004). "Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain: rotation mechanism for transmembrane signal transduction." J Biol Chem **279**(27): 28625-31.

Oliver, P. M., J. E. Fox, et al. (1997). "Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A." Proc Natl Acad Sci U S A **94**(26): 14730-5.

Pagano, M. and M. B. Anand-Srivastava (2001). "Cytoplasmic domain of natriuretic peptide receptor C constitutes Gi activator sequences that inhibit adenylyl cyclase activity." J Biol Chem **276**(25): 22064-70.

Pandey, K. N. (2005). "Biology of natriuretic peptides and their receptors." Peptides **26**(6): 901-32.

Pandey, K. N. (2005). "Natriuretic peptides and their receptors." Peptides **26**(6): 899-900.

Pandey, K. N., H. T. Nguyen, et al. (2002). "Ligand-regulated internalization, trafficking, and down-regulation of guanylyl cyclase/atrial natriuretic peptide receptor-A in human embryonic kidney 293 cells." J Biol Chem **277**(7): 4618-27.

Pedram, A., M. Razandi, et al. (2000). "Natriuretic peptides inhibit G protein activation. Mediation through cross-talk between cyclic GMP-dependent protein kinase and regulators of G protein-signaling proteins." J Biol Chem **275**(10): 7365-72.

Pfeifer, A., P. Ruth, et al. (1999). "Structure and function of cGMP-dependent protein kinases." Rev Physiol Biochem Pharmacol **135**: 105-49.

Piggott, L. A., K. A. Hassell, et al. (2006). "Natriuretic peptides and nitric oxide stimulate cGMP synthesis in different cellular compartments." J Gen Physiol **128**(1): 3-14.

Porter, J. G., A. Arfsten, et al. (1990). "Isolation and functional expression of the human atrial natriuretic peptide clearance receptor cDNA." Biochem Biophys Res Commun **171**(2): 796-803.

Potter, L. R. (1998). "Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptor B: dephosphorylation is a mechanism of desensitization." Biochemistry **37**(8): 2422-9.

Potter, L. R. (2005). "Domain analysis of human transmembrane guanylyl cyclase receptors: implications for regulation." Front Biosci **10**: 1205-20.

Potter, L. R. (2011). "Natriuretic peptide metabolism, clearance and degradation." FEBS J **278**(11): 1808-17.

Potter, L. R., S. Abbey-Hosch, et al. (2006). "Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions." Endocr Rev **27**(1): 47-72.

Potter, L. R. and D. L. Garbers (1992). "Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization." J Biol Chem **267**(21): 14531-4.

Potter, L. R. and D. L. Garbers (1994). "Protein kinase C-dependent desensitization of the atrial natriuretic peptide receptor is mediated by dephosphorylation." J Biol Chem **269**(20): 14636-42.

Potter, L. R. and T. Hunter (1998). "Phosphorylation of the kinase homology domain is essential for activation of the A-type natriuretic peptide receptor." Mol Cell Biol **18**(4): 2164-72.

Potter, L. R. and T. Hunter (1999). "A constitutively "phosphorylated" guanylyl cyclase-linked atrial natriuretic peptide receptor mutant is resistant to desensitization." Mol Biol Cell **10**(6): 1811-20.

Potter, L. R. and T. Hunter (2001). "Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation." J Biol Chem **276**(9): 6057-60.

Potthast, R., S. E. Abbey-Hosch, et al. (2004). "Calcium-dependent dephosphorylation mediates the hyperosmotic and lysophosphatidic acid-dependent inhibition of natriuretic peptide receptor-B/guanylyl cyclase-B." J Biol Chem **279**(47): 48513-9.

Potthast, R. and L. R. Potter (2005). "Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptors." Peptides **26**(6): 1001-8.

Rahmutula, D. and D. G. Gardner (2005). "C-type natriuretic Peptide down-regulates expression of its cognate receptor in rat aortic smooth muscle cells." Endocrinology **146**(11): 4968-74.

Roy, B. and J. Garthwaite (2006). "Nitric oxide activation of guanylyl cyclase in cells revisited." Proc Natl Acad Sci U S A **103**(32): 12185-90.

Ruskoaho, H. (1992). "Atrial natriuretic peptide: synthesis, release, and metabolism." Pharmacol Rev **44**(4): 479-602.

Rybalkin, S. D., C. Yan, et al. (2003). "Cyclic GMP phosphodiesterases and regulation of smooth muscle function." Circ Res **93**(4): 280-91.

Sandner, P., M. Kornfeld, et al. (1999). "Nitric oxide/cAMP interactions in the control of rat renal vascular resistance." Circ Res **84**(2): 186-92.

Sano, T., R. Imura, et al. (1992). "HS-142-1, a novel polysaccharide of microbial origin, specifically recognizes guanylyl cyclase-linked ANP receptor in rat glomeruli." Life Sci **51**(18): 1445-51.

Saouti, N., N. Westerhof, et al. (2010). "The arterial load in pulmonary hypertension." Eur Respir Rev **19**(117): 197-203.

Schachner, T., Y. Zou, et al. (2004). "Perivascular application of C-type natriuretic peptide attenuates neointimal hyperplasia in experimental vein grafts." Eur J Cardiothorac Surg **25**(4): 585-90.

Schroter, J., R. P. Zahedi, et al. (2010). "Homologous desensitization of guanylyl cyclase A, the receptor for atrial natriuretic peptide, is associated with a complex phosphorylation pattern." FEBS J **277**(11): 2440-53.

Schulz-Knappe, P., K. Forssmann, et al. (1988). "Isolation and structural analysis of "urodilatin", a new peptide of the cardiodilatin-(ANP)-family, extracted from human urine." Klin Wochenschr **66**(17): 752-9.

Schulz, S., M. J. Lopez, et al. (1997). "Disruption of the guanylyl cyclase-C gene leads to a paradoxical phenotype of viable but heat-stable enterotoxin-resistant mice." J Clin Invest **100**(6): 1590-5.

Schulz, S., S. Singh, et al. (1989). "The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family." Cell **58**(6): 1155-62.

Scotland, R. S., A. Ahluwalia, et al. (2005). "C-type natriuretic peptide in vascular physiology and disease." Pharmacol Ther **105**(2): 85-93.

Shakur, Y., L. S. Holst, et al. (2001). "Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family." Prog Nucleic Acid Res Mol Biol **66**: 241-77.

Shimizu, K., Y. Shintani, et al. (2002). "Potentiation of slow component of delayed rectifier K(+) current by cGMP via two distinct mechanisms: inhibition of phosphodiesterase 3 and activation of protein kinase G." Br J Pharmacol **137**(1): 127-37.

Shinomiya, M., J. Tashiro, et al. (1994). "C-type natriuretic peptide inhibits intimal thickening of rabbit carotid artery after balloon catheter injury." Biochem Biophys Res Commun **205**(2): 1051-6.

Shyjan, A. W., F. J. de Sauvage, et al. (1992). "Molecular cloning of a retina-specific membrane guanylyl cyclase." Neuron **9**(4): 727-37.

Singh, G., R. E. Kuc, et al. (2006). "Novel snake venom ligand dendroaspis natriuretic peptide is selective for natriuretic peptide receptor-A in human heart: downregulation of natriuretic peptide receptor-A in heart failure." Circ Res **99**(2): 183-90.

Soderling, S. H. and J. A. Beavo (2000). "Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions." Curr Opin Cell Biol **12**(2): 174-9.

Stein, B. C. and R. I. Levin (1998). "Natriuretic peptides: physiology, therapeutic potential, and risk stratification in ischemic heart disease." Am Heart J **135**(5 Pt 1): 914-23.

Steinhelper, M. E., K. L. Cochrane, et al. (1990). "Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes." Hypertension **16**(3): 301-7.

Steinmetz, M., R. Potthast, et al. (2004). "Diverging vasorelaxing effects of C-type natriuretic peptide in renal resistance arteries and aortas of GC-A-deficient mice." Regul Pept **119**(1-2): 31-7.

Stingo, A. J., A. L. Clavell, et al. (1992). "Presence of C-type natriuretic peptide in cultured human endothelial cells and plasma." Am J Physiol **263**(4 Pt 2): H1318-21.

Sudoh, T., N. Minamino, et al. (1990). "C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain." Biochem Biophys Res Commun **168**(2): 863-70.

Suga, S., K. Nakao, et al. (1992). "Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide." Endocrinology **130**(1): 229-39.

Suga, S., K. Nakao, et al. (1992). "Phenotype-related alteration in expression of natriuretic peptide receptors in aortic smooth muscle cells." Circ Res **71**(1): 34-9.

Takeuchi, H., K. Ohmori, et al. (2003). "Potentiation of C-type natriuretic peptide with ultrasound and microbubbles to prevent neointimal formation after vascular injury in rats." Cardiovasc Res **58**(1): 231-8.

Tamura, N., L. K. Doolittle, et al. (2004). "Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs." Proc Natl Acad Sci U S A **101**(49): 17300-5.

Thibault, G., A. Lacasse, et al. (1996). "Specific potentiation by cyclic AMP of natriuretic peptide-mediated cyclic GMP production in adipose tissue." Life Sci **58**(25): 2345-53.

Thompson, D. K. and D. L. Garbers (1995). "Dominant negative mutations of the guanylyl cyclase-A receptor. Extracellular domain deletion and catalytic domain point mutations." J Biol Chem **270**(1): 425-30.

Togashi, K., T. Kameya, et al. (1992). "Concentrations and molecular forms of C-type natriuretic peptide in brain and cerebrospinal fluid." Clin Chem **38**(10): 2136-9.

Tohse, N., H. Nakaya, et al. (1995). "Cyclic GMP-mediated inhibition of L-type Ca2+ channel activity by human natriuretic peptide in rabbit heart cells." Br J Pharmacol **114**(5): 1076-82.

Totsune, K., K. Takahashi, et al. (1994). "C-type natriuretic peptide in the human central nervous system: distribution and molecular form." Peptides **15**(1): 37-40.

Tremblay, J., R. Desjardins, et al. (2002). "Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases." Mol Cell Biochem **230**(1-2): 31-47.

Tsuji, T. and T. Kunieda (2005). "A loss-of-function mutation in natriuretic peptide receptor 2 (Npr2) gene is responsible for disproportionate dwarfism in cn/cn mouse." J Biol Chem **280**(14): 14288-92.

van den Akker, F. (2001). "Structural insights into the ligand binding domains of membrane bound guanylyl cyclases and natriuretic peptide receptors." J Mol Biol **311**(5): 923-37.

Vieira, M. A., M. Gao, et al. (2001). "Molecular and cellular physiology of the dissociation of atrial natriuretic peptide from guanylyl cyclase a receptors." J Biol Chem **276**(39): 36438-45.

Vlachopoulos, C., K. Aznaouridis, et al. (2010). "Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis." J Am Coll Cardiol **55**(13): 1318-27.

Waldman, S. A. and F. Murad (1987). "Cyclic GMP synthesis and function." Pharmacol Rev **39**(3): 163-96.

Waldman, S. A., R. M. Rapoport, et al. (1984). "Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues." J Biol Chem **259**(23): 14332-4.

Wall, M. E., S. H. Francis, et al. (2003). "Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase." Proc Natl Acad Sci U S A **100**(5): 2380-5.

Weber, S., D. Bernhard, et al. (2007). "Rescue of cGMP kinase I knockout mice by smooth muscle specific expression of either isozyme." Circ Res **101**(11): 1096-103.

Wen, J. F., X. Cui, et al. (2004). "High and low gain switches for regulation of cAMP efflux concentration: distinct roles for particulate GC- and soluble GC-cGMP-PDE3 signaling in rabbit atria." Circ Res **94**(7): 936-43.

Wilcox, J. N., A. Augustine, et al. (1991). "Differential regional expression of three natriuretic peptide receptor genes within primate tissues." Mol Cell Biol **11**(7): 3454-62.

Wilson, E. M. and M. Chinkers (1995). "Identification of sequences mediating guanylyl cyclase dimerization." Biochemistry **34**(14): 4696-701.

Winquist, R. J., E. P. Faison, et al. (1984). "Vasodilator profile of synthetic atrial natriuretic factor." Eur J Pharmacol **102**(1): 169-73.

Winquist, R. J., E. P. Faison, et al. (1984). "Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle." Proc Natl Acad Sci U S A **81**(23): 7661-4.

Wong, S. K., C. P. Ma, et al. (1995). "The guanylyl cyclase-A receptor transduces an atrial natriuretic peptide/ATP activation signal in the absence of other proteins." J Biol Chem **270**(51): 30818-22.

Wykes, V., T. C. Bellamy, et al. (2002). "Kinetics of nitric oxide-cyclic GMP signalling in CNS cells and its possible regulation by cyclic GMP." J Neurochem **83**(1): 37-47.

Yamamoto, S., F. Yan, et al. (2001). "Serine 331 is the major site of receptor phosphorylation induced by agents that activate protein kinase G in HEK 293 cells overexpressing thromboxane receptor alpha." Arch Biochem Biophys **393**(1): 97-105.

Yan, W., F. Wu, et al. (2000). "Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme." Proc Natl Acad Sci U S A **97**(15): 8525-9.

Yandle, T. G. (1994). "Biochemistry of natriuretic peptides." J Intern Med **235**(6): 561-76.

Yang, R. B., S. W. Robinson, et al. (1999). "Disruption of a retinal guanylyl cyclase gene leads to cone-specific dystrophy and paradoxical rod behavior." J Neurosci **19**(14): 5889-97.

Yasunari, K., M. Kohno, et al. (1992). "Phorbol ester and atrial natriuretic peptide receptor response on vascular smooth muscle." Hypertension **19**(4): 314-9.

Yoder, A. R., M. D. Stone, et al. (2010). "Mass Spectrometric Identification of Phosphorylation Sites in Guanylyl Cyclase A and B." Biochemistry.

Yuasa, K., K. Omori, et al. (2000). "Specific domain of cGMP-dependent protein kinase Ibeta but not Ialpha functions as a transcriptional activator in yeast." IUBMB Life **49**(1): 17-22.

Zaccolo, M. and M. A. Movsesian (2007). "cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology." Circ Res **100**(11): 1569-78.

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Bad Hersfeld, July 10, 2011

Guy Waisbrod

It is a pleasure to thank those who made this thesis possible. I am indebted to many people for their long-lasting support and encouragement which was invaluable for the successful completion of this research work.

Thanks to all my co-workers, without exception, of the Arbeitsgruppe Signaltransduktion of the Institut für Anatomie und Zellbiologie of the Justus-Liebig-University in Giessen for advice, scientific discussions and continous support during the long time this work came into being. My special thanks go also to the staff of the Institut of Physiologie, who provided a great deal of experimental resources.

I would like to thank *Marco Mewe* for introducing me so patiently into the intricacies of *in vivo* experiments using the organ bath technique.

To *Sabine Tasch* who introduced me to the secrets of ELISA as well as immunohistochemistry and contributed so much by originating many valuable ideas and suggestions.

I like to express further greatest thanks to *Mirjam Hildebrand* for her relentless help and contribution to my experiments particulary with regard to immunobloting and affinity labeling.

I am indepted particularly to ***Prof. Dr. Ralf Middendorff*** who asked me to join his staff and initiated the project. He offered me such a warm welcome in his growing team. His untiring help and great sense of humor were of priceless value.

I owe my deepest gratitude to my supervisor ***Dr. Dieter Müller***. This thesis would not have been possible without his continous and never ending support, cooperation and effort to read and re-read and help me edit every single draft of this thesis. He kept me always focused, unresting in his important criticism in order to meet his high set expectations. I must thank him for his inspiration and patience that helped me unlook so many doors in the world of science.

Last but surley not least, I wish to thank my family, especially my father Hannan and brother Sharon for their enduring support and many advices.