Cellular Mechanisms Regulating the Enzymatic Activities of cGMP-Producing Natriuretic Peptide Receptors: Studies in Mus musculus Cell Lines

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“One never notes what has been done;
One can only see what remains to be done…”

Marie Curie, 1894

Table of Contents

Abstract

The regulation of signalling pathways during and following their ligand-induced activation of cell surface receptors represents an important mechanism to ensure appropriate signal intensity and cellular response. In this regard, receptor down-regulation via internalization/endocytosis in response to chronic ligand exposure is known as a key reaction.

Three biologically-active peptides, designated as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) use plasma membrane receptors that contain intracellular domains capable of generating the second messenger cyclic GMP (cGMP) for eliciting effects in their target cells. The two cardiac hormones ANP and BNP bind to and stimulate guanylyl cyclase-A (GC-A), whereas GC-B is the specific receptor for CNP. Representing a particular phenomenon, the ligand-responsiveness of these two receptors is regulated by phosphorylation/dephosphorylation at intracellular residues without leaving the plasma membrane.

Since the natriuretic peptides play pivotal roles in a broad variety of tissues and cell types and considering that synthetic analogs have recently proven to be clinically beneficial, an understanding of receptor regulation mechanisms and cell type-specific phenomena is important from both a basic science and a clinical perspective.

In this thesis, I used two cell lines which either express only GC-A (MA-10 Leydig cells) or co-express GC-A and GC-B (αT3-1 pituitary cells) to specifically address certain questions of interest.

The experiments revealed that sustained exposure to either ANP itself or to the biologically-active phospholipid lysophosphatidic acid (LPA) elicits GC-A desensitization in MA-10 Leydig cells. Both reactions were found to have similar kinetics and finally resulted in an equal decrease (by 40%) in GC-A hormone-responsiveness. Homologous (ANP-induced), but not LPA-induced (so-called heterologous) desensitization was blocked by a cyclic AMP-dependent protein kinase (PKA) inhibitor, indicating distinct pathways and a crucial role for PKA in the process of homologous desensitization, where cGMP is generated as second messenger. LPA, but not ANP treatment enhanced extracellular signal-regulated kinase (ERK)

phosphorylation and induced a striking re-organization of actin filaments. The LPA effects suggested receptor-mediated pathways, consistent with the identification of type 2 (LPA_2) LPA receptor gene expression. At the molecular level, these findings showed for the first time (a) that homologous and heterologous desensitization are mediated by unique pathways, and (b) that PKA is essentially implicated in homologous (cGMP-dependent) desensitization processes. In addition, they provided novel evidence for cross-talks between natriuretic peptide and phospholipid signalling. Physiologically, the control mechanisms identified in MA-10 cells suggest distinct roles in native Leydig cells, related to regulation of steroidogenesis and cell growth/differentiation. The elucidation of pronounced cellular effects in response to LPA indicates for the first time that Leydig cells may represent targets for phospholipid signalling *in vivo.*

Questions that could be specifically addressed in αT3-1 cells were based on the co-expression of GC-A and GC-B in this cell type. The results obtained demonstrate for the first time (a) interactions (“cross-talks”) between ANP/GC-A and CNP/GC-B signalling at a cellular level and (b) that the hormone-responsiveness of natriuretic peptide receptors can be enhanced (“sensitization”) rather than reduced (“desensitization”) by signalling molecules acting via plasma membrane receptors. Since several cell types are known to express both ANP and CNP receptors *in vivo*, and considering that ANP represents a hormone and CNP a paracrine (locally-produced) factor, these results might be of a general physiological relevance.

In conclusion, this study has generated information thought to be significant for a better understanding of the mechanisms involved in regulation of natriuretic peptide signalling.

1 Introduction

1.1 Natriuretic peptides

The natriuretic peptide family consists of three structurally related peptides, designated as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). ANP and BNP are cardiac hormones that play pivotal roles in the regulation of blood pressure and cardiovascular homeostasis (Maack, 1996; Stein and Levin, 1998). ANP and BNP are secreted into the circulation in response to cardiac wall stretch and therefore act in a true endocrine fashion. In contrast, CNP is produced in vascular endothelial cells (Suga *et al*., 1992), chondrocytes (Hagiwara *et al*., 1994, 1996), and in high concentrations in the brain where it acts each locally in an autocrine and/or paracrine fashion (Sudoh *et al*., 1990; Komatsu, 1991).

Another related peptide is urodilantin, representing a variant of ANP which is exclusively localized in the kidney (Schulz-Knappe, 1988).

1.1.1 Structure and biosynthesis of natriuretic peptides

Each natriuretic peptide is encoded by a specific gene. The mRNA for ANP encodes a precursor protein (pro-ANP) of 126 amino acids. Cleavage of pro-ANP releases a 98-amino acid N-terminal fragment in addition to the 28-amino acid carboxyl-terminal fragment (ANP). Human pro-BNP contains 108 amino acids; processing releases the mature 32-amino acid BNP. CNP exists as 22- and 53-amino acid forms, each derived from the 103-amino acid pro-CNP. The 22-amino acid peptide CNP predominates in the brain, anterior pituitary, kidney and vascular endothelial cells (Sudoh *et al*., 1990; Suga *et al*., 1992; McArdle *et al*., 1994). Specific functions of the 53-amino acid form (Kelly and Struthers, 2001) are still poorly understood.

The natriuretic peptides share a common structural motif consisting of a 17-amino acid loop formed by an intramolecular disulfide linkage between 2 cysteine residues (Yandle, 1994; Levin, 1998). This disulfide loop as well as parts of their N- and C- terminal extensions are essential for the biological activity of the natriuretic peptides (Chen and Burnett ,1998).

1.1.2 Proposed roles for natriuretic peptides in testicular Leydig cells and gonadotrophs of the pituitary gland

Regulation of cardiovascular homeostasis represents a main function of natriuretic peptides within the organism, and in this context, vascular smooth muscle cells and the kidney are important peptide target sites (Potter and Hunter, 2001). However, receptors for natriuretic peptides are highly expressed in a variety of other cell types and tissues, and there is convincing evidence for significant roles in addition to the control of blood pressure and fluid/salt secretion (Müller *et al*., 2000, 2004; Walter and Stephan, 2004; Middendorff *et al*., 2002; El-Gehani *et al*., 2001; McArdle *et al*., 1993, 1994; Johnson *et al*., 1994; Schumacher *et al*., 1992; Mukhopadhyay *et al*., 1986a, 1986b; Pandey, 1994; Pandey *et al*, 1999).

In the testis, the testosterone-producing Leydig cells are characterized by high expression levels of the ANP receptor (reviewed by Middendorff *et al*., 2000). ANP-induced activation of this receptor has been reported to increase testosterone levels *in vitro* (Mukhopadhyay *et al*., 1986a, 1986b) and *in vivo* (Pandey, 1994; Pandey *et al*., 1999), indicating a specific role for testicular steroidogenesis.

In the pituitary gland, several lines of evidence point at important local functions of natriuretic peptides. For example, highest tissue concentrations of CNP were found in the anterior pituitary, and both ANP and CNP as well as their specific receptors are localized to the gonadotroph cells (Komatsu *et al*., 1991; McArdle *et al*., 1994). These findings, together with investigations which proved the functional activities of the receptors, strongly suggest that natriuretic peptides in the pituitary acts as neuroendocrine regulators, contributing to the neuroendocrine control of reproduction (reviewed comprehensively in Resch *et al*., 1997; and Fowkes and McArdle, 2000).

1.2 The Natriuretic Peptide Receptors

The principal mechanism by which natriuretic peptides exert their physiological effects involves binding to and activation of plasma membrane receptors that contain intrinsic guanylyl cyclase activity (Lucas *et al*., 2000). Thus, cGMP is the common “second messenger” in this signalling system.

Guanylyl cyclase-A (GC-A, also referred to as NPR-A) specifically binds to and is activated by ANP and BNP, whereas guanylyl cyclase-B (GC-B, also referred to as

NPR-B) is the specific receptor for CNP. GC-A is widely distributed in many tissues and cell types (Table 1, for a recent review, see Lucas *et al*., 2000). GC-B is expressed in a more restricted manner within the organism. In certain cell types, a co-expression of both receptors has been described (McArdle *et al*., 1994; Krause *et al*., 1997; DiCicco-Bloom *et al*., 2004; Jankowski *et al*., 2004).

Table 1: Natriuretic peptide receptors and their ligands, sites of expression and physiological roles

Receptor	**Ligand(s)**	**Sites of expression**	**Roles**
GC-A (NPR-A)	ANP>BNP	Vasculature, kidney, lung, heart, brain, pituitary, thymus, testis, smooth muscle cells (plus additional sites identified)	Vasodilation (relaxation of smooth muscle cells) Promotion of natriuresis/diuresis Inhibition of renin/aldosterone secretion Regulation of cell proliferation/differentiation
GC-B (NPR-B)	CNP	Vasculature, brain, pituitary, pineal gland spleen, lung, penis, endothelium, chondrocytes (certain additional sites described)	Vasodilation Regulation of bone growth Control of neuronal differentiation Regulation of hormone secretion
NPR-C	ANP, BNP, CNP	Kidney, lung, vasculature, testis, additional sites reported	Local modulator of natriuretic peptide effects

In addition to GC-A and GC-B, there is a third receptor, NPR-C, shown to have a natriuretic peptide clearance function (Maack *et al*., 1987). This receptor has equal affinities to all three natriuretic peptides (ANP, BNP and CNP) and does not have any guanylyl cyclase activity. This so-called clearance receptor controls the local availability of the natriuretic peptides via receptor-mediated internalization and

degradation (Maack, 1992). In addition, there are reports indicating that NPR-C can contribute to signalling by inhibition of adenylyl cyclase or activation of phospholipase C (Pagano and Anand-Srivastava, 2001; Murthy *et al*., 2000).

1.2.1 Structure of cGMP-generating natriuretic peptide receptors

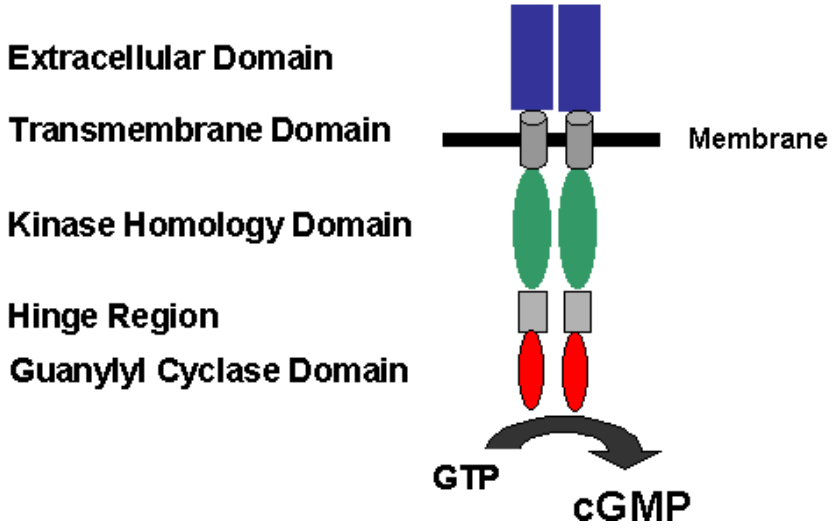

Figure 1: Common topology of the natriuretic peptide receptors, GC-A and GC-B
Receptor polypeptide chains consist of an extracellular ligand binding domain, a single transmembrane domain, a kinase homology domain, a hinge region, and a guanylyl cyclase catalytic domain. The scheme illustrates that the receptors are thought to occur as homodimers. (Modified from Kuhn, 2003)

The basic structures of GC-A and GC-B are very similar (Figure 1). They each contain a ~450-amino acid extracellular ligand binding domain at the N-terminus and a 21-residue hydrophobic transmembrane domain. The intracellular domain can be further subdivided into a kinase homology domain (KHD), a 41-amino acid hinge region and a 250-amino acid C-terminal guanylyl cyclase catalytic domain (Potter and Hunter, 2001).

1.2.1.1 Extracellular Domain

Extracellular domains of guanylyl cyclase receptors are the sites of peptide hormone binding. The diversity in primary structure is responsible for the specificity of ligand binding. The precise molecular mechanisms involved in ligand binding, and the subsequent activation of the catalytic domain remain to be defined (Duda *et al*., 1994).

1.2.1.2 Transmembrane domain

All membrane-bound guanylyl cyclases have a single transmembrane domain, representing a hydrophobic region that permits its insertion into the hydrophobic cell membrane lipid bilayer. The precise role of this domain besides membrane localization remains to be defined. However, it is notable that truncated receptor mutants, which have the extracellular domain but lack the transmembrane domain, are still capable of forming dimers and of binding ligands (for review, see Lucas *et al*., 2000).

1.2.1.3 Kinase homology domain

All membrane-bound guanylyl cyclases possess a ~250-amino acid kinase homology domain (KHD) located between the transmembrane and catalytic domains. The KHD exhibits a ~30% homology with a wide range of protein kinases but up to now, no kinase activity has been detected (Koller *et al*., 1992). The KHD has been proposed to normally repress the catalytic activity of the receptor (Chinkers and Garbers, 1989). However, the binding of ATP to KHD following receptor activation causes a derepression of the catalytic activity (Tremblay *et al*., 2002).

Hanks *et al*. (1988) discovered a sequence rich in glycine (GXGXXG) within the KHD of GC-A and GC-B which serves as an anchor for the terminal phosphate of ATP which is thought to be necessary for the maximal activation of the receptor (Chinker and Garbers, 1989; Goraczniak *et al*., 1992). A GC-A mutant devoid of this glycine-rich domain by site-directed mutagenesis became unresponsive to ATP (Duda, 1993), suggesting that the activation of the catalytic domain by ATP is mediated by KHD. The identification of phosphorylation sites, predominately serine and threonine residues within the KHD is an important aspect in the regulation mechanism involved in the desensitization of GC-A and GC-B (Potter and Hunter, 1998a, 1998b).

1.2.1.4 Hinge Region

The 41-amino acid hinge region is located between the kinase homology domain and the catalytic domain. Its structure is consistent with a coiled-coil configuration that favors specific protein-protein interactions (Thompson and Garbers, 1995; Wilson and Chinkers, 1995). It has been proposed that this region mediates the dimerization of catalytic subunits which is generally required for the catalytic activity of soluble and membrane-bound guanylyl cyclases (Thompson and

Garbers, 1995; Wilson and Chinkers, 1995). Thus, deletion of this region results in monomeric and inactive intracellular catalytic subunits (Potter and Hunter, 2001).

1.2.1.5 Guanylyl cyclase catalytic domain

The model of the catalytic mechanism of both adenylyl and guanylyl cyclases was developed based on the crystal structure of the rat type II C_2 adenylyl cyclase catalytic domain, considering the high sequence similarity between adenylyl and guanylyl cyclases. The model predicts that cyclases such as membrane guanylyl cyclases including GC-A and GC-B have two active sites per homodimer, thus capable of binding two substrate molecules (Liu *et al*., 1997).

In the basal state, GC-A and GC-B exist as homodimers in which each monomer is phosphorylated on certain residues in the KHD. Binding of the ligand to its receptor leads to transduction of an activatory signal through the plasma membrane which induces ATP to bind to KHD. ATP binding to KHD results in the derepression of the catalytic domain of the receptor, thus, rendering an active catalytic domain capable of converting guanosine triphosphate (GTP) into cGMP.

1.3 cGMP signalling

The discovery of 3',5' cyclic adenosine monophosphate (cAMP) by Sutherland in 1957 and 3',5' cyclic guanosine monophosphate (cGMP) (Waldmann *et al*., 1987) led to the formulation of the term second messenger, a central concept in understanding how information is transmitted into and within cells.

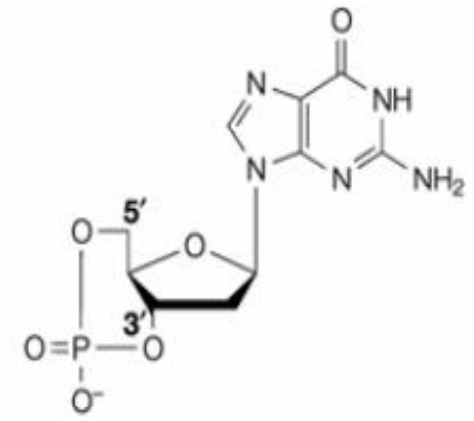

Figure 2: Structure of cyclic guanosine monophosphate (cGMP)
The chemical structure of cGMP is shown. The 3' and 5' C residues of ribose that are linked to the phosphate group are indicated.

Cyclic GMP (cGMP, Figure 2), which was first described in 1963 as a biological product, has emerged as an important focus in signal transduction. It is the key

messenger in a variety (for reviews, see Lucas *et al*., 2000; Münzel *et al*., 2003) of transduction pathways.

R.F. Furchgott, L.J. Ignarro and F. Murad received a Nobel Prize in 1998 for their pioneering studies concerning NO/cGMP signalling and its impact in the cardiovascular system. The advent of the medication, sildenafil (tradename VIAGRA™) for treatment of male impotence has attracted widespread attention. This agent potently inhibits PDE5, a cGMP-specific phosphodiesterase (Corbin and Francis, 1999).

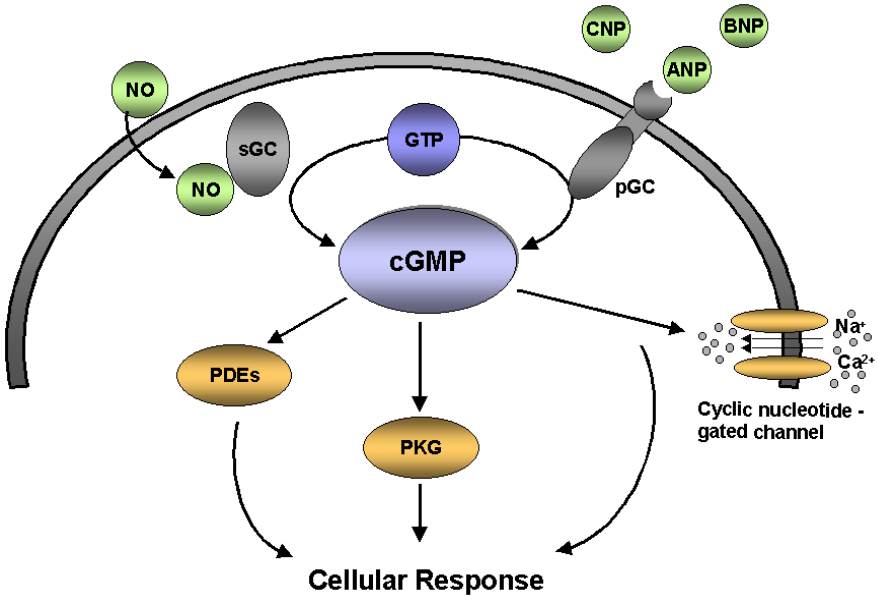

Figure 3: Generation mechanisms and cellular targets of cGMP
Two types of enzymes, designated as soluble guanylyl cyclase (sGC) and particulate guanylyl cyclase (pGC) catalyze the generation of cGMP from GTP. The most prominent activator of sGC is nitric oxide (NO), the major activators of pGCs are the natriuretic peptides, ANP, BNP, and CNP. Cellular effects of cGMP can be elicited by binding to either phosphodiesterases (PDEs), cGMP-dependent protein kinase (PKG) or cyclic nucleotide-gated ion channels.

1.3.1 cGMP-generating systems

Generally, cGMP is synthesized by a family of enzymes termed as guanylyl cyclases (GCs). These enzymes exist in two forms: the so-called soluble (sGC) and particulate (pGC) guanylyl cyclases. Both GCs synthesize cGMP in a similar way using GTP as a substrate and Mg^{+2} or Mn^{2+} as cofactors in a reaction that involves release of inorganic pyrophosphate (Senter *et al*., 1983). Soluble and particulate cyclases have different structures and are activated in different ways. Soluble GCs

can be activated by nitric oxide (NO) or carbon monoxide (CO) (Koesling, 1998; Schmidt *et al*., 1993), whereas the most prominent activators of particulate GCs are the natriuretic peptides (Figure 3 on previous page).

The amplitude and duration of cGMP signals are controlled by the rates of synthesis through guanylyl cyclases and the rates of degradation through 3',5'-cyclic nucleotide phosphodiesterases (PDEs).

1.3.2 cGMP-target proteins

The most important target/effector proteins of cGMP in cells are cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cGMP-regulated ion channels (Corbin and Francis, 1999).

1.3.2.1 cGMP-dependent protein kinase

cGMP-dependent protein kinase (GK, also known as PKG) is the major cellular target protein of cGMP and plays important roles in cGMP-dependent signal transduction pathways (Yuasa *et al*., 2000). GK is a member of a family of cyclic nucleotide-dependent protein kinases that also includes cAMP-dependent protein kinase (PKA). GK is a dimer composed of two identical monomers (Lohmann *et al*., 1997; Francis and Corbin ,1999; Sausbier *et al*., 2000). Each monomer contains a regulatory domain and a catalytic domain on a single polypeptide chain.

Activation of GK is mediated by cellular cGMP elevations in response to extracellular signals such as NO or natriuretic peptides. In its active form, GK can transfer the γ-phosphate from ATP to a serine or threonine residue of target proteins leading to physiological effects such as relaxation of vascular smooth muscles, airway distension, inhibition of cell proliferation, inhibition of platelet aggregation and apoptosis (Corbin and Francis, 1994).

There are two forms of GK: type I (GK I) contains an acetylated N-terminus and is usually localized in the cytoplasm; GK II has a myristoylated N-terminus, and is usually associated with membranes. Two closely related forms of type I are known: GK I-α and GK I-β which differ only within the first ~100 amino acids (Wall *et al*., 2003) of the N-terminal domain. This results in different binding affinities for cGMP. The molecular masses of these enzymes in mammals are ~76 kDa (GK I-α) and ~78 kDa (GK I-β). PKG II is a 86-kDa membrane-bound protein.

1.3.2.2 cGMP-regulated PDEs

Further effectors of cGMP are cGMP-regulated phosphodiesterases (PDEs). PDEs are a large group of structurally related enzymes that catalyze the hydrolysis of cyclic nucleotides to the corresponding inactive nucleoside 5'-monophosphates by cleaving the phosphodiester bond between the phosphorous and oxygen atoms at the 3'-position. They differ in their primary structure, tissue distribution, affinities for cyclic nucleotides and sensitivity to Ca^{2+} and various inhibitors (Corbin and Francis, 1999; Dousa, 1999; Soderling and Beavo, 2000).

The mammalian PDEs share a common structural organization consisting of a conserved catalytic domain located near the C-terminus, and regulatory domains located at the N-terminus of the protein. The catalytic domain is proposed to be the site of substrate binding and catalysis, whereas the N-terminus is though to regulate the catalytic domain, the subcellular localization of PDEs, and has allosteric binding sites for cyclic nucleotides. cGMP-binding at these sites stimulates (PDE2) or inhibits (PDE3) the catalytic activity. PDEs control the access of second messengers to their intracellular effectors (for review, see Mehats *et al*., 2002).

1.3.2.3 cGMP-regulated ion channel

Other targets of cGMP are cyclic nucleotide-gated (CNG) ion channels. The most prominent members of this ion channel family are the cGMP-gated channels located in the outer segment of the vertebrate photoreceptor cells which play a central role in the phototransduction process in rod and cone receptor cells.

Recent studies indicate that that the primary structure of the bovine cGMP-gated channel consists of a polypeptide chain of 690 amino acids (aa) with a calculated molecular weight of 79,601. The C-terminus of the channel has been identified as the cGMP binding domain. Binding of cyclic nucleotides to this domain results in a complex series of conformational rearrangements which lead to the opening of the channel. (Hsu and Molday, 1993).

1.3.2.4 cAMP-dependent protein kinase (PKA)

The holoenzyme form of PKA is a tetramer composed of two regulatory and two catalytic subunits (Francis and Corbin, 1994). Upon interaction with cAMP, the catalytic subunits dissociate from the regulatory subunits, and are then free to catalyze the phosphorylation of protein substrates. Because PKAs contain specific cyclic nucleotide binding domains with significant homology to PKGs, they may also be activated by cGMP, with a 50-fold lower selectivity as compared to cAMP (Pfeifer *et al*. 1999). This was further supported by the observations that both cGMP and cAMP are capable of cross-activating their respective kinases (Cornwell *et al*., 1994; Wang *et al*., 1999). In vitro studies have demonstrated that cGMP-cross activated PKA, which mediates the secretion of fluid induced by heat-stable enterotoxin (ST) in human intestinal cells (Forte *et al*., 1992; Chao *et al*., 1994). Cross-activation of PKA by ANP-induced cGMP in mouse Leydig cells has also been reported (Schumacher *et al*., 1992).

1.4 Regulation of cGMP-generating natriuretic peptide receptors

Despite the considerable progress on the structure-function studies of natriuretic peptide receptors, the regulation of its catalytic activity which affects the sensitivity of the receptor still remains a central issue of investigation. The following mechanisms have been implicated in the regulation of these receptors: receptor (1) regulation by extracellular tonicity, (2) regulation at the level of gene expression and (3) regulation by phosphorylation/dephosphorylation (for review, see Kuhn, 2003). Studies have established that the enzyme activity (hormone-responsiveness) of natriuretic peptide receptors GC-A and GC-B is mainly regulated by dephosphorylation (Potter and Garbers, 1992, 1994; Potter and Hunter, 1998a, 1998b; Potthast *et al*., 2004).

1.4.1 Regulation by Phosphorylation

Coordinated control of kinases and phosphatases provides the cell with a capacity to rapidly alter the phosphorylation state of cellular proteins (Figure 4) in order to meet different physiological demands (Mumby and Walter, 1993).

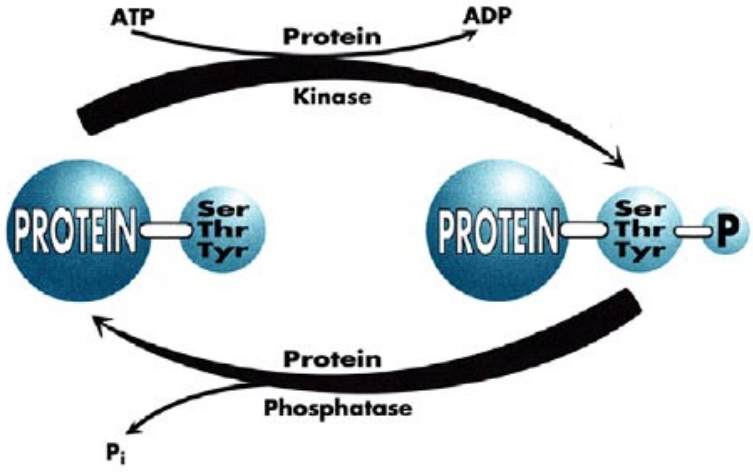

Figure 4: Regulation of proteins by phosphorylation
The activity of many cellular proteins is regulated by phosphorylation/dephosphorylation which takes place at either serine (Ser), threonine (Thr) or tyrosine (Tyr) residues. Phosphorylation (P) is catalyzed by kinases, dephosphorylation by phosphatases. (P indicates phosphate)

The first direct indication that natriuretic peptide receptors are regulated by protein phosphorylation was reported in 1992 (Potter *et al*., 1992 and Koller *et al.,* 1992) showing that GC-A purified from metabolically labelled HEK293 cells contain P^{32}. Later studies showed that this receptor was phosphorylated on six residues (Ser-497, Thr-500, Ser-502, Ser- 506, Ser-510, Thr-513) localized within the glycine-rich elbow of the ATP-binding region of KHD (Potter and Hunter, 1998a). Receptors lacking four or more of these sites are completely unresponsive to hormone (Potter and Garbers, 1992, 1994; Koller *et al*., 1993).

Similarly, GC-B activity is also regulated by phosphorylation of the following amino acid residues: Thr-513, Thr-516, Ser-518, Ser-523, Ser-526 which are also located within the KHD (Potter and Hunter, 1998b). The replacement of any of these residues with alanine to mimic a dephosphorylated state results in reductions of hormone-dependent guanylyl cyclase activities (Bryan and Potter, 2002). Thus, indicating that receptor phosphorylation is not merely modulatory but is absolutely necessary for GC activity. The kinase(s) responsible for GC-A and GC-B phosphorylation in the basal state are still undefined. Besides phosphorylation, dephosphorylation also regulates the sensitivity of GC receptors to ligand binding. Potter and Hunter (1998a, 1998b) proposed that prolonged activation of GC-A and

GC-B by natriuretic peptides leads to dephosphorylation resulting in subsequent desensitization.

1.4.2 Homologous Desensitization

Garbers (1989) demonstrated that membrane-bound guanylyl cyclase receptors in sperms of sea urchin were first activated, and then desensitized following interactions with their cognate egg peptides. These receptors were highly phosphorylated in the basal state, and were massively dephosphorylated following ligand-receptor interaction. Further investigations showed that such prolonged ligand-receptor interactions initiate a cascade leading to receptor dephosphorylation, known as homologous desensitization (Potter and Garbers, 1992). This acute form of desensitization is ligand-dependent because additions of cGMP analog, 8-bromo cGMP or dibutyryl-cGMP did not induce this kind of desensitization.

The ligand-dependency of both soluble and particulate GCs (i. e., GC-A) desensitization in human airway smooth muscle cells was reported by Hamad *et al.* (1999). Their findings showed that desensitization of sGCs occurred after pre-incubations with NO, whereas desensitization of GC-A occurred following pretreatments with ANP. That homologous desensitization is regulated by dephosphorylation was demonstrated by the addition of microcystin, a phosphatase inhibitor, which blocked the desensitization process (Potter and Hunter, 1999). In addition to microcystin-sensitive phosphatases, a recent report has shown that magnesium-sensitive phosphatases can also dephosphorylate GC-A in membranes of transfected mouse kidney 293 and NIH3T3 fibroblast cells (Bryan and Potter, 2002).

Receptor GC-B is also susceptible to homologous desensitization. Pre-exposure of GC-B to CNP caused a time-dependent dephosphorylation and homologous desensitization of the receptor in transfected NIH3T3 fibroblast cells which indicate that the catalytic activity of GC-B is tightly coupled to its phosphorylation state (Potter, 1998). The findings of Joubert *et al*. (2001) demonstrated that ANP-induced GC-A desensitization resulted from an inactivation of a GC-A-associated kinase rather than an increase in phosphatase activity. An indication that phosphorylation also plays a crucial role in the process of homologous desensitization. The principle of homologous desensitization is shown in Figure 5.

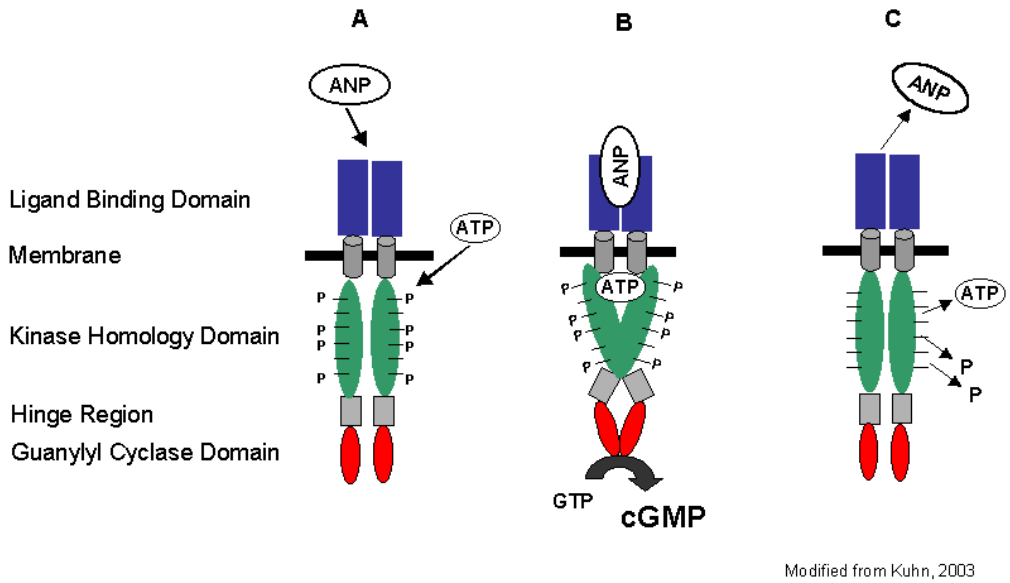

Figure 5: Current model of ANP-induced activation and homologous desensitization of GC-A

In the absence of ANP, GC-A is phosphorylated at several serine and threonine residues located in the kinase homology domain (A). ANP-binding in the presence of ATP leads to conformational changes resulting in strongly enhanced guanylyl cyclase activity (B). Prolonged exposure to ANP can induce receptor dephosphorylation and increased dissociation rates of ANP (C). The dephosphorylated receptor is unresponsive to further hormonal stimulation. P indicates phosphate.

1.4.3 Heterologous Desensitization

Natriuretic peptide-dependent activation of GC-A and GC-B can be reduced not only by chronic exposure to their specific ligands (homologous desensitization) but also by exposure to other agents like endothelin, vasopressin and angiotensin. The latter is referred to as heterologous desensitization (Potter and Garbers, 1994).

A number of reports have demonstrated this form of desensitization. Studies on a phorbol 12-myristate 13-acetate (PMA)-induced GC-A desensitization indicated that this process is directly associated with substantial decreases of ^{32}P content of the receptor that coincides with an attenuation of GC activity (Potter and Garbers, 1994). Other experiments have shown that arginine-vasopressin, angiotensin II and endothelin also induced desensitization of GC-A through the activation of PKC in cultured cell lines (Nambi *et al.*, 1986, 1987; Haneda *et al.*, 1991; Jaiswal, 1992). A recent report on vasopressin-dependent inhibition of GC-B in A10 rat aortic smooth muscle cells provided evidence for a calcium-dependent desensitization pathway distinct from that of PKC (Abbey and Potter, 2002).

Recently, bioactive phospholipids have been reported to elicit desensitizing effects in GC-B. Sphingosine-1-phosphate (S1P) was shown to desensitize GC-B in transfected NIH3T3 fibroblasts cells (Chrismann *et al*., 2003), and proposed that the controlling mechanism of this phenomenon is different from that of the known S1P signalling pathway. Another report on the S1P-dependent desensitization of GC-B in transfected NIH3T3 fibroblasts and A10 vascular smooth muscle cells was accounted for an increase in intracellular calcium rather than a degradation of the receptor (Potthast *et al*., 2004). Lysophospholipid acid (LPA), another bioactive phospholipid has also been reported to inhibit GC-B activity in transfected NIH3T3 cells in which the regulatory mechanism has to be identified (Abbey and Potter, 2003). The principle of heterologous desensitization is illustrated in Figure 6.

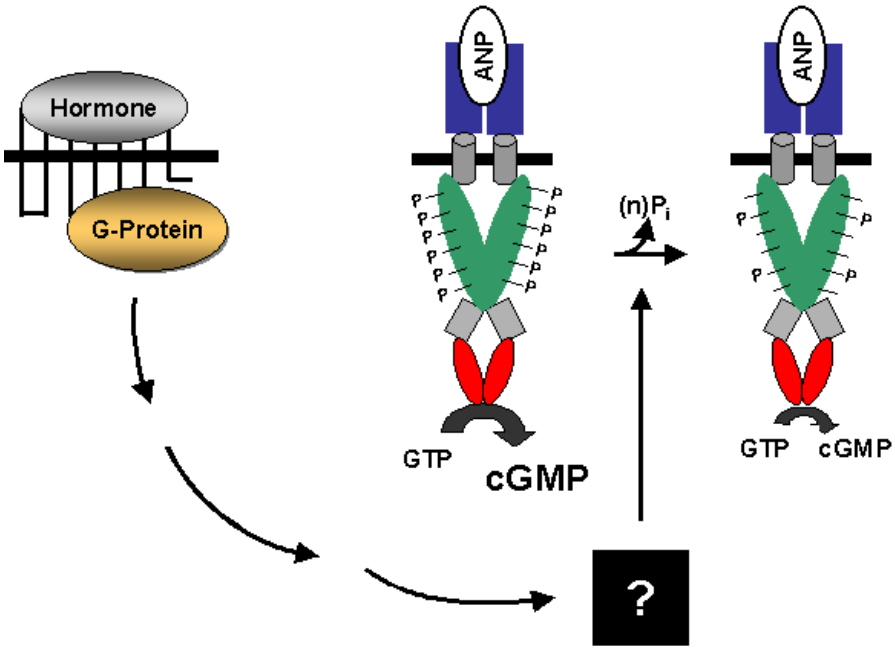

Figure 6: Heterologous desensitization of GC-A
In this scheme, it is indicated that an ANP-unrelated hormone, acting via a 7-transmembrane receptor, elicits GC-A dephosphorylation. The intracellular signalling cascades and, in particular, the phosphatases finally activated, are not yet elucidated and may differ depending on the hormone involved. In any case, GC-A dephosphorylation results in a desensitized (less responsive to ANP stimulation) receptor.

1.5 Lysophosphatidic acid

Although lipids were previously considered as building blocks of the cell membrane, they are now recognized also as important cell signalling molecules. The group of Moolenaar established in 1989 (van Corven *et al*., 1989) that lysophosphatidic acid (LPA) and other phospholipids are not only simply structural components of the cell membrane, but also biological mediators.

LPA is a bioactive lipid with a wide variety of biological actions, particularly as an inducer of cell proliferation, differentiation, migration, adhesion, invasion, survival and morphogenesis. Links to LPA signalling have been implicated in such diverse biological processes such as tissue remodeling, neurogenesis, myelination and olfaction as well as immune and reproductive functions (for reviews, see Moolenaar *et al*., 2004; Ishii *et al*., 2004).

In the male reproductive system, some possible roles of LPA have been reported. The findings of Garbie *et al*. (2000) demonstrated an induction of the acrosome reaction through LPA-activated PKCα in bovine sperm. Tanaka *et al.* (2004) reported that human seminal plasma contains lysophospholipase D (lysoPLD) which converts lysophospholipids to LPA, indicating an active LPA metabolism in the seminal plasma, and a possible role of LPA in the male reproductive tract including prostate cancer. Additional studies demonstrated that LPA is directly generated by human prostate cancer cells (CaP cell line) suggesting an autocrine function in the prostrate. (Xie *et al*., 2002). In the female reproductive system, elevated LPA levels were detected in patients suffering from early-stage ovarian cancer compared with controls and therefore, the plasma level may be considered as a potential biomarker for ovarian cancer (Xu *et al*., 1998).

Besides the numerous mitogenic effects of LPA, the phospholipid is also well known for its striking effects on actin cytoskeleton (Moolenaar *et al*., 1999). There are in addition remarkable effects of LPA on nervous system-derived cells where it induces growth cone collapse and neurite retraction. In brain-derived astrocytes, LPA causes reversal of process outgrowth (“stellation”) and in brain-derived endothelial cells, LPA enhances tight junction permeability, suggesting that the lipid may modulate blood-brain barrier function (for review, see Moolenaar *et al*., 2004).

1.5.1 Structure and biosynthesis of LPA

LPA (Figure 7) consists of three structural domains: the phosphate head group, a linker region, and a lipophilic tail (Hopper *et al*., 1999). The phosphate group is proposed to be essential for receptor activation, whereas the lipophilic tail is suggested to be responsible for its biological activity (Santos *et al*., 2000).

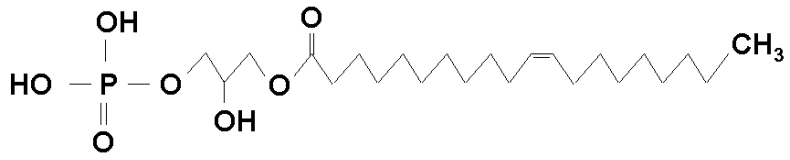

Figure 7: The chemical structure of LPA

LPA can be generated in activated cells by the hydrolysis of phospholipids pre-existing in the cellular membranes (Budnik and Mukhophadyay, 2002), although the exact mechanism of LPA biosynthesis is still undefined (Moolenaar *et al*, 2004).

Serum LPA is produced by multiple enzymatic pathways that involve monoacylglycerol kinase, phospholipase $A_{1,}$ secretory phospholipase $A_{2,}$ and lysophospholipase D (lysoPLD), including autotaxin. Several enzymes are involved in LPA degradation which include: lysophospholipase, lipid phosphate phosphatase, and LPA acyl transferase such as endophilin. Serum LPA is bound to albumin and low-density lipoproteins, which possibly protect LPA form rapid degradation (for review, see Ishii *et al*., 2004).

1.5.2 LPA receptors

The identification of G-protein-coupled receptors for LPA was a major contribution that provided a mechanistic basis for many of their effects on cells in culture (Luquain *et al*., 2003).There are four identified LPA receptors in mammals. The first three, LPA $_{1-3}$, share sequence homology with one another, whereas LPA_4 is divergent in sequence. LPA_1 contains 364 amino acids (aa), a 7-transmembrane structure and couples to multiple G proteins. It has an apparent molecular mass of ~41 kDa. LPA_2 which also couples to G proteins contains 351 aa (human) or 348 aa (mouse) with a predicted molecular mass of ~39 kDa. LPA_3 differs from the two previous LPA receptors (LPA_1 and LPA_2) by not coupling to $G_{12/13}$ and has a preference for LPA molecules with unsaturated acyl chains. LPA_4 shows similarity to

the platelet activating factor (PAF) G-protein coupled receptor (GPCR). This receptor consists of 370 aa with a molecular mass of ~42 kDa (for review, see Anliker and Chun, 2004).

The LPA receptors are distributed widely in the organism, and each subtype induces a variety of cellular responses (see Table).

Table 2: Selected responses mediated by LPA receptor types in culture

Receptors	**Responses**	**Cell Types**	**References**
LPA_1	Cell rounding	TSM (immortalized neuroblast) B103 (neuroblastoma) B103	Hecht *et al*., 1996 Fukushima *et al*., 1998 Ishii *et al*., 2000
	AC inhibition	TSM HTC4 (hepatoma) B103	Hecht *et al*., 1996 An *et al*., 1998b Ishii *et al*., 2000
	$[Ca^{2+}]_i$ increase	Jurkat T (lymphoma) HTC4	An *et al*., 1998b An *et al*., 1998b
	MAP kinase activation	Yeast (*S. cerevisiae)* B103	Erickson *et al*., 1998I Ishii *et al*., 2000
	Stress-fiber formation	RH7777 (hepatoma)	Fukushima *et al*., 1998
LPA_2	$[Ca^{2+}]_i$ increase	Jurkat T HTC4 Sf9 (insect) RH7777 (hepatoma)	An *et al*., 1998b An *et al*., 1998b Bandoh *et al*., 1999 Im *et al*., 2000b
	AC inhibition	HTC4 B103	An *et al*., 1998b Ishii *et al*., 2000
	AC stimulation	Sf9	Bandoh *et al*., 1999
	MAP kinase activation	PC12 (pheochromo-cytoma) B103	Bandoh *et al*., 1999 Ishii *et al*., 2000
	Cell rounding	B103	Ishii *et al*., 2000
LPA_3	$[Ca^{2+}]_i$ increase	Sf9 RH7777	Bandoh *et al*., 1999 Im *et al*., 2000b
	AC stimulation	Sf9	Bandoh *et al*., 1999
	AC inhibition	B103	Ishii *et al*., 2000

AC, adenylyl cyclase

1.5.3 LPA and natriuretic peptide signalling

In recent studies, the two bioactive phospholipids, S1P and LPA have been shown to elicit a potent acute inhibitory effect on GC-B activity in untransfected fibroblast NIH3T3 cells, NIH3T3 cells overexpressing GC-B and in A10 vascular smooth muscle cells through the induction of receptor desensitization (Chrisman *et al*., 2003; Abbey and Potter, 2003; Abbey-Hosch *et al*., 2004). So far, these are the first reports demonstrating cross-interactions between natriuretic peptides and phospholipids.

Cross-talks between LPA and GC-B signalling may have important physiological implications. Such interactions suggest that GC-B is under a constant repression by LPA serum levels, and that GC-B inhibition is a rapid response to LPA signalling (Abbey and Potter, 2003). One hypothesized functional role of this interaction is that GC-B desensitization maximizes the response of fibroblast in the wound healing process.

The intracellular pathways that trigger LPA-mediated GC-B desensitization are still poorly understood. While previous studies have proposed a role for PKC in the process of GC-B desensitization (Potter and Garbers, 1994; Potter and Hunter 2000), a very recent report indicated that LPA-induced GC-B desensitization is PKC-independent but is linked to elevations in calcium levels, and that calcium-dependent receptor dephosphorylation is the underlying mechanism (Potthast *et al*., 2004). In all these studies, an involvement of LPA receptors has not yet been addressed.

1.6 Aims of the Study

1.6.1 Background

Although the principle of natriuretic peptide receptor regulation has been well established by basic studies performed with receptor-transfected cell lines, a variety of crucial questions remain to be addressed. Hitherto, the signalling pathways mediating homologous and heterologous desensitization are still poorly understood. Furthermore, the receptor-specificity (GC-A versus GC-B) of such processes has not yet been examined. In addition, potential cell-type specific phenomena need to be elucidated with regard to assessments of a physiological relevance. A further

question of particular interest is whether GC-A- and GC-B-mediated signalling might cross-react when both receptors are co-expressed in the same cell.

To be able to address some of these questions in a specific manner, I selected two cell lines for my investigations which either express only GC-A (MA-10 cells) or do express GC-A and GC-B (αT3-1). In these cells, the receptors are endogenously expressed, and both cell lines represent well established models for the native (*in vivo*) cells they derive from. Thus, in addition to allow investigations on molecular mechanisms, the studies were expected to provide information of physiological relevance.

1.6.2 Experiments with MA-10 cells

The immortalized MA-10 cell line has been derived from a hormonally-responsive mouse Leydig cell tumor (Hoelscher and Ascoli, 1996). MA-10 cells have been frequently used as a model system for studying Leydig cell physiology, in particular regarding the regulation of steroidogenesis by different biological factors (Ascoli, 1981; Pereira *et al*., 1988; Manna *et al*., 1999; Liu *et al*., 2001; Tsuchiya *et al*., 2003; Chen *et al*., 2005). These cells, like native Leydig cells, highly express GC-A, and many studies have used this cell type to examine receptor-mediated functions of ANP (Khurana and Pandey, 1994, 1995; Kumar *et al*., 1997; Pandey *et al*., 2002). The endogenous expression of GC-A in this cell line thus provided an appropriate basis to investigate the following items (see Figure 8):

1. Whether GC-A is desensitized during prolonged exposure to ANP (homologous desensitization).
2. To study the signalling pathways in this process.
3. To determine whether other agents/receptors evoke inhibition of GC-A activity (heterologous desensitization).
4. To comparatively characterize the mechanisms involved in homologous and heterologous desensitization processes.
5. To examine whether LPA is capable of inducing GC-A desensitization.

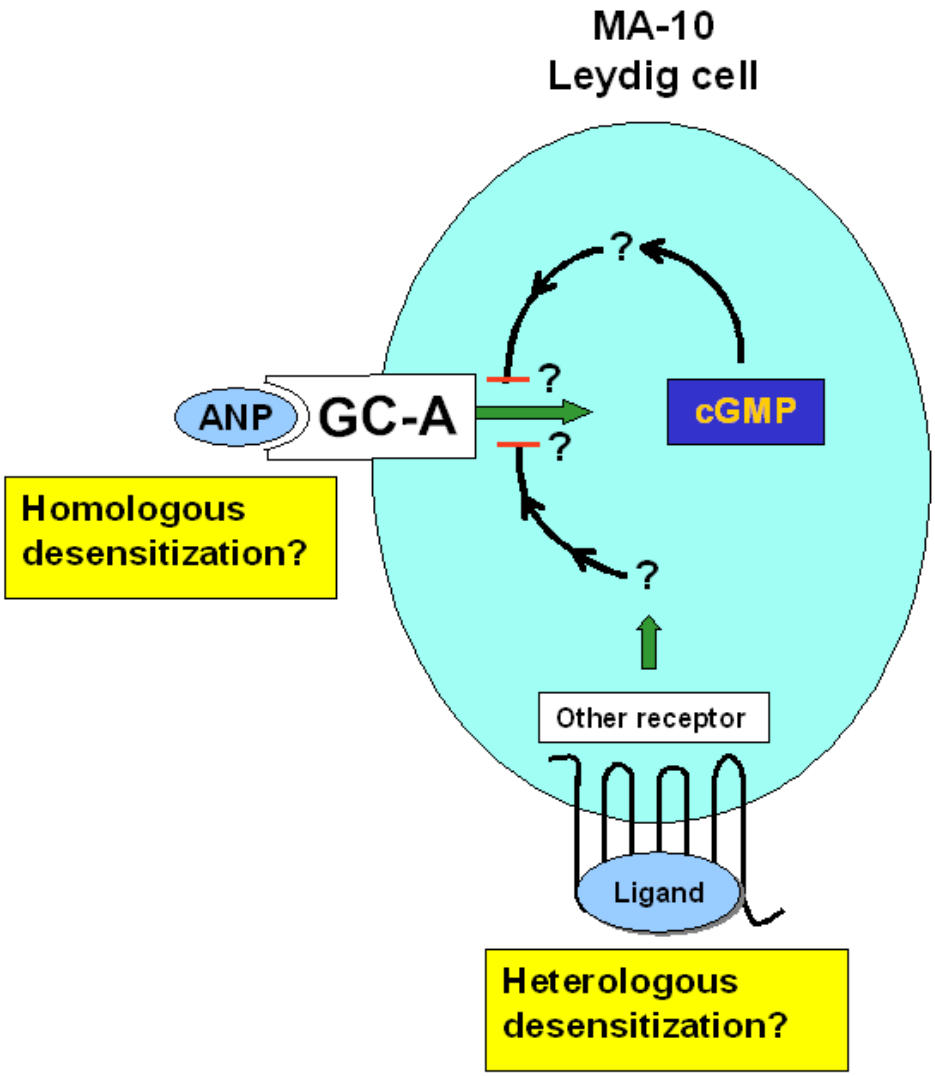

Figure 8: Overview of the experiments and their underlying questions in MA-10 cells

The studies served to examine homologous and heterologous desensitization of GC-A and to characterize the pathways involved.

1.6.3 Experiments with αT3-1 cells

The mouse pituitary tumor αT3-1 cell line is derived from the anterior lobe of the pituitary (Windle *et al.*, 1990). αT3-1 cells have the typical characteristics of gonadotrophs and are an established model system for studying pituitary functions (Willars *et al.*, 2001). Moreover, both native gonadotrophs and αT3-1 cells are characterized by the expression of GC-A and GC-B as well as by a local production of CNP (Fowkes and McArdle, 2000), suggesting that natriuretic peptides are neuroendocrine regulators in the pituitary (see Resch *et al.*, 1997 and Fowkes and McArdle, 2000 for comprehensive reviews). Thus, αT3-1 cells were thought to be

particularly well suitable to address experimentally the following questions (outlined in Figure 9):

1. Whether ANP and/or CNP can induce homologous desensitization of their respective (GC-A, GC-B) receptors.
2. Whether such effects are selectively directed against their specific receptor proteins (i. e., ANP and GC-A, CNP and GC-B), despite the generation of a common second messenger (cGMP).
3. Whether other signaling pathways can elicit (heterologous) desensitization and whether such reactions are or are not natriuretic peptide receptor (GC-A, GC-B)-specific.

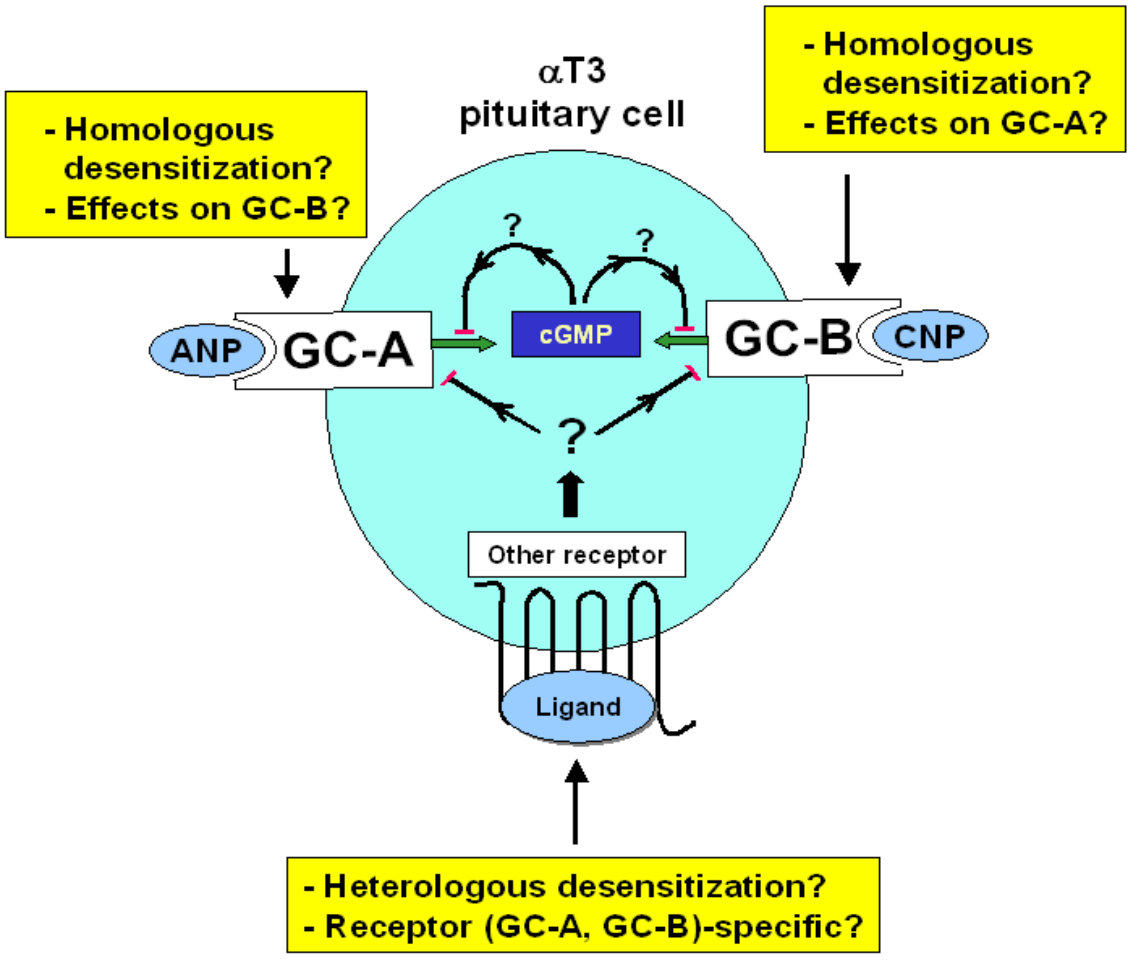

Figure 9: Overview of the experiments and their underlying questions in αT3-1 cells
These studies served to examine (a) whether ANP and/or CNP can induce homologous desensitization, (b) whether such reactions are receptor-specific, and (c) whether heterologous desensitization, induced by a different receptor/ligand signalling system, would affect ANP and CNP receptors in an equal manner or not.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

MA-10 mouse Leydig tumor cells were a kind gift from Dr. M. Ascoli from the Dept. of Pharmacology, University of Iowa, Iowa City, USA

Mouse pituitary tumor αT3-1 cells were kindly provided by Dr. P. Mellon from the Dept. of Reproductive Medicine, University of California, San Diego, USA

2.1.2 Wistar rats

Healthy 2-3 months old Wistar rats were purchased from Charles River Laboratories, Sulzfeld, Germany

2.1.3 Reagents and solutions

All reagents were purchased from Sigma unless otherwise stated.

Solutions used

Medium without serum (MA-10 cells)	1:1 Dulbecco's minimal essential medium and HAM F-12 nutrient mixture 2 mM L-glutamine 100 IU/ml penicillin;100 µg/ml streptomycin 0.1% BSA (w/v)
Medium without serum (αT3-1)	Dulbecco's modified Eagles medium (high glucose) 100 IU/ml penicillin;100 µg/ml streptomycin 0.1% BSA (w/v)
Buffered minimal essential medium	9.61 g/L Minimal essential medium 5.98 g/L HEPES adjust pH to 7.4 prior to addition of 0.5 g/L BSA
Earl's salt solution	55 mM glucose 8 mM $MgS0_4$ (heptahydrate) 50 mM KCl 1.2 M NaCl 10 mM sodiumdihydrogenphosphate 1% BSA (w/v) 25 mM HEPES

Homogenizing buffer	50 mM Tris-HCl 1 mM EDTA 1 mM DTT 0.1 mM PMSF (added before use)
4x Separating gel buffer	1.5 M Tris-HCl, pH 8.8 0.4% (w/v) SDS
4x Stacking gel buffer	0.5M Tris-HCl, pH 6.8 0.4% (w/v) SDS
PAGE buffer	0.25 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
TBS tween buffer	1x TBS buffer + 0.05% Tween 20
Blocking buffer	1% Boehringer blocking reagent 0.01 M maleic acid 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
Tris-EDTA buffer	10 mM Tris-HCl, pH 7.5 1 mM EDTA
TAE (Tris-acetate EDTA)	40 mM Tris-acetate 1 mM EDTA
Gel loading buffer	25% glycerine 10 mM EDTA 0.25% (w/v) bromphenol blue 0.25% (w/v) xylene cyanol

2.2 Methods

2.2.1 Cell culture

MA-10 mouse Leydig tumor cells were grown in a 1:1 mixture of Dulbecco's minimal essential medium and Ham F-12 nutrient mixture supplemented with 7.5% horse serum, 2.5% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

Mouse pituitary tumor αT3-1 cells were maintained in Dulbecco's modified Eagle's medium (4500 mg/L glucose, 110 mg/L pyruvate, 548 mg/L glutamine), 10% fetal calf serum ,100 units/ml penicillin and 100 µg/ml streptomycin.

Depending on the experiments intended, cells were grown in either T_{25} or T_{75} culture flasks (Nunc), 12-well plates (Costar) or 21.5-cm^2 plates (Nunc).

2.2.1.1 Cryoconservation and thawing

Freshly prepared cell suspensions were centrifuged at 250 x g for 5 min at 4°C in culture medium. The supernatant was aspirated, and the cell density was adjusted to 2-8 x 10^6 cells/ml with ice-cold culture medium containing 10% dimethylsulfoxide (DMSO, Sigma). Cells were transferred into sterile vials (1ml/vial) and pre-incubated in an ice bath for 30 min before freezing at -80°C. For longer preservation periods, cells were kept in liquid nitrogen.

Frozen cells were thawed quickly, transferred into 50-ml Falcon tubes containing 10 ml cold growth medium and spun down as described above. Supernatants were aspirated, and the cell pellets were resuspended with a suitable amount of growth medium and seeded in T_{25} or T_{75} culture flasks (Nunclon ™ Surface).

2.2.1.2 Surface trypsination of cells

Ma-10 cells and αT3-1 cells in T_{25} or T_{75} culture flasks were washed twice with sterile PBS and treated with 1-2 ml trypsin–EDTA (0.5 g/L trypsin and 0.2 g/L EDTA, Gibco BRL). Cells were incubated for either 3 min at 37°C (MA-10 cells) or for 1 min at RT (αT3-1 cells). Trypsination was stopped by the addition of PBS with moderate agitation of the flask to facilitate detachment of cells. Cell suspensions were transferred to sterile tubes and centrifuged at 250 x g for 3 min. Cell pellets were

resuspended with culture medium, counted using a haemacytometer (Neubauer) and seeded into 12-well plates (Costar) or 21.5-cm^2 culture dishes (Nunc) for further experimental usage.

2.2.1.3 Preparation of cells for in vitro stimulation

MA-10 and αT3-1 cells were maintained as described in Section 2.2.1. At about 80% confluency, cells were trypsinated, washed once with PBS and counted in a haemacytometer (Neubauer). In general, experiments were performed in 12-well plates in volumes of 0.5 ml/ well. MA-10 cells were seeded with a density of $5x10^4$ cells/ml medium (without serum), whereas α T3-1 were plated with a density of $1x10^5$ cells/ml medium (with serum). Cells were incubated overnight in an atmosphere of 95% air and 5% CO_2 at 37°C (Heraeus Instruments) and then stimulated directly or after serum starvation for 2 h (αT3-1 cells).

2.2.2 Isolation, purification and culture of Leydig cells

Wister rats were killed by decapitation under CO_2-induced anesthesia. The Leydig cell isolation procedure was based on the method of Schumacher *et al*. (1978). Briefly, testes were removed, decapsulated, and placed in buffered minimal essential medium (MEM, Sigma) containing 0.25 mg/ml Collagenase IA (Sigma) in a shaking water bath at 100 cycles/min for 20 min at 34°C (GFL 1083). After incubation, the tubes were shaked manually to obtain a homogenous cell suspension. Cell suspensions were filtered through a nylon gauze and centrifuged at 100 x g for 10 min. Resulting pellets were resuspended in buffered MEM containing 0.05% BSA (w/v) and applied to 50 ml discontinuous Percoll (Pharmacia, Uppsala, Sweden) with a density gradient ranging from 1.01-1.12 g/ml. The tubes were subjected to centrifugation at 1,000 x g for 30 min at RT. After centrifugation, cell layers containing Leydig cells (density ~1.05 g/ml) were collected with a capillary tube and subsequently washed once with buffered MEM (0.05% BSA) by centrifugation at 100 x g for 10 min at RT. The resulting cell pellets were resuspended in a known volume of the same medium. Cell viability was > 95% as measured by tryphan blue (Sigma) exclusion tests. Cells were counted with a Neubauer counting chamber and dispensed in 12-well plates at a density of $5x10^4$ cells/ml/well. Plates were incubated overnight at 37°C in an atmosphere of 95% O_2 and 5% CO_2 to allow cells to recover and attach to the plates. The next day the

medium was aspirated, and cells were used for specific treatments. In certain cases, fresh isolated Leydig cells were directly used for experiments performed in polystyrol tubes 75x12mm (Sarstedt).

2.2.3 Whole Cell Stimulation

αT3-1 and MA-10 cells were grown in T_{75} culture flasks and harvested at 70-80% confluence. MA-10 cells were plated in 12-well plates with a density of $5x10^4$ /well /ml serum-free medium containing 0,1% BSA whereas αT3-1 cells were plated with $1x10^5$ /well /ml serum-containing medium (these cells do not survive in medium depleted of serum). Cells were incubated overnight at 37°C in an atmosphere of 95% air and 5% $C0_2$. The following day, the medium was aspirated and cells were treated with different substances (Table 3) in the presence of 0.25 mM 1-methyl-3-isobutyl-xanthine (IBMX, a phosphodiesterase inhibitor used to avoid cGMP degradation). αT3-1 cells were serum starved for two hours at 37°C prior to different LPA cell treatments (αT3-cells were not starved in treatments not involving LPA) in the presence of 0.25 mM IBMX. After the corresponding cells treatments, reactions were terminated by the addition of 1.5 ml of ice-cold 100% ethanol (v/v). Plates were re-incubated at –20°C for 1 h to facilitate the extraction of intracellular cGMP. Ethanol extracts were collected and centrifuged at 2,000 x g for 30 min at 4°C to get rid of protein and cell fragments. Supernatant fractions were transferred into glass tubes and evaporated to dryness in a vortex evaporator (Evapotex™) for 1-1.5 h at 60°C. Resulting pellets were reconstituted with TRFIA assay buffer. Total cGMP (cells and medium) was measured by a commercial cGMP-TRFIA kit (Section 2.2.3.1) within 24h.

Table 3: Substances used in cell treatments of MA-10 and αT3-1 cells

Substance, Manufacturer	**Final concentration**	**Stimulation time**
Atrial natriuretic peptide (ANP), Bachem	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M	0, 1, 3, 5, 15, 30, 60 min
C-type natriuretic peptide (CNP), Bachem	10^{-9}, 10^{-8}, 10^{-7} M	30 min
Brain natriuretic peptide (BNP), Bachem	10^{-7} M	30 min
Sodium nitroprusside (SNP), Sigma	10^{-5}, 10^{-4}, 10^{-3} M	30 min
Lysophosphatidic acid (LPA), Sigma	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M	30 min
Forskolin , Calbiochem	0.1 µM, 0.4 µM, 1.0 µM 4.0 µM	30 min, 3h
Pituitary adenylate cyclase-activating peptide (PACAP-27), Novabiochem	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	5, 30, 60 min
Pituitary adenylate cyclase-activating peptide (PACAP-38), Novabiochem	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	5, 30, 60 min, 3h
Vasointestinal peptide (VIP), Novabiochem	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	30 min
Human choriongonadotropin (hCG), Boehringer	20 ng/ml, 50 ng/ml	5, 30, 60 min
Phorbol 12-myristate 13-acetate (PMA), Sigma	0.1 µM	30 min
PD 98059 (MEK inhibitor), Calbiochem	40 µM	15, 30 min
BAY 11-7082 (I Kappa B inhibitor), Biomol	10 µM	15, 30 min
Caffeic acid phenylethylester (CAPE), Alexis Biochemicals	10 µg/ml	15, 30 min
H 89 (PKA inhibitor), Biomol	0.1µM, 1.0 µM, 10µM 50 µM	15, 30 min
1 – butanol, Merck	0.3%	15, 30 min
2 – butanol, Sigma	0.3%	15, 30 min

1-methyl - 3-isobutyl-xanthine (IBMX), Sigma	0.25 mM	present in all cell treatments
Okadaic acid (Phosphatase inhibitor), Calbiochem	0.1 nM, 20 nM	30 min
Microcystin-LR (Phosphatase inhibitor), Calbiochem	0.5 µM	30 min
Rp-8-Br-PET- cGMPs (cGMP analog), Biolog	5 µM	15, 30 min
^{125}I-ANP, Amersham	0.5 nM	15 min at 20^{0}C
^{125}I-[Tyr^0]CNP32-53, Amersham	2 nM	15 min at 20^{0}C

2.2.3.1 cGMP assay

The amount of cGMP after cell treatments (Table 3) was determined by a **t**ime-**r**esolved **f**luoro **i**mmuno**a**ssay (TRFIA). It represents a competitive solid phase assay based on a cGMP-specific rabbit antiserum, an Eu^{+3}-chelate labelled cGMP tracer, and microtiter strips coated with goat-anti-rabbit-antibodies. The cGMP in the sample competes with an europium (Eu^{+3}) chelate-labelled cGMP tracer for binding to the antibody. The resulting cGMP-antibody and tracer-antibody complexes are simultaneously bound to the surface of the wells via the secondary antibody. This assay (available as kit) includes the following reagents: assay buffer, cGMP antiserum, cGMP-PL-Eu^{+3} as tracer, enhancement solution and cGMP standard solutions.

cGMP standards were prepared at the following concentrations: 14.58, 4.86, 1.62, 0.54, 0.18, 0.06, 0.00 pmol/ml assay buffer (detailed instructions provided in kit). Samples were diluted with assay buffer (0.10 M sodium phosphate, 0.15 M NaCl, 1.0 mM EDTA, 0.2% BSA, 0.01% Tween 20, 0.01% thimerosal, pH 7.0) if necessary. Before starting the assay, microtiter strips (Fluoro Nunc^{TM} module) coated with goat-anti-rabbit-gamma globulin were prewashed with a wash solution (0.02% Tween in H_20, 0.5% NaCl). Fifty microliters of standards and samples were pipetted first, followed by 50 µl of cGMP-PL-Eu^{+3} tracer and finally 100µl cGMP-antiserum. After incubation, microtiter strips were washed 4 times with the wash solution at room temperature using an automatic plate washer (Wellwash AC). Two hundred microliters of enhancement solution (15 µM napthoyltrifluoroacetone, 50 µM

trioctylphosphinoxid, 0.1 M acetic acid, 0.1% Triton X-100, potassium hydrogen phthalate) was pipetted and incubated >= 60min at room temperature on a plate shaker in a dust-free chamber (ziplock bag). Time-resolved fluorescence was measured in a Wallac 1420 multilabel counter at 340 nm excitation and 615 emission. Results were evaluated by Workout software (Wallac/Perkin Elmer).

2.2.3.2 cAMP assay

An analogous, but cAMP-specific TRFIA, was used to determine cAMP levels after cell treatments (Table 3). The principle of the assay, the reagents and the procedure are similar to that of cGMP assay described above (Section 2.2.3.1) except for the different tracer and antiserum. cAMP standards were prepared from stock solutions containing 10 nmol/ml assay buffer at the following concentrations: 9.72, 3.24, 1.08, 0.36, 0.12, 0.04, and 0.00 pmol/ml assay buffer (detailed instructions provided in kit). Assays were measured in a Wallac 1420 multi-labelled counter at 340 nm excitation and 615 emission. Results were evaluated by Workout software (Wallac /Perkin Elmer).

2.2.4 Protein extraction by subcellular fractionation

Confluent (MA-10 and αT3-1) cells in T_{75} culture flasks were washed twice with ice-cold PBS and scrapped off into 3 ml PBS containing 2 µM microcystin and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Cells were centrifuged at 500 x g for 5 min at 4°C, and the resulting pellet was resuspended in a suitable amount of freshly prepared homogenizing buffer (50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1µM microcystin and 0.1mM PMSF). Cells were disrupted using a Potter-Elvehjem homogenizer by 20 to 30 strokes. Resulting homogenates were centrifuge at 3,000 x g for 6 min at 4°C to remove cell debris. The supernatants containing cytosol and particulate fractions were re-centrifuged at 100,000 x g for 30 min at 4°C (Beckmann L7 ultracentrifuge, rotor 70.ITI). Resulting pellets containing membrane vesicles were resuspended with a suitable volume of 50 mM Tris-HCl, pH 7.5 to obtain a membrane protein concentration of ≅ 5 µg/µl. Protein concentrations of both membrane and cytosolic fractions were estimated according to Bradford (Section 2.2.6). Fractions were stored at -80°C until use.

2.2.5 Protein extraction with Poppers™ cell lysing reagent

The Poppers™ cell lysing reagent (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%Triton X-100, 2,5 mM sodium pyrophosphate, 1 mM ß-glycerolphosphate, 1 mM Na_3VO_4, 1 µg/ml Leupeptin, and 0.1 mM PMSF, the latter directly added before use) allows an efficient lysis of cultured cells. The method separates crude protein extracts (lysates) from cellular debris.

After treatment with the signalling molecules indicated, cells were rinsed once with ice-cold PBS. Cells were scraped off the plate with rubber scrapers into 300 µl ice-cold Poppers reagent and transferred to Eppendorf tubes. Eppendorf tubes were kept on ice for 30 min with frequent vortexing. The mixture was centrifuged at 10,000 x g for 10 min at 4°C. The supernatant fractions both containing membrane and cytosolic protein fractions, and the pellets, containing the nuclear fraction, were stored at -80°C until use. Protein content of both fractions were determined by Bio-Rad Protein Assay (Section 2.2.6).

2.2.6 Quantitative protein determination according to Bradford

The Bradford method (Bradford, 1976) is based on the observation that the absorbance maximum for an acidic solution of Coomasie Brilliant Blue G-250 shifts from 465 nm to 598 nm when protein binding occurs. A standard curve is generated by assessing known amounts of a standard protein. Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Munich, Germany). Standards were prepared using bovine serum albumin (BSA fraction V, Sigma) at the following concentrations: 4, 8, 12, and 16 µg/µl. After preparing the assay solutions according to the manufacturer's protocol, 350 µl-aliquots of samples and standards were dispensed into 48-well plates (Nunc), and the absorbance was measured at 590 nm by a spectrophotometer (SLT Lab Instruments, Austria) using the software Easy-fit, version 7.02.

2.2.7 Guanylyl cyclase assays

These assays served to determine the hormone-dependent activity of guanylyl cyclases by measuring the generation of cGMP from GTP as substrate. The whole nucleotide generating system consisted of 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 50 mM NaCl, 1 mg/ml BSA, 0.5 mM IBMX, 0.2mM EGTA, 10 mM creatine phosphate, 13.2 IU/ml creatine phosphokinase, and 1 mM GTP. Hormone-

independent (basal) activities were measured in the absence of guanylyl cyclase-activating agents (ANP, CNP, SNP). The specific effects of these agents were determined by co-incubations with 1mM ATP and 5 mM $MgCl_2$. To assess total amounts of particulate guanylyl cyclases (GC-A, GC-B), reactions were performed in the presence of 1% Triton and 5 mM $MnCl_2$. This treatment ligand-independently activates these enzymes to a maximal extent (Potter, 1998).

Assays were performed with 5 µg of protein in total volumes of 75 µl by incubation for 12 min at 37°C in a thermoblock (Eppendorf). After centrifugation at 11,500 x g for 4 min at 4°C, supernatant fractions were collected and either kept frozen at -80°C or immediately used for cGMP measurements (Section 2.2.3.1). GC activity was expressed in nmol cGMP/mg protein/12 min.

2.2.8 SDS – Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE serves to separate proteins based on their size. During heating in the presence of the anionic detergent, SDS, proteins become unfolded and acquire a net negative charge that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode.

Discontinues SDS-PAGE according to Laemmli (1970) used in my studies consisted of separating gels containing 7.5 – 9% acrylamide, 0.375 M Tris-HCl, pH 8.8 in addition to stacking gels of 4% acrylamide, 0.125 M Tris-HCl, pH 6.8. Separating gel solutions were slowly pipetted between glass plates, thereafter overlayed with butanol and allowed to polymerize at room temperature overnight. After removal of butanol, stacking gel solutions were pipetted down on top of the separating gel and the comb was inserted. Gels were allowed to polymerize for 60 min, the comb was removed, and the wells were rinsed with running buffer using a suitable syringe. Prior to loading, samples were boiled at 100°C for 3 min in SDS-PAGE sample buffer consisting of 0.125 M Tris-HCl, pH 6.8, 66.6 mM dithiothreitol, 6.6% (v/v) glycerine, 5% (w/v) SDS, and 0.02% bromphenol blue. Electrophoresis was performed in Hoefer SE 600 units at 140 V for 3 to 4 h in the presence of PAGE buffer (see 2.1.3). Molecular weight standards (Sigma, SDS-6H) were used as size references.

2.2.9 Protein transfer to membranes (Blotting)

The transfer of size-separated proteins from gels to nitrocellulose membranes was done generally in a tank blot apparatus (Hoefer Scientific) using 100 mM Tris-HCl / 193 mM glycine as transfer buffer.

Membranes (Hybond-ECL, Amersham) and filter paper (6 sheets/gel) were cut to the dimension of the gel. The gel, the membrane and the sheets of filter paper (3MM, Whatman) were pre-equilibrated with transfer buffer. The gel/blot sandwiches, each consisting of 3 sheets of Whatman paper, the gel, the membrane, and again 3 sheets of Whatman paper were layed onto a glass plate. A glass pipette was gently rolled on top of the sandwich to remove any bubble that may be trapped underneath the gel. The transfer sandwiches were assembled in the holder cassettes and installed in the blot tank filled with transfer buffer. Electroblotting was performed overnight at 30 V for 12-13 h at 4° C. To control the efficiency of the protein transfer, and to mark the migration of protein size standards, blotted membranes were stained with Ponceau S (Sigma) for 15 min. After rinsing the membranes 4-5 x with H_20, blots were placed in a transparent sheet protector and immediately photocopied. Thereafter, blots were washed with Tris buffered saline (pH 7.6) for 5 min prior to immunodetection. The membranes were either used directly or dried and stored at 4°C prior to immunostaining.

2.2.10 Immunostaining of membranes

To protect against unspecific interactions with the antibody solutions, membranes were first incubated with blocking buffer (see 2.1.3) for 1 h followed by three 5-min washings in TBST. After blocking, primary antibodies appropriately diluted with antibody dilution buffer (see 2.1.3) were added and incubated for 1 h at RT. Thereafter membranes were washed 3 times for 10 min each with 1x TBST and then incubated with peroxidase-linked secondary antibodies for 1 h at RT. After washing as before, antibody binding was visualized using enhanced chemiluminiscence (ECL) detection kits (Amersham) according to the manufacturer's protocol. Blots were wrapped with a plastic foil and gently pressed by rolling a glass pipette over it to remove trapped bubbles. The wrapped blots were placed into a film cassette and exposed to autoradiography films (X-Omat AR, Kodak). Films were exposed for different time periods (1 min to 24 h) at 4°C prior to processing in an automatic film processor (Agfa CP100).

Table 4: Primary antibodies used in immunoblotting

Primary Antibody	Host	Dilution	Manufacturer
Phospho-Akt (Ser473)	Rabbit	1:1000	Biolab
Phospho-p44/p42 MAPK (Thr202/Try204)	Mouse	1:1000	Cell Signalling Technology
Anti-MAP kinase 1/2(Erk1/2-CT)	Rabbit	1:5000	Biomol

Table 5: Secondary antibodies used in immunoblotting

Secondary Antibody	Dilution	Manufacturer
Peroxidase-conjugated goat anti-rabbit IgG	1:2000	Sigma
Peroxidase-conjugated goat anti-mouse IgG	1:10,000	Rockland

2.2.11 Stripping

Stripping serves to remove bound antibodies from membranes, in order to allow membrane reprobing with the same or different antibodies. Membranes were washed shortly with distilled water, and then incubated in 200 ml freshly prepared 5% non-fat milk powder (Roth) for 30 min. After washing with distilled water the membranes were treated with 0.5 M NaCl and 0.5 M acetic acid for 30 min. Membranes were washed shortly with water and then neutralized with 1.5 M Tris-HCl, pH 7.5 for 1 min. followed by thorough washing. All incubations were performed in an orbital shaker (Heidolph Polymax 1040) at room temperature. After stripping, membranes were re-blocked for 1 to 2 hours or longer in blocking buffer (see 2.1.3) followed by extensive washing in TBST.

2.2.12 Affinity crosslinking

Membranes (10 µg of protein) were incubated with either ^{125}I-(Tyr^{O})-CNP (2 nM) or ^{125}I-ANP (0.5 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 consisting of 0.375 M Tris-HCl, pH 6.8, 200 mM dithiothreitol, 20% glycerine, 15% sodium dodecyl sulfate, and 0.06% bromphenol blue. Prior to analysis by SDS-PAGE under reducing conditions according to Laemmli (1970) in 7.5% 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 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.

2.2.13 Isolation of total RNA from cells

Total RNA was isolated from MA-10 or αT3-1 cells using PeqGold RNA (PeqLab, 25-2040) purification kit following the manufacturer's instruction. Briefly, cells were cultured as described (Section 2.2.1), washed twice with PBS (PAA), scrapped off with a rubber scrapper and centrifuged at 300 x g for 5 min. Pellets were homogenized in 1 ml of PeqGold solution. RNA was then extracted by adding 1/10 volume of chloroform. Tubes were vortexed for 15 sec and incubated for 5 min at 4°C followed by centrifugation at 12,000 rpm (Eppendorf 54151) for 20 min at 4°C. The upper aqueous phase, containing the RNA, was aspirated, mixed with an equal volume of isopropyl alcohol (Merck) then centrifuged at 12,000 rpm for 15 min at 4°C. Resulting pellets were washed twice with 80% ethanol (Merck). The RNA pellets were dried shortly at 68°C, dissolved in TE (10 mM Tris-HCl, pH 7.4; 1 mM EDTA), and RNA concentrations were determined by measuring the spectrophotometric absorbance at 260 nm (Ultrospec 3000, Pharmacia Biotech). To estimate RNA integrity, aliquots were electrophoretically separated in 1.5% agarose gels and visualized by staining with ethidium bromide.

2.2.14 Reverse transcription and PCR analysis of LPA-receptors

The first-strand cDNA was synthesized from 3 µg total RNA using Superscript II reverse transcriptase (Invitrogen), 0.5 mM of each deoxyribonucleoside triphosphate

and 500 ng oligo(dt)$_{12-18}$ (Roche) as primer. After completion of the reaction, 80 µl of nuclease-free water was added to yield a final volume of 100 µl. Samples were kept at -80°C or immediately used for PCR analysis.

For PCR analyses five microliters of cDNA were used as templates for PCR amplification of either LPA-receptor transcripts or of mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene in the above mentioned cell lines. In total volumes of 50 µl, the PCR reaction mixtures contained 0.5 units Bio Therm Taq-polymerase, 5 µl 10x BioTherm buffer, 1.5% Ficoll 400, 100 pmol of each primer and 0.2 mM dNTP (Peqlab). For GAPDH, only 1 µl cDNA and 50 pmol of each primer were used in the PCR reaction. Primers were designed on the basis of known sequences obtained from the NCBI data bank and were commercially synthesized (MWG Biotech). The following primers were employed.

Table 6: Primers used for amplification of LP receptor subtypes by PCR analysis

	Primer sequence
LPA_1 receptor, sense	5’ ATC TTT GGC TAT GTT CGC CA 3’
LPA_1 receptor, antisense	5’ TTG CTG TGA ACT CCA GCC A 3’
LPA_2 receptor, sense	5’ TGG CCT ACC TCT TCC TCA TGT TCC A 3’
LPA_2 receptor, antisense	5’ GGG TCC AGC ACA CCA CCA AT 3’
LPA_3 receptor, sense	5’ AGT GTC ACT ATG ACA AGC 3’
LPA_3 receptor, antisense	5’ GAG ATG TTG CAG AGG C 3’
GAPDH- specific primer, sense	5’ GTC TTC ACC ACC ATG GAG 3’
GAPDH- specific primer, antisense	5’ GTC ATG GAT GAC CTT GGC 3’

PCR conditions were as follows: after a 2-minute denaturation step at 95°C, samples were subjected to 30 cycles at 95°C for 1 min, 48°C (LPA_3), 58°C (LPA_2), or 53°C for 1 min (LPA_1) and (GAPDH), and 72°C for 1min, followed by an additional elongation step at 72°C for 10 min. As negative controls, cDNA was replaced by water in the reaction mixture. The PCR products were separated in 1.5% agarose gels in 1x TE buffer containing ethidium bromide (0.5 µg/ml). The fragment lengths were determined by co-analyses with a 100- bp size ladders (PeqGold Leiter Mix).

Reaction products were visualized under UV light by means of the Compact Imaging System (Imago).

2.2.15 Immunohistochemistry and confocal microscopy

MA-10 cells (12,000/chamber) were grown on 4-well thin #1 borosilicate cover slides (LAB-TEK, NUNC) for confocal analysis. After cell treatment with either 10 μM LPA or 0.1 μM ANP, cells were washed twice with phosphate buffered saline and fixed with 3% paraformaldehyde for 30 min followed by thorough washing with PBS. Cells were permeabilized with 0.5% Triton X-100 for 5 min, then blocked with 5% rabbit non-immune serum (Sigma). The cells were then incubated with primary antibody (anti-smooth muscle α-actin, Sigma diluted 1:400) for 60 min, followed by a 60-min incubation with Cy3-conjugated secondary antibody (anti-rabbit, Jackson Immunochemicals diluted 1:200) in a dark chamber. To counterstain the cell nuclei, 4'6'-diamidino-2-phenylindole (DAPI, Sigma diluted 1:200) was applied after the second antibody for 10 min, then washed three times with PBS before viewing with a Nikon laser scanning confocal microscope. Nikon filter blocks (330-380 nm) was used for DAPI imaging and FITC filter (450-490 nm) for Cy3-immunoflourescence. Micrographs were taken by a Leica digital camera.

2.2.16 Data presentation and statistical analysis

The data were graphed and analyzed with Prism 3.02 (Graph Pad Software, Inc. San Diego, Ca). The figures show results as mean ± SD of individual experiments done in triplicates which were representative of at least two additional experiments performed. The significance of effects was assessed by Student's *t* test.

Significant effects ranging from P value of <0.05 to <0.001, are indicated by asterisks. Standard deviations are indicated by vertical bars. In some cases, the bars are not visible due to small SD values.

3 Results

3.1 Characterization of natriuretic peptide receptor expression in MA-10 and αT3-1 cell lines

Initial studies served to identify appropriate model systems for studying the regulation of natriuretic peptide receptor desensitization *in vivo.* Based on different criteria (see introduction), the MA-10 and αT3-1 cell lines were thought to represent particularly useful experimental models for this kind of investigation.

To further characterize these cell lines in terms of natriuretic peptide receptor expression, membranes prepared from either MA-10 or αT3-1 cells were incubated with radiolabelled ANP (the ligand for GC-A) or CNP (the GC-B ligand), respectively, and receptor expression was analyzed by UV light-induced crosslinking followed by SDS-PAGE and autoradiography. This method has been proved to be a reliable approach for the examination of such receptors in cells and tissues (Müller *et al*., 2000, 2002, 2004; Middendorff *et al*., 2002; Willipinski-Stapelfeldt *et al*., 2004).

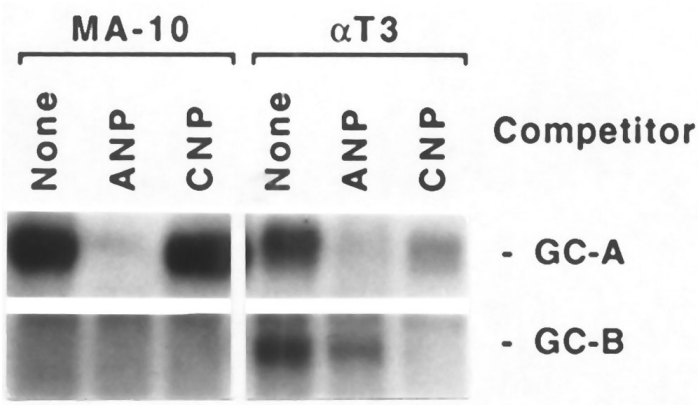

Figure 10: Analysis of GC-A and GC-B expression in MA-10 and αT3-1 cells
Membrane proteins were isolated from MA-10 or αT3-1 cells and incubated with either ^{125}I-ANP or ^{125}I-(Tyr^{0})CNP in the absence (None) or presence (1 µM) of unlabelled ANP or CNP (Competitor). Crosslinking was induced by UV irradiation, and reaction products were analyzed by SDS-PAGE and autoradiography. Bands at approximately 125 kDa, representing ^{125}I-ANP-labelled (GC-A) or ^{125}I-Tyr^{0}-CNP-labelled (GC-B) receptors are indicated.

These experiments revealed (Figure 10) that MA-10 cells selectively express GC-A, whereas both receptor types (GC-A and GC-B) are present in membranes prepared from αT3-1 cells.

In addition, these studies also revealed (not shown) the absence in both cell lines of detectable amounts of the third natriuretic peptide receptor, NPR-C, which is labelled by both radioligands (Müller *et al*., 2004; Willipinski-Stapelfeldt *et al*., 2004) and has an apparent molecular mass of 60 kDa.

The results obtained were consistent with previous findings that MA-10 cells express GC-A (Pandey *et al*., 1986,1988) and reports indicating the presence of both GC-A and GC-B in αT3-1 cells by RT-PCR analyses (McArdle *et al*., 1994). The expression of NPR-C in these cell lines has not been examined before.

3.2 Characterization of the functional activities of natriuretic peptide receptors in MA-10 and αT3-1 cells

To further prove the expression of natriuretic peptide receptors in the two cell lines, experiments in order to assess their functional activities (ligand-dependent stimulation of cGMP production) were carried out.

3.2.1 MA-10 cells

MA-10 cells were incubated in either the absence or presence (0.1 µM) of natriuretic peptides for 30 min, and the amounts of cGMP produced were determined by TRFIA (see 2.2.3.1).

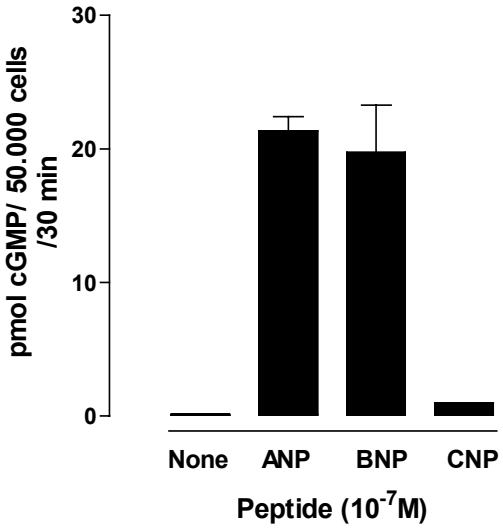

Figure 11: Production of cGMP in MA-10 cells in response to exposure to ANP, BNP and CNP

Serum-starved MA-10 cells were incubated in the absence (None) or presence of the natriuretic peptides indicated followed by measurement of cellular cGMP (see section 2.2.3.1). ANP and BNP elicited 430-fold and 395-fold increases as compared to control. Data shown represents means ± SD of three experiments .

The results (Figure 11) show that ANP and BNP (the ligands for GC-A) are highly effective in stimulating cGMP production, whereas CNP only slightly induces this reaction. Thus, these results confirmed that GC-A is the only (or at least

predominant) natriuretic peptide receptor in this cell line and that it is functionally-active.

Analogous experiments were performed with freshly-isolated rat Leydig cells. These studies (Figure 12) showed up to 300-fold increases in cGMP values in response to ANP but not to CNP.

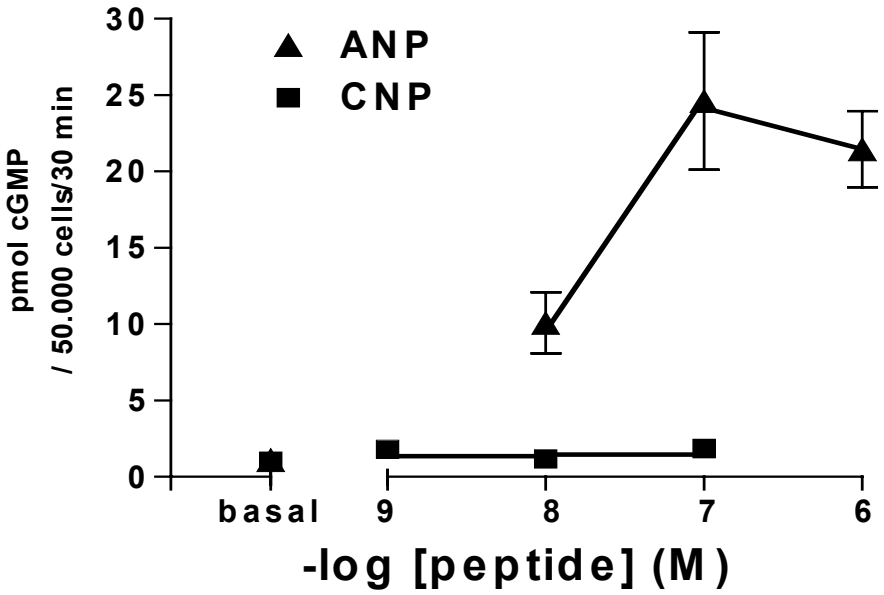

Figure 12: Effects of ANP and CNP on cGMP production in isolated rat Leydig cells
Rat Leydig cells were incubated with the indicated concentrations of ANP or CNP for 30 min followed by measurement of cellular cGMP (2.2.3.1). Data shown represents means ± SD of three experiments.

These results showed close similarity between rat Leydig cells and MA-10 cells with regard to the ANP-sensitivity of GC-A and supported the intention of using MA-10 cells instead of isolated rat Leydig cells in the course of this project. Incubations with CNP, a specific activator of GC-B, produced only slight amounts of cGMP possibly due to a cross activation of GC-A in MA-10 and Leydig cells. Based on the results obtained, ANP was chosen as the ligand for stimulating GC-A in this study. The data was consistent with a report by Goeddel (1991) who showed previously that ANP is more effective in stimulating GC-A than BNP.

3.2.2 αT3-1 cells

After establishing the expression of GC-A and GC-B in αT3-1 cells (see section 3.1), further studies served to assess the functional activities of these receptors.

Cells were incubated with varying concentrations of ANP or CNP, ranging from 10^{-10} M to 10^{-6} M, and cGMP accumulations after 30 min were determined.

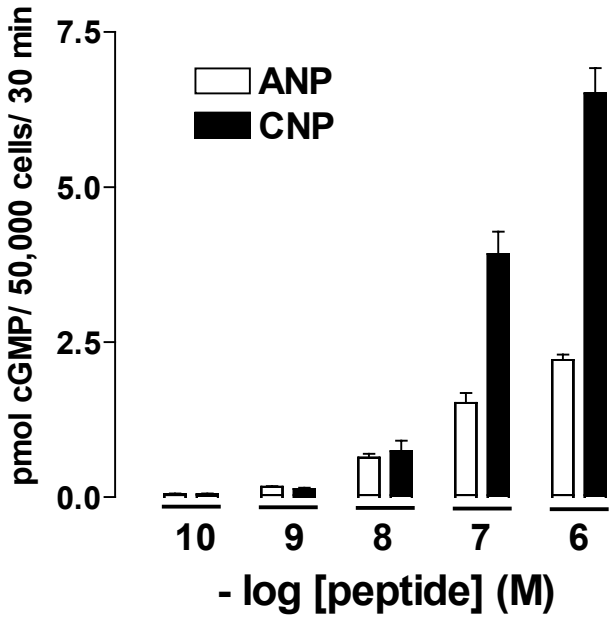

Figure 13: Dose- dependent ANP- and CNP-induced cGMP production in αT3-1 cells

αT3-1 cells were incubated with the indicated concentrations of ANP or CNP prior to the measurement of cellular cGMP (see section 2.2.3.1). Values are means ± SD of triplicate assays with duplicate determinations of cGMP each.

The results (Figure 13) demonstrate that ANP evoked dose-dependent increases in cGMP levels from 10^{-9} M (4-fold) up to 10^{-6} M (55-fold). CNP also elicited concentration-dependent increases in cGMP ranging from 10^{-9} M (3-fold) up to 10^{-6} M (130-fold) increase peptide concentrations. Thus, both peptides were found to stimulate cGMP production in αT3-1 cells, proving the expression and functional activities of both GC-A and GC-B. These results also demonstrated that CNP was three times more effective than ANP suggesting higher expression levels and/or activity of GC-B as compared to those/that of GC-A in αT3-1 cells. Peptide concentrations of 10^{-7} M (0.1 µM) were selected for the succeeding experiments because these concentrations are close to physiological concentrations.

3.3 Homologous desensitization of GC-A in MA-10 cells

Based on the above findings, MA-10 cells should represent a useful model to study GC-A desensitization phenomena, since (a) these cells express GC-A endogenously (i. e., not artificially due to receptor transfection), (b) there is no interference by other natriuretic peptide receptors (GC-B, NPR-C), and (c) the cells are similar to native Leydig cells where physiological effects of ANP and GC-A have been described (Khurana and Pandey, 1995; Kumar and Pandey, 1997).

Thus, one goal of my study was to examine whether prolonged exposure of ANP to MA-10 cells could induce GC-A desensitization, representing a question of potential physiological significance. In case of positive results, further experiments should try to identify molecular signalling events involved.

3.3.1 Preliminary examinations

Since binding of ANP to GC-A initially elicits the generation of cGMP, it is likely that cGMP is involved in intracellular signalling events leading to GC-A desensitization. cGMP-dependent protein kinase I (GK I) is the major intracellular mediator of cGMP signals in many cell types (Francis and Corbin, 1999; Kotera *et al*., 2003). In this context, findings that human Leydig cells do not express GK I (Middendorff *et al*., 2002) are of potential interest. To examine whether GK I absence is a general characteristic of Leydig cells, GK I-specific immunoblot analyses of rat Leydig cells and MA-10 cells were performed.

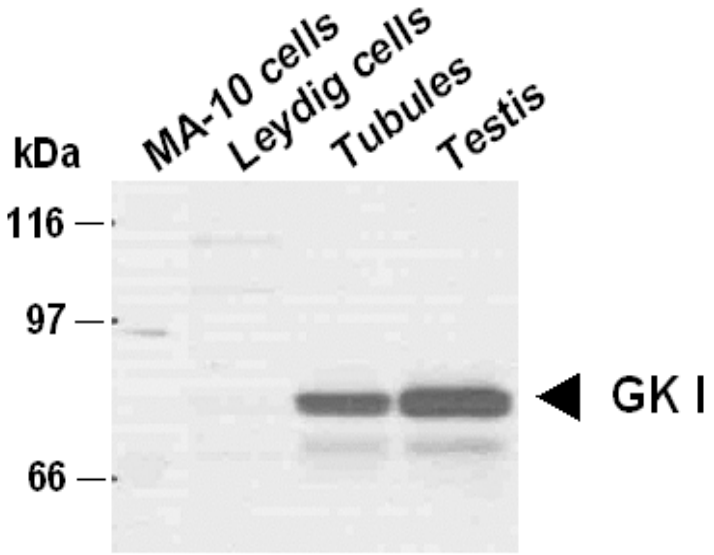

Figure 14: Immunoblot analysis of GK I expression
Soluble fractions (30 µg of protein each) of homogenates prepared from MA-10 cells, rat Leydig cells, rat seminiferous tubules and testes were fractionated by SDS-PAGE and analyzed by immunoblotting using antibodies against GK I. Immunoreactivity associated with a protein of 74 kDa (arrow), is detectable only in reference tissues but not in MA-10 or Leydig cells. The positions of molecular weight markers (in kDa) are indicated.

Figure 14 shows that expression of GK I is undetectable both in MA-10 and Leydig cells, whereas the enzyme is well detectable in two positive control tissues. Based on these results, GK I apparently can not be involved in cGMP signalling in MA-10 cells.

To investigate the time-dependency of ANP-induced cGMP production in MA-10 cells, incubations with 0.1 µM ANP were carried out for different time periods prior to measurements of cGMP.

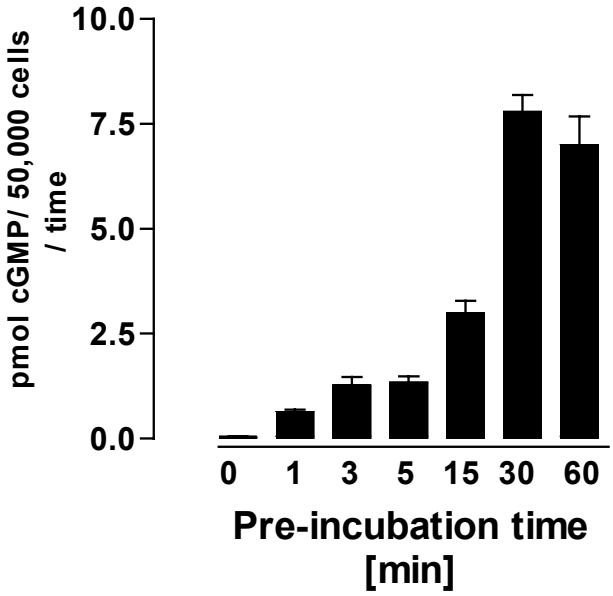

Figure 15: Incubation of MA-10 cells with ANP induces time-dependent cGMP production.
Serum-starved MA-10 cells were incubated with 0.1 µM ANP for the time periods indicated prior to measurement of cellular cGMP accumulation (described in 2.2.3.1). Data represents means ± SD of triplicate assays with duplicate determinations of cGMP each.

As shown in Figure 15, ANP-induced increases in cGMP values were fast and detectable after 1 min incubation (12-fold increase). Highest values were obtained after 30 min (156-fold increase), and more prolonged incubation times did not further enhance the concentration of cGMP.

Thus, in the following experiments, ANP incubation times of 30 min were used to generate maximum cGMP accumulations.

3.3.2 Pre-treatment of MA-10 cells with ANP seems to lead to GC-A desensitization

To examine whether GC-A in MA-10 cells is desensitized in response to prolonged exposure to ANP, cells were pre-exposed to ANP for different time periods, then washed and re-incubated with ANP for 30 min to assess cGMP production.

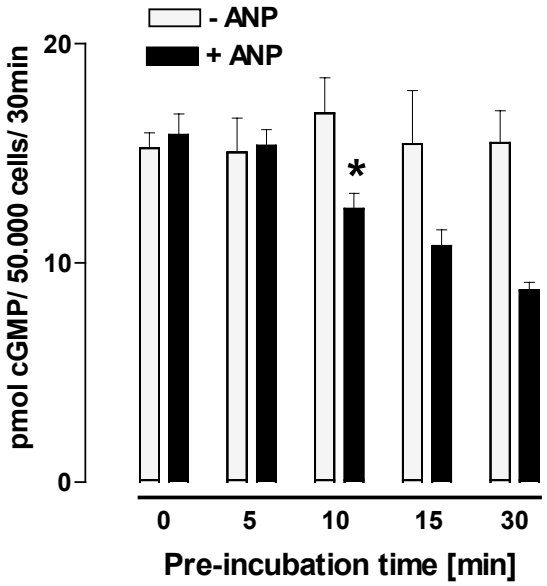

Figure 16: Pretreatment of MA-10 cells with ANP causes time-dependent reductions in subsequent ANP-dependent cGMP production

Serum-starved MA-10 cells were pretreated in the presence (0.1 µM) or absence of ANP for the time periods indicated. The medium was aspirated, cells were washed once with PBS and then re-incubated for 30 min with 0.1 µM ANP. Cellular cGMP was measured by TRFIA (see section 2.2.3.1) . Results are means ± SD of triplicate assays with duplicate determinations of cGMP each.

The results (Figure 16) demonstrate that pre-exposure of MA-10 cells to ANP triggers in a time-dependent manner reductions in subsequent ANP-dependent responses. A significant reduction by 26% ($P < 0.01$) was observed already after a 10-min pre-incubation time. This reduction increased to 43.5% during 30 min. No further attenuation in cGMP levels was observed after a 60-min pre-exposure (results not shown). cGMP values of non-treated cells (-ANP) did not show any alterations at all time points tested. These results indicate that ANP-dependent cGMP elevations were reduced after pre-exposure of cells to ANP, a phenomenon designated as homologous desensitization (Potter, 1998).

3.3.3 ANP-dependent desensitization of GC-A is based on a decrease in hormone-dependent guanylyl cyclase activity

In principle, the above (3.3.2) described findings could be explained by different molecular mechanisms, only one of which is based on a decrease in hormone-responsiveness of GC-A. Other conceivable mechanisms include receptor degradation, receptor internalization or increases in intracellular cGMP degradation. To address these questions appropriately, MA-10 cells were incubated for 30 min in either the absence or presence of ANP, then homogenized, and proteins were separated into particulate (membrane) and soluble (cytosolic) fractions. Membrane fractions were then assayed for basal and ANP-dependent guanylyl cyclase activities.

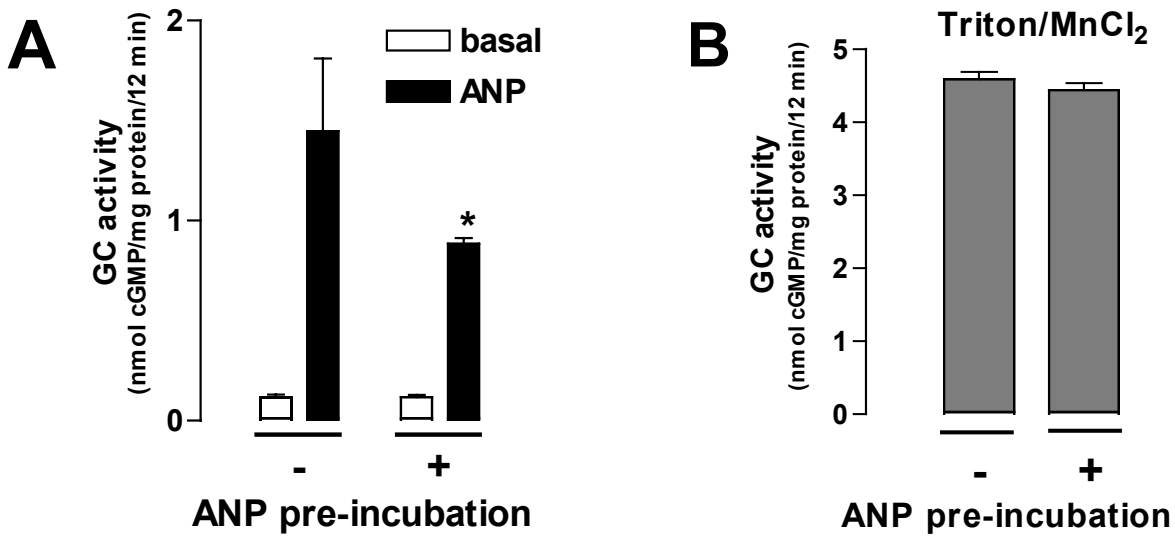

Figure 17: Comparative guanylyl cyclase assays with membranes prepared from MA-10 cells after pre-incubations of cells in the absence or presence of ANP
Serum-starved MA-10 cells in T_{75} culture flasks were incubated in either the absence or presence (0.1 µM) of ANP for 30 min at 37°C. Cells were then homogenized in the presence of phosphatase inhibitors, and the homogenates were separated into membrane and cytosolic protein fractions. The membrane fractions were assayed for basal and ANP-dependent (A) or for detergent-stimulated (B) guanylyl cyclase activities.

As demonstrated in Figure 17A, membranes prepared from ANP-pretreated cells were less responsive to ANP stimulation in this assay as membranes derived from control cells. This decrease (by 39% as compared to control) was very similar to that observed in experiments with intact cells, while basal values of membrane GC activity were unaffected. Thus, these findings ruled out an involvement of cytosolic cGMP-degrading enzymes and strongly suggested that the effect was fully accounted for by a decrease in ANP-dependent GC-A activity.

To further prove that the ligand-dependent activity (and not the molecular concentration) of GC-A was reduced in response to ANP pretreatment, membranes prepared as before were assayed for GC activity in the presence Triton/$MnCl_2$. Under these conditions, membrane guanylyl cyclases are most efficiently and ligand-independently activated and hence indicate the total amount of these enzymes present in a given preparation (Potter, 1998). As shown in Figure 17B, ANP pretreatment did not reduce significantly the capability of generating cGMP, indicating that the hormone-responsiveness but not the membrane concentration of GC-A was affected.

Thus, prolonged exposure of MA-10 cells to ANP elicits desensitization of GC-A by mechanisms directly reducing its ligand-dependent activity.

3.3.4 The PKA inhibitor, H89, blocks homologous desensitization of GC-A

Since homologous desensitization of GC-A is based on an alteration of its intrinsic guanylyl cyclase activity, it is expected that intracellular signalling mechanisms are involved. Previous reports have indicated (Potter and Garbers, 1992; Potter and Hunter, 1998) that dephosphorylation at certain sites, localized in the intracellular kinase-homology domain of GC-A and GC-B, is responsible for receptor desensitization phenomena.

Since cGMP is the first signalling molecule generated during ANP-dependent ("homologous") desensitization processes, a crucial involvement of cGMP target proteins is likely. Considering that cGMP-dependent protein kinase I (GK I) is apparently not present in MA-10 cells (Figure 14) and based on findings (Schumacher *et al*., 1992) that cAMP-dependent protein kinase (PKA) acts as a cGMP target protein in Leydig cells, I examined whether inhibition of PKA could affect the ANP-dependent desensitization of GC-A.

Thus, cells were pretreated with ANP in either the absence or presence (1 µM) of the the PKA inhibitor, H89, prior to washing and following assessments of ANP-induced cGMP production.

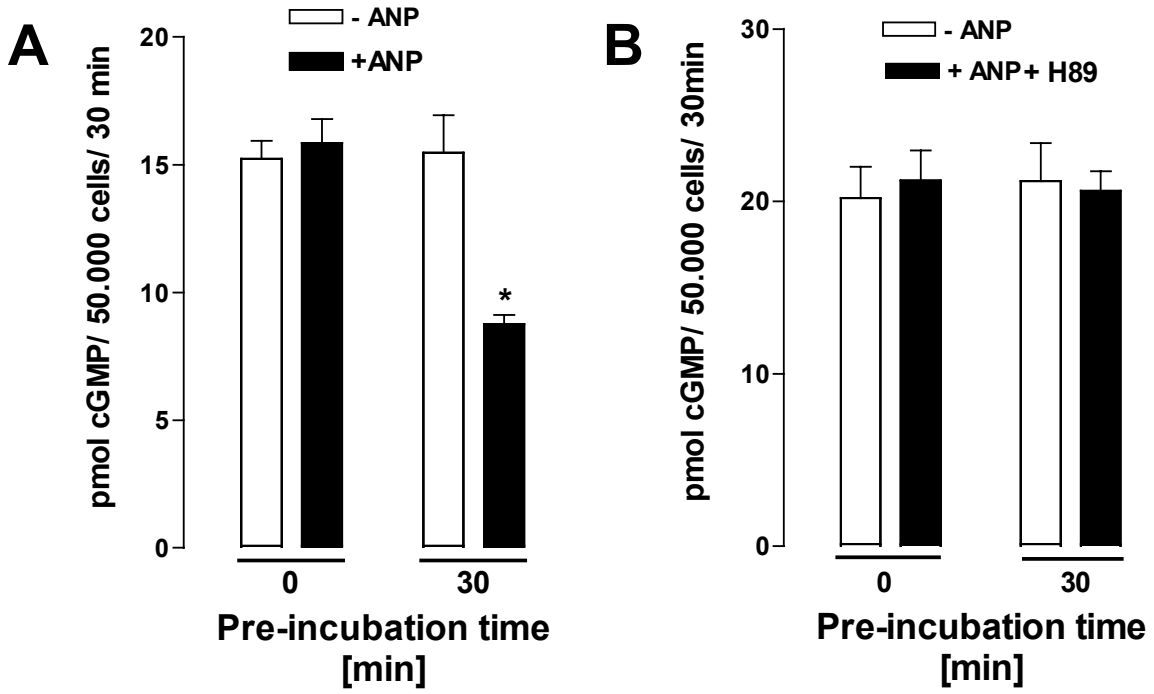

Figure 18: Co-incubation with the PKA inhibitor, H89, abrogates ANP-induced desensitization of GC-A

Serum-starved MA-10 cells were pretreated with either ANP (0.1 µM) alone (A) or with ANP plus 1 µM H89 (B). Incubations in the absence of ANP (-ANP) served as controls. After washing, subsequent ANP-induced cGMP accumulation was measured by TRFIA (section 2.2.3.1). Data represents means ± SD of triplicate assays with duplicate determinations of cGMP each.

As shown (Figure 18), ANP pretreatment of MA-10 cells for 30 min elicited GC-A desensitization (A), whereas the same reaction was completely blocked when ANP was co-incubated with H89 (B). As proved by control experiments, any specific effects of 1 µM H89 remained undetectable when cells were pretreated without ANP or directly exposed to the peptide (not shown). Thus, this study demonstrates that PKA mediates the ANP-induced desensitization of GC-A in MA-10 cells. This is a remarkable finding, showing for the first time an implication of this kinase during cellular signalling events leading to homologous desensitization of natriuretic peptide receptors.

3.4 LPA-induced ("heterologous") desensitization of GC-A in MA-10 cells

After establishing homologous desensitization of GC-A in MA-10 cells, it was of particular interest to look for heterologous desensitization phenomena in this cell line and to characterize comparatively the kinetics and intracellular signalling mechanisms (e.g., PKA involvement ?) implicated.

3.4.1 Initial studies with isolated Leydig cells

To look for agents which could induce a heterologous desensitization, I initially examined whether pituitary adenylate cyclase activating peptide (PACAP), recently shown to desensitize GC-B in cultured penis corpus cavernosum smooth muscles (Guidone *et al*., 2002), is also capable of inducing GC-A desensitization in cultured Leydig cells. Incubations, performed with both the 38- and 27-amino acid forms (PACAP-38, PACAP-27) of the peptide, revealed dose-dependent increases in cAMP production by Leydig cells (Figure 19A), indicating the presence and activity of specific PACAP receptors. However, pre-incubations of Leydig cells with these peptides for different time periods failed to affect following ANP-induced cGMP accumulations (Figure 19B) suggesting that PACAP can desensitize only GC-B and/or that signalling pathways present in penis corpus cavernosum smooth muscle cells are not active in Leydig cells. Analogous assays were also performed with hCG, a well established stimulator of cAMP production and steroidogenesis in Leydig and MA-10 cells (Ascoli, 1981; Saez, 1994). Like PACAP, hCG (50 ng/ml), pre-exposed for up to 60 min to Leydig cells, failed to interfere with ANP-dependent accumulations of cGMP (data not shown).

Therefore, I looked for other signalling molecules which could induce heterologous desensitization of GC-A in MA-10/Leydig cells.

For this investigation, an attractive agent with the capability of inducing heterologous desensitization was the bioactive lipid LPA. Recent publications had shown that bioactive lipids can elicit GC-B desensitization in fibroblasts and vascular smooth muscle cell lines (Abbey and Potter, 2003; Chrisman *et al*., 2003; Potthast *et al*., 2004; Abbey-Hosch *et al*., 2004). Thus, questions of major physiological significance that could be addressed were (a) whether MA-10 cells are targets for LPA effects and (b) whether cross-talks between LPA and natriuretic peptide signalling are GC-B specific or also existent in case of GC-A.

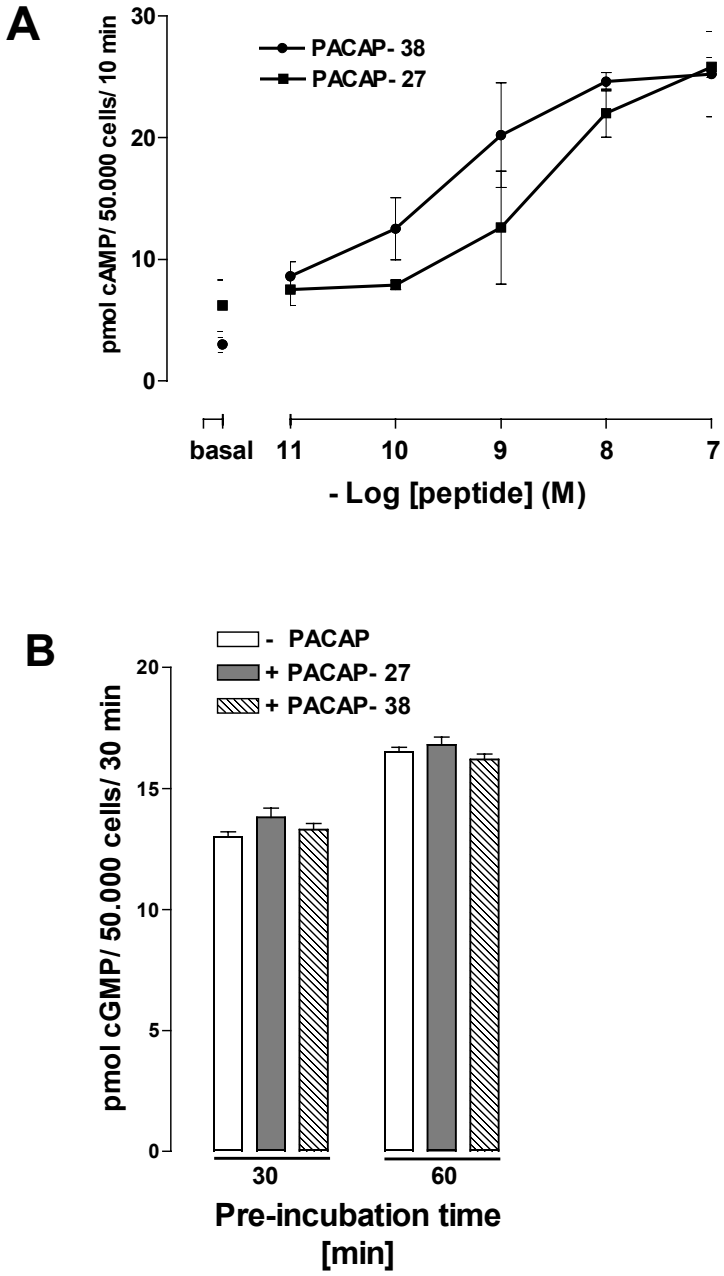

Figure 19: Characterization of the effects of PACAP on cAMP levels (A) and on ANP-dependent GC-A activity (B) in rat Leydig cells

A: Aliquots ($5x10^4$ cells per assay) of rat Leydig cells were incubated for 10 min in the absence (basal) or presence of different concentrations of either PACAP-38 or -27. Accumulations of cAMP were determined by using a cAMP-specific TRFIA (see 2.2.3.2).

B: Leydig cells were pre-incubated for 30 or 60 min in the absence or presence (0.1 µM) of either PACAP-38 or -27. After washing, cells were re-incubated with ANP (0.1 µM) for 30 min. Amounts of cGMP were measured by cGMP-specific TRFIAs (see 2.2.3.1). Results shown represent means ± SD of two experiments each.

3.4.2 LPA exposure to MA-10 cells inhibits ANP-induced cGMP elevations in a dose- and time- dependent manner

To investigate LPA effects on GC-A activity, MA-10 cells were pre-incubated with LPA before treatments with ANP. LPA pre-incubations for 30 min prior to assessment of ANP-induced cGMP generation revealed inhibitory effects of LPA which are exerted in a dose-dependent manner (Figure 20A). Nearly maximal effects were obtained at 10 µM LPA, and I selected this LPA concentration, like in previous studies with GC-B (Abbey and Potter, 2003; Potthast *et al*., 2004), for the following experiments. Pre-incubations with 10 µM LPA for different time periods demonstrated that LPA effects are rapidly (after 5 min) evident and further increase up to pre-exposure times of 30 to 60 min (Figure 20B). More prolonged pretreatments were found to be less effective (not shown), in agreement with observations in case of LPA-induced desensitization in NIH3T3 cells overexpressing GC-B (Abbey and Potter, 2003)

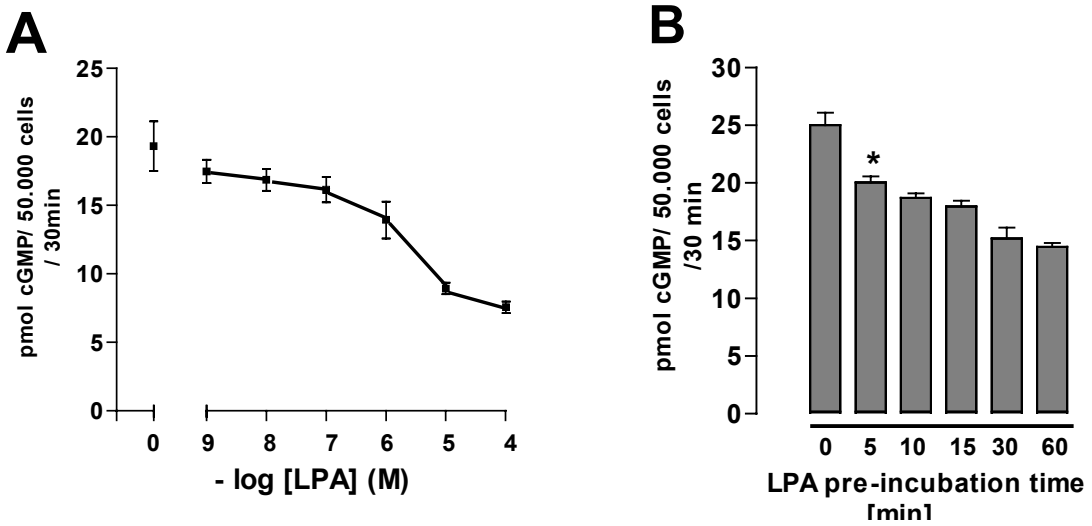

Figure 20: LPA inhibits ANP-induced cGMP elevations in a dose- (A) and time-dependent manner (B)

Serum- starved MA-10 cells were pretreated with increasing concentrations of LPA (as indicated) for 30 min (A). Other sets of MA-10 cells were pretreated with 10 µM LPA for different time periods (B). Media were aspirated, cells were washed once with PBS and subsequently stimulated with 0.1 µM ANP. Cellular cGMP accumulations were determined by TRFIA (section 2.2.3.1) Results represent means ± SD of five (A) or two (B) experiments.

When cells were pre-incubated for 30 min in either the absence or presence of LPA and then exposed to different levels of ANP, the desensitizing effect of LPA was detectable at all ANP concentrations (10^{-6} to 10^{-10} M) examined (Figure 21).

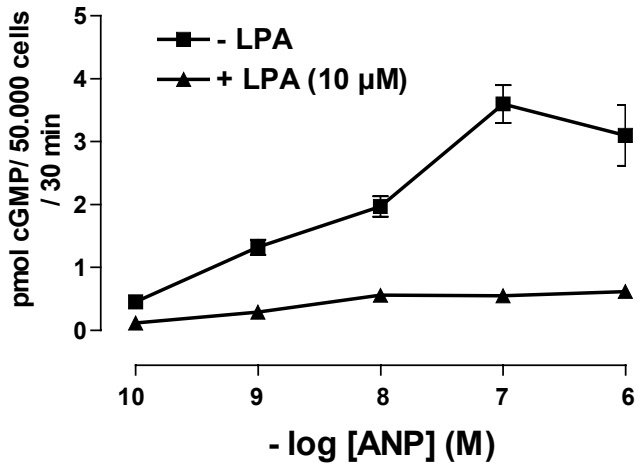

Figure 21: Characterization of the LPA effect at different ANP concentrations: Serum-starved MA-10 cells were pre-incubated in the absence or presence (10 µM) of LPA for 30 min. Media were aspirated, cells were washed once with PBS and subsequently stimulated with increasing concentrations of ANP (as indicated). Reactions were terminated, and cellular cGMP was measured (see section 2.2.3.1). Results are means ± SD of triplicate assays with duplicate determinations of cGMP each.

Thus, these findings strongly suggested that LPA exposure to MA-10 cells is capable of eliciting desensitization of GC-A.

3.4.3 LPA-induced desensitization of GC-A is based on a decrease in hormone-dependent guanylyl cyclase activity

To prove that the LPA effect is indeed membrane-localized, I assayed comparatively guanylyl cyclase activity in membranes prepared from cells that have been or have not been pre-exposed to LPA. These experiments (Figure 22) showed LPA-dependent reductions (by nearly 40%) in ANP-stimulated GC activity, while basal (i. e., unstimulated) activities appeared unaffected (Figure 22A). As revealed by further assays carried out in the presence of Triton/$MnCl_2$ (see 2.2.7), the LPA-elicited decline in ANP-dependent GC activity was not accounted for by a decrease in total receptor amount (Figure 22B). Thus, these findings demonstrate that cellular LPA exposure reduced receptor hormone sensitivity and show for the first time cross-talks between LPA and GC-A signalling, indicating that biologically-active lipids can interact with both types of the cGMP-generating natriuretic peptide receptors.

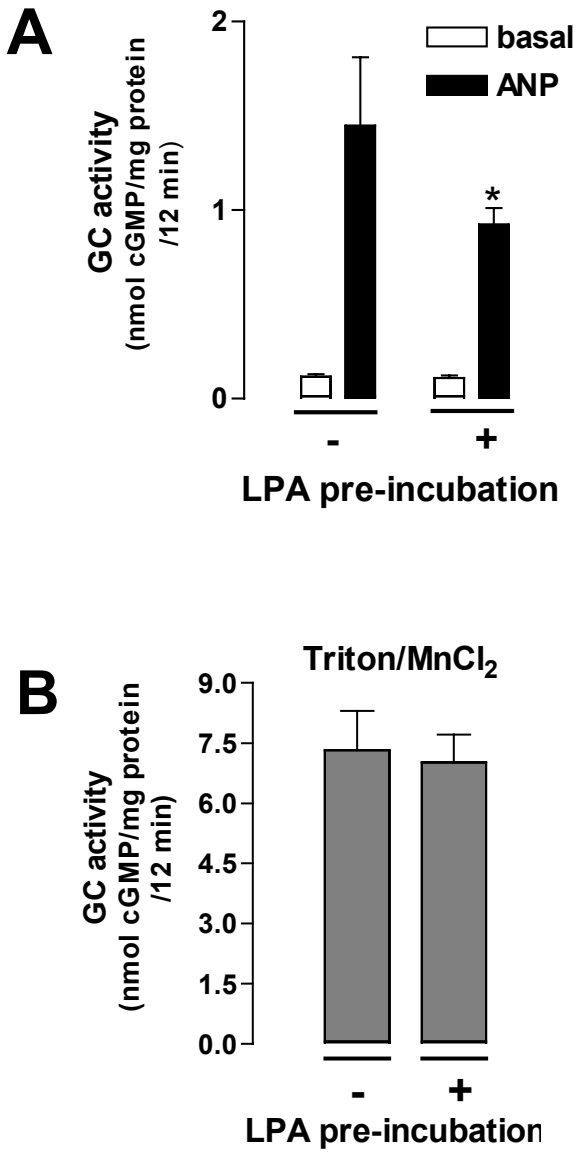

Figure 22: Comparative guanylyl cyclase assays with membranes prepared from MA-10 cells after pre-incubations of cells in the absence or presence of LPA
Serum starved MA-10 cells in T_{75} culture flasks were incubated in either absence or presence (10 µM) of LPA for 30 min at 37°C. Cells were then homogenized in the presence of phosphatase inhibitors, and the homogenates were separated into membrane and cytosolic protein fractions. The membrane fractions were assayed for basal and ANP-dependent (A) or for detergent-stimulated (B) guanylyl cyclase activities.

3.4.4 The LPA-induced desensitization of GC-A is not mediated by PKA

Previous results of this study have shown the involvement of PKA in homologous desensitization of GC-A in MA-10 cells. In the following experiments, the question of whether PKA is also involved in mediating heterologous desensitization of GC-A was investigated.

MA-10 cells were pre-exposed to LPA in either the absence of presence of the PKA inhibitor, H89, prior to determinations of ANP-induced cGMP production. As shown (Figure 23), LPA pretreatments elicited massive reductions (by ~ 40%) in GC-A activity. However, and in contrast to assessments of homologous desensitization (see Figure 16), H89 completely failed to inhibit this reaction. These experiments also further proved that H89 per se (LPA-, H89+) does not affect GC-A activity. Thus, these results convincingly demonstrated that PKA is not involved in mediating the heterologous desensitization of GC-A in MA-10 cells and corroborated the specificity of the effect of H89 during inhibition of homologous desensitization.

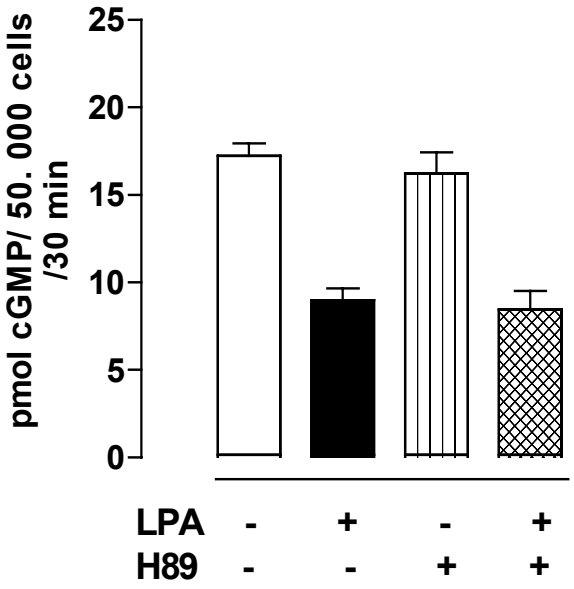

Figure 23: The PKA inhibitor H89 does not block LPA-induced desensitization of GC-A in MA-10 cells

MA-10 cells were pre-incubated for 30 min in the absence or presence of LPA (10 µM) and/or H89 (1 µM) as indicated. Media were aspirated, washed once with PBS, and cells were re-incubated with 0.1 µM ANP prior to measurement of cellular cGMP (section 2.2.3.1). Results are means ± SD of triplicate assays with duplicate determinations of cGMP each.

3.4.5 Experimental variability in experiments with LPA

During the course of these investigations, I observed a considerable variability when experiments with LPA were not performed in parallel but were repeated some weeks later. While the kind of effects were always reproducible, the degree of effects sometimes differed significantly.

Since LPA represents a relatively unstable agent, one possible explanation was that LPA looses activity during prolonged storage. Another potential explanation was that MA-10 cells alter their LPA-responsiveness during the course of repeated passaging. To investigate this issue, comparative experiments with either different batches of LPA or with different batches of cells were performed.

To address a potential role of LPA, three different solutions of the agent (freshly-dissolved in methanol; dissolved in methanol but stored for 3 months at –20°C; freshly-dissolved in aqueous solution) were used in desensitization experiments performed with aliquots of the same MA-10 cell batch. This study (Figure 24A) revealed very similar effects elicited by all three preparations at all LPA concentrations tested. Thus, LPA instability apparently was not responsible for the observed assay variability.

To examine a possible role of the MA-10 cell populations, comparative assays were performed with cells after either 36 (P36) or 70 passages (P70). These experiments revealed two significant alterations. First, ANP-dependent cGMP production by MA-10 cells decreases during progressive passaging (Figure 24B), indicating decreases in the amount and/or activity of GC-A. Secondly, LPA-induced desensitization of GC-A was lower in P70 as compared to P36 cell batches (Figure 24C). The latter effect could be explained by either reductions in the amount and/or activity of LPA receptors or by alterations within intracellular signalling pathways.

Thus, these experiments demonstrated that the observed assay variability was due to alterations at the cellular level and not accounted for by LPA instability. It has to be emphasized, however, that the effects per se were reproducible in any case.

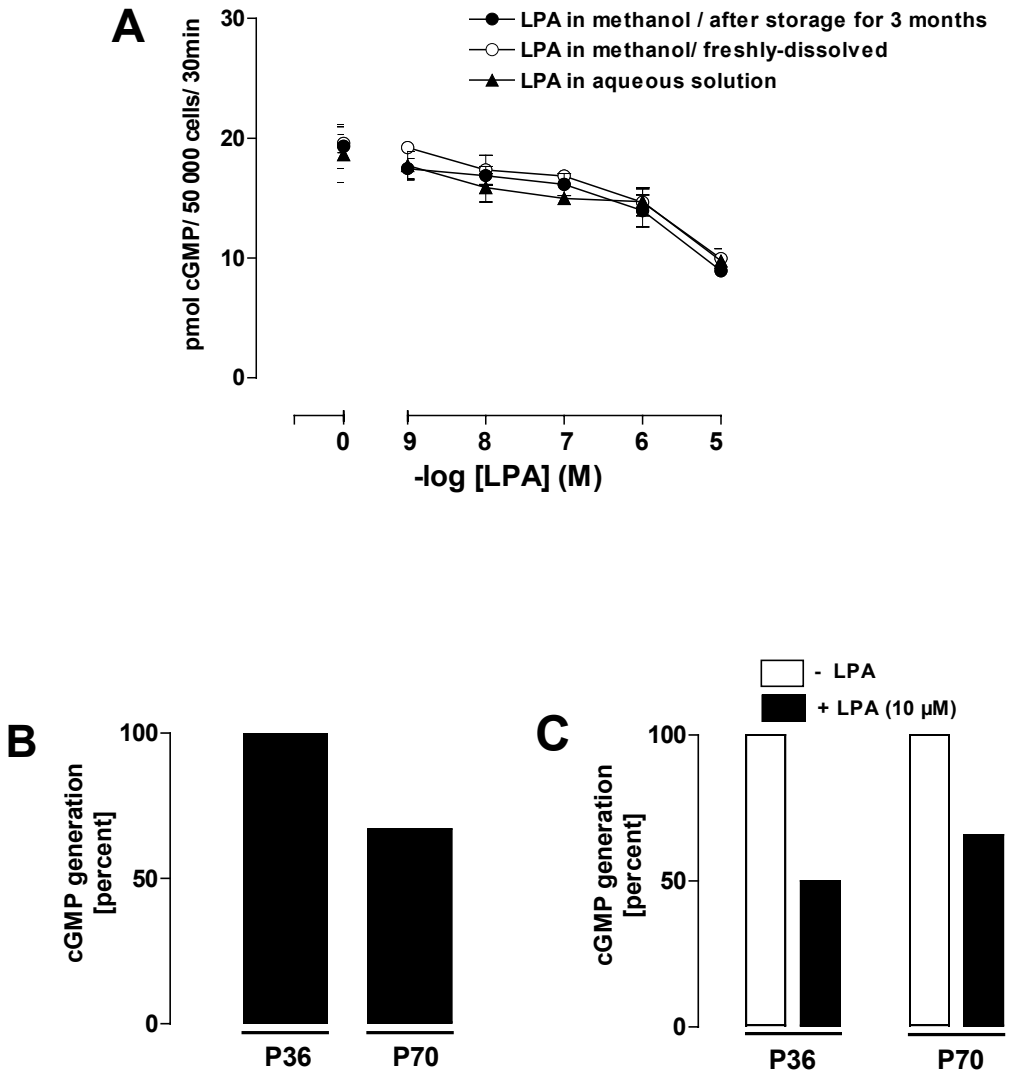

Figure 24: Experiments to identify the reasons for assay variability in desensitization studies with LPA

A: Desensitization experiments as described in Figure 20A were carried out with different batches of LPA. The aqueous solution consisted of 100 µM NaCl and 10 mM Hepes, pH 7.4 (Jalink *et al*., 1990).

B: ANP-induced cGMP production was assayed as described in Figure 11 but with different batches (P36, P70) of MA-10 cells. The amounts of cGMP measured in P36 were indicated as 100%.

C: LPA-induced desensitization of GC-A (in the presence of 10 µM LPA freshly dissolved in methanol) was assayed as described in A but with different batches (P36, P70) of MA-10 cells. In each case, cGMP production in control reactions in the absence of LPA was indicated as 100%.

3.4.6 LPA induces ERK phosphorylation in MA-10 cells

Above described findings (3.4.4) ruled out an involvement of PKA in promoting LPA-induced desensitization of GC-A. Since extracellular signal-regulated kinase (ERK) 1 and ERK 2 are known potential targets for LPA-mediated cellular effects (Budnik *et al*., 2003; Luquain *et al*., 2003). I next tested whether LPA exposure to MA-10 cells can alter the activity of these enzymes. LPA was found to induce ERK phosphorylation rapidly and in a time-dependent manner (Figure 25), whereas ANP exposure for 30 min (Figure 25) or other time periods examined (15, 60 min; not shown) failed to elicit this reaction. By being detectable already after 5 min, the effect of LPA on ERK phosphorylation was comparable to that exerted on GC-A activity (see Figure 20B). These results confirmed that LPA elicits cellular effects in MA-10 cells in a receptor-mediated manner and suggested a possible involvement of ERK 1/ 2 during the process of LPA-induced GC-A desensitization.

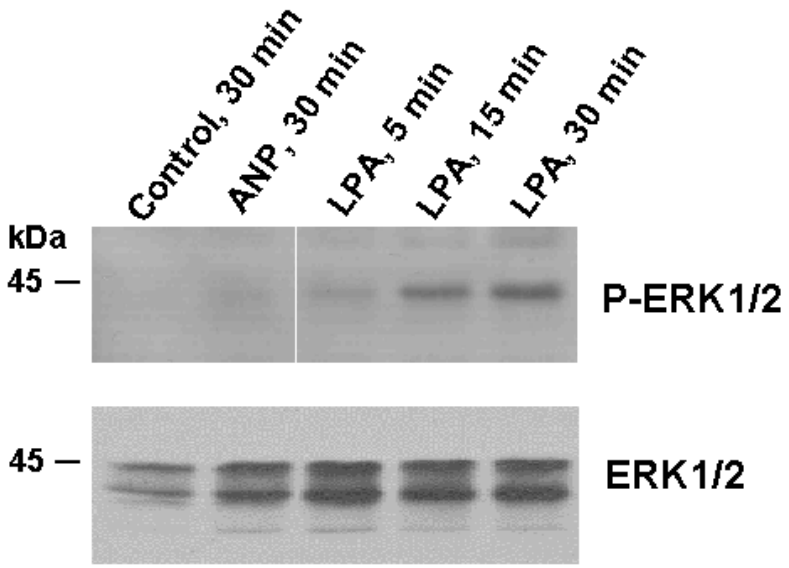

Figure 25: Immunoblot analyses of ERK phosphorylation in response to LPA in MA-10 cells

MA-10 cells were incubated for the time periods indicated in either the absence (Control) or presence (10 µM) of LPA. Additional controls were performed with ANP (0.1 µM) instead of LPA. After cell lysis, lysates (containing 80 µg proteins each) were subjected to immunoblot analyses using antibodies directed against phosphorylated ERK 1/2 (P-ERK 1/2) or recognizing the antigens in a phosphorylation state-independent manner (ERK 1/2). The migration of a molecular size marker (in kDa) is indicated.

3.4.7 The MEK/ERK pathway is not involved in LPA-induced desensitization of GC-A in MA-10 cells

To examine whether ERK phosphorylation is functionally linked to GC-A desensitization, I performed experiments in the presence (40 µM) of the MEK inhibitor, PD 98059, previously shown to efficiently prevent LPA-induced ERK phosphorylation (Budnik *et al*., 2003). These studies (Figure 26) revealed that MEK inhibition does not protect against the LPA-mediated decrease in GC-A hormone sensitivity.

During this investigation, I noticed a certain inhibitory effect of PD 98059 alone on ANP-dependent GC-A activity. However, the effect of LPA on GC-A activity was in the same range (inhibition by 40%) when incubations were performed in either the absence or presence of the MEK inhibitor (Figure 26). Thus, the LPA-elicited desensitization of GC-A is not mediated by the MEK/ERK pathway.

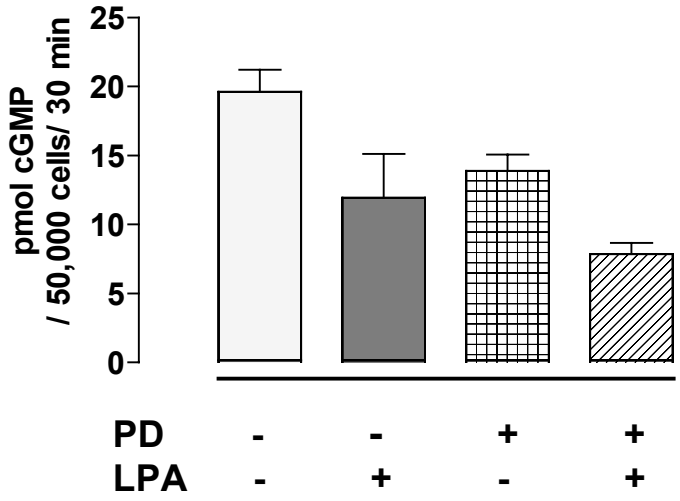

Figure 26: The LPA-induced desensitization of GC-A is insensitive to MEK inhibition by PD 98059

Serum-starved MA-10 cells were pre-incubated for 30 min in either the absence or presence of LPA (10 µM) and/or PD 98059 (PD, 40 µM) as indicated. In reactions with PD, cells had been initially pre-exposed to the inhibitor alone for 15 min. After washing, cells were then incubated with ANP (0.1 µM) for 30 min prior to measurements of cGMP. Results shown are means ± SD of triplicate assays with duplicate determinations of cGMP each.

3.4.8 Gene expression of LPA receptors in MA-10 cells

Since LPA is known to induce cellular effects via interaction with specific plasma membrane receptors (Budnik and Mukhopadhyay, 2002) and considering that different types of LPA receptors have been described which may be differentially linked to intracellular signalling pathways (Moolenaar *et al*., 2004; Anliker and Chun, 2004), experiments were performed to examine the gene expression in MA-10 cells of the two well characterized LPA receptors, LPA_1 and LPA_2. In analogous studies, LPA receptor gene expression in αT3-1 cells was investigated. Based on RT-PCR analyses (Figure 27), these experiments revealed the presence of transcripts for LPA_2 but not LPA_1 in MA-10 cells. In contrast, gene expression of both receptors was detectible in αT3-1 cells. These findings suggest that MA-10 (Leydig) cells and αT3-1 (pituitary) cells are targets for LPA-regulated activities *in vivo* and that the observed effects in MA-10 cells (heterologous desensitization of GC-A, phosphorylation of ERK) are mediated by LPA_2 rather than LPA_1 receptors.

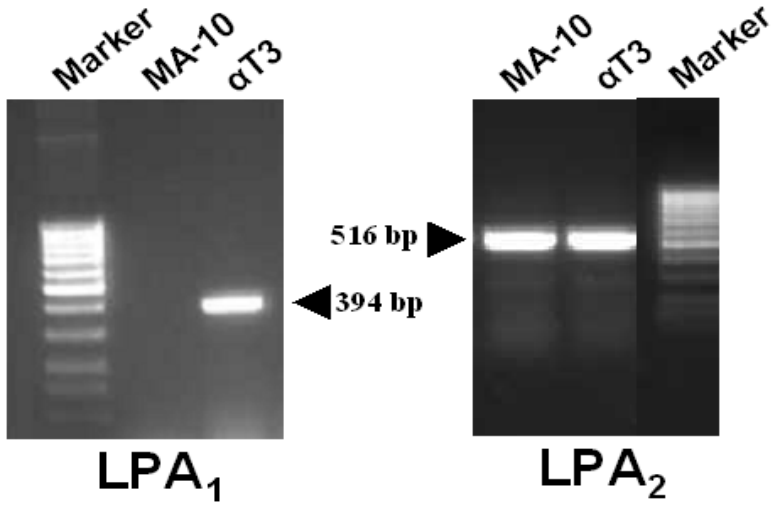

Figure 27: RT-PCR analysis of LPA receptor (LPA_1, LPA_2) expression in MA-10 and αT3-1 cells

RNA was isolated from MA-10 or αT3-1 cells, reverse-transcribed into cDNA and then subjected to PCR in the presence of either LPA_1- or LPA_2- specific primers. The migration in agarose gels of ethidium bromide-stained PCR products of the expected sizes (LPA_1:394 bp; LPA_2:516 bp) in relation to that of 100-bp ladders (Marker) is shown.

3.4.9 LPA induces morphological alterations in MA-10 cells

The identification of LPA-induced decreases in GC-A activity and increases in ERK phosphorylation provided evidence for significant and receptor-mediated activities of the lipid in MA-10 cells.

To further evaluate potential physiological responses to LPA in MA-10 cells, I examined whether LPA exposure can induce alterations in morphology and cytoskeletal remodeling, representing well established actions of LPA in many cell types (Moolenaar *et al*., 1999, 2004).

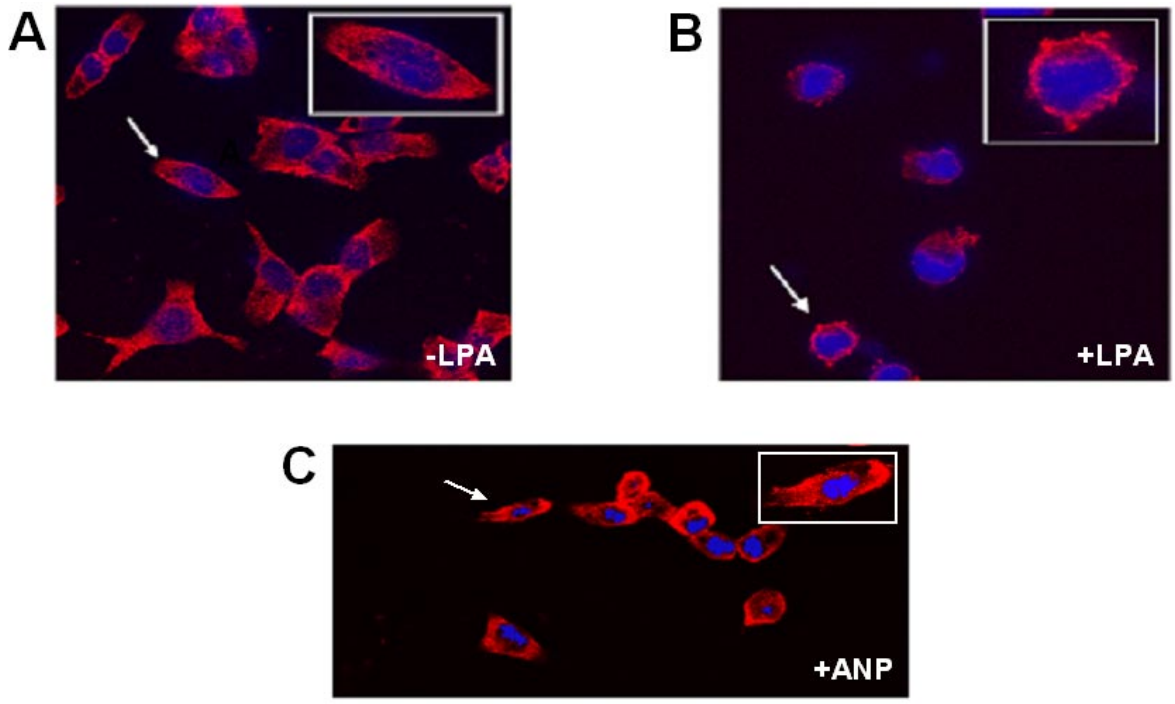

Figure 28: Immunocytochemical analysis of LPA effects in MA-10 cells
MA-10 cells, plated on glass slides, were serum-starved overnight and then incubated for 30 min in either the absence or presence (10 µM) of LPA as indicated (A,B). Control incubations with ANP (0.1 µM) are shown in C. After fixation, cells were treated with an antibody directed against α-actin, and immunoreactivity was visualized using Cy3-conjugated secondary antibody (red). Cell nuclei were counter-stained with DAPI (blue). Insets show typical cells (arrows) in higher magnification.

To address this issue, MA-10 cells were grown on chamber slides and incubated in either the absence or presence of LPA for up to 30 min. Then, cells were fixed, permeabilized and stained with an antibody that recognizes α-actin. Incubations with Cy3-conjugated secondary antibodies served to visualize the

immunoreactivity. For comparison, analogous assays were also performed with ANP instead of LPA. These studies demonstrated dramatic morphoregulatory effects induced by LPA, resulting in cell rounding, loss of intercellular connections and a striking re-organization of actin filaments (Figure 28B). These changes, evident within 20 to 30 min during LPA exposure, were found to be reversed after withdrawal of the agonist (not shown) and were not induced by exposure to ANP (Figure 28C). Thus, the results confirmed the generation of profound effects by LPA in MA-10 cells, raising the possibility that Leydig cell physiology *in vivo* is markedly influenced by LPA signalling.

3.5 Regulation of natriuretic peptide receptors in αT3-1 cells

The investigations with MA-10 cells, selectively expressing GC-A, served to identify and characterize comparatively homologous and heterologous desensitization of the ANP receptor, GC-A.

The following studies, performed with αT3-1 cells (expressing both GC-A and the CNP receptor, GC-B), served to focus on a number of interesting questions which are related to the co-expression in one cell type of both cGMP-generating natriuretic peptide receptors. Specific questions that could be addressed by this model were

(a) whether ANP and/or CNP can induce homologous desensitization of their respective (GC-A, GC-B) receptors,

(b) whether such ANP- and/or CNP-induced effects are selectively directed against their specific receptor proteins (i. e., ANP and GC-A, CNP and GC-B) despite the generation of a common second messenger (cGMP), and

(c) whether heterologous desensitization (if detectable) affects both receptors equally or not.

3.5.1 The activities of both GC-A and GC-B are unaffected by LPA

Considering that LPA was highly effective in desensitizing GC-A in MA-10 cells, I next examined whether the bioactive lipid can induce natriuretic peptide receptor desensitization in αT3-1 cells.

Cell aliquots were pretreated for 30 min at different concentrations of LPA, then washed and re-incubated with either 0.1 μM ANP or CNP prior to determinations of cGMP content. At all LPA concentrations tested (10^{-9} to 10^{-5} M), any effects on either ANP-induced or CNP-induced cGMP production remained undetectable (Figure 29).

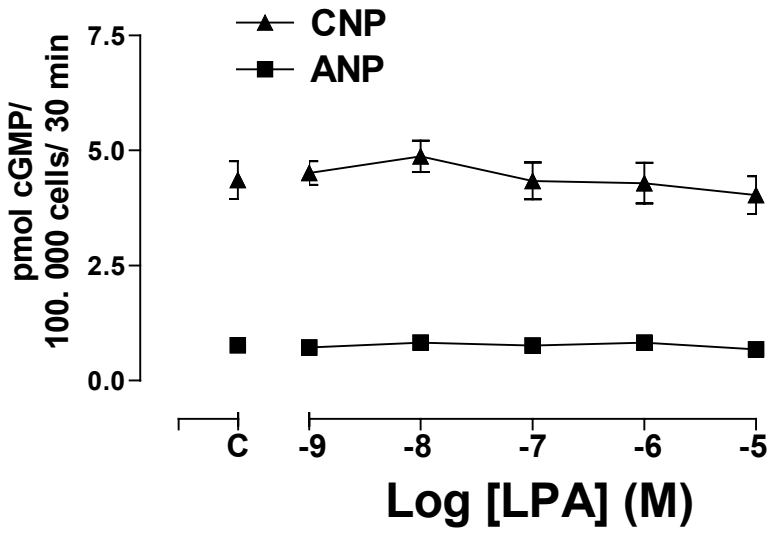

Figure 29: LPA pre-exposure does not affect the ligand-dependent activities of GC-A and GC-B in αT3-1 cells

αT3-1 cells were pretreated with the indicated concentrations of LPA for 30 min. The media were aspirated, and cells were washed once with PBS. Cells were subsequently stimulated with (0.1 µM) ANP or CNP to induce cGMP production after which cellular cGMP accumulations were measured. Control assays, pre-incubated for 30 min in the absence of LPA, are shown (C). Results shown represent means ± SD of three experiments each.

Thus, LPA, despite the gene expression of its two major receptor types, LPA_1 and LPA_2 (see Figure 27), is unable to influence natriuretic peptide receptor activities in αT3-1 cells. These findings suggested that intracellular signalling pathways, mediating LPA-induced desensitization of GC-A in MA-10 cells, are not present or active in αT3-1 cells.

3.5.2 Examination of homologous desensitization

As shown before (Figure 13), both ANP and CNP dose-dependently induce cGMP production in αT3-1 cells. To investigate the time dependency of these reactions, cells were incubated with either ANP or CNP (0.1 µM each) for different time periods prior to assessments of cGMP levels.

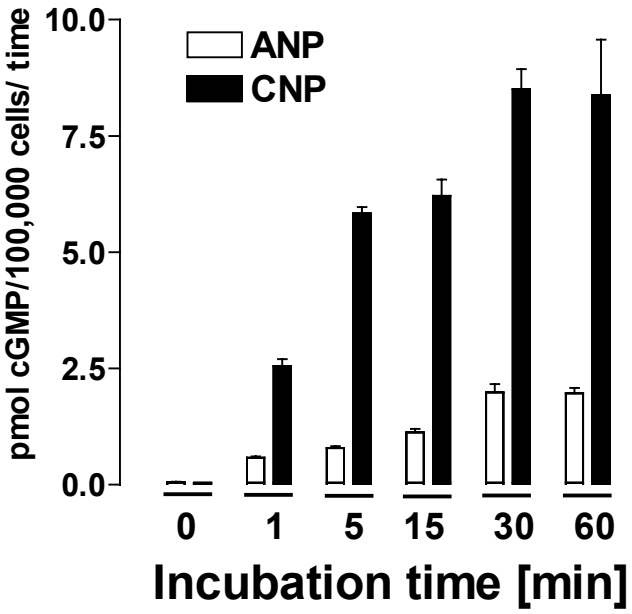

Figure 30: Time-dependency of ANP- and CNP-induced cGMP production in αT3-1 cells

Cells were incubated in the presence (0.1 µM) of either ANP or CNP at time periods indicated prior to measurements of cGMP accumulations (see section 2.2.3.1). Values represent means ± SD of triplicate assays with duplicate determinations of cGMP each.

As shown (Figure 30), both peptides rapidly (already after 1 min incubation time) induced cGMP accumulation, and maximum values were observed in each case after 30 min. As compared to cGMP levels in untreated cell (0 min incubation time), ANP elicited 40-fold and CNP 283-fold increases after 30 min. These results suggested that CNP receptors are more abundant or more active than ANP receptors in αT3-1 cells.

As with MA-10 cells, homologous desensitization assays were carried out using pre-incubation times of up to 30 min and peptide concentrations of 0.1 µM. To examine homologous desensitization of GC-A, ANP-pretreated cells were washed and re-incubated with ANP. To assess such effects on GC-B, analogous assays were performed with CNP instead of ANP.

These studies revealed a relatively moderate but significant homologous (i. e., ANP-induced) desensitization of GC-A (Figure 31A), whereas CNP clearly failed to induce desensitization of GC-B (Figure 31B). The results are remarkable with regard to physiological and molecular aspects: 1. They demonstrate that cells co-expressing

ANP and CNP receptors can be distinctly influenced during prolonged exposure to the two peptide ligands (one receptor becomes desensitized, the other not). 2. Despite the generation of the same second messenger (cGMP), homologous desensitization is receptor-specific, indicating a compartmentalization of the signalling pathways involved.

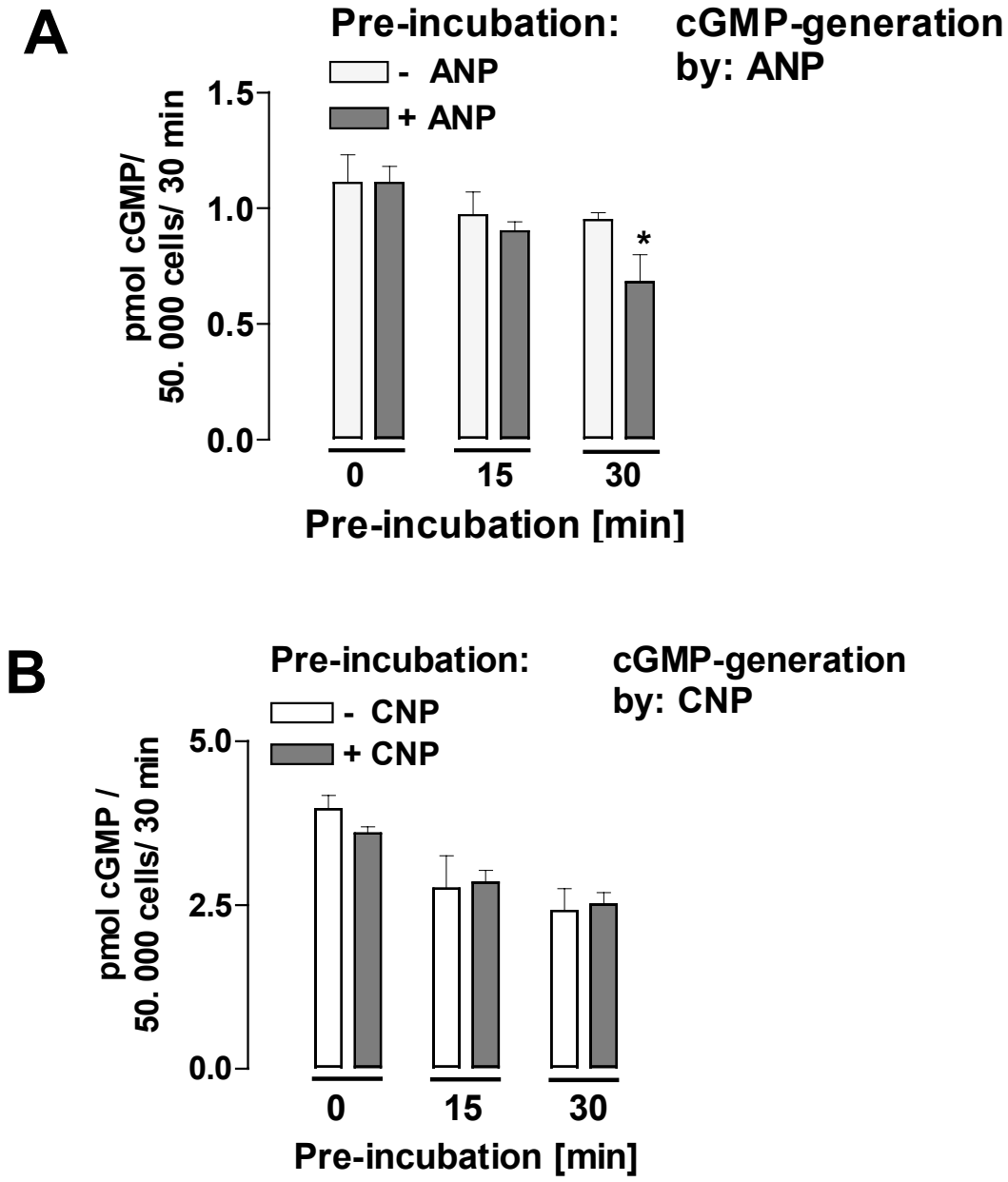

Figure 31: Investigations of homologous desensitization of GC-A and GC-B in αT3-1 cells

Cells were pre-incubated in either the absence or presence (0.1 µM) of ANP for the time periods indicated, washed, and ANP-dependent cGMP generation during a following incubation for 30 min was determined (A). Analogous assays, performed with CNP instead of ANP, are shown in B. Values are means ± SD of three experiments each.

3.5.3 Identification of cross-reactions between GC-A and GC-B signalling

After investigating the homologous desensitization of GC-A/GC-B in αT3-1 cells, it was of particular interest to examine whether prolonged exposure of ANP might have any effects on the CNP receptor (GC-B) and vice versa (CNP to GC-A). To address this issue, cells were pretreated as before with either ANP or CNP but then re-incubated each with the other peptide.

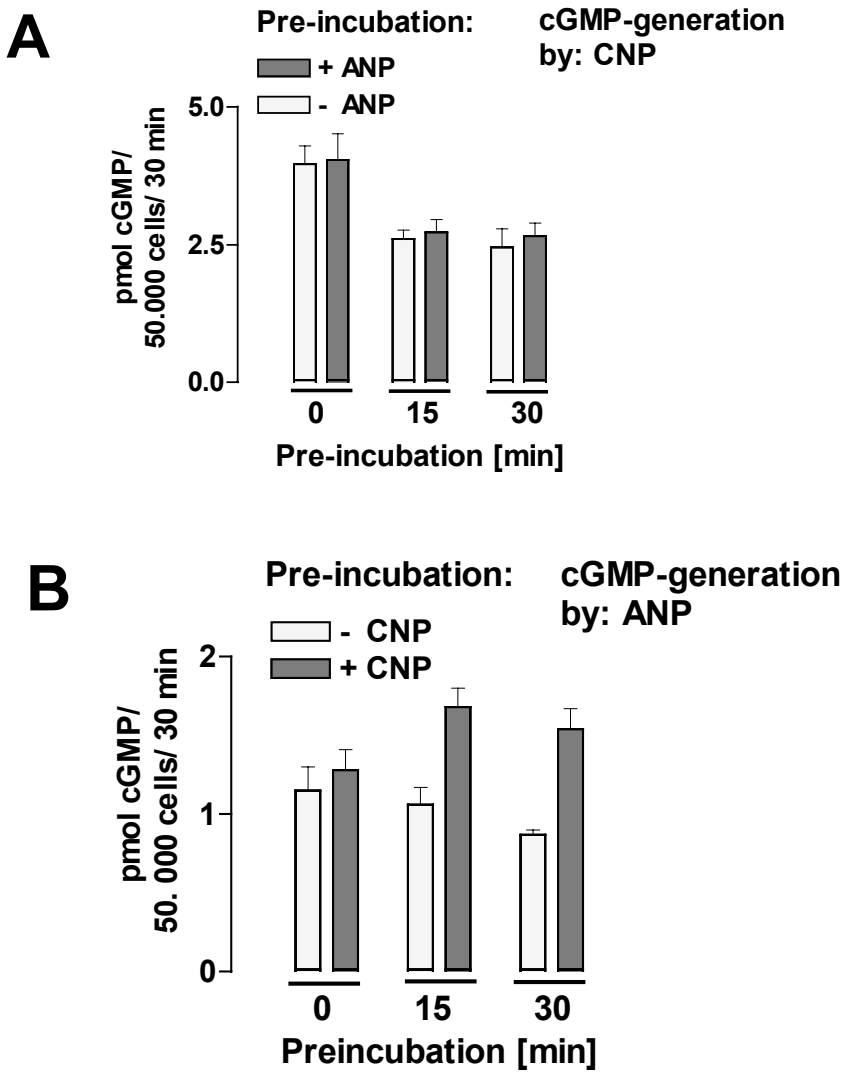

Figure 32: Effects of ANP-pre-exposure on GC-B (A) and of CNP-pre-exposure on GC-A (B) activity in αT3-1 cells

A: Cells were pre-incubated for the time periods indicated in either the absence or presence (0.1 µM) of ANP. After washing, cGMP generation by CNP was determined. B: Cells were pre-incubated with CNP, and cGMP generation by ANP was measured. Values are means ± SD of five experiments each.

As shown (Figure 32A), pretreatments with ANP had no effects on subsequent CNP-induced cGMP accumulations. However, when cells had been pre-exposed to CNP, the following stimulation of GC-A activity by ANP was much more effective that in controls pre-incubated in the absence of CNP (Figure 32B). These unexpected findings, indicating an enhanced hormone-responsiveness of GC-A in response to CNP exposure, were corroborated in many independent experiments, carried out with different batches of αT3-1 cells. Thus, this study shows for the first time (a) direct interactions between ANP/GC-A and CNP/GC-B signalling at a cellular level and (b) that the hormone-responsiveness of natriuretic peptide receptors can be enhanced ("sensitization") rather than reduced ("desensitization") by signalling molecules acting via plasma membrane receptors.

3.5.4 Characterization of "resensitization" of GC-A by CNP/GC-B signalling

The following studies served to characterize the molecular mechanisms underlying the CNP-induced ("re"-)sensitization of GC-A in αT3-1 cells.

3.5.4.1 The effect is based on an increase in ANP-dependent guanylyl cyclase activity

As in studies described before which addressed the mechanisms responsible for receptor desensitization, the effect of CNP pretreatment on GC-A activity was examined on cell membrane preparations. Cells were exposed to 30 min to CNP, then homogenized, and guanylyl cyclase assays were performed with membrane fractions of the cellular proteins.

These studies demonstrated that ANP-induced cGMP accumulations were increased after such treatments (Figure 33A), while CNP-elicited cGMP generation remained unaffected (Figure 33B). In each case, basal guanylyl cyclase activities (i. e., cGMP production by membranes in the absence of ANP/CNP) were not influenced by cellular pretreatment with CNP. In addition, detergent-dependent (Triton/$MnCl_2$)-stimulations of membrane guanylyl cyclases resulted in equal values of cGMP irrespective of CNP-pretreatments or not (Figure 33C).

Thus, these findings confirmed the results obtained with intact cells and provided evidence that the CNP-induced enhancement of GC-A activity was in fact accounted for by specific increases in hormone-responsiveness of the ANP receptors.

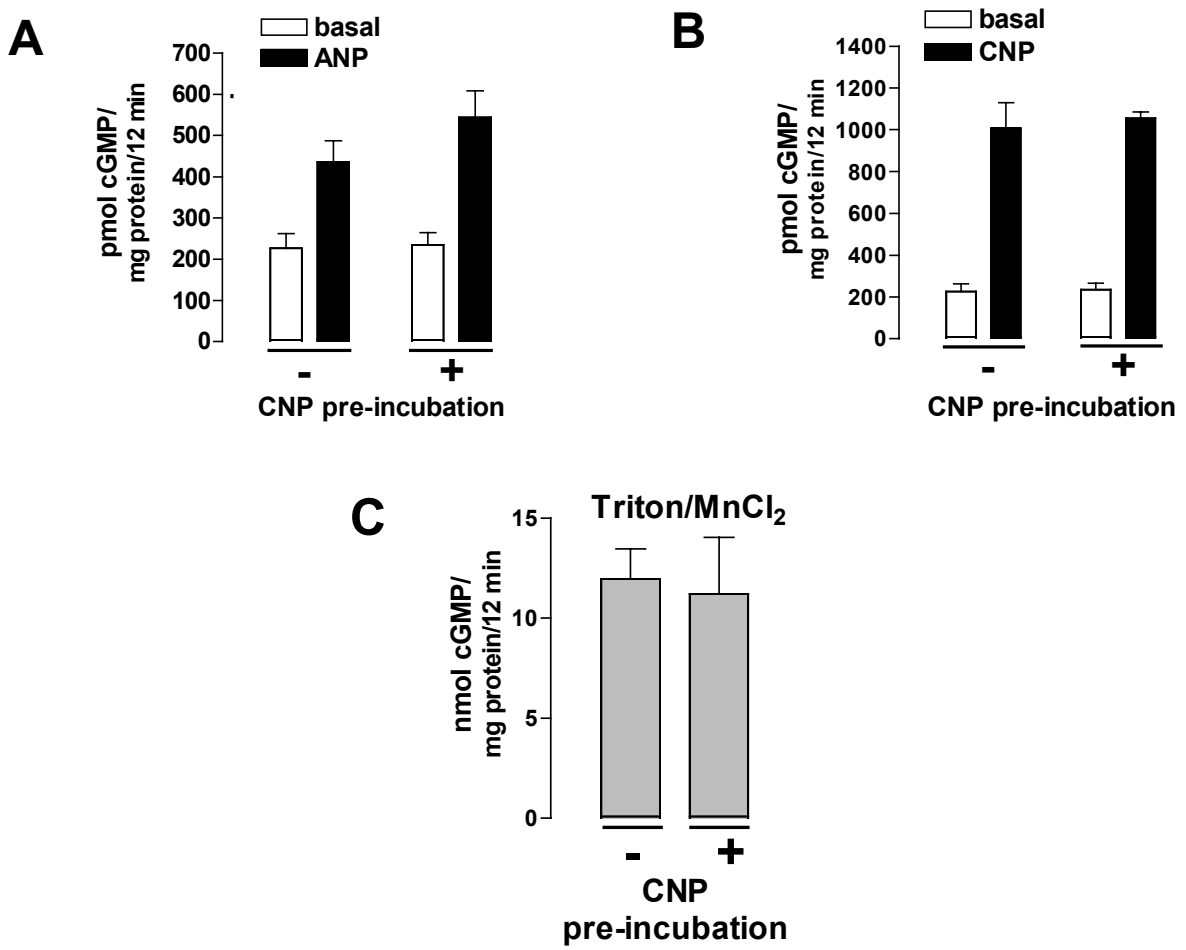

Figure 33: Guanylyl cyclase assays with membranes prepared from αT3-1 cells after pre- incubations in the absence or presence of CNP

αT3-1 cells were pre-incubated for 30 min in either the absence or presence (0.1 µM) of CNP. After homogenization, membrane fractions of cellular proteins were prepared and assayed for ANP- (A), CNP- (B) or detergent (Triton/$MnCl_2$)-dependent (C) guanylyl cyclase activity. The data represents the means ±SD of triplicate assays with duplicate determinations of cGMP each.

3.5.4.2 CNP-induced GC-A sensitization is mediated by PKA

As shown before, homologous (Figure 18) but not heterologous (Figure 23) desensitization of GC-A in MA-10 cells requires the activity of PKA. Hence, it was of interest to examine whether this kinase might or might not play a role in mediating the CNP-induced sensitization of GC-A in αT3-1 cells. Different from GC-A desensitization in MA-10 cells, where PKA is thought to act as component of a signalling cascade which finally activates a GC-A-dephosphorylating enzyme, a further and/or alternative role for PKA is conceivable in case of (re-)sensitization processes: the kinase could be directly implicated in GC-A phosphorylation.

To investigate a potential role of PKA, αT3-1 cells were incubated for different time periods with CNP in either the absence or presence (1 µM) of the PKA inhibitor H89. For control, analogous incubations were performed without CNP. After washing, cells were then re-incubated with ANP to assess GC-A-mediated cGMP production.

As demonstrated (Figure 34), CNP pretreatments for 15 or 30 min massively enhanced (by 63% or 49% respectively) the ANP-dependent GC-A activity. These effects, however, were completely blocked when CNP was co-incubated together with H89. Thus, these findings show that the CNP-induced sensitization of GC-A in αT3-1 cells is PKA-dependent.

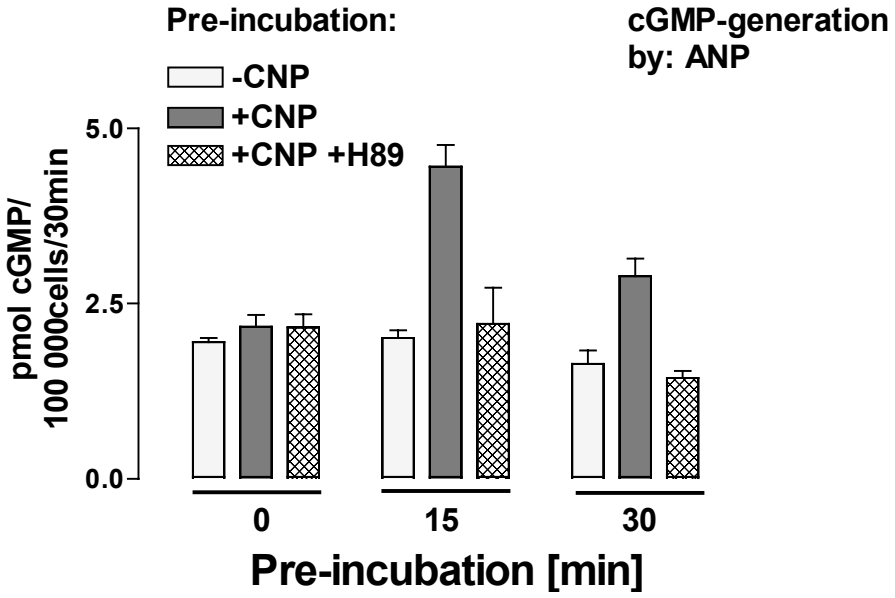

Figure 34: The effect of the PKA inhibitor H89 on CNP-induced sensitization of GC-A in αT3-1 cells

αT3-1 cells were incubated for the time periods indicated in either the absence of CNP, the presence (0.1 µM) of CNP, or the presence of CNP plus H89 (1 µM). Cells were washed once with PBS and reincubated with 0.1 µM ANP to induce cGMP production prior to measurement of cellular cGMP. Values represent means ± SD of three experiments each.

3.5.5 Experimental prove that GC-A activity in αT3-1 cells is regulated by both ANP- and CNP- mediated signalling

While GC-B activity in αT3-1 cells was unaffected by cell agents (CNP, ANP, LPA) tested, the above described findings revealed a remarkable sensitivity of GC-A: its hormone-responsiveness was reduced by pre-exposure to ANP and increased by pre-exposure to CNP. Considering the current model that the activity of natriuretic

peptide receptors is regulated by phosphorylation/dephosphorylation at a set of intracellular serine/threonine residues (Potter and Hunter, 1998a,1998b) and that the extent of receptor phosphorylation directly correlates with the extent of ligand-dependent activity (Potter, 1998), my results obtained for GC-A could be used to make certain predictions: Under normal culturing conditions (i. e., in the absence of ANP/CNP in the medium), GC-A was neither fully phosphorylated (this would prevent a further enhancement of activity) nor fully dephosphorylated (this would prevent an experimental desensitization). Since the extent of GC-A desensitization detectable in αT3-1 cells was rather low as compared to the extent of sensitization attainable, it could also be suggested that the phosphorylation state of the receptor under normal culture conditions is poor. If these predictions are correct, one would expect that pretreatments with CNP (enhancing receptor phosphorylation) should result in a more pronounced desensitization during following incubations with ANP.

To test this hypothesis, αT3-1 cells were pretreated with CNP prior to assessments of ANP-induced (homologous) desensitization of GC-A. As shown (Figure 35A), CNP treatment alone strongly enhanced GC-A activity. When these cells were subsequently exposed to ANP, the desensitizing effect (by ~50%) was much higher than that in experiments (by ~20%) without CNP pretreatments. Controls performed in the absence of natriuretic peptides (Figure 35B) did not show any significant alterations.

Thus, these findings proved that GC-A in αT3-1 cells is regulated in a specific and opposite manner by ANP and CNP. Intracellular cross-reactions between ANP/GC-A and CNP/GC-B signalling have not yet been described before. Since several cell types are known to express both ANP and CNP receptors (McArdle *et al*., 1994; Krause *et al*., 1997; DiCicco-Bloom *et al*., 2004; Jankowski *et al*., 2004) and considering that ANP represents a hormone and CNP a paracrine (locally-produced) factor, the results might be of major physiological relevance.

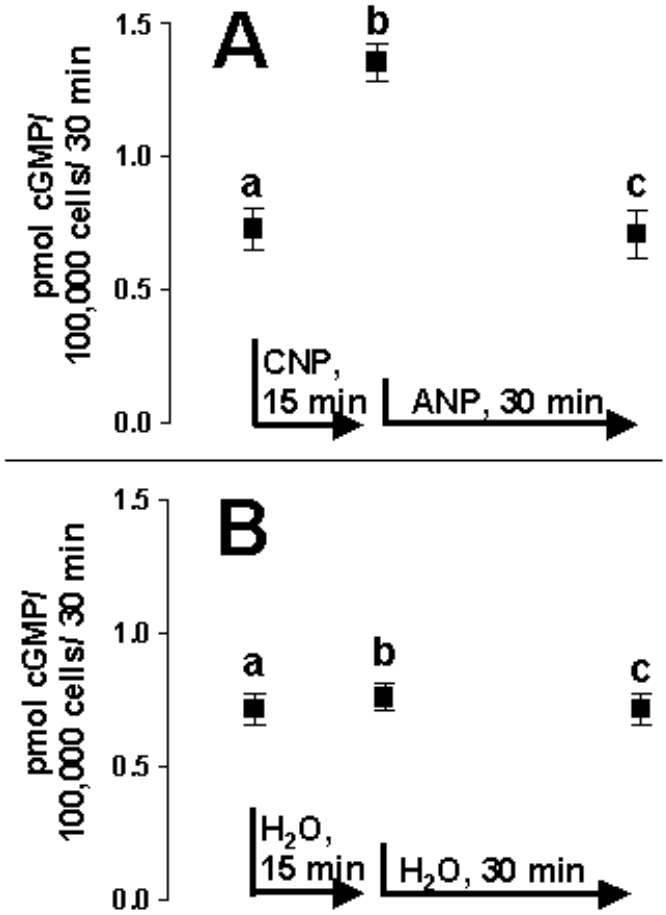

Figure 35: Demonstration of initial enhancement (by CNP) and subsequent reduction (by ANP) of GC-A activity in αT3-1 cells

A: Aliquots of cells were either not pre-incubated (a), pre-incubated for 15 min with CNP (b), or pre-incubated as in b and subsequently exposed for 30 min to ANP (c). Cells were then washed, and ANP-dependent cGMP production was measured. In B, control assays (pre-incubated in the absence of natriuretic peptides) are shown. Results shown are means ± SD of triplicate assays with duplicate determinations of cGMP each.

4 Discussion

4.1 Experiments with MA-10 cells

The present study addressed questions related to physiological and molecular aspects of natriuretic peptide signalling. Using MA-10 cells as an experimental model, I show that the hormone responsiveness of GC-A in Leydig cells decreases during prolonged exposure to ANP and is also affected in a very similar manner by LPA. Considering (i) reports on functional activities of ANP/GC-A in Leydig cells (Mukhopadhyay *et al*., 1986a; Schumacher *et al*., 1992; Pandey and Singh, 1990; Pandey *et al*., 1999) and (ii) that LPA-induced effects in this cell type have not yet been described before, these findings provide novel information of potential physiological relevance. At the molecular level, results regarding the role of PKA in mediating ANP- but not LPA-induced desensitization of GC-A, are thought to be of major significance. For an overview, the main findings are schematically summarized in Figure 36 (see page 86).

4.1.1 ANP-induced desensitization

That prolonged exposure to ANP can induce GC-A desensitization has been well established in a variety of studies performed in different GC-A-transfected cell lines (Potter and Garbers, 1992; Koller *et al*., 1993; Potter and Garbers, 1994; Joubert *et al*., 2001; Bryan and Potter, 2002). In addition, homologous desensitization of GC-A has been identified in cultured vascular smooth muscle cells (Cahill *et al*., 1990; Yasunari *et al*., 1992). The present findings, elucidating GC-A desensitization in MA-10 Leydig cells, support that this kind of regulation represents a widespread phenomenon, presumably active in many cell types. Consistent with other investigations (Kato *et al*., 1991; Vieira *et al*., 2001; Fan *et al*., 2005), GC-A desensitization in MA-10 cells is not due to receptor endocytosis and/or degradation but seems to be entirely explained by a decreased receptor hormone-responsiveness. Receptor-mediated endocytosis is the cellular mechanism by which clearance (C) type receptors of natriuretic peptides exert their clearance function (Cohen *et al*., 1996).

In the present work, cross-linking experiments revealed the absence of the natriuretic peptide clearance receptor (NPR-C) which rules out the involvement of receptor endocytosis. In addition, the basal cyclase (ligand-independent) activities

measured in membranes prepared from ANP-pretreated cells showed no reductions compared to controls (ANP-nontreated cells). This ligand-independent cyclase activity is an excellent indicator of the total amount of GC-A present in any given membrane preparation (Potter, 1998) thus indicating the absence of a receptor protein degradation. However, a 40% reduction of hormone-dependent cyclase activities measured in membranes of ANP-pretreated cells implies a decrease in ligand-receptor sensitivity and is therefore highly suggestive of an intracellular modulation.

Early studies on GC-A desensitization revealed receptor dephosphorylation as the underlying mechanism (Potter and Garbers, 1992). GC-A was shown to exist as a phosphoprotein in resting HEK293 cells, and studies showed that CG-A in membrane preparations can be dephosphorylated by protein phosphatases which is directly correlated with the cyclase activity of the receptor (Potter and Garbers, 1992; Potter, 1998; Potter and Hunter, 1999; Bryan and Potter, 2002). However, the phosphatases involved in the desensitization process are still unknown. Considering the blocking effects of okadaic acid in both GC-A and GC-B desensitization (Potter and Garbers, 1992; Potter, 1998), this suggest that such desensitization processes may be caused by certain okadaic-sensitive phosphatases. Other studies have however demonstrated that a serine/threonine protein phosphatase inhibitor, microcystin could also block the *in vitro* desensitization of GC-A in crude membranes of transfected NIH fibroblast cells which substantiate the idea that GC-A may also be desensitized by microcystin-sensitive protein phosphatases (Potter and Hunter, 1999; Bryan and Potter, 2002).

In the present study, experiments performed in the presence of phosphatase inhibitors failed to block the ANP-dependent GC-A desensitization. Additions of 20 nM okadaic acid or 0.5 μM microcystin-LR failed to block ANP-induced desensitization in intact MA-10 cells, thus indicating that the protein phosphatase(s) which may be involved in this process is/are neither microcystin- nor okadaic-sensitive protein phosphatase(s). Microcystin-LR is a hepatotoxic peptide associated with most strains of the blue algae *Microcystis aeruginosa* and is a potent inhibitor of type I protein phosphatase (PP) and type 2A PP, whereas okadaic acid (OA) is extracted from cultures of dinoflagellate, *Prorocentrum Sp*. and is also a potent inhibitor of serine- and threonine-specific PP1 and PP2A (Geringer, 2004). The

above results are comparable to those reported by Potter and Hunter (1999) which indicated that additions of different concentrations of okadaic acid which should be effective in inhibiting PP1, PP2A, PP4 and PP5 did not block the homologous desensitization of GC-A in whole cell experiments. Interestingly, these findings are inconsistent to analogous experiments performed by the same authors in membrane preparations where a blocking effect of okadaic acid was observed. According to the group, such a discrepancy may be due to the fact that the amounts of okadaic acid used (intact cell experiments) may not be sufficient to inhibit the relevant PPs over the entire assay period, or that the physiological PPs may be okadaic acid-insensitive.

Further findings on the identification of specific protein phosphatases involved in GC-A desensitization was recently reported. Compelling evidence have documented that GC-A desensitization in membranes of transfected mouse kidney 293 and NIH3T3 fibroblast cells, was accounted for an effect of distinct microcystin-sensitive and magnesium-dependent protein phosphatases in which the latter is proposed to be a member of the protein phosphatase 2C family (Abbey and Potter, 2002).

Notably, experiments performed in phosphorylating (sensitizing) GC-A, it was shown that GC-A-associated ^{32}P was increased when microcystin was included in the reaction mixture. These increases in ^{32}P is not due to an increased amounts of GC-A in the immunoprecipitates but rather to a sensitization by ATP and microcystin which correlates well with phosphorylation (Foster and Garbers, 1998). To this end, another report has indicated a significant reduction in the activity or affinity of a receptor-associated kinase (probably a GC-A kinase) which contributed to a low phosphorylation level and a subsequent receptor (GC-A) dephosphorylation (Joubert *et al*., 2001). Considering the above observations, it appears that both phosphorylation and dephosphorylation are important mechanisms in the regulation of GC-A desensitization.

Since binding of ANP to GC-A initially elicits the generation of cGMP, it is likely that cGMP is involved in intracellular signalling events leading to GC-A desensitization. cGMP-dependent protein kinase I (GK I) is the major intracellular mediator of cGMP signals in many cell types (Francis and Corbin, 1999; Kotera *et al*., 2003). In this context, findings that human Leydig cells do not express GK I (Middendorff *et al*., 2002) were of potential interest. By immunoblot analyses, GK I

expression was also undetectable in mouse (not shown), rat and MA-10 Leydig cells (Figure 14), suggesting that GK I deficiency represents a characteristic of Leydig cells. This may be the reason why GK I has never been involved in the natriuretic peptide signalling in Leydig cells. In this context, the absence of this enzyme highly suggests that it is not implicated in the ANP-induced desensitization of GC-A. However, it is worth mentioning that GK I has been involved in the regulation of GC-A. Recent study has demonstrated that ANP-stimulated GC-A recruits GK I to the plasma membranes and promotes its activation via ANP/GC-A-generated cGMP in transfected and untransfected HEK 293 cells. Thus, proposing an ANP-induced GC-A/GK I association which may represent a novel mechanism for both compartmentation of cGMP-mediated signalling and regulation of receptor sensitivity (Airhart *et al*., 2003).

The findings that the PKA inhibitor H89 completely blocked the ANP-induced desensitization of GC-A in MA-10 cells pointed at the possibility that PKA rather than a cGMP-dependent protein kinase acts as the functional target for cGMP in these cells. In fact, this kind of interaction is strongly supported by previous studies in mouse Leydig cells, which revealed (i) that cGMP, in addition to cAMP, is capable of stimulating testosterone production, (ii) that both gonadotropin- and ANP-induced steroidogenesis is inhibited by a cAMP antagonist, (iii) that this antagonist and cGMP competitively reduce [^{3}H]cAMP binding to Leydig cell proteins, and (iiii) that in lysates prepared from either ANP- or gonadotropin-stimulated cells, PKA was activated, as assessed by functional assays (Schumacher *et al*., 1992). Thus, there is considerable evidence that homologous desensitization of GC-A in MA-10 Leydig cells is mediated by a direct interaction between cGMP and PKA.

The regulation of vascular endothelial growth factor (VEGF) in mouse Leydig cells has also been shown to be regulated in part by ANP/cGMP signalling through which cGMP cross-activates PKA (Anand *et al*., 2003). Furthermore, studies in other cell types have also demonstrated that cGMP cross-activated PKA, which mediates the secretion of fluid induced by heat-stable enterotoxin (ST) in human intestinal cells (Forte *et al*., 1992; Chao *et al*., 1994). Likewise, the overexpression of nitric oxide synthase (NOS) resulting to an increased cGMP levels resulted in a cross-activation of PKA in rat aortic smooth muscle cells (Lincoln *et al*., 1995). Such cross-interactions were thought to be due to the primary sequence homology between PKA

and PKG particularly in areas that encode the nucleotide binding sites thus accounting for the cross-activation of PKA by cGMP (Hofmann *et al*., 2000; Vaandrager *et al*., 1996). These results are of interest with regard to the debate whether cGMP in fact could signal via PKA *in vivo* (discussed in Lucas *et al*., 2000). It has to be noted that H89 failed to inhibit the LPA-induced (i. e., cGMP-independent) desensitization of GC-A in MA-10 cells and that the same PKA inhibitor has been successfully used in other studies to assess PKA-mediated effects in this cell line (e. g., Wang *et al*., 2000; Hirakawa and Ascoli, 2003).

4.1.2 LPA-induced desensitization

This study demonstrates that LPA can induce desensitization of GC-A. Considering that previous publications have exclusively reported on GC-B desensitization by LPA (Abbey and Potter, 2003; Potthast *et al*., 2004) or sphingosine-1-phosphate (Chrisman *et al*., 2003; Abbey-Hosch *et al*., 2004), the present results establish that cross-talks between phospholipid and natriuretic peptide signalling do not represent a GC-B-specific phenomenon.

The elucidation in MA-10 cells of effects such as ERK phosphorylation and cell rounding in response to LPA indicates receptor-mediated activities (Ishii *et al*, 2004). This was supported by the identification of LPA receptor gene expression. Thus, it appears reasonable to assume that the LPA effect on GC-A is also receptor-mediated, being consistent with a rapidly-elicited response (both ERK phosphorylation and GC-A desensitization are detectable already after 5 min LPA treatment). That direct additions of LPA to membrane preparations of transfected NIH3T3 fibroblast cells does not affect GC-B activity (Abbey and Potter, 2003) clearly indicates that the molecule does not have a direct intracellular mode of action. Hence, the nature of the signalling route between LPA and ANP transductions would then be expected to be dependent on LPA-receptor associated molecules.

The RT-PCR analyses strongly suggest that LPA_2 but not LPA_1 receptors are involved, although contributions by two additional (LPA_3, LPA_4) receptor types described cannot be excluded (Ishii *et al*., 2004). Biological responses for LPA are mediated through signalling via activation of specific 7-transmembrane G-protein receptors (GCPRs) (Mills and Moolenaar, 2003). At present, there are four identified mammalian LPA receptors. The first three, LPA_{1-3} share sequence homology with one another, whereas LPA_4 is divergent in sequence. LPA_1 is the most widely

expressed and the best characterized receptor, although LPA_2 is also widely distributed being expressed in the testis, kidney, lung, thymus, spleen, and stomach of adult mice and in the human testis, pancreas, prostrate, thymus, spleen and leukocytes. In primary cultures of mouse embryonic fibroblasts, targeted deletion of LPA_2 has shown significant loss of normal LPA signalling which includes Ca^{2+} mobilization, stress fiber formation and PLC activation (for review, see Ishii *et al*., 2004). So far the expression of LPA receptor(s) in Leydig cells has not yet been reported.

Experiments performed in the presence of kinase inhibitors revealed that neither PKA nor the MAPK/ERK pathway are functionally involved in the process of GC-A desensitization. These are rather interesting findings considering the reported implications of PKA and ERK signalling in rat Leydig cell physiology. For instance, in MA-10 cells expressing recombinant human luteinizing hormone receptor (hLHR) and in primary cultures of rat Leydig cells, the activation of ERK1/2 cascade was via a PKA-dependent activation of Ras (Hirakawa and Ascoli, 2003). Martinelle *et al*. (2004) have shown a gonadotropin receptor-mediated activation of ERK cascade involving PKA and PKC as upstream kinases in rat Leydig cells. Based on these observations, it is plausible to think of a PKA involvement in the LPA-mediated desensitization. Although PKA was shown to play a pivotal role in the homologous desensitization of GC-A in this study, this enzyme is apparently not involved in LPA-induced desensitization as the addition of H89 did not abolish the inhibiting effect of LPA. These findings highly imply that LPA-dependent desensitization is modulated by a distinct pathway different from that of ANP-induced desensitization.

In the present study, phosphorylation of ERK1/2 was detected in lysates prepared from LPA-pretreated cells which showed an increasing degree of ERK1/2 phosphorylation consistent to that exerted in GC-A activity in intact cells. Based on the fact that LPA can evoke diverse biological responses associated with activation of the MAPK signalling cascade (Kranenburg and Moolenaar, 2001), these results raised the possibility that ERK1/2 phosphorylation might be involved in LPA-induced inhibition of GC-A. However, the failure of PD 98059 (a specific inhibitor of MEK1/2) to abolish the desensitization reaction clearly excluded such a molecular link.

LPA-induced GC-B desensitization has recently found to be linked to intracellular calcium elevations (Potthast *et al*., 2004). Because the intracellular amino acid domains of GC-A and GC-B are 78% identical, studies have shown that both receptors are desensitized through dephosphorylation in a similar manner (Potter, 1998). In addition, the KHDs of these receptors have shown to be functionally equivalent (Koller *et al*., 1992). Based on these data, LPA-dependent GC-A desensitization may also be linked to increases in intracellular calcium.

Generally, calcium increase is a well established cellular response mediated by LPA receptors (Contos *et al*., 2000), and targeted deletion of LPA_2 in mice results in a significant loss of LPA-induced calcium mobilization (Ishii *et al*., 2004). The former is supported by the fact that LPA is known to mobilize calcium in different cell types (Ishii *et al*., 2004). For instance, increases in calcium by human LPA_2 and LPA_3 expressed in HTC4, Sf9 or RH77 cells have been shown which were mediated by pertussis toxin (PTX)-sensitive G proteins and PLC (Meyer zu Heringdorf, 2004). In addition, LPA has also been reported to mobilize intracellular calcium in the following organs: rat hippocampal neurons, neural retina of chick embryo, human neutrophiles, rat cortical oligodendrocytes and human fibroblasts (Jalink *et al*., 1990; Holtsberg *et al*., 1997; Zhou *et al*., 1999; Itagaki *et al*., 2004; Yu *et al*., 2004; Meyer Zu Heringdorf, 2004).

For several years, PKC was thought to be an obligatory component for inhibiting GC-A and GC-B activity (Jaiswal *et al*, 1988; Marala *et al*., 1993; Crooke and Chang, 1997; Potter and Garbers, 1992, 1994; Potter and Hunter, 2000). Interestingly, the more recent investigations on heterologous desensitization of GC-B do not implicate PKC anymore. Recent studies on vasopressin-, S1P-, and LPA-induced GC-B desensitization in A10 vascular smooth muscle cells, transfected and non-transfected NIH3T3 fibroblast cells have ruled out its involvement (Abbey and Potter, 2002; Abbey-Hosch *et al*., 2004; Potthast *et al*., 2004). Instead, the authors demonstrated the predominant role of a calcium-dependent dephosphorylation in the desensitization process.

Assuming that intracellular calcium mediates LPA-induced desensitization, another intriguing question is how calcium dephosphorylate GC-A. In this context, the role of calcium-binding proteins has been implicated (Abbey and Potter, 2002). Inhibitory effects of calcium on retinal guanylyl cyclases RetGC-1 and RetGC-2 with

a predicted structural topology similar to GC-A and GC-B were reported to be regulated by guanylyl cyclase activator proteins (GCAPS). GCAPS stimulate these receptors at low calcium concentrations, and inhibit them at high calcium concentrations (Olshevskaya *et al*., 2002).

Another protein known to modulate a calcium-dependent cGMP signalling in living neural cells is the visin-like protein-1 (VILIP-1). This protein was observed to have a direct physical interaction with recombinant GC-A and GC-B and native pGCs enriched from rat brains. However, no observations were made regarding the calcium fluctuations which may influence such effect (Braunewell *et al*., 2001; Spilker *et al*., 2002). Based on the above discussion, the observed LPA-dependent desensitization in this study may be proposed as a result of a LPA-induced increases in intracellular calcium which may activate an as yet unknown calcium-binding protein. This in turn may have a direct contact with the catalytic domain thus directly or indirectly mediating receptor desensitization.

Cytomorphological responses to LPA have been reported through its striking effects on actin-based cytoskeleton (Moolenaar *et al*., 2004). In the present study, pretreatments of MA-10 cells with LPA showed dramatic changes in the actin cytoskeleton and induced cell rounding. Budnik *et al*. (2003) also demonstrated morphoregulatory effects of LPA on actin cytoskeleton of luteal cells, an effect which was mimicked by CNF1, a bacterial toxin known to activate small G-proteins of the Rho family. Similar effects were also observed in Swiss mouse embryonic fibroblast 3T3 cells, in which incubations with LPA induced actin stress fiber formation and assembly of focal adhesions in a Rho-dependent manner (Gohla *et al*., 1998). The mechanism of LPA-dependent cytoskeletal morphoregulation is thought to proceed via $G\alpha_{12/13}$ subunits which bind directly to Rho-specific guanine nucleotide exchange factors (GEFs) which then activate Rho. Such an activation is followed by downstream Rho kinase which drives actomyosin-based contractile events i. e., neurite retraction, endothelial tight junction opening and cell rounding (for review, see Moolenaar *et al*., 2004). Considering that GC-A is membrane-bound, LPA-induced cell rounding may directly or indirectly affect its receptor density and/or its cyclase activity.

As observed with ANP-induced desensitization, the LPA-elicited decrease in hormone-dependent GC-A activity was verified to an equal extent each in

experiments with intact cells and in guanylyl cyclase assays with membrane preparations, while basal activities and receptor levels remained unaltered. Thus, the desensitization seems to be entirely explained by reactions directly affecting the receptor hormone-responsiveness. Although experiments with phosphatase inhibitors failed to provide decisive results (see below), receptor dephosphorylation is thought to represent the only currently-conceivably mechanism underlying this desensitization process.

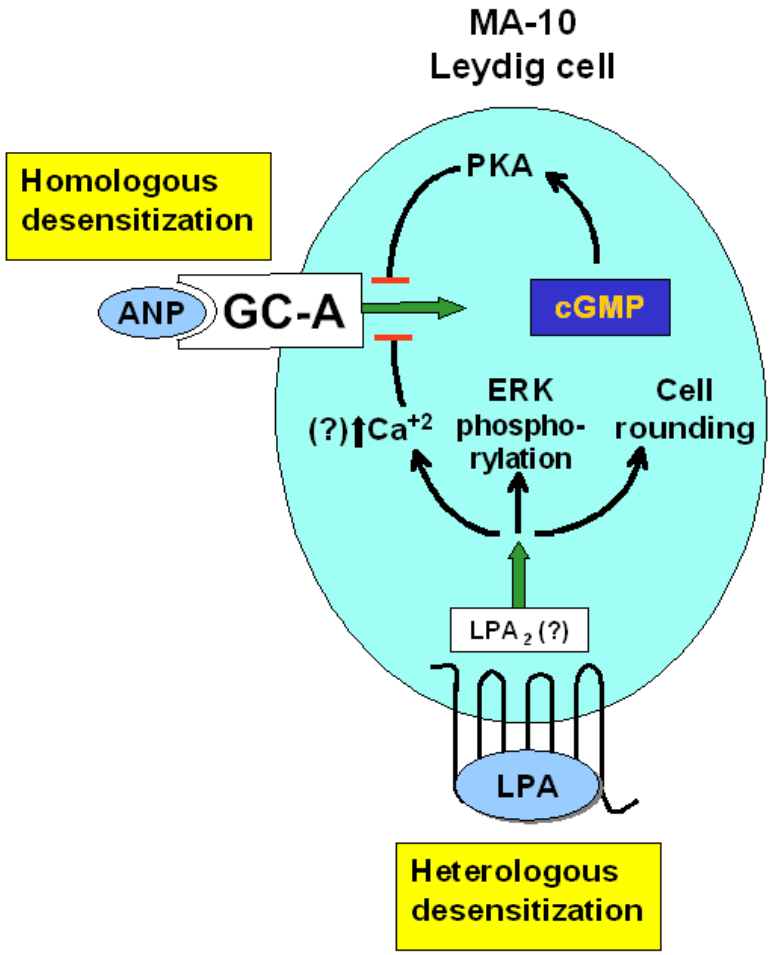

Figure 36: Results obtained with MA-10 cells
Both ANP and LPA are capable of inducing desensitization of GC-A. The ANP-induced (homologous) desensitization is mediated by PKA, presumably by a direct interaction with cGMP. The LPA-induced (heterologous) desensitization is PKA-independent, does not involve the MAP kinase pathway but may be linked to intracellular increases in Ca^{2+}. In addition to GC-A desensitization, LPA elicits ERK phosphorylation and cell rounding, presumably via interaction with type 2 (LPA_2) receptors. ANP does not elicit ERK phosphorylation or cell rounding.

4.1.3 Comparison between homologous and heterologous desensitization

Both the ANP- and LPA-induced effects observed in this study are based on endogenously-expressed signalling components, allowing comparative characterizations in an in vivo-like context. Importantly, the differential sensitivities of ANP- versus LPA-elicited desensitization against PKA inhibition (the former is prevented, the latter is unaffected) provides unequivocal evidence for distinct pathways each. On the other hand, the time-dependency of desensitization in response to both agents was remarkably similar, with maximum effects generated after 30 min each. The only significant difference was that LPA effects (first detectable after 5 min) were earlier recognizable as those elicited by ANP (10 min). Of particular interest, however, was the observation that both agents finally caused equal decreases (by 40% each) in ANP-dependent GC-A activity. On the assumption that receptor dephosphorylation is responsible for GC-A desensitization, this implies an equal extent of dephosphorylation activity each. Considering previous findings that homologous desensitization involves global receptor dephosphorylation, whereas the heterologous process results in the loss of a single phosphate (discussed in Potter and Hunter, 2001), the present results are noteworthy.

In this regard, my observation that different signalling pathways give rise to the same extent of GC-A desensitization, raised a number of interesting questions. In particular, it has to be asked whether the same phosphatase or whether distinct phosphatases might be finally activated in these processes. Unfortunately, experiments carried out with phosphatase inhibitors failed to provide satisfiable results. Desensitization assays (data not shown) in the presence of okadaic acid did not reveal any effects on either ANP- or LPA-induced GC-A desensitization at inhibitor concentrations of 0.1 nM (inhibits PP2A) or 20 nM (inhibits PP2A plus PP1). Analogous experiments performed in the presence of 0.5 µM microcystin-LR (effective on both PP1 and PP2A) demonstrated inhibitory (by 40%) effects on both ANP- and LPA-induced desensitization, but the inhibitor per se was found to reduce ANP-dependent GC-A activity to a similar extent (not shown). Although all of these results could be explained by desensitization processes in which neither PP2A nor PP1 are involved, clear-cut results are thought to require the identification of functionally involved phosphatases. Based on a recent investigation (Bryan and Potter, 2002), a member of the PP2C protein phosphatase family might represent a candidate enzyme.

4.1.4 Potential relevance for Leydig cell physiology

The Leydig cells of the testis act to produce testosterone and were recently shown to exhibit striking neuroendocrine properties (Davidoff *et al*., 2004). Immortalized mouse Leydig tumor "MA-10" cells are gonadotropin-responsive and well established as an experimental model in studies of Leydig cell physiology (Hoelscher and Ascoli, 1996). Gonadotropins, using cAMP-mediated pathways, are the principle regulators of Leydig cell steroidogenesis. However, ANP-induced stimulation of GC-A in Leydig cells has been reported to increase testosterone levels *in vitro* (e. g., Mukhopadhyay *et al*., 1986; Schumacher *et al*., 1992) and *in vivo* (Pandey *et al*., 1999), indicating an additional, modulatory role. In this context, the findings that GC-A becomes desensitized during prolonged exposure to ANP identify a control mechanism by which signal intensity and cellular response can be appropriately attenuated. Such a mechanism could play a regulatory role not only in the adulthood but also during postnatal developmental stages (Müller *et al*., 2004) and in the fetal (El-Gehani *et al*., 2001) testis.

In this study, ANP failed to increase ERK phosphorylation. Since ANP-induced activation of ERK has been functionally linked to anti-hypertrophic effects in neonatal rat ventricular myocytes (Silberbach *et al*., 1999), the present findings suggest that such a pathway is not active in Leydig cells. On the other hand, the present data cannot exclude inhibitory rather than activating effects. In fibroblasts, CNP-induced accumulations of cGMP were found to block activation of the mitogen-activated protein kinase cascade (Chrisman and Garbers, 1999), and several studies revealed analogous effects generated by ANP (Silberbach and Roberts, 2001).

LPA was found to elicit pronounced responses in MA-10 cells. To my knowledge, this is the first report on activities of LPA in this cell type, and the present findings strongly suggest that Leydig cells may represent targets for phospholipid signalling *in vivo*. LPA concentrations effective in the present experiments were similar to those (1 to 5 µM) estimated to be present in human serum (Ishii *et al*., 2004).

The specific effects of LPA on GC-A activity must not necessarily indicate an exclusive or primary cellular role in the context of regulation of steroidogenesis. ANP-induced cGMP in Leydig cells may have other, not yet recognized, functions, e. g., related to the control of cell growth/differentiation (Silberbach and Roberts, 2001).

Most remarkably, the identification of pronounced morphoregulatory activities of LPA in MA-10 cells strongly suggest a significant role in controlling the phenotype/differentiation of these cells. In this context, it has to be noted, that Leydig cells are characterized by the occurrence during postnatal development of three distinct phenotypes, including hypertrophied and so-called spindle-shaped forms. They all derive from the same (vascular) progenitor cell type but at different sites (Davidoff *et al*., 2004), suggesting local environmental effects. Based on the activities observed in this study, it is well conceivable that LPA signalling is functionally involved in regulating such processes as well as under conditions of Leydig cell hyperplasia/tumorigenesis.

4.2 Experiments with αT3-1 cells

Questions that have been specifically addressed in this study were based on the co-expression of GC-A and GC-B in αT3-1 cells. The results obtained demonstrate for the first time (i) interactions (“cross-talks”) between ANP/GC-A and CNP/GC-B signalling at a cellular level and (ii) that the hormone-responsiveness of natriuretic peptide receptors can be enhanced (“sensitization”) rather than reduced (“desensitization”) by signalling molecules acting via plasma membrane receptors. The key findings of the experiments with αT3-1 cells are schematically summarized in Figure 37 (see page 92).

4.2.1 Molecular aspects

Homologous desensitization processes of both natriuretic peptide receptors, GC-A and GC-B, are characterized by an initial generation of cGMP as second messenger. Assuming that this cyclic nucleotide acts as a component of the signalling cascades eliciting receptor desensitization, it was of particular interest to examine whether ANP- and/or CNP-induced effects are specifically directed against their receptors (GC-A, GC-B) or not. Moreover, the proved co-expression and functional activities of the two receptor types in αT3-1 cells raised the physiologically-important question whether (or not) the activities of both signalling systems (ANP/GC-A; CNP/GC-B) can be regulated/controlled via receptor desensitization.

Results of this study revealed highly specific reactions and pathways. For example, GC-A but not GC-B becomes desensitized in response to sustained ligand exposure. And ANP-induced desensitization of GC-A, which is accompanied by

accumulations of cGMP, does not affect the activity of GC-B. Thus, these findings indicate a cellular compartmentation of the two receptors together with other signalling factors, in which (a) cGMP produced in response to ANP selectively mediates desensitization of GC-A and (b) cGMP produced in response to CNP fails to induce analogous effects on GC-B.

Most strikingly, however, and representing an observation of particular potential relevance, pre-incubations of cells with CNP elicited pronounced increases (up to 60%) in the hormone-responsiveness of GC-A. This data strongly confirms the existence of compartmentalized pathways by demonstrating that the same second messenger (cGMP) can induce either a desensitization (when generated by GC-A) or an enhanced activity (when generated by GC-B) at the same receptor protein.

The existence of specialized plasma membrane structures may contribute to compartmentalization of distinct pathways. This include caveolae and lipid rafts which may contain a variety of molecules i. e., GCPRs, adenylyl cyclases, PKCs, nitric oxide synthase (NOS) and other proteins. Although the significance of the association of signalling proteins within the microdomains is not clear, one of the theories is that interactions in such microdomains might compartmentalize, modulate and integrate signal events at the cell surface. (Hur and Kim, 2002).

In agreement with unique pathways, the kinetics of ANP-induced desensitization and CNP-induced sensitization of GC-A are dissimilar. Sensitization rapidly attained maximal values (after 15 min) and declined thereafter, whereas ANP-induced receptor desensitization, like in MA-10 cells, is only partially detectable after 15 min and requires longer treatments for a maximum expression.

As assessed by guanylyl cyclase assays with membrane preparations, the CNP-induced sensitization of GC-A was accounted for by specific increases in hormone responsiveness and not explainable by elevated receptor levels. Although the present data is consistent with a mechanism by which two different pathways (GC-A- versus GC-B–mediated) can elicit opposite effects on the same receptor molecule, I cannot exclude the alternate (but less probable) possibility that subpopulations of GC-A may exist which are differentially affected.

Remarkably, CNP-induced sensitization of GC-A was found to be completely blocked by inhibition of PKA activity, establishing an essential involvement of this

kinase. Reports have implicated PKA as a mediator in gonadotrophs such as in the regulation of Ca^{2+} influx brought about by a forskolin-, 8-bromo-cAMP- and PACAP-38-stimulated cAMP/PKA system which mediates changes in $[Ca^{2+}]i$ (Hezareh *et al*., 1997). Another role of activated PKA in pituitary cells has been shown to cause a slow, but sustained increase in gonadotropin synthesis and secretion in pituitary cell cultures (Gharib *et al*., 1990). Despite of the reported involvement of PKA in pituitary cells, a direct interaction between the enzyme and natriuretic peptide signalling in the same cells has not yet been reported. Whether PKA is part of a signalling cascade finally leading to the activation of a GC-A kinase or whether PKA itself executes GC-A phosphorylation represents an interesting question which remains to be explored. Since the enzyme(s) responsible for GC-A phosphorylation *in vivo* have not yet been defined (Potter and Hunter, 2000), the present findings are thought to be of major future significance.

An enhanced ANP-dependent cGMP production by GC-A, potentially indicating a receptor sensitization, has been reported in three previous studies, where cellular effects of the beta-adrenergic agonist isoproteronol (Thibault *et al*., 1996; Kishimoto *et al*., 1994) or of NaCl-treatments (Katafuchi *et al*., 1992) have been examined. However, all three studies were carried out with cells (adipocytes, vascular smooth muscle cells, vascular endothelial cells) containing large quantities of the natriuretic peptide clearance receptor (NPR-C), raising the possibility of effects induced NPR-C rather than by GC-A itself. In fact, down-regulation of NPR-C levels, which leads to attenuated clearance of ANP, was found to be fully responsible for the increased GC-A activity in one study (Kishimoto *et al*., 1994). In case of NaCl treatments, GC-A sensitization was observed also on membrane preparations, suggesting that the effect cannot be exclusively explained by reductions in NPR-C concentration (Katafuchi *et al*., 1992). Thus, in addition to demonstrate GC-A sensitization by receptor-mediated signalling, the present findings are the first to prove such a reaction in the absence of NPR-C expression.

By receptor crosslinking to ^{125}I-labeled ANP, this study convincingly demonstrates GC-A expression in αT3-1 cells, an issue of debate in some previous publications (McArdle *et al*., 1993, 1994). Although the crosslinking experiments indicated similar plasma membrane levels of GC-A and GC-B, functional studies revealed a markedly higher potency of CNP (as compared to ANP) to stimulate

cGMP production. This suggested that GC-A and GC-B may exist under normal culturing conditions in different activation (sensitization) states, i. e., that GC-A is distinguished by a relatively poor hormone-responsiveness in comparison to that of GC-B. This hypothesis could be circumstantiated by experiments in which (i) the ANP-dependent GC-A activity was strongly up-regulated in response to CNP treatments and (ii) subsequent homologous desensitization processes were much more effective than in cells without prior CNP pre-exposure. Thus, these findings establish that the activities of natriuretic peptide receptors in a cellular context are regulated in a quite complex but highly specific manner.

In contrast to the situation in MA-10 cells, LPA does not promote any alterations in the activity of natriuretic peptide receptors in αT3-1 cells. Since gene expression of the two major LPA receptors (LPA_1 and LPA_2) is clearly detectable, these findings suggest that intracellular pathways capable of mediating effects on GC-A and/or GC-B are not present and/or active in this cell type. Thus, cross-talks between natriuretic peptide and phospholipid signalling do not represent a general but a cell type-specific phenomenon.

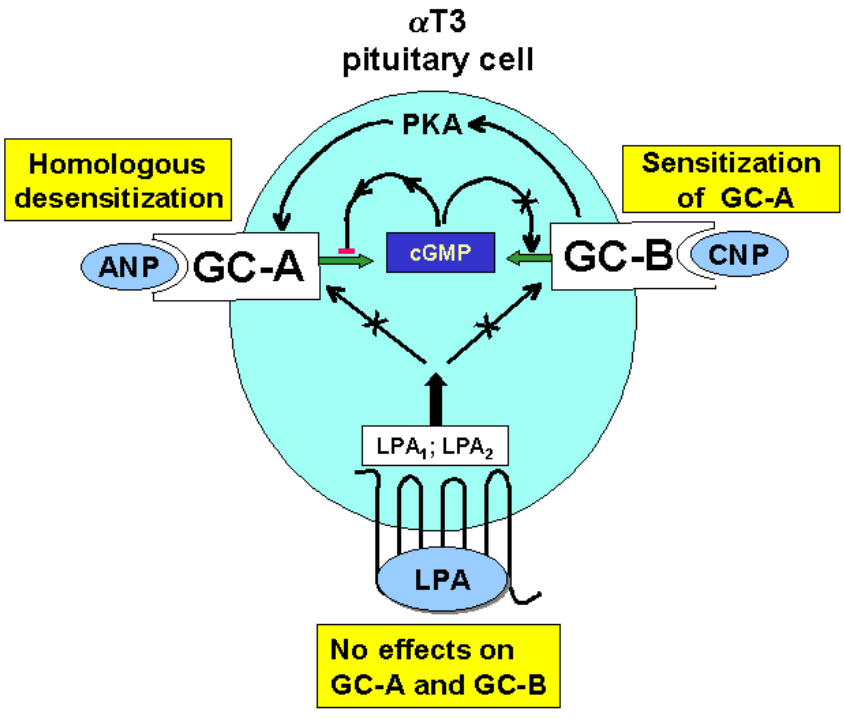

Figure 37: Results obtained with αT3-1 cells

ANP but not CNP induces homologous desensitization of the specific receptors, GC-A and GC-B, respectively. CNP elicits, in a PKA-dependent manner, enhanced activity (sensitization) of GC-A. Despite the gene expression of LPA_1 and LPA_2, LPA does not affect in this cell type the activity of natriuretic peptide receptors.

4.2.2 Physiological aspects

The present findings, identifying intracellular cross-talks between ANP and CNP signalling, are thought to be of a broad significance. Various cell types have been characterized to express both ANP and CNP receptors (McArdle *et al*., 1994; Krause *et al*., 1997; DiCicco-Bloom *et al*., 2004; Jankowski *et al*., 2004), and interactions between the two signalling systems (ANP primarily acts as a hormone, CNP in a paracrine/autocrine manner) could be used to locally modulate cellular responses.

In this regard, the pituitary gland deserves attention. A great number of studies provided information suggesting local functions of both natriuretic peptides in this organ (comprehensively reviewed by Resch *et al*., 1997; Fowkes and McArdle, 2000). The co-expression of GC-A and GC-B in cells of the anterior lobe of the pituitary as well as in the gonadotroph-derived αT3-1 cell line indicates that these cells represent targets for both ANP and CNP actions. Remarkably, the highest tissue concentration of CNP was found in the anterior pituitary (Komatsu *et al*., 1991), and immunocytochemical analyses localized CNP immunoreactivity to gonadotroph cells (McArdle *et al*., 1994). Moreover, it has been shown that αT3-1 cells produce CNP, identified as the mature 22-amino acid form (Fowkes and McArdle, 2000). Thus, there is considerable evidence that CNP in these cells plays an important role as an autocrine and/or paracrine regulator. Functionally, reported data suggests that CNP-induced cGMP accumulations in gonadotropin-releasing hormone (GnRH)-responsive pituitary cells can decrease the GnRH-stimulated gonadotroph secretion (McArdle *et al*., 1994), consistent with a role in the neuroendocrine control of reproduction. Specific functions for ANP in these cells have not yet been explored. The present findings, however, clearly indicate that both CNP and ANP effects are specifically transmitted and identify a further local role for CNP, namely to activate the ANP/GC-A signalling pathway.

Abbreviations

ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5' monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine 3', 5' monophosphate
CNP	C-type natriuretic peptide
DNA	deoxyribonucleic acid
ERK	extracellular-regulated protein kinase
FCS	fetal calf serum
GC	guanylyl cyclase
GC-A	guanylyl cyclase-A
GC-B	guanylyl cyclase-B
GH	gonadotropic hormone
GK I	G kinase I (cGMP-dependent protein kinase I)
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
h	hour
hCG	human choriongonadotropin
HEK	human embryonic kidney cell line
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	half of maximal inhibitory concentration
kDa	kilodalton
LH	luteinizing hormone
LPA	lysophosphatidic acid

LPA_1	lysophosphatidic acid receptor type 1
LPA_2	lysophosphatidic acid receptor type 2
LPA_3	lysophosphatidic acid receptor type 3
LPA_4	lysophosphatidic acid receptor type 4
M	molarity
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated kinase kinase
min	minute
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
PACAP	pituitary adenylate cyclase activating peptide
PACAP-27	27-amino acid form of PACAP
PACAP-38	38-amino acid form of PACAP
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pGC	particulate guanylyl cyclase
PKA	cAMP-dependent protein kinase
PLC	phospholipase C
PLD	phospholipase D
PP	protein phosphatase
PP1	protein phosphatase 1
PP2	protein phosphatase 2
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature

RT-PCR	reverse transcriptase-polymerase chain reaction
S1P	sphingosine-1-phosphate
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TBST	Tris buffered saline-Tween 20
TE	Tris/EDTA buffer
TEMED	N,N,N’,N’-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminoethane
U	unit
UV	ultraviolet
V	volt
v/v	volume per volume
VSMC	vascular smooth muscle cell
w/v	weight per volume

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