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Regulation of vascular smooth muscle cell growth by cyclic nucleotides and cGMP-dependent protein kinase I

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IV. Abbreviations

$[Ca^{2+}]_i$	cytosolic calcium concentration
8-Br-cAMP	8-Bromoadenosine-3', 5'-cyclic monophosphate
8-Br-cGMP	8-Bromoguanosine-3', 5'-cyclic monophosphate
8-Br-PET-cGMP	β-Phenyl-1,N^2-etheno-8-bromoguanosine-3',5'-cyclic monophosphate
8-pCPT-cGMP	8- (-Chlorophenylthio) guanosine-3',5'-cyclic monophosphate
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride
ANP	atrial natriuretic peptide
APS	ammonium persulfate
ATP	adenosine-5'-trisphosphate
bFGF	basic fibroblast growth factor
BK_{Ca}	large-conductance Ca^{2+}-activated K^+ channel
BNP	brain natriuretic peptide
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium
cAK	cAMP-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CFTR	cystic fibrosis transmembrane conductance regulator
cGKI	cGMP-dependent protein kinase
cGMP	cyclic guanosine monophosphat
CNF	cytotoxic necrotizing factor
CNG	cyclic nucleotide-gated
CNP	C-type natriuretic peptide
CRIB	Cdc42-Rac-interacting binding domain of human PAK
ctr	control
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
DETA/NO	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOC	deoxycholate
DTT	1,4-Dithiothreit
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
f.c.	final concentration
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GDI	guanine nucleotide dissociation inhibitor
GPCR	G-protein coupled receptor
GST	gluthathione S-transferase
GTP	guanosine-5'-trisphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT	hypoxanthine-guanine phosphoribosyl transferase
IP_3	inositol-1,4,5-trisphosphate
IP_3R	IP_3 receptor
IPTG	isopropyl-β-D-thiogalactopyranoside

IRAG	IP_3 receptor-associated cGMP-kinase substrate
ko	knockout
LIMK	LIM-kinase
MAPK	p42/44 mitogen activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMLV	moloney murine leukemia virus
MYPT	myosin targeting subunit
NO	nitric oxide
NOS	NO synthase
OD	optical density
PAGE	polyacrylamid gel electrophoresis
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet derived growth factor
pGC	particulate guanylyl cylcase
PI	propidium iodide
PK	proteinase K
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinyliden difluoride
RBD	Rho-binding domain
RGS	regulator of G protein signaling
RLC	regulatory light chain
RNA	ribonucleic acid
ROCK	Rho kinase
Rp-8-Br-PET-cGMPs	Rp-isomer of 8-Br-PET-cGMP
Rp-8-pCPT-cGMPs	Rp-isomer of 8-pCPT-cGMP
RT	room termperature
RT-PCR	reverse transkriptase – polymerase chain reaction
SDS	sodium dodecyl sulfate
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
sGC	soluble guanylyl cylcase
SRF	serum free
TB	toluidine blue
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol
VASP	vasodilator-stimulated phosphoprotein
VSMC	vascular smooth muscle cell
wt	wild-type

A. Introduction

1. cGMP Signaling

Cyclic guanosine monophosphate (cGMP) is an ubiquitous intracellular second messenger in the cardiovascular system. cGMP is generated from guanosine-5'-trisphosphate (GTP) by two types of guanylyl cyclases (GCs) that differ in their cellular location and activation by specific ligands: (1) the particulate GCs (pGCs) present at the plasma membrane, which are activated by natriuretic peptides (NPs) such as atrial (ANP), brain (BNP), and C-type natriuretic peptide (CNP) (Garbers and Lowe, 1994; Kuhn, 2003; Padayatti et al., 2004) and (2) the soluble guanylyl cyclase (sGC) present in the cytosol and activated by nitric oxide (NO) (Gross and Wolin, 1995; Hofmann F, 2004; Padayatti et al., 2004) (Fig. 1).

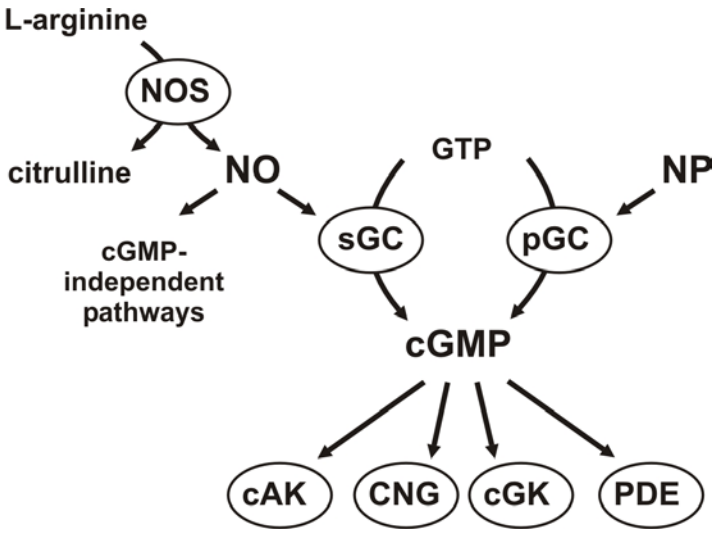


Fig. 1: Generation of cGMP and some downstream targets. For details see text

Almost 30 years ago, Murad and co-workers identified NO as an agent that is released from glycerol trinitrate and other vasodilating compounds, and stimulates cGMP production in crude preparations of GC (Katsuki et al., 1977). Ten years later, several groups showed that NO is produced in biological systems and is identical to endothelium-derived relaxing factor, which decreases vascular tone (Ignarro et al., 1987; Palmer et al., 1987). Since then, it has been shown that NO is a signal molecule of key importance for blood pressure regulation, immune response and learning (Feil et al., 2005a; Feil et al., 2003; Ignarro et al., 1999).

NO is unstable and decomposes within seconds. It is generated by NO synthases (NOS) that catalyze the conversion of the amino acid arginine to citrulline (Loscalzo and Welch, 1995) (Fig. 1). Three different isoforms are known. nNOS (NOS-1) isolated from neurons and eNOS (NOS-3) isolated from endothelia, are constitutively expressed and are dependent on

Ca^{2+}/calmodulin. The third isoform is the “inducible” NOS (iNOS, NOS-2), which is expressed upon stimulation with e.g. cytokines in many different tissues and cell types, such as macrophages, endothelial cells and vascular smooth muscle cells. The iNOS is independent of Ca^{2+} and generates NO for a prolonged period and in higher amounts as compared to the other two isoforms (Michel and Feron, 1997). The generated NO diffuses from its originating cell to surrounding cells, regulating a variety of cellular functions. Identified regulatory mechanisms involving NO include the stimulation of sGC, leading to the generation of cGMP as well as cGMP-independent processes like the production of reactive nitrogen and oxygen radicals (Feil et al., 2005a; Feil et al., 2003; Furchgott and Zawadzki, 1980; Ignarro et al., 1999).

As mentioned above, cGMP can also be generated via activation of pGC by NPs. The NPs constitute a family of polypeptides that regulate mammalian blood volume and blood pressure by effects on the kidney and the systemic vasculature. More recently, the ability of NPs to modulate cell growth, both cell proliferation and cardiomyocyte hypertrophy, has received attention. The biological activities of the NPs are initiated by their binding to cell surface receptors of two types: R1 receptors that contain a cytoplasmic C-terminal guanylyl cyclase domain (NPR-A, NPR-B) and R2 receptors that have no intrinsic cyclase activity (NPR-C). The NPR-C acts as a clearance receptor for NPs and may have additional functions that are cGMP-independent (Silberbach and Roberts, 2001).

In eukaryotic cells, different target proteins have been identified for cGMP: (1) In the retina and the olfactory system cyclic nucleotide-gated channels (CNGs) are opened in response to cGMP (Biel et al., 1998; Biel et al., 1999). (2) cGMP-regulated phosphodiesterases (PDEs) are - among others - important for regulating the cyclic adenosine monophosphate (cAMP) and cGMP level (Rybalkin et al., 2003; Sonnenburg and Beavo, 1994). For example PDE 3 hydrolyzes cAMP and is inhibited by cGMP. This mechanism might be a possible phathway for organic nitrates to mediate their effects by activating cAMP-dependent protein kinase (cAK) (Osinski et al., 2001). (3) cAMP-dependent protein kinase (Worner et al., 2006). (4) cGMP-dependent protein kinases (cGKs). cAMP as well as cGMP is hydrolyzed by PDEs. In many cell types, the main part of cGMP is degraded by the cGMP-activated PDE 5 (Mullershausen et al., 2003; Sonnenburg and Beavo, 1994).

2. cGMP-Dependent Protein Kinases (cGKs)

2.1 Structure, Tissue Distribution, and Function

The discovery of the cGKs more than 30 years ago by Kuo and Greengard (Kuo and Greengard, 1970) and by Hofmann and Sold (Hofmann and Sold, 1972) lead to the view that the cGKs might mediate numerous physiological responses to cGMP, although the cGKs are not the only receptors for cGMP (Pfeifer et al., 1999; Ruth, 1999).

Mammals express two forms of cGKs: a soluble form – cGKI with a molecular weight of 77 kDa, and a membrane bound form – cGKII (87 kDa). The structures of cGKI and cGKII are closely related to each other, but the enzymes differ in their tissue distribution and physiological function. Both belong to the family of serine/threonine kinases and exist as homodimers. Each monomer consists of two functional domains (Fig. 2): A regulatory domain and a catalytic domain. (1) The regulatory domain comprises (a) the amino-terminal leucine zipper that is responsible for the homodimerization, (b) the pseudo-substrate site, an autoinhibitory domain, and (c) the two cGMP binding sites. (2) The catalytic domain includes the MgATP and peptide-binding pockets, and the catalytic center that transfers the γ phosphate from ATP to a serine/threonine residue of the target protein (Hofmann et al., 2000; Hofmann et al., 1992; Pfeifer et al., 1999). The two isoforms cGKIα and cGKIβ (Fig. 2) differ only in their amino-terminal sequence and are generated by two alternatively used promoters from the *prkg1* gene.

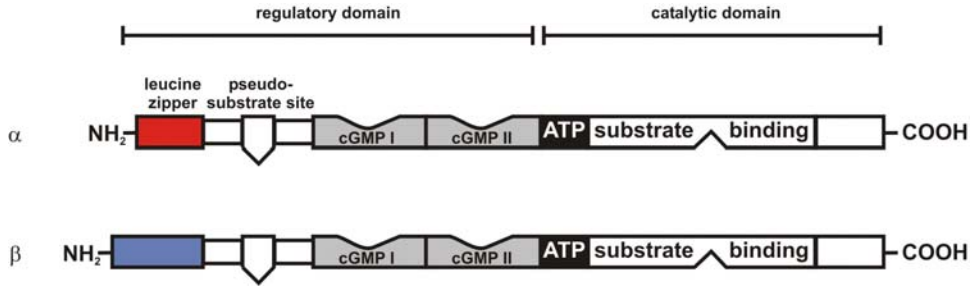


Fig. 2: Structure of cGKI (adopted from Kleppisch (Kleppisch, 1999)). cGKIα and cGKIβ only differ in their amino-terminus (shown in red and blue). For further explanation see text.

cGKI is present in high concentrations (>0.1 µM) in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular endplate and the kidney vasculature (Hofmann et al., 2000). A new study of Feil et al. showed that cGKI is expressed in various regions of the mouse brain and in the retina (Feil et al., 2005b). Low levels have been identified in vascular endothelium, granulocytes, chondrocytes and osteoclasts. The Iα

isoenzyme is mainly found in lung, heart and cerebellum. Together with the Iα isoenzyme, the Iβ isoenzyme is highly expressed in smooth muscle, including uterus, vessels, intestine, trachea and hippocampus (Hofmann et al., 2000; Keilbach et al., 1992). The Iα and Iβ cGKs interact with different proteins through their distinct N-termini (see below) and show different activation kinetics. cGKIβ needs 5-15 times higher concentrations of cGMP to be activated in comparison to cGKIα (Ruth et al., 1991). cGKI is involved in many different processes throughout the whole body. In the nervous system cGKI was found to be involved in the sensitization of nociceptive neurons and distinct forms of synaptic plasticity and learning (Feil et al., 2005a; Kleppisch et al., 2003). Moreover, using cGKI-deficient mice, it could be shown that cGKI inhibits platelet aggregation (Massberg et al., 1999). In the isolated murine heart the negative inotropic effect of NO/cGMP was dependent on cGKI (Feil et al., 2003; Wegener et al., 2002). Most important, the analysis of cGKI-knockout mice demonstrated that cGKI contributes to the cGMP-dependent relaxation of blood vessels, and, therefore, might play an important role in the regulation of blood pressure (Koeppen et al., 2004; Pfeifer et al., 1998; Sausbier et al., 2000). In particular, activation of cGKI was shown to interfere with the vasoconstrictory signaling that leads to an increase of the cytosolic calcium concentration ($[Ca^{2+}]_i$) in vascular smooth muscle cells (VSMCs) (Munzel et al., 2003).

cGKII has been reported to be expressed in several brain nuclei, intestinal mucosa, kidney, chondrocytes and lung (Hofmann et al., 2000; Lohmann et al., 1997). It is anchored at the plasma membrane by myristoylation of the N-terminal glycine residue. cGKII regulates intestinal fluid secretion by phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR), bone growth and renal renin secretion by phosphorylation of unknown proteins (Hofmann et al., 2000; Vaandrager et al., 1998). Furthermore, cGKII was shown to be important for resetting the circardian clock (Oster et al., 2003), and to modulate anxiety-like behavior and neurobehavioral effects of alcohol (Werner et al., 2004).

2.2 cGKI Signaling in the Vascular System

2.2.1 Smooth Muscle Contraction

The contractile state of VSMCs is regulated dynamically by hormonal and neuronal inputs. Contraction and relaxation of VSMCs is initiated by a rise and fall of the $[Ca^{2+}]_i$ (Berridge et al., 2000). Agonists like carbachol, norepinephrine and bradykinin activate G-protein coupled receptors (GPCRs) (G_q and G_{11}). Activation of these GPCRs causes an activation of phospholipase Cβ (PLCβ). The activated PLCβ in turn generates diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) whereas IP_3 binds to the IP_3-receptor (IP_3R) at the endoplasmic reticulum (ER) causing an efflux of Ca^{2+} from the

ER into the cytosol. Moreover, Ca^{2+} can be released from the ER through activation of the ryanodine receptor (Berridge, 2002; Lee, 1997) (Fig. 3).

A second mechanism to increase $[Ca^{2+}]_i$ is via an influx of extracellular Ca^{2+} via voltage-dependent and –independent Ca^{2+} channels (Hofmann et al., 1999; Moosmang et al., 2003; Wegener et al., 2004). The rise in $[Ca^{2+}]_i$ activates the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), which phosphorylates the regulatory myosin light chain (MLC) leading to activation of myosin ATPase, actomyosin cross bridging, and an increase in tension (Fig. 3).

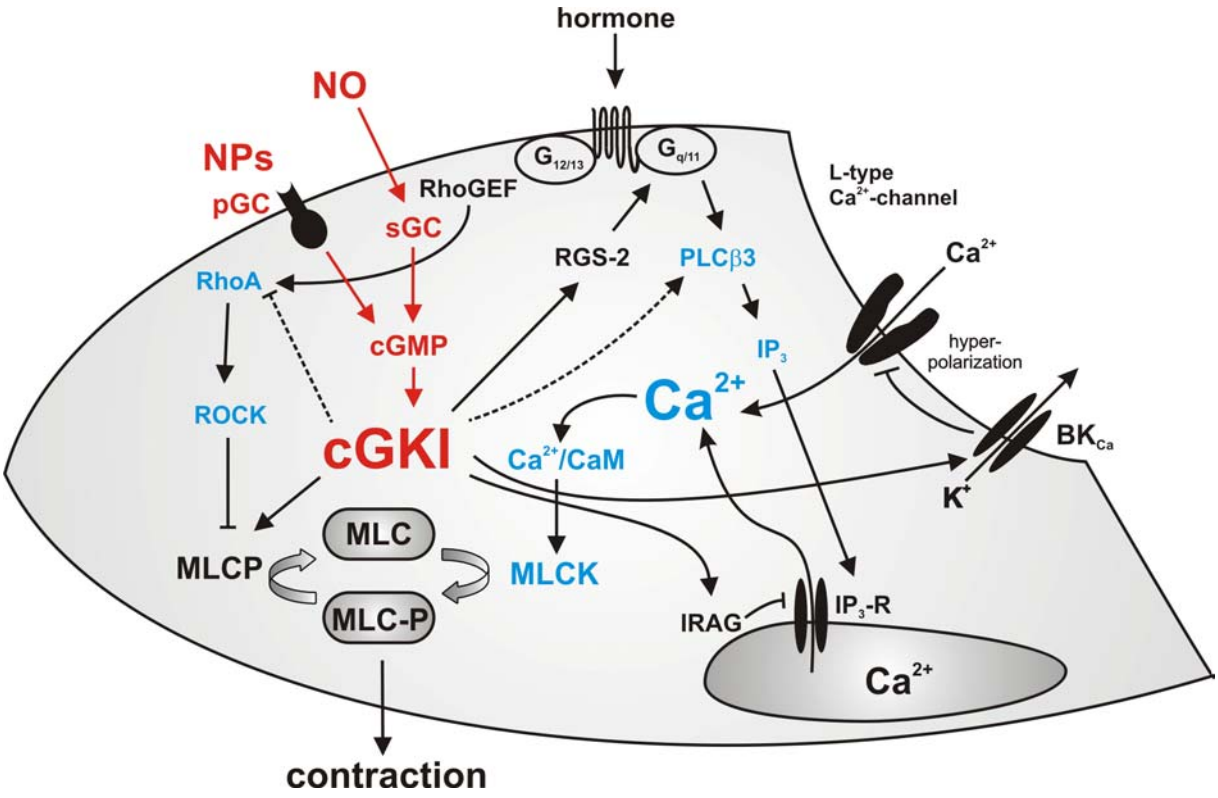


Fig. 3: Contraction and cGMP/cGKI signaling in smooth muscle. Major components for smooth muscle contraction are shown in blue. The activation of cGKI – leading to relaxation of smooth muscle - is shown in red Further explanation see text.

Relaxation occurs when $[Ca^{2+}]_i$ decreases, resulting in inactivation of MLCK and dephosphorylation of the MLC by MLC phosphatase (MLCP) (Hofmann et al., 2006). Furthermore, smooth muscle contractility can also be modulated at constant $[Ca^{2+}]_i$. The Rho/Rho kinase (ROCK) pathway inhibits MLCP activity leading to increased levels of phosphorylated MLCs and tension at a given $[Ca^{2+}]_i$. This process is known as Ca^{2+} sensitization of contraction (Somlyo and Somlyo, 2003) (Fig. 3). Thus, the contractile state of the SMC is determined by the level of MLC phosphorylation, which in turn is regulated by signaling pathways that affect the balance of MLCK and MLCP activity (Hofmann et al., 2006; Ito et al., 2004; Murthy, 2006; Weisbrod et al., 1998).

2.2.2 cGMP/cGKI-Mediated Vasorelaxation

It is well established that cGMP-elevating agents promote smooth muscle relaxation. Recent studies with cGKI-knockout mice have shown that NO can induce the relaxation of vascular smooth muscle by activation of sGCs, production of cGMP, and activation of cGKI (Koeppen et al., 2004; Pfeifer et al., 1998; Sausbier et al., 2000). Furthermore, NO and cGMP may regulate vascular tone by signaling pathways that do not require cGKI (Sausbier et al., 2000; Weisbrod et al., 1998; Worner et al., 2006). cGKI has been shown to catalyze the phosphorylation of a number of physiologically relevant proteins, which adjust the contractile activity of the SMCs, including proteins that regulate free $[Ca^{2+}]_i$, the cytoskeleton and the phosphorylation state of the regulatory light chain (RLC) of smooth muscle myosin (Hofmann et al., 2006; Lincoln et al., 2001).

cGKI inhibits both hormone receptor-triggered and depolarization-induced contraction by several mechanisms (Fig. 3). A major effect is the decrease of $[Ca^{2+}]_i$ (Cornwell and Lincoln, 1989; Pfeifer et al., 1998; Schlossmann et al., 2000). cGKI may attenuate hormone receptor-activated contraction by inhibition of PLCβ activity and IP_3 synthesis, through phosphorylation of the regulator of G protein signaling (RGS) proteins (Tang et al., 2003) or PLCβ (Xia et al., 2001). A specific target for cGKIβ is the IP_3R-associated cGMP kinase substrate (IRAG), which has been identified in a complex with cGKIβ and the smooth muscle IP_3 receptor type 1. Phosphorylation of IRAG by cGKIβ inhibits IP_3 induced Ca^{2+} release from intracellular stores (Geiselhoringer et al., 2004; Schlossmann et al., 2000). An additional target for cGKI is the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel. Direct phosphorylation or indirect regulation of a protein phosphatase leads to increased open probability of the channel, which results in a hyperpolarization of the membrane and closing of voltage-dependent Ca^{2+} channels, thereby reducing Ca^{2+} influx (Sausbier et al., 2000). The cGKI may also activate the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) by phosphorylation of the SERCA regulator phospholamban (Koller et al., 2003), which causes an increased reuptake of cytosolic Ca^{2+} into the ER.

Another target for cGKI-mediated vasorelaxation is the MLCP. cGKIα interacts with the myosin targeting subunit (MYPT1) and activates MLCP. Increased MLCP activity reduces the level of phosphorylated MLC and causes relaxation at constant $[Ca^{2+}]_i$ (Surks et al., 1999; Wooldridge et al., 2004). Further mechanisms that might be involved in cGKI-dependent smooth muscle relaxation might affect RhoA (Sauzeau et al., 2000) and telokin (Walker et al., 2001) (Fig. 3). It has been described that cGKI phosphorylates and thereby inactivates RhoA-GTP (Sauzeau et al., 2000; Sawada et al., 2001). This phosphorylation reduces the activity of ROCK, which causes increased MLCP activity and subsequent decreased contractility.

2.2.3 Isoform Specificity

As mentioned above, cGKIα and cGKIβ differ in their amino terminus. This leads to different substrate recognition (Lohmann et al., 1997). It was shown that cGKIα specifically binds to MYPT of myosin phosphatase (MLCP) (Surks et al., 1999). Schlossmann et al. could show that activation of cGKIβ in transfected COS cells blocked the Ca^{2+} release from the ER through phosphorylation of the substrate protein IRAG (Schlossmann et al., 2000), whereas Feil et al. revealed that in primary VSMCs, the hormone-induced Ca^{2+} release was blocked by activation of cGKIα (Feil et al., 2002). Whether these isoform specific functions of cGKIα and cGKIβ are relevant for the regulation of smooth muscle tone *in vivo* could not be clarified. To learn more about the isoform specificity of cGKIα and cGKIβ in smooth muscle, Weber generated mice that specifically express either the Iα or the Iβ isoform selectively in smooth muscle (Weber, 2006).

While the role of cGKI in smooth muscle relaxation is quite well understood (Hofmann et al., 2000; Lincoln et al., 2001; Pfeifer et al., 1999), its function in vascular remodeling, phenotypic modulation and proliferation is discussed controversially (Feil et al., 2003; Hofmann et al., 2006; Lincoln et al., 2001).

3. Vascular Remodeling and Phenotypic Modulation

Proliferation, dedifferentiation, and migration of VSMCs contributes to the formation of vascular diseases like hypertension and atherosclerosis (Owens et al., 2004; Ross, 1999). The analysis of transgenic mice showed that NO can both promote and inhibit pathological vascular remodeling (Chen et al., 2001; Detmers et al., 2000).

Regardless of the origin of the intimal cells during the response to injury, it is well established that VSMCs, which represent the major cell type present in the vessel wall, acquire the capacity to proliferate and synthesize extracellular matrix (ECM) proteins (Lincoln et al., 2001; Owens et al., 2004). One key process in vascular remodeling is the phenotypic modulation of VSMCs from contractile to proliferating/dedifferentiated (synthetic) cells. The synthetic phenotype of VSMCs - associated with the activation of cell proliferation and characteristic morphological changes to elongated cells with a “hill-and-valley” growth pattern - is also acquired during the *in vitro* culturing of the cells. Therefore, cultured VSMCs have become an accepted model for examining mechanisms of phenotypic modulation (Chamley-Campbell et al., 1979). Some studies with cultured VSMCs suggest that cGKI is a key factor regulating the establishment of a contractile-like phenotype and migration (Eigenthaler et al., 1999; Lincoln et al., 2001). cGMP/cGKI signaling regulates VSMC contractility, but also influences the phenotype. It was reported that both RhoA-dependent Ca^{2+}-sensitization of the contractile apparatus and actin cytoskeleton organization - e.g. stress fiber formation - in vascular smooth muscle are inhibited by cGMP through cGKI-mediated phosphorylation of RhoA (Sauzeau et al., 2000).

Furthermore, phenotypic modulation might also be associated with a loss of cGKI expression during prolonged culture (Boerth et al., 1997; Dey et al., 1998) or in response to inflammatory cytokines (Browner et al., 2004b). In line with these findings Anderson et al. (Anderson et al., 2000) could show that cGKI expression decreases in coronary artery in pig in response to injury. This is associated with a loss of calponin expression, a marker protein for the contractile phenotype. Other studies analyzing cGMP signaling suggest an inhibitory role for cGMP signaling on VSMC growth (Garg and Hassid, 1989). Direct activation of sGC using YC-1 caused an increased cGMP level, reduced proliferation of VSMCs and reduced arterial neointima formation following experimental balloon injury (Tulis et al., 2002). Furthermore, Sinnaeve et al. could show in a balloon-injured rat carotid artery model that sGC gene transfer – and subsequent stimulation with Molsidomine - leads to an increase in the cGMP level and a subsequent reduction in neointima formation (Sinnaeve et al., 2001). In a successive study, using the same injury model in porcine coronary arteries, they delivered constitutively active cGK by adenoviral gene transfer, which also resulted in a reduced neointimal area. In contrast, transfection with full-length cGKIβ had no effect on neointimal

area (Sinnaeve et al., 2002). In summary, the above mentioned studies propose a growth inhibitory effect of cGMP(/cGKI) signaling and in consequence a vasculo-protective function of this pathway.

In contrast to the vasculo-protective model of cGMP/cGKI signaling, the analysis of atherosclerosis in control and smooth muscle-specific cGKI-knockout animals – on an ApoE-deficient background - revealed a reduced lesion area in cGKI-deficient mice, indicating that endogenous smooth muscle cGKI promotes atherogenesis *in vivo* (Wolfsgruber et al., 2003). In line with these findings, the analysis of primary VSMCs from wild-type and cGKI-deficient animals revealed that activation of cGKI stimulates the growth of these cells (Wolfsgruber et al., 2003). cGKI might modulate cell growth through effects on cell adhesion, migration, proliferation and apoptosis (Brown et al., 1999; Pollman et al., 1996; Smolenski et al., 2000; Wolfsgruber et al., 2003).

Phenotypic Modulation: Rho/ROCK Signaling and Adhesion

The phenotype of VSMCs is dependent on the cytoskeleton. It is well known that for the formation of stress fibers RhoA/ROCK signaling is important (Ridley and Hall, 1992; Rottner et al., 1999; Worth et al., 2004). RhoA stimulates actomyosin-based contractility through its downstream target ROCK, and this is required for stress fiber formation in cultured cells. ROCKs control the formation of stress fibers by inactivating MLCP (Kimura et al., 1996).

As mentioned earlier cGKI relaxes smooth muscle, in part by inhibiting Ca^{2+}-sensitization via inhibition of RhoA/ROCK signaling. cGKI activates MLCP by phosphorylation of MYPT at Ser695, thereby blocking RhoA/ROCK signaling (Surks et al., 1999). Furthermore, cGKI has been described to inhibit RhoA by phosphorylating it at Ser188. Phosphorylation of RhoA at Ser188 prevents the translocation of RhoA to the membrane and causes a stabilization of the protein in the cytosol bound to the guanine nucleotide-dissociation inhibitor (GDI) (Murthy, 2006; Sauzeau et al., 2000; Sawada et al., 2001). Inhibition of RhoA by phosphorylation has been described to cause a reduction of stress fibers *in vitro* (Sauzeau et al., 2000). The ends of stress fibers are anchored to adhesion plaques, specialized sites that attach the ventral plasma membrane to the extracellular matrix. Clustered within an adhesion plaque are integral membrane proteins, the integrins. Actin filaments of the stress fibers are attached to integrins through adapter proteins, including α-actinin and vinculin. Many other proteins like actin-binding proteins, kinases, and membrane-binding proteins are also localized in cell adhesion plaques, although their precise functions are still unknown.

The integrins are a large class of cell surface receptors that bind different components of the extracellular matrix, thereby playing an essential role for adhesion. Integrins are heterodimers of α and β subunits, and the ligand-binding site is composed of parts of both

chains. In mammals, at least 20 integrin heterodimers, comprised of 14 types of α subunits and 8 types of β subunits, are known. A single β chain can interact with multiple α chains, forming integrins that bind different ligands. Even though integrins are present on the cell surface, they may require activation in order to bind their ligand and thus to anchor the cell to the extracellular matrix or to another cell.

4. Pharmacological and Genetic Analysis of cGKI Function

The pharmacological analysis of cultured cells with the use of different agonists and antagonists and the identification of cGKI substrate proteins suggested multiple and sometimes contradictory cellular functions and mechanisms of cGKI-mediated signaling (Hofmann et al., 2000; Lincoln et al., 2001; Lohmann et al., 1997; Pfeifer et al., 1999). However, our understanding of the significance of cGKI as mediator of NO/cGMP signaling *in vivo* is only at the beginning. The analysis of which cellular functions are dependent on cGKI is complex for several reasons: (1) Several receptors have to be considered as potential mediators of cGMP effects; (2) cGKI expression might be lost during passaging (Cornwell et al., 1994b); (3) many studies were performed with transfected cells that overexpressed cGKs at levels that may not represent physiological conditions; (4) the value of a number of “specific” cGK inhibitors may be limited (Burkhardt et al., 2000).

To study the (patho)physiological roles of cGKs *in vivo*, conventional (Pfeifer et al., 1998) and conditional (Wegener et al., 2002; Wolfsgruber et al., 2003) cGKI knockout mice were generated. These mice provide a useful tool for analyzing cGKI functions *in vivo* and *in vitro*. By comparing wild-type with cGKI-deficient cells, a clear interpretation of the data is possible.

5. Aim of this Work

The aim of this work was to elcuidate the effects of cyclic nucleotide signaling on the growth of VSMCs. To reveal whether cGKI is indeed growth-promoting or an inhibitor of VSMC growth, the properties of primary and subcultured VSMCs isolated from wild-type and cGKI-deficient mice were compared. In addition, to study a possible cross-talk of cGMP and cAMP signaling, cells were also treated with 8-Br-cAMP to activate cAK, a known inhibitor of growth. The strategy to compare the effects of drugs in wild-type and cGKI-deficient cells also allowed for a validation of the specificity of several frequently used “cGKI-specific” agonists and antagonists. The major aim of this study was to decipher the molecular mechanisms of VSMC growth regulation via the cGMP/cGKI signaling pathway. To this end, the effects of cGKI on proliferation, apoptosis, and cell adhesion were studied in primary VSMCs.

B. Materials and Methods

1. Materials

If not mentioned otherwise, all used reagents and chemicals were purchased from Roth, Invitrogen or Sigma. All cGKI agonists and antagonists were purchased from Biolog. DETA/NO, Y27632, and U-46619 were purchased from Alexis. H1152 was supplied from Calbiochem.

2. Mouse Breeding and Genotyping

A conditional cGKI allele (L2) was obtained by flanking exon 10 with loxP sites. Excision of exon 10 from the L2 allele by Cre-mediated recombination of the *lox*P sites produced an L-allele (Wegener et al., 2002). Homozygous $cGKI^{L-/L-}$ mice did not express cGKI protein and were phenotypically indistinguishable from a cGKI-deficient mouse line reported previously (Pfeifer et al., 1998). Mice were bred on a SV129 background. Mice used in this work were generated from heterozygous $cGKI^{+/L-}$ mice.

2.1 Tail Tip Biopsy

For genotyping 2 mm of mouse tail tip biopsy material from 10-14 day old animals was used. Tips were incubated over night at 55°C in 50 µl proteinase K (PK) working solution (Tab. 2). Next, samples were centrifuged at 18,000 xg for 1 min at room temperature (RT). The supernatant was transferred into a clean polymerase chain reaction (PCR) test tube. Remaining PK activity was inactivated by heating the samples to 95°C for 15 min. In general, the DNA solution was stored at –20°C until the genotyping PCR was performed on 1 µl of the samples.

TE buffer

	stock	final concentration (f.c.)
TrisHCl, pH 8.0	1 M	0.1 M
EDTA pH 8.0	0.5 M	10 mM

Tab. 1: TE buffer.

PK working solution		
	stock	f.c.
PK	50 mg/ml	1 mg/ml in 1x TE-buffer
Taq DNA Polymerase buffer	10x	1x

Tab. 2: PK working solution.

2.2 PCR

The PCR is an enzymatically method to amplify defined DNA sequences *in vitro*. For the amplification of the isolated DNA from the mouse tail biopsy, a DNA polymerase (Taq; Promega), primer (Tab. 4), the four deoxynucleotide triphosphates (dNTPs), and the template DNA are needed (Tab. 5).

10x PCR buffer		
	stock	f.c.
KCl	1 M	500 mM
Tris/HCl pH 8.0	1 M	100 mM
$MgCl_2$	1 M	15 mM
dNTPs	100 mM	2 mM

Tab. 3: 10x PCR-buffer.

Primer for cGKI genotyping	
Primer	*Sequence*
RF53	5´-cct ggc tgt gat ttc act cca-3´
RF118	5´-aaa tta taa ctt gtc aaa ttc ttg-3´
RF125	5´-gtc aag tga cca cta tg-3´

Tab. 4: Primer for cGKI genotyping.

PCR reaction			
DNA	tail biopsy		1 µl
PCR buffer	10x	with dNTPs	2.5 µl
Primer	25 µM	0.25 µl each	0.75 µl
Taq DNA polymerase	5 U/µl		0.25 µl
H_2O			20.5 µl
			25 µl

Tab. 5: PCR reaction.

The PCR is a cyclic process and involves three steps carried out in the same test tube at different temperatures:

Standard PCR conditions

Initial denaturation	94°C, 5 min	
Denaturation	94°C, 15 sec	35x
Annealing	55°C, 30 sec	
Elongation	72°C, 30 sec	
Final elongation	72°C, 5 min	

Amplification was performed in a Biometra Thermocycler. PCR fragements of tial biopsy DNA were diluted with 6x DNA loading dye (Tab. 6) and subjected to agarose gel electrophoresis.

2.3 Agarose Gel Electrophoresis (Sambrook, 1989)

Nucleic acids possess a negative charge due to their sugar-phosphate backbone. Due to this property, nucleic acids move to the anode in an electric field. DNA fragments are resolved according to their mass and conformation. Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. Through intercalation of ethidium bromide nucleotide fragments are made visible under UV-light. To compare fragment length, a 1kb DNA ladder was used (Gibco-BRL).

6x DNA loading dye		
	stock	f.c.
Ficoll Typ400		18% (w/v)
EDTA, pH 8.0	0.5 M	0.12 M
10x TBE	10x	6x
Bromphenol Blue	50 mg/ml	0.1% (w/v)
Xylencyanol FF	50 mg/ml	0.1% (w/v)

Tab. 6: 6x DNA loading dye.

10x TBE gel buffer	
Tris/HCl	0.9 M
EDTA, pH 8.0	20 mM
Boric acid	0.9 M

Tab. 7: 10x TBE gel buffer.

DNA electrophoresis standard	
1kb DNA ladder (1 µg/µl)	100 µl
6x DNA loading dye	1 ml
10x TE buffer	0.6 ml
Add 6 ml H_2O	

Tab. 8: DNA electrophoresis standard.

In general, the agarose (SeaKem LE Agarose; Biozym) concentration in the gel was 1-2% (w/v) in 1x TBE gel buffer. Gel solutions were heated in a microwave oven before ethidium bromide (10 mg/ml) was added (f.c. was 0.5 µg/ml). The electrophoresis was performed in 1x TBE buffer at 150 V for 30 min depending on the size of the separated fragments. Figure 4 shows a typical result for the genotyping of offspirngs that were bred from heterozygous $cGKI^{+/L-}$ mice.

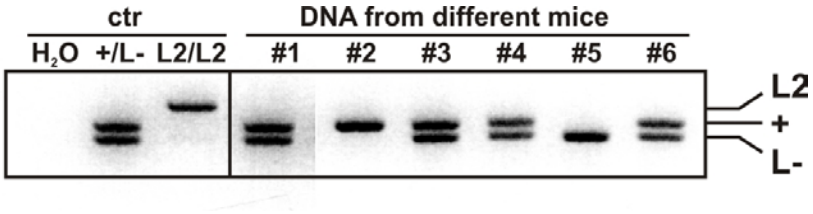


Fig. 4: Representative mouse genotyping PCR of cGKI. Three primer are used in one PCR reaction to generate the L- (RF53 + RF118 – 250bp), the + (RF53 + RF 125 – 284bp) or the L2 band (R F53 + RF125 – 338bp). Mice numbers 1, 3, 4, and 6 are heterozygous, mouse 2 is a wild-type (+/+) and mouse 5 is a cGKI-knockout (L-/L-).

3. Cell Culture

For primary VSMC culture, thoracacic aortae were obtained from mice aged three to eight weeks. For cell culture several aortae are pooled. Cells were grown in culture medium (Tab. 10). Murine subcultured VSMCs were generated by subsequent passaging. Rat VSMCs (generated from aortic media and intima) and human aortic, umbilical vein and neointimal VSMCs were a generous gift of Dr. W. Erl (Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, LMU). Rat cells were incubated in DMEM:F12 (Gibco) supplemented with 10% FCS and human VSMCs were cultured in smooth muscle cell growth medium 2 (Promocell) with an additional 10% FCS. All VSMCs were cultured at 37°C with 6% CO_2.

3.1 VSMC Preparation

Reagents

- nominated Ca^{2+}-free medium
- Culture medium
- Phosphate buffered saline (PBS)
- Trypan blue (Trypan blue solution 0.4%)
- Collagenase, Hyaluronidase, Papain (Sigma)
- DTT, BSA

Nominal Ca^{2+}-free medium			
	M [g/mol]	conc. [mM]	amount [g]
Na-Glutamate	169.1	85	14.37
NaCl	58.44	60	3.5
HEPES	238.3	10	2.38
KCl	74.56	5.6	0.42
$MgCl_2 \bullet 6H_2O$	203.3	1	0.20
Add 1 l H_2O	**pH 7.4**	**autoclave**	

Tab. 9: Ca^{2+}-free medium.

Culture medium for murine VSMCs			
	stock	volume [ml]	f.c.
Dulbecco's modified eagle medium (DMEM)		500 ml	
Fetal Calf Serum (FCS)		50 ml	10%
Penicillin / Streptomycin (Pen/Strep)	1000 U/ml / 1000 µg/ml	5 ml	1%

Tab. 10: Culture medium for murine VSMCs. All ingredients were purchased from Gibco.

PBS, pH 7.4	
	f.c.
NaCl	135 mM
KCl	3 mM
$Na_2HPO_4 \bullet 2H_2O$	8 mM
KH_2PO_4	2 mM

Tab. 11: PBS.

(Enzyme-) Stocks	
	conc. [mg/ml Ca^{2+}-free medium]
Papain (P4762 – Sigma)	7
Sigma Blend Collagenase (C-7926 – Sigma)	10
Hyaluronidase (H-3506 - Sigma)	10
BSA	100
DTT	100

Tab. 12: Enzyme stocks.

Enzyme working solution A			**Enzyme working solution B**		
	vol [µl]	f.c.		vol [µl]	f.c.
Papain	100	0.7 mg/ml	Hyaluronidase	100	1 mg/ml
BSA	10	1 mg/ml	Collagenase	100	1 mg/ml
DTT	10	1 mg/ml	BSA	10	1 mg/ml
Add 1 ml Ca^{2+}-free medium			**Add 1 ml Ca^{2+}-free medium**		

Tab. 13: Enzyme working solutions.

Enzyme working solutions were filtered sterile. For up to eight aorta 1 ml of enzyme working solution A and B was used (Tab. 13). Aortae were digested in a 1.5 ml reagent cap at 37°C. For the digestion, aortae were dissected from the mice and washed in 1x PBS (Tab. 11). The vessels were cleaned of adjacent fatty tissue and blood was removed. Aortae were treated 40-45 min with enzyme working solution A. Afterwards, aortae were centrifuged for 2 min at 300 xg. Solution A was removed and solution B (prewarmed) was added. The incubation time of the pre-digested aortae with enzyme working solution B varied from 10-20 min (depending on age of the mice and quality of enzymes). For a high yield of cells it is important to triturate the solution several times with a 1 ml pipette tip. The digestion reaction was stopped with 10 ml of culture medium. Cells were centrifuged at 900 rpm (Hettich ROTANTA/AP) for 7 min. The cell pellet was resuspended in an appropriate[1] volume of culture medium for counting in a Haemacytometer. Viability was controled by Trypan Blue exclusion. Therefore the Trypan blue solution (0.4%) was diluted 1:10 in cell suspension.

[1] To determine to celll number, the cells should have a density of ~1x10^6 cells/ml. Based on a yield of ~0.4x10^6 cells/aorta, a digest of 10 aortae should be resuspendend in 4 ml of culture medium.

3.2 Passaging of VSMCs

Reagents

- Culture medium
- Trypsin-EDTA (Gibco) (10x solution)
- PBS

Cells were passaged at a confluence of approximately 80-90%. Cells were washed twice with prewarmed PBS to remove serum components. 1 ml of 1x Trypsin (diluted 1:10 in PBS) was applied per culture dish (55 cm^2). Trypsin digestion was performed at 37°C. Trypsinization was stopped by adding 5 ml culture medium when most cells were detatched. For primary cells, this process may take up to 30 min whereas highly passaged cells detach within a couple of minutes. Additional 5 ml of culture medium were added to rinse the plate again. Cells were centrifuged at 900 rpm (Hettich ROTANTA/AP) for 7 min and cell number was determined using a Heamacytometer (see 3.1). Cells were replated at a density of 5,000 cells/well of a 96 well plate for growth assays or 200,000 cells/10 cm plate for further passaging.

4. Immuncytochemistry

Cells were seeded on glass cover slides in a 24 well plate at a cell density of 100,000 cells/well. After two to three days of growth, cells were washed twice with PBS and fixed for 10 min in 3.7% formaline in PBS. Afterwards, cells were permeablized with ice cold (-20°C) acetone for 5 min and washed with 1% BSA in PBS. Unspecific binding sites were blocked with 5% serum in PBS for 10 min. For staining, 30 µl of 1:100 diluted primary antibody was pipetted on parafilm and the glass slides were turned upside down on the drop for 30 min. After three successive washing steps (5 min each), cells were labelled with the secondary fluorescent conjugated antibody. Therefore, the antibody was diluted 1:200 in PBS and the glass slides were again turned upside down on 30 µl of antibody solution for 30 min.

For F-actin staining, cells were stained with Rhodamine-Phalloidine (Invitrogen) for 20 min. Therefore, the Rhodamine-Phalloidine was diluted 1:200 in PBS and the glass slides were turned upside down on 30 µl of staining solution. For double labeling of the cells with an antiboday and staining for F-actin, the Rhodamine-Phalloidine was applied in parallel with the secondary antibody. For further information on the used antibodies see B.10.

After staining, the cells were embedded in Moviol (Calbiochem) with p-phenylendiamine (Sigma) as anti-fading substance or Permaflour (BeckmanCoulter) with Hoechst dye (H33258, Sigma) to stain the nuclei on microscope slides. Pictures were taken with either a

confocal microscope (Leica TCS NT) or a fluorescence microscope (Zeiss). The stained cells were kept at 4°C for up to three months.

5. Cell-based Assays

5.1 Analysis of Apoptosis by Flow Cytometry

Reagents: human Annexin V FITC Kit ; Bender MedSystems: Cat. No BMS306FI
Labeled Annexin V can be used to detect phosphatidylserine on the outer leaflet of the cell membrane using flow cytometry. Presentation of Annexin V on the surface of the cell is an indicator of early apoptosis.
For the analysis of apoptosis of freshly isolated primary VSMCs, cells were held in suspension in culture medium at a cell density of $0.5x10^6$ cells/ml +/- 1 mM 8-Br-cGMP for up to 22 hours. For each time point, a sample of 100,000 cells was taken und stained for Annexin V. Before the samples were subjected to flow cytometry, cells were stained with propidium iodide[2] (PI). Flow cytometry was performed using a FACS Calibur (Becton Dickinson). Of each sample 10,000 cells were counted. Data was analyzed with Cell Quest Pro (4.62). Annexin V positive and PI negative cells were defined as apoptotic cells and used for statistical analysis.

5.2 Integrin Analysis by Flow Cytometry

FACS buffer	
PBS, pH 7.4	500 ml
FCS	5%
NaN_3	0.02%

Tab. 14: FACS buffer.

All antibodies used for the analysis of integrins were purchased from Biolegend. To validate integrin presentation on the cell surface and control conditions and in response to 1 mM 8-Br-cGMP, cells were held in suspension in a falcon tube or in a 96 well round bottom plate for 24 hours. 150,000 cells were used per sample. The staining of the cells as well as the analysis by flow cytometry was performed in FACS buffer (Tab. 14). Cells were stained for β_1 and β_3 integrins (5 µg/ml antibody each). For detection, a secondary FITC-labelled antibody

[2] PI is also used as marker for apoptotic and necrotic cells. The dye intercalates into the DNA. This can only happen when the plasma membrane is not intact anymore.

was used (1.25 µg/ml). Dead cells were excluded by PI staining. 10,000 cells per sample were counted. Data was analyzed with Cell Quest Pro (4.62).

5.3 Growth Assays

CellFix		
	stock	volume [ml]
Formaldehyde	37%	27
Glutaraldehyde	25%	4
Add 500 ml PBS, pH 7,4		

Tab. 15: Cell Fix.

For all growth assays with murine VSMCs, cells were cultured for three days in a 96 well plate with 20,000 cells/well for primary cells, and 5,000 cells/well for passaged cells. Subcultured rat and human cells were cultured at different cell densities and measured between day two to four. The growth, respectively the cell number was determined by using the MTS assay (Promega) according to manufacturer's protocol. The MTS assay is based on the activity of cell metabolism. The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product. This is an indirect measure for the cell number. The second assay applied was the Toluidine blue (TB) assay. This assay determines the cell quantity by staining the cells in each well, which can be used as indirect measure for the number of cells. The TB assay was performed subsequently to the MTS assay, using the same cells. Briefly, cells were washed once with serum-free medium. 100 µl serum-free medium were added to each well followed by 20 µl of MTS solution. OD_{495} was measured after 30 and 60 min. Subsequent to the MTS assay, cells were washed twice with PBS at room temperature. Afterwards cells were fixed and stained for 10 min at room temperature. Therefore, 0.5% TB (Sigma) was dissolved in icecold CellFix (Tab. 15). After fixation and staining, the cells were washed five times with PBS to remove excess staining. To determine the number of cells, the TB stained cell membranes were destained by adding 100 µl 1% SDS in water to each well for 5 min, resulting in a blue coloured supernatant. The OD was measured at 620 nm with a plate reader (Titertek Multiscan MCC/340).

Integrin Blocking Assay

The assay was performed according to the growth assay. Integrin blocking antibodies were added at different concentrations to the cells before seeding them, to block adhesion. For antibody concentrations see B.10.

6. Protein Analysis

6.1 Generation of Protein Extracts

SDS protein lysis buffer		
	stock	f.c.
TrisHCl, pH 8.0	1 M	21 mM
SDS	10%	0.7%
β-Mercaptoethanol		1.7%
PMSF	100 mM	0.2 mM

Tab. 16: SDS protein lysis buffer.

For the generation of protein extract, VSMCs that have previously been washed twice with PBS were lysed by adding 150 µl SDS protein lysis buffer (Tab. 16) to each well in a 6 well plate. After lysis the extracts were heated to 95°C for 5 min. Protein extracts were stored at –20°C.

6.2 Determination of Protein Concentration

The protein concentration was determined using a protein assay kit from Sigma (Cat. No. P5656) according to the manufacturer's protocol. The kit uses protein determination according to Lowry and is based on the following principle: An alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple colour when the phenol reagent is added. Absorbance was read at 750 nm. The protein concentration was determined from a BSA calibration curve.

Because the lysis buffer for the generation of protein extracts (Tab. 16) disturbs the concentration measurement, the protein was precipitated before determining the concentration. All necessary reagents were supplied within the kit.

6.3 Protein Precipitation (Wessel and Flugge, 1984)

4x TrisHCl/SDS, pH 6.8	
Tris	6 g
SDS	0.4 g
Add 100 ml H_2O, pH 6.8, filter sterile	

Tab. 17: 4x TrisHCl/SDS, pH 6.8.

6x SDS sample buffer	
4xTrisHCl/SDS, pH 6.8	7 ml
Glycerol	3.6 g
SDS	1 g
1,4-Dithiothreit (DTT)	0.93 g
Bromphenol blue	1.2 mg

Tab. 18: 6x SDS sample buffer.

The amount of protein to be precipitated should not exceed 400 µg. The precipitation takes place at room temperature. 150 µl sample volume were mixed with 600 µl of methanol and 150 µl chloroform. Subsequently 450 µl water were added to each tube and the samples were mixed. Tubes were centrifuged at 18,000 xg for 2 min. The upper phase was carefully removed without disturbing the pellet in the interphase. 450 µl of methanol were added and samples were centrifuged at 18,000 xg for 2 min. The supernatant was discarded and the pellet was air dried at RT. The pellet was resuspended in an appropriate volume of 1x SDS sample buffer to obtain the desired concentration (Tab. 18). In general the samples were adjusted to 2 µg/µl.

6.4 Western Blot

Westernblot was performed according to standard procedures. The following chemicals and buffers were used for SDS-PAGE:

- Molecular weight standards (See-Blue®, See Blue Plus2®; Invitrogen)
- Polyvinyliden difluoride (PVDF) membrane (Millipore, Immobilin-P)
- Tween
- Milk powder
- ECL western blotting analysis system (Amersham)

4x TrisHCl/SDS, pH 8.8	
Tris	18.2 g
SDS	0.4 g
Add 100 ml H_2O, pH 8.8, filter sterile	

Tab. 19: 4x TrisHCl/SDS, pH 8.8.

Separating gel			
Stock solutions	Final acrylamide concentration in the separation gel		
	8%	**10%**	**12%**
30% acrylamide/ 0.8% bisacrylamide	4 ml	5 ml	6 ml
4x TrisHCl/SDS, pH8.8	3.75 ml	3.75 ml	3.75 ml
H_2O	7.25 ml	6.25 ml	5.25 ml
Amonium persulfate (APS)	50 µl	50 µl	50 µl
Temed	10 µl	10 µl	10 µl

Tab. 20: Separating gel.

Stacking gel	
30% acrylamide/ 0.8% bisacrylamide	0.65 ml
4x TrisHCl/SDS, pH6.8	1.25 ml
H_2O	3.05 ml
APS	12.5 µl
Temed	5 µl

Tab. 21: Stacking gel.

10x SDS electrophoresis buffer	
Tris/HCl, pH 8.3	250 mM
Glycin	1.92 M
SDS	1% (w/v)

Tab. 22: 10x SDS electrophoresis buffer.

Transfer buffers			
	Anode I, pH 10.4	**Anode II, pH 10.4**	**Cathode, pH 7.6**
	f.c.	f.c.	f.c.
TrisHCl	0.3 M	20 mM	20 mM
Methanol	20%	20%	20%
6-Aminocaproic acid	-	-	40 mM

Tab. 23: Transfer buffers for semi-dry blotting.

10x TBS, pH 8.2	
Tris/HCl	50 mM
NaCl	750 mM
Methanol	20%

Tab. 24: 10x Tris buffered saline (TBS).

Samples of 10-30 µg protein were loaded on a gel. This amount resembles about ½ of the protein extract generated from cells that were grown in one well of a 6 well plate. Therefore cells were lysed with an appropriate volume of SDS lysis buffer (~150 µl). The lysate was precipitated and resuspended in an appropriate volume of SDS sample buffer (~40 µl/well of a 6 well plate). To increase the amount of protein extract, several wells were pooled.

The samples were heated at 95°C for 5 min before loading. Proteins were separated by their molecular weight using denaturing SDS polyacrylamide gel electrophoresis. Next, the separated proteins were transferred (blotted) to a PVDF membrane using a semi-dry transfer chamber. The transfer unit is composed of two closely spaced electrodes separated by filter papers, saturated with transfer buffer, including the gel and a PVDF membrane. The following setup was used for blotting:

Anode plate, 3x filter papers saturated with anode transfer buffer I (Tab. 23), 2x filter papers saturated with anode transfer buffer II, PVDF membrane soaked in 100% methanol and saturated with anode transfer buffer II, gel, 5x filter papers saturated with cathode transfer buffer, and cathode plate. The transfer was performed for 1 h with 50 mA per gel.

To block unspesific binding sites, the membrane was blocked with 5% milk powder in 1x TBS-T (TBS + 0.1% Tween) for 1 hour at room temperature. After blocking the membrane was washed three times in 1x TBS-T and afterwards incubated with the primary antibody solution over night at 4°C. After three additional washing steps, the membrane was incubated with a horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature. The secondary antibody was prepared freshly every time needed (1:2000 in 1% milk powder diluted in 1x TBS-T). For detection of the proteins that were recoginzed by the antibodies, the enhanced chemiluminescent (ECL) method was used. The detection is based on the peroxidase-catalyzed oxidation of the chemiluminescent substrate luminol. 1 ml of a 1:1 mixture of the detection solutions A and B was used for each membrane. Following exposure of the soaked membrane to a X-ray film the protein antigen was visualized as a band. A molecular weight standard containing proteins of known size provided information about the molecular weight of the protein.

6.5 Phosphorylation of VASP

VASP was originally identified as a substrate for both cGK and cAK. Three phosphorylation sites on VASP have been identified, Ser157, Ser239 and Thr278. Ser239 is described to be the preferential phosphorylation site for cGK, whereas Ser157 is described to be the preferential phosphorylation site for cAK (Butt et al., 1994a). For phosphorylation of VASP, 100,000 cells/well were seeded in a 6 well culture plate. Cells were grown to 80-90% confluence and serum starved for 48 hours. Afterwards cells were treated with different compounds for 30 min. Cells were lysed followed by western analysis. For detection of VASP, an antibody that recognizes total VASP was used. The purified protein migrates as a 46 kDa protein in SDS/PAGE. After phosphorylation by cGK or cAK at Ser157, VASP migrates in SDS/PAGE as a 50 kDa protein (Halbrugge and Walter, 1989). Using this antibody only provides information about phosphorylation at Ser157. Whether VASP is also phosphorylated at Ser239 remains unknown. The termination of VASP as “p-VASP” and “VASP” in the results part only refers to the phosphorylation at Ser157.

7. Analysis of small GTPases

For the Rac and Rho pulldown, different GST-tagged constructs coding either for a Rac- or a Rho-binding domain were used.

7.1 Expression and Evaluation of RBD- and PAK-CRIB-Constructs

Bacteria were grown in Luria-Bertani (LB-)Medium[3] in a shaker at 37°C. Bacteria were grown in the presence of ampicillin (f.c. 100 µg/ml), to select for the bacteria which express the ampicillin resistance gene. The resistance gene is encoded on the plasmid, that also encodes for the GST fusion construct. All used constructs were sequenced and plasmid DNA was isolated. The following sequencing primer were used for all constructs:

forward: 5’- ggc tgg caa gcc acg ttt ggt g -3’

reverse: 5’- cgg gag ctg cat gtg tca gag g-3’

[3] for 1l LB-Medium: Trypton 10 g, Yeast extract 5 g and NaCl 5 g

GST-C21 ((Reid et al., 1996), generous gift of John Collard)

- Rho-binding domain (RBD) from Rhotekin (270bp)
- vector: pGEX-3X (Amersham), inserted between BamHI and EcoRI restriction sites
- host: BL21(DE3) (E. coli) (Stratagene)
- resistance: ampicillin

The GST-C21 construct was sent on a filter. The obtained plasmid was transfected in BL21 (DE3) by electroporation.

GST-RBD (Rho-binding domain) (gift of S. Linder)

- RBD from ROCK2 (Rho kinase) (m-RNA of bos Taurus) gi|31241963 (bp2821-3228)
- sequence homology with murine sequence >90%
- vector: pGEX-2T (Amersham), inserted into the BamHI restriction site
- host: DH5α (E. coli)
- resistance: ampicillin

GST-PAK-CRIB ((Sander et al., 1998), gift of John Collard)

- CRIB from human PAK (Cdc42-Rac-interacting binding domain of human p21-activacted kinase 1B, mRNA) gi|3265159 (350bp)
- vector: pGEX-2TK (Amersham), inserted between BamHI and EcoRI restriction sites
- host: DH5α (E. coli)
- resistance: ampicillin

7.2 DNA Isolation

For isolation of plasmid DNA commercially available kits were used (Miniprep Kit – Peqlab; Plasmid Maxi Kit – Qiagen). All used buffers and solutions were supplied by the manufacturer. The principle is alkaline lysis (Birnboim and Doly, 1979) of the cells and subsequent purification of DNA by chromatography.

DNA concentration was determined by photometry at a wavelength of 260 nm. An OD of 1 at 260 nm and 1cm cuvette thickness resembles 50 µg/ml dsDNA. The purity of the isolated DNA can be checked by the ratio of OD_{260}/OD_{280}[4]. This ratio should be higher than 1.7.

[4] protein concentration is determined at this wavelength

7.3 Transformation

Bacteria were transformed by electroporation. 150 µl of electro-competent cells were transfected with ~10 ng plasmid DNA. During the whole procedure the cells were kept on ice. For transfection, the mixture was pipetted in a cuvette. The electroporation was performed using a GenePulser™ (BioRad) and Puls Controller (BioRad) with the indicated instrument settings:

Voltage 2.5 kV

Capacity 25 µF

Resistance 200 Ω

The average time constant should be 4.5 ms. After transfection, bacteria were incubated for 1 hour at 37°C in 1 ml LB-medium in a shaker. Afterwards cells were plated on LB-plates with ampicillin selection over night at 37°C. The attained clones were analyzed with the use of restriction enzymes and by sequencing.

7.4 Fragmentation of DNA with the Use of Restriction Enzymes

Restriction endonucleases (also called restriction enzymes) are bacterial enzymes that cut nucleic acids specifically according to their sequence[5]. These enzymes recognize and cut a palindromic sequence. In this work BamHI and EcoRI (NEB) have been used to check GST-constructs. For each reaction 20 U[6] enzyme were added to 1 µg DNA. Restriction reactions were accomplished at 37°C for 1-2 hours. The reactions were applied to agarose gel electrophoresis to check the length of the fragments.

7.5 Sequencing

Sequencing was performed according to Sanger (Sanger et al., 1977). DNA fragments are generated by “Terminator Cycle Sequencing”. The integration of fluorenscence labelled dideoxynucleotides (ddNTPS) leads to cycle termination and the generation of fragments with different length. The sequence was analyzed with an ABI Prism™ Sequence-Analyzer (Perkin-Elmer Applied Biosystems). With the use of a computer, the sequence was calculated from the raw data (Multiscan 100Es, Sony).

[5] Restriction enzymes are traditionally classified into three types on the basis of subunit composition, cleavage position, sequence specificity and cofactor requirements. Type II enzymes cut DNA at defined positions close to or within their recognition sequences. They produce discrete restriction fragments and distinct gel banding patterns.

[6] 1U = amount of enzyme to cut 1 µg DNA/h under optimal conditions

"Terminator Cycle Sequencing"

Reaction

DNA (50-500 ng)	2 µl
Ready Reaction Mix (RRM)[7]	4 µl
Primer (0,8 pmol/µl)	4 µl
H_2O	ad 20 µl

Synthesis of the labelled DNA fragments:

Denaturation	95°C, 2 min	
Denaturation	95°C, 30 sec	25x
Annealing	50°C, 40 sec	
Polymerisation	60°C, 4 min	

For the purification of fragments, "Centri Sep Spin columns" (Perkin-Elmer Applied Biosystems) were used according to the manufacturer's instruction. The dried DNA was resuspended in 20 µl "Template Suppression Reagent" (TSR) (Perkin-Elmer Applied Biosystems). Before sequencing, the sample was denatured at 95°C for three min.

7.6 Rho- and Rac-Pulldown

7.6.1 Expression of Constructs and Isolation of GST-Fusion Proteins

For a pulldown experiment 500 ml LB-Medium were used to express the GST-fusion construct. Expression of GST-fusion constructs was induced with IPTG[8] (f.c. 0.5 mM) when bacteria reached an OD_{600nm} of 0.5. Afterwards bacteria were incubated for further 3 hours at 37°C in a shaker. Bacteria were sedimented at 5,000 rpm (CENTRIKON H-401, Hermle) for 5 min at 4°C. Cells were pooled in ice cold PBS. Cells were sedimented by centrifugation for 15 min at 4°C. Bacteria were resuspended in 10 ml lysisbuffer (Tab. 25) and subsequently lysed by sonication (6x for 15 seconds). TritonX100 was added to a final concentration of 1% and the lysate was put for 30 min on ice on a shaker. Afterwards the lysate was centrifuged at 4°C for 20 min at 20,000 rpm in an ultracentrifuge (L80, Beckmann).

[7] Contains AmpliTaq DNA polymerase, buffer, dNTPs, fluorescence labelled ddNTPs (Perkin Elmer Applied Biosystems)

[8] Isopropyl-beta-D-thiogalactopyranoside

Lysis buffer for bacteria		
	stock	f.c.
TrisHCl, pH 7.4	1 M	50 mM
NaCl	4 M	150 mM
$MgCl_2$	1 M	5 mM
DTT		1 mM
Aprotinin	5 mg/ml	5 µg/ml
Leupeptin[9]	5 mg/ml	5 µg/ml
AEBSF	0.2 M	0.5 mM

Tab. 25: Lysis buffer for bacteria.

Coomassie staining solution		
	stock	solution
Coomassie (ServaBlueR)		1.5 g
Methanol	100%	455 ml
Acetic acid	100%	80 ml
Add 2 l H_2O		

Tab. 26: Coomassie staining solution.

Destaining solution		
	stock	f.c.
Methanol	100%	10%
Isoporpanol	100%	10%
Acetic acid	100%	10%

Tab. 27: Destaining solution.

Next, the Rho or Rac binding domain, expressed as GST-fusion protein, was linked to glutathione sepharose beads 4B (Amersham). Therefore, about 1 ml beads (enough for ~5 samples of VSMC protein extract) were washed twice with cold PBS (~5 ml) at 4°C and once with lysis buffer (~5 ml) for bacteria. Subsequently, the sepharose beads were incubated with the bacterial lysate, including the GST-fusion protein (~11 ml), for 1 hour on ice on a shaker. After five washing steps with washing buffer (~ 5 ml each) (Tab. 29), the beads were stored on ice over night. Whether the binding of the GST-fusion protein to the glutathione sepharose beads was succesfull, was checked with SDS-PAGE. Therefore a sample of the beads (~15 µl) was diluted in 6x SDS sample buffer and heated to 95°C for 5 min. Subsequent to SDS-PAGE the gel was stained with Coomassie (Tab. 26) over night at RT. The next day, excess staining of the gel was removed by incubating the gel for 1 hour in destaining solution (Tab. 27). One band, representing the GST-fusion protein (~40-50 kDa, depending on the size of

[9] in 50% EtOH

the binding domain linked to the 26 kDa GST) demonstrated that the beads were coupled to the GST-fusion protein.

Native lysis buffer for VSMCs

	stock	f.c.
TrisHCl, pH 7.4	1 M	50 mM
NaCl	4 M	500 mM
$MgCl_2$	1 M	10 mM
TritonX100	10%	1%
DOC	10%	0.5%
SDS	10%	0.1%
EGTA	0.1 M[10]	5 mM
Aprotinin	5 mg/ml	10 µg/ml
Leupeptin[11]	5 mg/ml	10 µg/ml
AEBSF	0.2 M	0.5 mM

Tab. 28: Native lysis buffer for VSMCs.

Pulldown washing buffer

	stock	f.c.
TrisHCl, pH 7.4	1 M	50 mM
NaCl	4 M	150 mM
$MgCl_2$	1 M	10 mM
TritonX100	10%	1%
EGTA	0.1 M	5 mM
Aprotinin	5 mg/ml	10 µg/ml
Leupeptin	5 mg/ml	10 µg/ml
AEBSF	0.2 M	0.5 mM

Tab. 29: Pulldown washing buffer.

7.6.2 Pulldown

To activate Rac respectively RhoA - as positive control - cells were treated with 2 µg/ml cytotoxic necrotizing factor (CNF) for three hours prior lysis. Primary VSMCs were grown for three days on 55 cm^2 culture dishes. 2x 10^6 cells were seeded per culture dish (ctr 4x; 8-Br-cGMP 2x). Subcultured VSMCs were used close to confluence (two culture dishes (55 cm^2) per condition). Cells were harvested on ice with ice cold native lysis buffer (Tab. 28) and a cell scraper in a final lysis buffer volume of 500 µl per experimental condition. Lysates were centrifuged at 4°C for 10 min at 18,000 xg. A small fraction (~30 µl) of the supernatant was removed for determination of total Rac or RhoA and the residual VSMC lysate (~ 450µl) was pooled with the GST-fusion protein loaded beads (~ 200 µl). Beads were incubated at 4°C on a shaker for 1 hour. The supernatant was removed and beads were washed three times

[10] EGTA dissolved in 220 mM NaOH – pH 7.8
[11] in 50% EtOH

with ice cold washing buffer (Tab. 29). An appropriate volume (~45 µl) of 2x SDS sample buffer (Tab. 16) was added. Samples were boiled at 95°C for 10 min.
As an alternative method, another assay was established to analyze the activity of RhoA (G-Lisa – Cytoskeleton). The assay was used according to the manufacturer's manual and is based on the same principle as a "traditional" pulldown assay, but is described to be more sensitive. As positive control, cells were treated with the thromboxane mimetic U-46619 at a concentration of 2 µM for 5 min prior lysis.

8. RNA Isolation and Reverse Transcriptase (RT-) PCR

VSMCs were harvested after three days of growth. Cells were washed twice with PBS. Afterwards, an appropriate volume of Trizol (peqGold RNAPure, Peqlab) was added to the cells (~2 ml per 55 cm^2 culture dish). It took about 5 min to lyse the cells. 1 ml of lysed cells was added to each 1.8 ml cap. Subsequently 200 µl of chloroform was added to each tube. Caps were mixed and left for 5 min at room temperature. Samples were centrifuged for 5 min at 18,000 xg at room temperature. The upper phase (aqueous ~600 µl) was transferred to a new cap. 500 µl of isopropanol were added and the samples were vortexed. RNA was precipitated overnight at 4°C.
Samples were sedimented at 18,000 xg at 4°C for 10 min. The pellets were washed twice with 75% ethanol. The pellets were air dried and resuspended in an appropriate volume (~25 µl per 55 cm^2 plate) of DEPC treated water for 10 min at 55°C.

Determination of RNA Concentration

A quartz cuvette was used and the OD at 260 nm was measured. The concentration was calculated as follows: RNA [µg/µl] = 40 µg/ml[12] x OD_{260} x dilution factor / 1000
After determining the RNA concentration an DNAse digest was performed. Therefore 20 U DNAse (Roche) were added to each preparation (Stock 10 units/µl – diluted 1:5 in RT buffer (Tab. 3)). Then the caps were put in a Thermocycler: 30 min at 37°C – 5 min at 80°C – 4°C. After digestion, the RNA concentration was adjusted to 0.1 µg/µl.

[12] 40 µg/ml RNA = 1 OD_{260}

RT-PCR Reaction

RT-PCR reaction			
RNA	0.5 µg		5 µl
RT-Buffer	10x PCR-Buffer	with dNTPs	5 µl
Primer A + B	25 µM	0.5 µl each	1 µl
QG 197 / 198[13]	*depending on Primer A+ B*		
H_2O			34 µl
			45 µl

Tab. 30: RT-PCR reaction.

Reverse Transcription

Denaturation	94°C, 5 min
Slowly cool down to 50°C	0,07°C/sec
Add 5 µl MMLV-RT (10 U/µl) (Invitrogen)	50°C, 20 min (Stock 200 U/µl)
Add 5µl Taq-Polymerase (0.5U/µl) (Promega)	(Stock 5U/µl)

PCR (for DNA-fragments up to 1kb)

Initial Denaturation	94°C, 5min	
Denaturation	94°C, 10sec	} 35x
Annealing	55°C, 30sec	
Polymerisation	72°C, 30sec	
Final Polymerisation	72°C, 5min	

For fragments up to 500 bp the polymerisation step at 72°C for 30 seconds can be omitted. After the RT-PCR has been performed, the samples were mixed with 6x DNA loading dye and loaded on a gel. The bands were detected under UV-light and analyzed with GelDoc2000 and QuantityOne4.1.1. (BioRad).

To verify that the RNA is free of DNA contamination, a test reaction is performed. Two similar reactions were prepared. In each reaction four primer were added: two which amplify a fragment coded by one exon and two primer that amplify a fragment that is encoded on two exons (intron flanking). The two exon fragment can only be amplified when the exons have been spliced. Otherwise the fragment is too long and cannot be amplified during polymerisation. Then the reverse transcription is started with and without reverse transcriptase. Figure 5 gives a representative example. The upper band is generated with two primer (PW1 and PW2, primer for a sequence in ferritin light chain (FLC)), which amplify a fragment in an intron-free sequence. The lower band is generated with two other primer (QG197 and QG198, primer for an intron flanking sequence in HPRT), which amplify a fragment that can only be generated upon correct splicing. Consequently, as shown in Figure

[13] QG 197 / 198 – Primer for Hypoxanthine-Guanine Phosphoribosyl Transferase (HPRT) – serves as internal standard – Intron flanking

5, in the presence of RT (+RT), a band is visible for FLC in DNA and RNA, whereas a band for HPRT is only visible in the RNA. In the absence of RT (-RT) no cDNA can be generated, resulting in a band for ferritin light chain only in the DNA samples. For pirmer sequences see B.11.

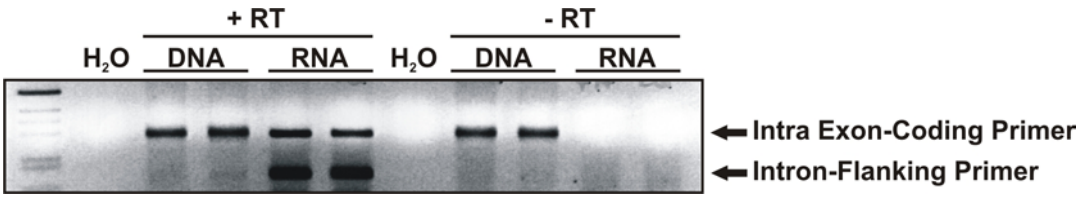


Fig. 5: Check for RNA purity. The tested RNA is free of DNA contamination. Further explanation see text.

9. Statistical analysis

The OriginPro-Software, version 6.1, was used for statistical analysis. Data are presented as mean±SEM. In order to compare groups an unpaired Student's t-test was used. To analyize the results obtained from apoptosis, a two-way anova was applied. Therefore, the Prism-Software, version 4.0, was used.

For analysis of p-MLC fluorescence digital images of fluorescence, labled VSMCs were analyzed by ImageJ 1.34s. The total fluorescence was determined by multiplying the cell area with the mean of the signal intensitiy for each cell.

10. Antibodies

Primary antibodies					
	Distributor	**Host**	**[kDa]**	**Dilution**	**Application**
cGKI	Prof. F. Hofmann	rabbit	~75	1:200	western blot
RhoA	Santa Cruz	mouse	~24	1:1000	western blot
Rac	Upstate	mouse	~21	1:1000	western blot
β-Actin	Abcam	rabbit	~45	1:50.000	western blot
VASP	Alexis	rabbit	~45	1:4000	western blot
Vinculin	Santa Cruz	goat	~114	1:500	western blot
FAK	Cell Signaling	rabbit	~125	1:500	western blot
phospho-FAK	Chemicon	mouse	~125	1:1000	western blot
phospho $RhoA_{Ser188}$	Calbiochem	rabbit	~24	1:1000	western blot
p38-MAPK	Cell Signaling	rabbit	~38	1:1000	western blot
Isotype control	BioLegend	a. hamster		5 µg/ml	FACS
β_1 Integrin (CD29)	BioLegend	a. hamster		25 µg/ml	blocking
				5 µg/ml	FACS
β_3 Integrin (CD61)	BioLegend	a. hamster		25 µg/ml	blocking
				5 µg/ml	FACS
AKT	Cell Signaling	rabbit	~60	1:1000	western blot
MLC_{20}	Cell Signaling	rabbit	~18	1:1000	western blot
Phospho-MLC_{Ser19}	Cell Signaling	mouse	~18	1:1000	western blot
RhoE	Upstate	mouse	~29		western blot
pan-MAPK	Cell Signaling	rabbit	~42/44	1:1000	western blot
Cofilin	Cytoskeleton	rabbit	~19	1:1000	western blot
phospho-Cofilin	Cell Signaling	rabbit	~19	1:2000	western blot
LIMK 1	Cell Signaling	rabbit	~70	1:1000	western blot
LIMK 2	Cell Signaling	rabbit	~70	1:1000	western blot

Secondary antibodies			
	Distributor	**Dilution**	**Application**
α armenian hamster FITC conjugated	Biolegend	1,25 µg/ml	FACS
α mouse HRP conjugated	Santa Cruz	1:2000	western blot
α rabbit HRP conjugated	Cell Siganling	1:2000	western blot
α goat HRP conjugated	Santa Cruz	1:2000	western blot

11. Oligonucleotides for RT-PCR

Primer

	Name		Sequence
FLC	PW1	for	5′- TTG CAC CTG CGG GCC TCC TAC –3′
	PW2	rev	5′- ACC CAG GGC ATG CAG ATC CAA –3′
HPRT	QG197	for	5′- GTA ATG ATC AGT CAA CGG GGG AC –3′
	QG198	rev	5′- CCA GCA AGC TTG CAA CCT TAA CCA –3′
Integrin β_1	PW35	for	5′- AAT GGC GTG TGC AGG TGT CGT -3′
	PW36	rev	5′- TGC AAT GGG TCA CAG GAT CGA -3′
Integrin β_3	PW37	for	5′- CCG ACA ACC ACT ACT CTG CCT -3′
	PW38	rev	5′- ACG CAC CTT GGC CTC GAT ACT -3′
RhoA	PW55	for	5′- ATG TGG CGG ATA TCG AGG TGG –3′
	PW56	rev	5′- AAC TCC CGT CTC GTG TGC TCG –3′
RhoE	RL1	for	5′- GTC CCA ATA CCA AGA TGC TGT -3′
	RL2	rev	5′- TGC TGA GAG TTC TGG TCT GC -3′
Vinculin	PW21	for	5′- CTC CCT TGG AGA GAT AGC TGC -3′
	PW22	rev	5′- TTA GCT GGT CTA CAC GGT CAC -3′

All oligonucleotides were purchased from MWG Biotech.

Primer

	Name	Conc.	Conc. - HPRT	Fragment Length
FLC	PW1 + PW2	6.25 µM	25 µM	286 bp
Integrin β_1	PW35 + PW36	6.25 µM	50 µM	364 bp
Integrin β_3	PW37 + PW38	6.25 µM	25 µM	471 bp
RhoA	PW55 + PW56	25 µM	25 µM	286 bp
RhoE	RL1 + RL2	25 µM	25 µM	314 bp
Vinculin	PW21 + PW22	12.5 µM	25 µM	356 bp
HPRT	QG197 + QG198	co-amplified as internal standard		177 bp

C. Results

A previous *in vivo* analysis of endogenous cGKI function in atherosclerosis (Wolfsgruber et al., 2003) suggested a proatherogenic role of VSMC cGKI. Treatment of primary (unpassaged) VSMCs from wild-type (wt) and cGKI-deficient (ko) animals with 8-Br-cGMP caused a strong increase in the growth of wild-type cells (Fig. 6). This effect of 8-Br-cGMP was absent in cGKI-deficient cells, demonstrating that the increase in growth is mediated via cGKI (Fig. 6). These results indicate that primary culture can be used to study the cGMP/cGKI-mediated growth-promoting mechanisms.

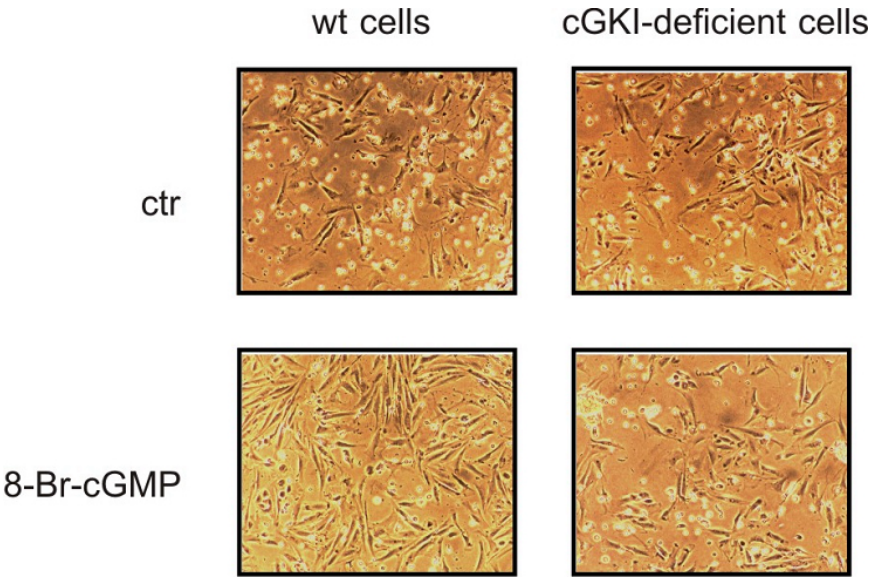


Fig. 6: Primary VSMCs, three days after seeding. Wt and ko cells were treated with 1mM 8-Br-cGMP.

Many studies that examined the growth effects of cGKI on VSMCs were performed with cell lines or highly passaged VSMCs in combination with different cGKI agonists and “specific” cGKI inhibitors. In contrast, the comparison of primary wild-type and cGKI-deficient cells allows clear cut interpretation of data.

Another kinase that has major impact on VSMCs growth is the cAK. It is well established that cAK inhibits VSMC growth (Bonisch et al., 1998; Bornfeldt and Krebs, 1999; Chen et al., 2004; Osinski et al., 2001). In addition, there is increasing evidence that high levels of cGMP might lead to cross-activation of cAK (Bonisch et al., 1998; Bornfeldt and Krebs, 1999; Chen et al., 2004; Osinski et al., 2001; Worner et al., 2006) (Fig. 7).

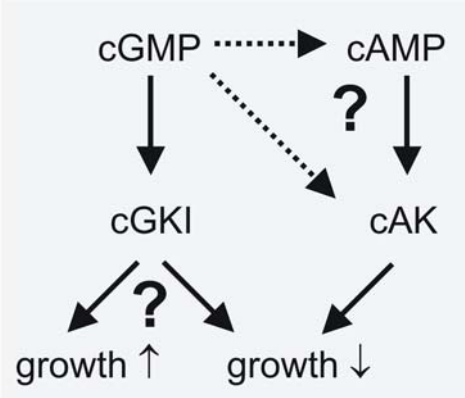


Fig. 7: The effect of cGMP/cGKI signaling on VSMC growth is not well understood.

The effect of cGKI on VSMC growth is not well understood (Fig. 7). As already mentioned in the introduction, cGMP signaling in general, but also cGMP signaling via cGKI is thought to have an anti-proliferative effect. To clarify these opposing functions of cGKI, we used the model of primary VSMCs from wild-type and cGKI-deficient cells to investigate cGKI functions and to validate different cGKI "agonists" and "antagonists".

To measure the growth of VSMCs in response to different stimuli, two growth assays were established. The first assay used was the MTS assay (Promega) (B.5.3), which is commonly used and based on the activity of cell metabolism. The second assay used was the TB assay (B.5.3). It was performed subsequently to the MTS assay, with the same cells. Furthermore, to monitor cGKI activity, phosphorylation of VASP was examined, a substrate protein for cGKI as well as cAK. Figure 8 demonstrates that both assays give comparable results and that phosphorylation of VASP can be used to monitor cGKI activity.

Stimulation of primary wild-type VSMCs with 8-Br-cGMP caused a strong increase in cell number as compared to control (Fig. 8a, b; black bars), whereas this effect was absent in cGKI-deficient cells (Fig. 8a, b; white bars). Moreover, stimulation of wild-type cells with 8-Br-cGMP led to a strong phosphorylation of VASP at Ser157. This phosphorylation could not be seen in cGKI-deficient cells with 100 µM 8-Br-cGMP. Although, a slight phosphorylation could be observed with high concentrations of 8-Br-cGMP (1 mM), suggesting a cross-activation of cAK (Fig 8c).

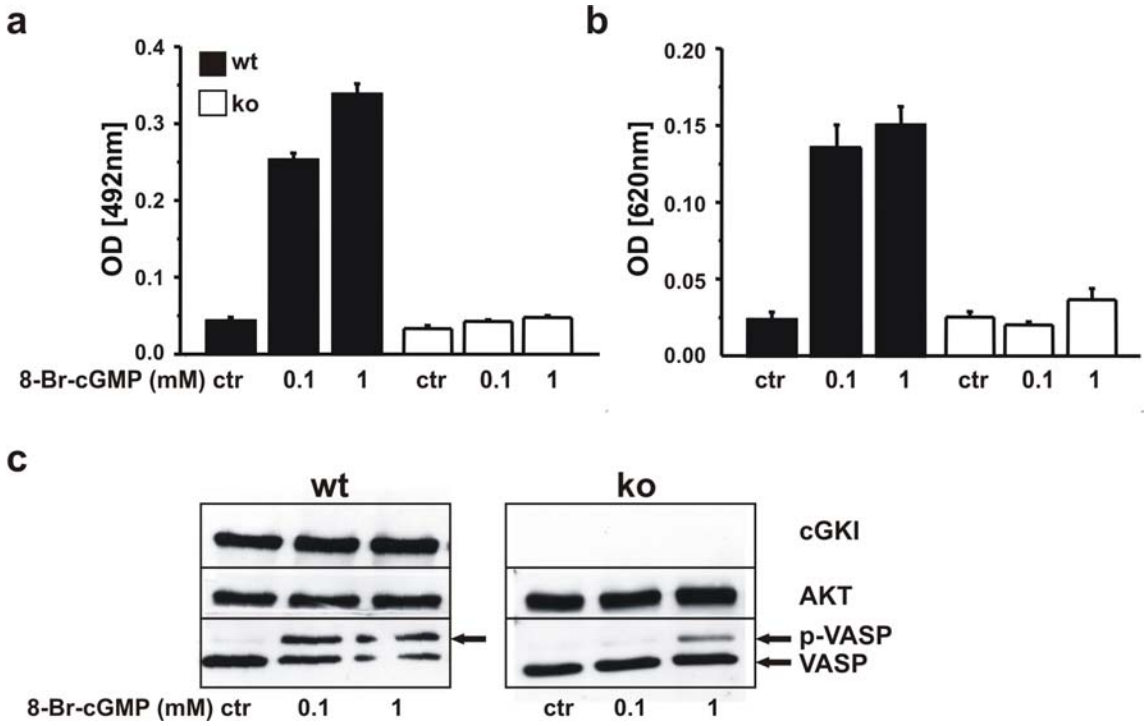


Fig. 8: Growth assays and phosphorylation of VASP in primary VSMCs. (a, b) Assays were performed three days after seeding the VSMCs. Drug treatment occurred over 72 hours. mM concentrations of 8-Br-cGMP were used. Both assays (a) MTS assay – measured at 492nm - and (b) TB assay – measured at 620nm - give similar results. Each condition was tested in n=8 wells. (c) Western blot of wt and ko cells. Serum-starved primary VSMCs (2 days) were treated for 30 min with the indicated concentrations of 8-Br-cGMP. The antibody detects total VASP. The 50 kDa (upper) band indicates phosphorylation at Ser157. The determination of VASP and p-VASP considers only phosphorylation at Ser157. No conclusion can be made for phosphorylation at Ser239. AKT was used as loading control. The antibody for cGKI gives only a signal in wt cells and no signal in ko cells.

In summary, the analysis of primary VSMC growth by (1) light microscopy (Fig. 6), (2) growth assays (Fig. 8a, b), and (3) phosphorylation of VASP (Fig. 8c), demonstrates that cGKI is activated in response to 8-Br-cGMP and causes a strong increase in growth.

1. cGKI Agonists and Antagonists

To clarify the opposing results about cGKI function on VSMC growth, the effects of various cyclic nucleotide analogs and cGKI inhibitors on the growth of primary aortic VSMCs were studied. Besides 8-Br-cGMP, two other commonly used cGMP analogs were tested, namely 8-pCPT-cGMP and 8-Br-PET-cGMP. As can be seen in Figure 9, all tested agonists stimulated the growth of primary wild-type cells as compared to control, whereas these drugs did not reveal a growth effect in cGKI-deficient cells. For all further experiments 8-Br-cGMP was used as cGKI agonist.

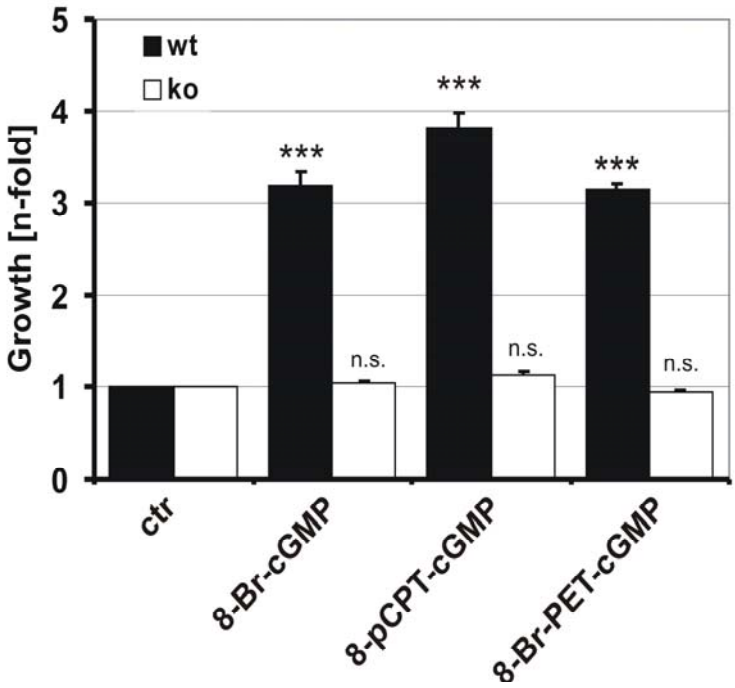


*Fig. 9: Growth assay of primary VSMCs (TB) in response to various cGKI agonists. Drug treatment (100 µM) occurred over 72 h. Each condition was tested in n=8 wells. All tested agonists increased growth in wt cells significantly (***, p<0.001) in comparison to untreated (ctr) cells (ctr 1.0; 8-Br-cGMP 3.2±0.16; 8-pCPT-cGMP 3.8±0.16; 8-Br-PET-cGMP 3.1±0.07). All tested agonists had no significant (n.s.) effect on the growth of ko cells. Growth was normalized to control. One representative example of three experiments is shown. Error bars represent SEM.*

The following experiments were performed to examine the inhibitory effect of Rp-8-pCPT-cGMPs, Rp-8-Br-PET-cGMPs (Butt et al., 1994b; Butt et al., 1990; Zhuo et al., 1994), and DT-2 (Dostmann et al., 2000; Taylor et al., 2004), three commonly used cGKI inhibitors. As shown in Figure 10a, Rp-8-pCPT-cGMPs revealed only a slight growth suppressing effect on 8-Br-cGMP stimulated VSMC growth. This substance did not affect growth under control conditions. Rp-8-Br-PET-cGMPs failed to inhibit 8-Br-cGMP stimulated growth of VSMCs. Indeed, Rp-8-Br-PET-cGMPs revealed a slight growth-promoting effect on basal growth of wild-type cells (Fig. 10b). These findings are supported by *in vitro* kinase assays from Valcheva et al. (unpublished data). Addition of Rp-8-Br-PET-cGMPs to the purified cGKI activated the kinase in the absence of 8-Br-cGMP, indicating that Rp-8-Br-PET-cGMPs is a partial agonist rather than an antagonist. DT-2, another cGKI inhibitor tested, revealed

neither an effect on basal nor on stimulated growth of wild-type VSMCs (Fig. 10c). None of the tested inhibitors had an effect on the growth of cGKI-deficient VSMCs, neither on unstimulated nor on 8-Br-cGMP treated cells.

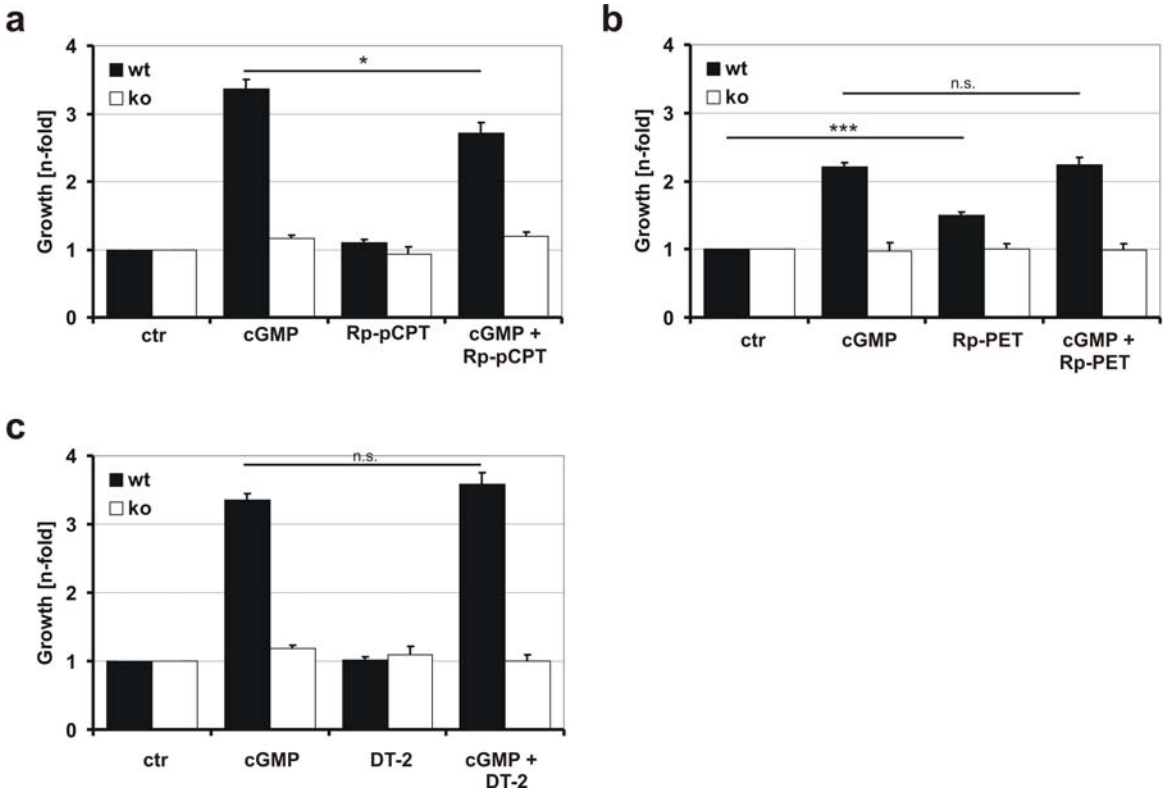


Fig. 10: Growth assays (TB) of primary VSMCs in response to various cGKI antagonists. Data derived from five independent experiments are shown. 8-Br-cGMP (cGMP) was used at 0.1 mM. (a) Rp-8-pCPT-cGMPs (Rp-pCPT) (0.1 mM). 8-Br-cGMP induced growth in wt cells was significantly (, p<0.05) reduced in response to Rp-8-pCPT-cGMPs (ctr 1.0 n=24 wells; cGMP 3.7±0.13 n=20 wells; Rp-pCPT 1.1±0.05 n=16 wells; cGMP + Rp-pCPT 2.7±0.14 n=5 wells). Rp-8-pCPT-cGMPs had no effect on the growth of ko cells (ctr 1.0 n=13 wells; cGMP 1.2±0.04 n=11wells; Rp-pCPT 0.9±0.11 n=11 wells; cGMP + Rp-pCPT 1.2±0.06 n=7 wells) (b) Rp-8-Br-PET-cGMPs (Rp-PET) (0.1 mM). The assay was performed in 1% DMSO f.c.. Rp-8-Br-PET-cGMPs (stock 10 mM) was dissolved in 100% DMSO. cGMP induced growth in wt cells was not significantly (n.s.) reduced in response to Rp-8-Br-PET-cGMPs. Rp-8-Br-PET-cGMPs stimulated basal growth (***, p<0.001) in comparison to control (ctr 1.0 n= 36 wells; cGMP 2.2±0.07 n=23 wells; Rp-PET 1.5±0.05 n=30 wells; cGMP + Rp-PET 2.2±0.11 n=14 wells). Rp-8-Br-PET-cGMPs had no effect on the growth of ko cells (ctr 1.0 n=12 wells; cGMP 1.0±0.12 n=6 wells; Rp-pPET 1.0±0.07 n=10 wells; cGMP + Rp-pPET 1.0±0.08 n=5 wells). (c) DT-2 (10 µM). DT-2 revealed neither an effect on growth in wt cells (ctr 1.0 n= 31 wells; cGMP 3.4±0.10 n=27 wells; DT-2 1.0±0.04 n=18 wells; cGMP + DT-2 3.6±0.16 n=15 wells) nor in ko cells (ctr 1.0 n= 15 wells; cGMP 1.2±0.10 n=11 wells; DT-2 1.1±0.11 n=11 wells; cGMP + DT-2 1.0±0.10 n=7 wells). Growth was normalized to control. Error bars represent SEM.*

In summary, only Rp-8-pCPT-cGMPs slightly inhibited cGKI-mediated growth, whereas the other tested cGKI inhibitors failed to suppress cGKI-induced growth. As described above, VASP can be used to monitor cGKI activity. To verify whether the inhibitors failed to inhibit cGKI activity, phosphorylation of VASP was examined. An inhibition of cGKI activity should

result in a reduced phosphorylation of VASP. 8-Br-cGMP induced a strong phosphorylation of VASP at Ser157 in wild-type cells (Fig. 8, 11), but Rp-8-Br-PET-cGMPs as well as Rp-8-pCPT-cGMPs failed to reduce VASP phosphorylation at the indicated concentrations in wild-type cells (Fig. 11a). No effect on phosphorylation of VASP could be observed in cGKI-deficient cells (Fig. 11b).

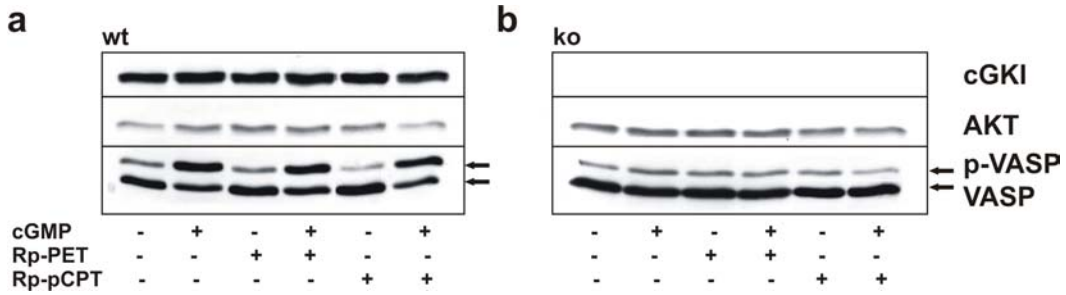


Fig. 11: Effect of cGKI antagonists on VASP phosphorylation. Serum starved primary VSMCs were treated for 30 min with either Rp-8-Br-PET-cGMPs (Rp-PET) (0.1 mM) or with Rp-8-pCPT-cGMPs (Rp-pCPT) (0.1 mM) in the absence or presence of 8-Br-cGMP (cGMP) (0.1 mM). The VASP antibody detects total VASP (for further explanation see Fig. 8). AKT was used as loading control. The antibody against cGKI was used to differentiate wt from ko cells. (a) In wt cells phosphorylation of VASP is not increased under non-stimulatory conditions in response to Rp-8-Br-PET-cGMPs or Rp-pCPT-cGMPs. As well, phosphorylation of VASP at Ser157 is not decreased in the presence of 8-Br-cGMP in response to Rp-8-Br-PET-cGMPs or Rp-pCPT-cGMPs. (b) In ko cells no effect of Rp-8-Br-PET-cGMPs and Rp-pCPT-cGMPs can be observed in the absence or presence of 8-Br-cGMP. One representative blot of three experiments is shown.

Taken together, all tested agonists stimulated the growth of primary VSMCs via activation of cGKI. The tested “inhibitors” failed to inhibit cGKI activity in the present study. These results demonstrate that the genetic strategy to study cGKI function is superior to an approach solely based on pharmacological tools, and that the tested cGKI “inhibitors” should only be used in carefully controlled experiments.

2. Growth of Primary vs. Subcultured VSMCs

2.1 Comparison of Primary and Subcultured VSMCs from the Mouse Aorta

In addition to regulating the contractile phenotype, the cGMP/cGKI and cAMP/cAK signaling pathways appear to also modulate the proliferative phenotype, as many agents that relax blood vessels through these two pathways also inhibit VSMC growth (Garg and Hassid, 1989; Indolfi et al., 2000; Koyama et al., 2001). To examine, whether the growth properties differ between primary and subcultured cells, primary VSMCs were repeatedly passaged and the growth was examined in response to 8-Br-cGMP in wild-type and cGKI-deficient cells. Furthermore, the effect of 8-Br-cAMP – an activator of cAK – on the growth of VSMCs was tested, to examine the described anti-proliferative effect of 8-Br-cAMP. In addition, it should be elucidated whether 8-Br-cGMP is able to cross-activate cAK by analyzing cGKI-deficient VSMCs.

As shown previously, 8-Br-cGMP strongly promotes the growth of primary VSMCs through activation of cGKI (Fig. 12a, c). Surprisingly, activation of cGKI in subcultured cells resulted in a slight growth suppression (Fig. 12b, d). As expected, 8-Br-cAMP treatment reduced the growth of primary (Fig. 12a) and subcultured cells (Fig. 12b) as compared to control, in wild-type as well as in cGKI-deficient cells. 8-Br-cAMP activated cAK in primary as well as in passaged cells as revealed by phosphorylation of VASP (Fig. 12c, d). Interestingly, the phosphorylation of VASP at Ser157 was much stronger through activation of cGKI than cAK. This was unexpected, because Ser157 is described to be the preferential phosphorylation site for cAK (Butt et al., 1994a; Smolenski et al., 2000).

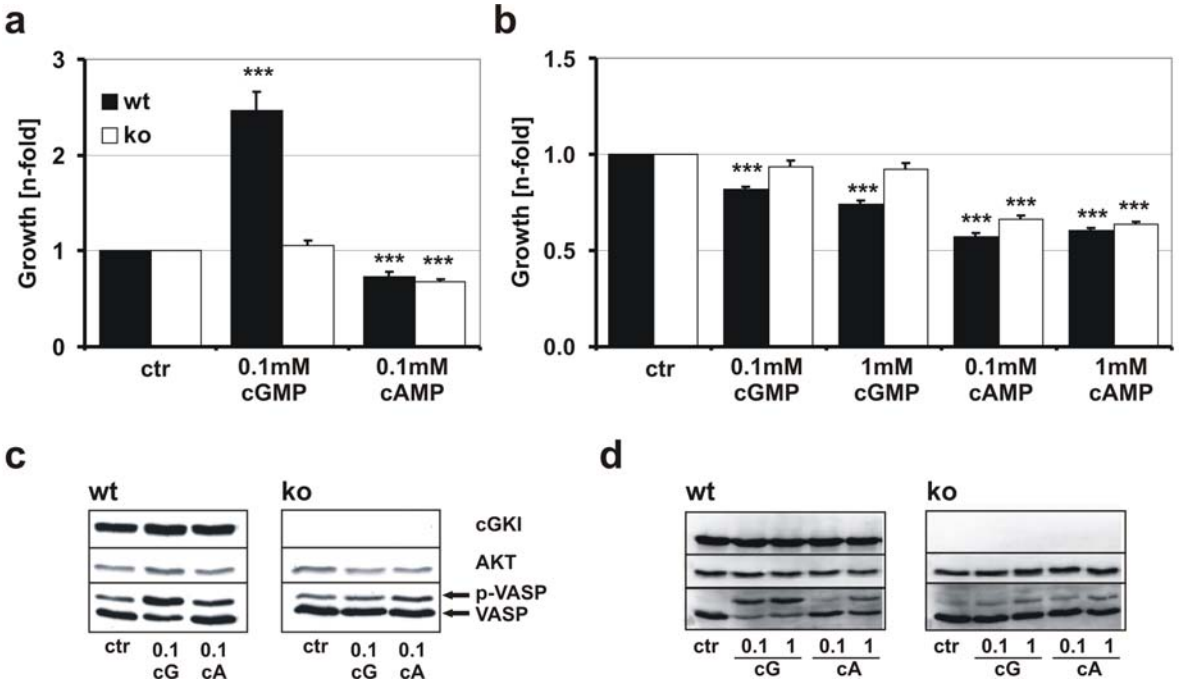


*Fig. 12: Comparison of the growth of primary (a, c) and subcultured VSMCs (P5) (b, d). (a) Growth assay (MTS) with primary VSMCs in response to 8-Br-cGMP (cGMP) and 8-Br-cAMP (cAMP). One of at least three different experiments is shown. In primary wt VSMCs 8-Br-cGMP significantly promotes growth in comparision to control (***, p<0.001), whereas no effect can be observed in ko cells. 8-Br-cAMP inhibits growth in wt and ko cells (***, p<0.001) (wt: ctr 1.0 n=8 wells; cGMP 2.5±0.20 n=8 wells; cAMP 0.7±0.05 n=8 wells; ko: ctr 1.0 n=5 wells; cGMP 1.1±0.05 n=6 wells; cAMP 0.7±0.02 n=6 wells). (b) Growth assay (MTS) with subcultured VSMCs. In passaged wt cells 8-Br-cGMP significantly inhibits growth in comparison to ko cells (***, p<0.001). 8-Br-cAMP inhibits growth in wt and ko cells in comparison to control (***, p<0.001) (wt: ctr 1.0 n=7 wells; cGMP (0.1mM) 0.8±0.01 n=7 wells; cGMP (1mM) 0.7±0.02 n=8 wells; cAMP (0.1mM) 0.6±0.02 n=8 wells; cAMP (1mM) 0.6±0.02 n=8 wells; ko: ctr 1.0 n=8 wells; cGMP (0.1 mM) 0.9±0.03 n=8 wells; cGMP (1 mM) 0.9±0.03 n=7 wells; cAMP (0.1 mM) 0.7±0.02 n=8 wells; cAMP (1 mM) 0.6±0.01 n=8 wells). Growth was normalized to control. Error bars represent SEM. (c) VASP phosphorylation in response to 8-Br-cGMP (cG) and 8-Br-cAMP (cA) in primary and (d) subcultured VSMCs. Serum starved cells (2 days) were treated with 8-Br-cGMP or 8-Br-cAMP for 30 min with the indicated concentrations. (c) In primary VSMCs 0.1 mM 8-Br-cGMP induces a strong phosphorylation of VASP at Ser157 that is absent in ko cells. 0.1 mM 8-Br-cAMP induces phosphorylation of VASP at Ser157 in wt and ko cells. (d) In subcultured VSMCs 8-Br-cGMP (0.1 and 1 mM) induces a strong phosphorylation of VASP at Ser157 in wt cells. In ko cells only a slight phosphorylation of VASP at Ser157 can be observed at high concentrations of 8-Br-cGMP (1 mM), which might be caused by cross-activation of cAK. 8-Br-cAMP only induces a phosphorylation of VASP at Ser157 in high concentrations (1 mM) in wt and ko cells.*

In contrast to several studies, which show that cGKI expression in VSMCs is lost through passaging (Boerth et al., 1997; Cornwell and Lincoln, 1989; Dey et al., 1998), cGKI was strongly expressed in our VSMCs at least up to passage 11 (Fig. 12d, data not shown). Furthermore, as revealed by phosphorylation of VASP, cGKI is activated in response to 8-Br-cGMP in passaged murine VSMCs (Fig. 12d, data not shown).

In summary, activation of cGKI revealed bivalent effects: In primary murine VSMCs, activation of cGKI causes a strong increase in growth, whereas its activation in subcultured

cells leads to a slight growth inhibition (Fig. 13a). In contrast, activation of cAK in primary as well as in passaged VSMCs leads to growth inhibition in wild-type cells and in cGKI-deficient cells (Fig. 13b), demonstrating that the effects mediated by 8-Br-cAMP are independent of cGKI. Furthermore, cAK reveals a stronger growth inhibitory effect as compared to cGKI (Fig. 13). The results obtained in this study on subcultured VSMCs are in line with most studies that analyzed established cell lines or subcultured cells.

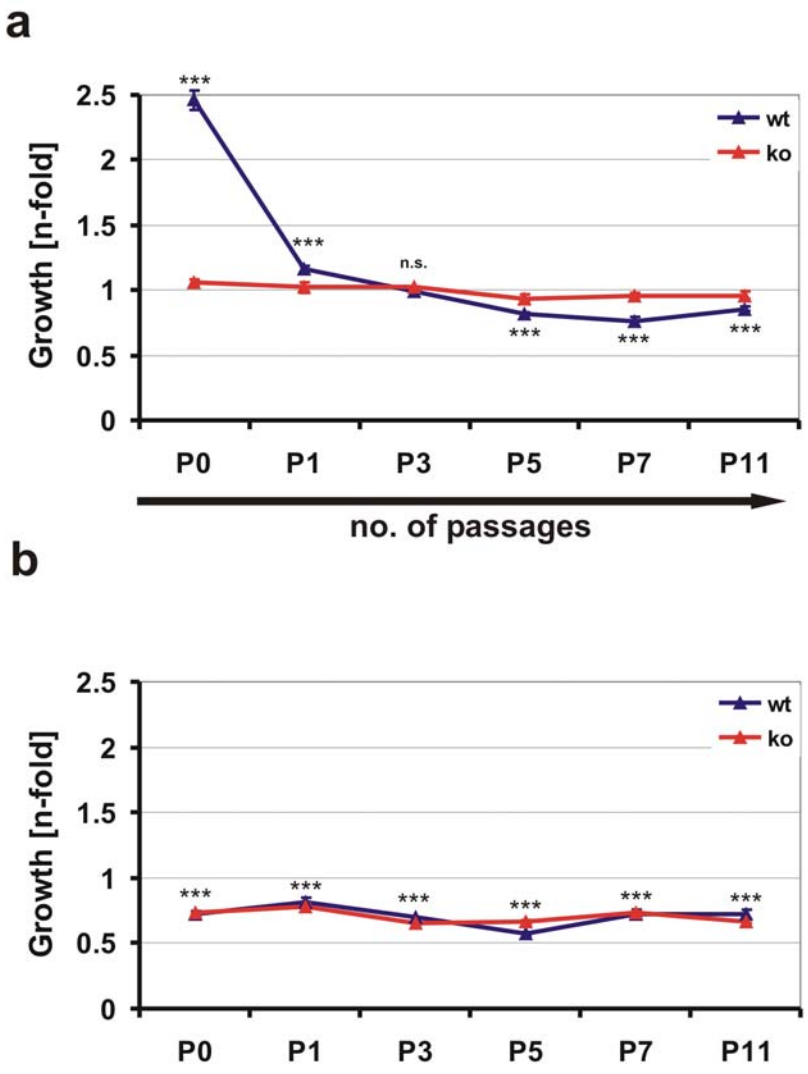


*Fig. 13: Growth performance of primary (P0) up to passage 11 (P11) VSMCs in response to 8-Br-cGMP and 8-Br-cAMP (MTS assay). (a) Cells were treated with 0.1 mM 8-Br-cGMP or (b) 0.1 mM 8-Br-cAMP, respectively. Growth was normalized to control (untreated cells) (>1 = increased growth, 1 = no growth, <1 = decreased growth in comparison to control). (a) Growth is significantly increased in primary wt cells in response to 8-Br-cGMP (***, p<0.001), whereas growth is significantly reduced in subcultured (>P5) cells. Treating ko cells with 8-Br-cGMP has no effect on growth. (b) 8-Br-cAMP significantly inhibits growth in wt and ko cells. n=5-8 wells. Error bars represent SEM.*

Figure 13 demonstrates that wild-type VSMCs in response to 8-Br-cGMP change their growth properties with increasing numbers of passages. A strong increase in growth in response to 8-Br-cGMP in primary VSMCs changes to a slight growth inhibition at passage 5 as compared to control. cGKI-deficient VSMCs do not change their growth in response to 8-Br-cGMP, demonstrating that the effects observed in wild-type cells are mediated via cGKI. According to these findings, the growth inhibiting effect in subcultured wild-type cells is mediated via cGKI and not via cross-activation of cAK by 8-Br-cGMP. Nevertheless, activation of cAK by 8-Br-cGMP and vice versa has been reported by others (Barman et al., 2003; Cornwell et al., 1994a; Lin et al., 2001; Osinski et al., 2001). Based on the current findings, we can redraw figure 7 showing the growth effects of cGMP/cGKI and cAMP/cAK signaling (Fig. 14).

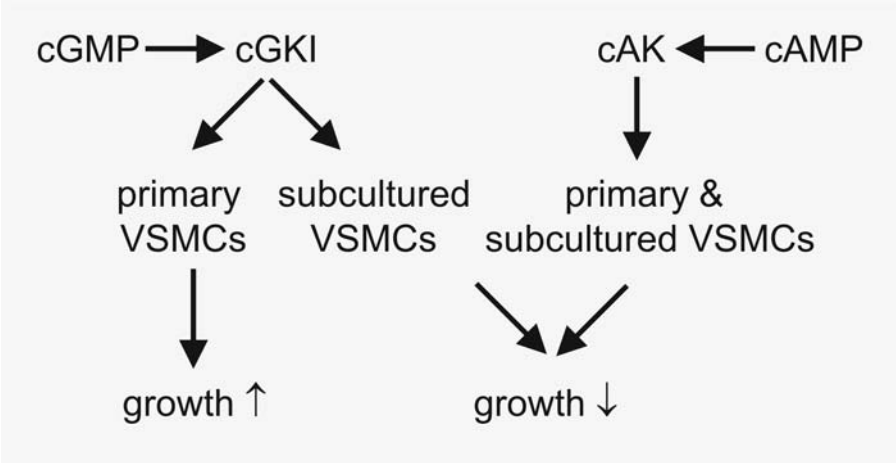


Fig. 14: Model for VSMC growth in response to cGKI and cAK. cGMP/cGKI signaling promotes growth in primary VSMCs. In contrast, growth is slightly suppressed in subcultured VSMCs. cAMP/cAK signaling acts – independent of cGKI – growth suppressing in primary as well as in subcultured cells. Further explanation see text.

Finally, it should be elucidated whether the growth-promoting effect of 8-Br-cGMP in primary VSMCs could also be mimicked by endogenous cGMP generated via activation of sGC or pGC. Therefore, a growth assay was performed with primary VSMCs that were treated with several cGMP-elevating drugs, such as ANP, CNP, and DETA/NO.

As shown in Figure 15, treatment of primary VSMCs with ANP or CNP resulted in a moderate growth-promoting effect in wild-type cells, whereas no effect on growth could be detected in cGKI-deficient cells. Interestingly, DETA/NO induced opposing effects in wild-type and cGKI-deficient cells. It promoted growth in wild-type cells and reduced growth in cGKI-deficient cells. Feil et al. (Feil et al., 2002) showed previously that the cGMP level rises in wild-type and cGKI-deficient cells (P1 cells) upon stimulation with DEA-NO, whereas cAMP levels remained unaltered. Thus, endogenous cGMP – generated via activation of sGC - might stimulate growth of primary wild-type VSMCs via activation of cGKI. In contrast,

deletion of cGKI may uncover a direct interaction of endogenous cGMP with cAK or cGMP-independent effects of NO, resulting in growth suppression. In addition, comparing the strong growth-promoting effect of DETA/NO on primary wild-type VSMCs with the rather small growth effect of ANP and CNP suggests that the source of endogenous cGMP might be important for the resulting effect on growth.

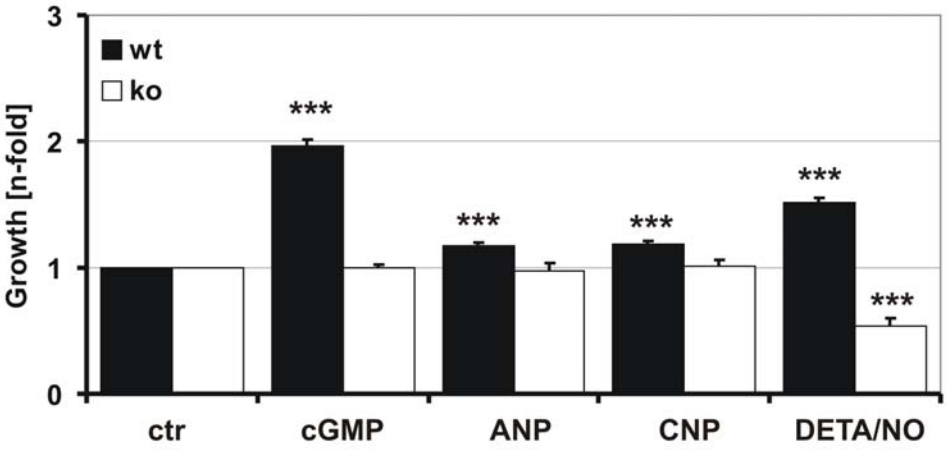


*Fig. 15: Growth of primary VSMCs in response to various cGMP-elevating drugs (MTS assay). In wt cells 8-Br-cGMP (cGMP) (0.1 mM), ANP and CNP (1 µM), and DETA/NO (0.1 mM) significantly (***, p<0.001) increase the growth of VSMCs in comparison to control (1.0 n=8 wells; cGMP 2.0±0.06 n=8 wells; ANP 1.2±0.03 n=8 wells; CNP 1.2±0.03 n=8 wells; DETA/NO 1.5±0.03 n=8 wells). In ko cells 8-Br-cGMP, ANP and CNP reveal no effect on growth of VSMCs, whereas DETA/NO significantly (***, p<0.001) suppresses growth of VSMCs in comparison to control (1.0 n=8 wells; cGMP 1.0±0.03 n=8 wells; ANP 1.0±0.06 n=5 wells; CNP 1.0±0.05 n=8 wells; DETA/NO (1.5±0.03 n=8 wells). Growth was normalized to control. Error bars represent SEM.*

These results indicate that NO can exert a growth-promoting effect by activating cGKI, and that the anti-proliferative effect of NO is not mediated by cGKI (Ignarro et al., 2001). In summary, we propose that the observed effects of 8-Br-cGMP on primary VSMCs would be caused *in vivo* by NO rather than by NPs.

2.2 Growth of Subcultured Rat and Human VSMCs

To validate the results obtained with the murine subcultured VSMCs (C.2.1), the growth effects of 8-Br-cGMP and 8-Br-cAMP were tested in subcultured VSMCs from rat and human. All examined VSMCs from rat respectively human were subcultured cells. Rat VSMCs were analyzed, derived from the media of the aorta and neointimal cells from the carotids, and human VSMCs derived from the media of coronary artery as well as neointimal VSMCs from the carotis were analyzed. The tested rat VSMCs did not show any change in growth in response to 8-Br-cGMP treatment (Fig. 16a, b), although cGKI was expressed and could be activated as demonstrated by phosphorylation of VASP (Fig. 16c, d). In contrast, treating these cells with 8-Br-cAMP revealed a growth suppressing effect compared to control (Fig. 16).

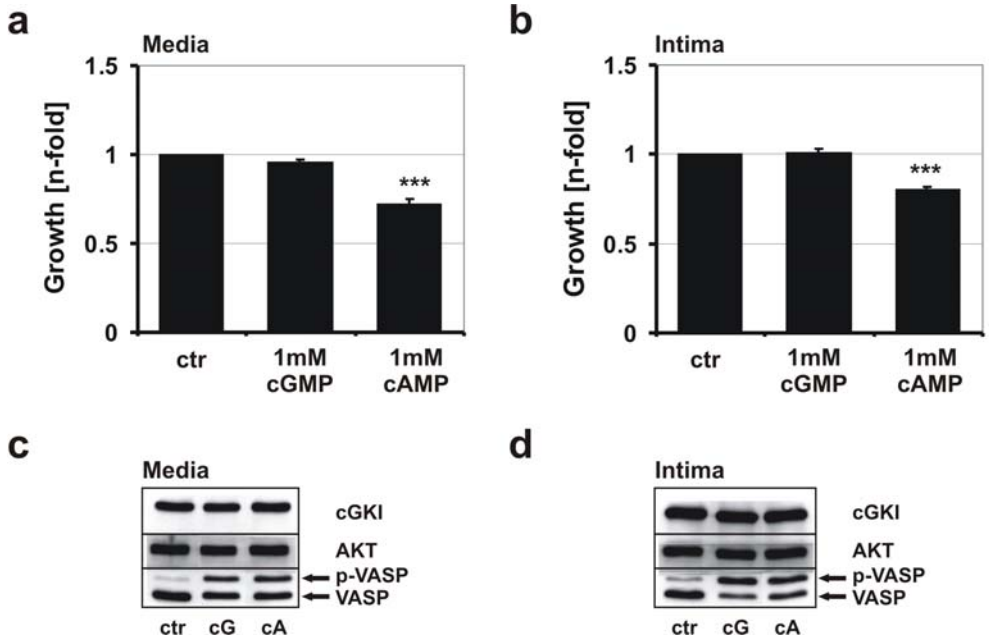


*Fig. 16: Growth of subcultured rat VSMCs. (a) VSMCs derived from the media (P8) and (b) the intima (P13). (c, d) Corresponding western blots showing VASP phosphorylation at Ser157. (a, b) Growth assays (MTS) of rat VSMCs in response to 8-Br-cGMP (cGMP) and 8-Br-cAMP (cAMP) after 2 days of growth. One of three similar experiments is shown. 8-Br-cGMP does not influence growth as compared to control. 8-Br-cAMP significantly (***, p<0.001) inhibits growth in comparison to control (media: ctr 1.0 n=8 wells; cGMP 1.0±0.02 n=8 wells; cAMP 0.7±0.03 n=7 wells; intima: ctr 1.0 n=8; cGMP 1.0±0.02 n=8; cAMP 0.8±0.01 n=8). Growth was normalized to control. Error bars represent SEM. (c, d) Phosphorylation of VASP at Ser157 is induced in response to 8-Br-cGMP (cG) and 8-Br-cAMP (cA). AKT was used as loading control. 8-Br-cGMP and 8-Br-cAMP induce a strong phosphorylation of VASP at Ser157 as compared to control.*

Determination of the growth properties of passaged human VSMCs in response to 8-Br-cGMP and 8-Br-cAMP revealed a similar picture as for the rat cells (Fig. 17). Again, 8-Br-

cGMP had no effect on growth, although cGKI was still present and activated in response to 8-Br-cGMP, whereas 8-Br-cAMP revealed a strong growth suppressing effect.

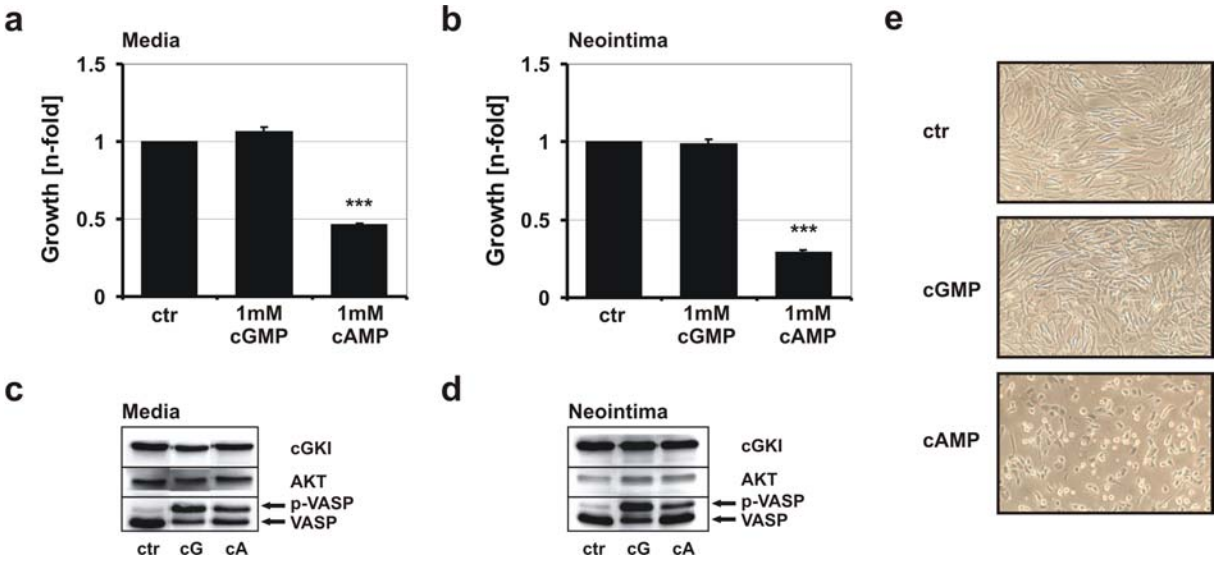


*Fig. 17: Growth of subcultured human VSMCs. (a) VSMCs from the media (P10) and (b) the neointima (P13). (c, d) Corresponding blots showing VASP phosphorylation. (e) Neointimal VSMCs after four days treatment with 1mM drugs. (a, b) Growth assays (MTS, after 4 days of growth) with subcultured human VSMCs in response to 8-Br-cGMP (cGMP) and 8-Br-cAMP (cAMP). One of three different experiments is shown. 8-Br-cGMP does not influence growth in response to control. 8-Br-cAMP significantly (***, p<0.001) inhibits growth in comparison to control (media: ctr 1.0 n=7 wells; cGMP 1.0±0.03 n=7 wells; cAMP 0.5±0.01 n=8 wells; neointima: ctr 1.0 n=8; cGMP 1.0±0.03 n=8; cAMP 0.3±0.01 n=8). Growth was normalized to control. Error bars represent SEM. (c, d) Phosphorylation of VASP at Ser157 is induced in response to 1 mM 8-Br-cGMP (cG) and 1 mM 8-Br-cAMP (cA).*

8-Br-cAMP treatment and potential activation of cAK led to a strong reduction in subcultured rat and human VSMC number. This is in line with the analysis of the murine cells. However, in contrast to mouse VSMCs, cGKI had no effect on the growth of the tested rat or human VSMCs. This might be due to slightly different experimental conditions, or different growth behavior of passaged VSMCs from rat and humans in comparison to murine cells. In addition, the human cells grew very slowly and we do not know about the former treatment of the cells.

The previous experiments confirmed that cAMP/cAK signaling acts growth suppressing on VSMCs from mouse, rat and human - in passaged cells as well as in primary mouse VSMCs. Interestingly, the growth effects of cGMP/cGKI signaling differ in primary vs. subcultured cells. cGKI mediates a strong growth-promoting effect in primary VSMCs, whereas it has no or a growth inhibiting effect on subcultured cells. Because cGKI revealed its most prominent growth effect in primary VSMCs and because the increased growth correlates well with the *in vivo* findings on atherosclerosis (Wolfsgruber et al., 2003), the further analysis concentrated on cGMP/cGKI signaling in primary VSMCs.

3. cGKI-Mediated Growth Effects in Primary VSMCs

After evaluating our primary culture system, the underlying mechanisms for the increased growth of primary VSMCs in response to cGMP/cGKI signaling were studied. Therefore, proliferation, apoptosis and adhesion were analyzed.

3.1 Analysis of Proliferation

To analyze the growth progression of primary VSMCs during the first 72 hours after their isolation from the aorta, time-lapse microscopy was performed. Figure 18 shows the growth progression of freshly isolated VSMCs under control conditions (unstimulated) and in the presence of 8-Br-cGMP. The 8-Br-cGMP treated cells attach faster to the culture dish in comparison to untreated cells. After 48 hours, many 8-Br-cGMP treated cells have already attached, whereas almost none of the untreated VSMCs have attached (Fig 18). Moreover, the analysis of the time-lapse recordings suggests that the freshly isolated VSMCs have to attach to survive. To attach, the VSMCs have to adopt to the culture conditions within 72 hours. Using time-lapse microscopy, no mitotic events were observed in the time-period analyzed. This observation indicates that primary VSMCs do not proliferate during the first 72 hours of in vitro culture. Thus, the increased growth potential observed in wild-type cells after stimulation with 8-Br-cGMP is not attributable to increased proliferation.

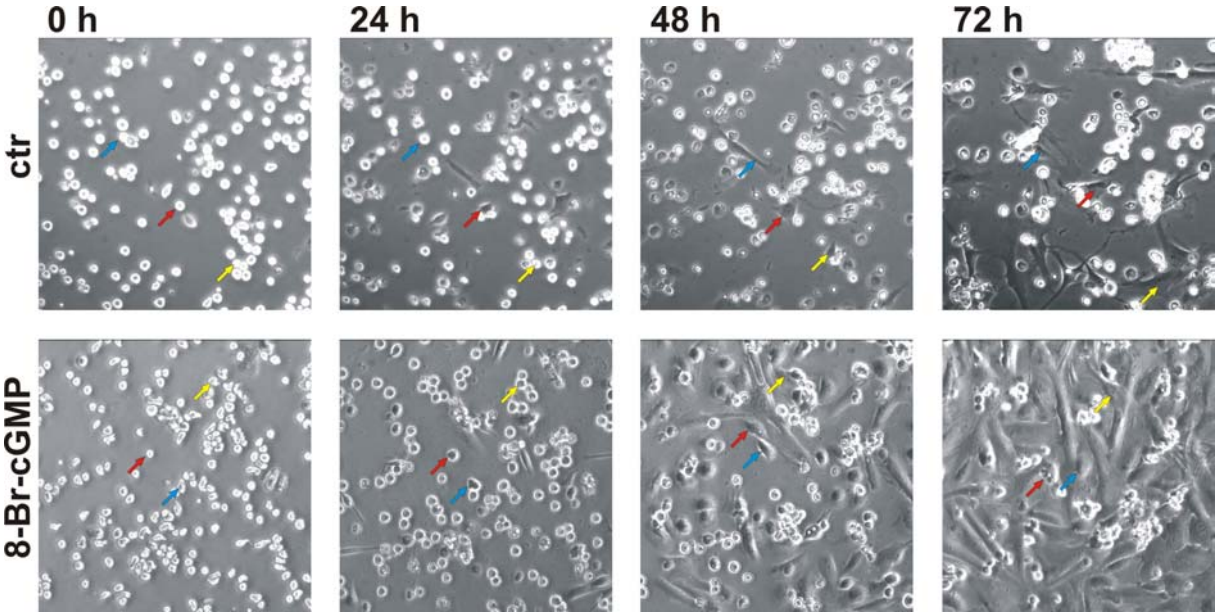


Fig. 18: Analysis of VSMC proliferation by time-lapse microscopy. 8-Br-cGMP (1 mM) treated cells attach faster and in higher quantity as compared to control. After 48 h many 8-Br-cGMP treated cells have attached while only a couple of cells under control conditions have attached. Arrows highlight individual cells during the indicated time period.

3.2 Analysis of Apoptosis

As revealed by time-lapse microscopy, proliferation as possible reason for the increased cGKI-mediated growth can be excluded. Increased growth could also be a result of decreased apoptosis in response to 8-Br-cGMP. Less dead cells were observed in the presence of 8-Br-cGMP in comparison to untreated cells after 72 hours (Fig. 18), indicating that cGMP/cGKI signaling might influence apoptosis. Freshly isolated VSMCs were held in suspension for up to 22 hours in the absence and presence of 8-Br-cGMP. At each time-point indicated, a sample of cells was stained for annexin V, a marker for early apoptosis, and propidium iodide, a marker for late apoptosis and necrosis. Figure 19 shows that cGMP/cGKI signaling causes a reduction of Annexin V positiv and PI negativ VSMCs, indicating a slight suppression of apoptosis. An anti-apoptotic action of cGMP/cGKI signaling has also been described by others (Fiscus, 2002; Ha et al., 2003).

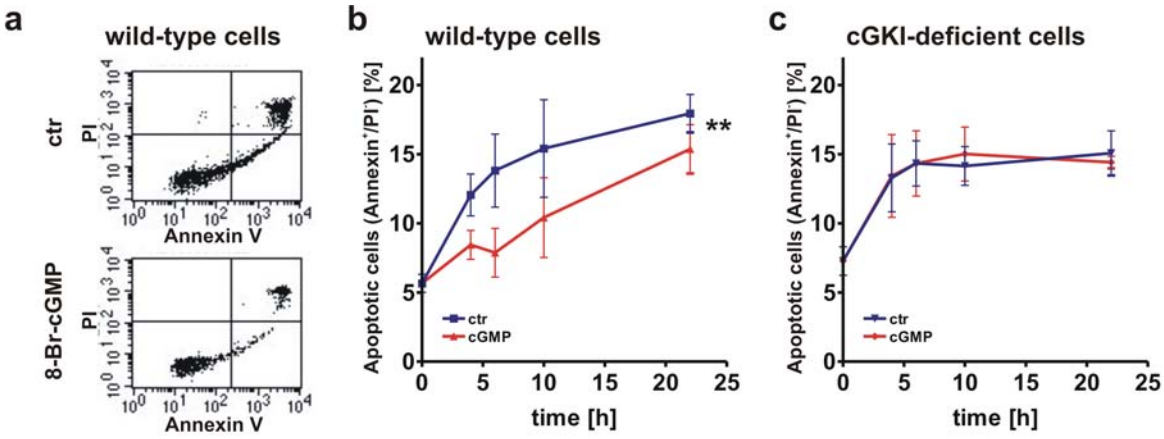


*Fig. 19: Analysis of apoptosis of primary VSMCs in suspension in the absence and presence of 1 mM 8-Br-cGMP. Summary of two different experiments with each n=2 for every time point. Cells were labeled with Annexin V and PI. (a) Original measurements of primary wild-type VSMCs after 6 hours in suspension in the absence and presence of 8-Br-cGMP. (b) In wild-type cells 8-Br-cGMP (cGMP) mediates a slight anti-apoptotic effect on – Annexin V positive and PI negative labeled - VSMCs in comparison to control (**, p<0,01; two-way ANOVA). (c) The anti-apoptotic effect mediated by 8-Br-cGMP in wt cells is absent in ko cells.*

Although an anti-apoptotic effect by stimulating cGKI in primary VSMCs could be detected, this effect appeared to be relatively weak. Thus, it is presumably not the major mechanism that mediates the strong growth-promoting effect of cGKI. An alternative mechanism that could account for the increased growth might be a cGKI-dependent effect on adhesion. Therefore, adhesion was investigated in the following experiments.

3.3 cGMP/cGKI Signaling in Adhesion

3.3.1 Cytoskeletal Staining

VSMCs change their phenotype upon adhesion to the culture dish. The cells spread and change their shape from round to more elongated cells (Fig. 18). Wild-type VSMCs treated with 8-Br-cGMP, show a more homogenous phenotype in comparison to VSMCs under control conditions (Fig. 18). The observed changes probably include changes in the cytoskeleton. Therefore, primary VSMCs were stained for F-actin and Vinculin – as marker for focal adhesions. Wild-type and cGKI-deficient VSMCs were cultured for 24 hours, 48 hours and 72 hours in the absence and presence of 8-Br-cGMP (Fig. 20).

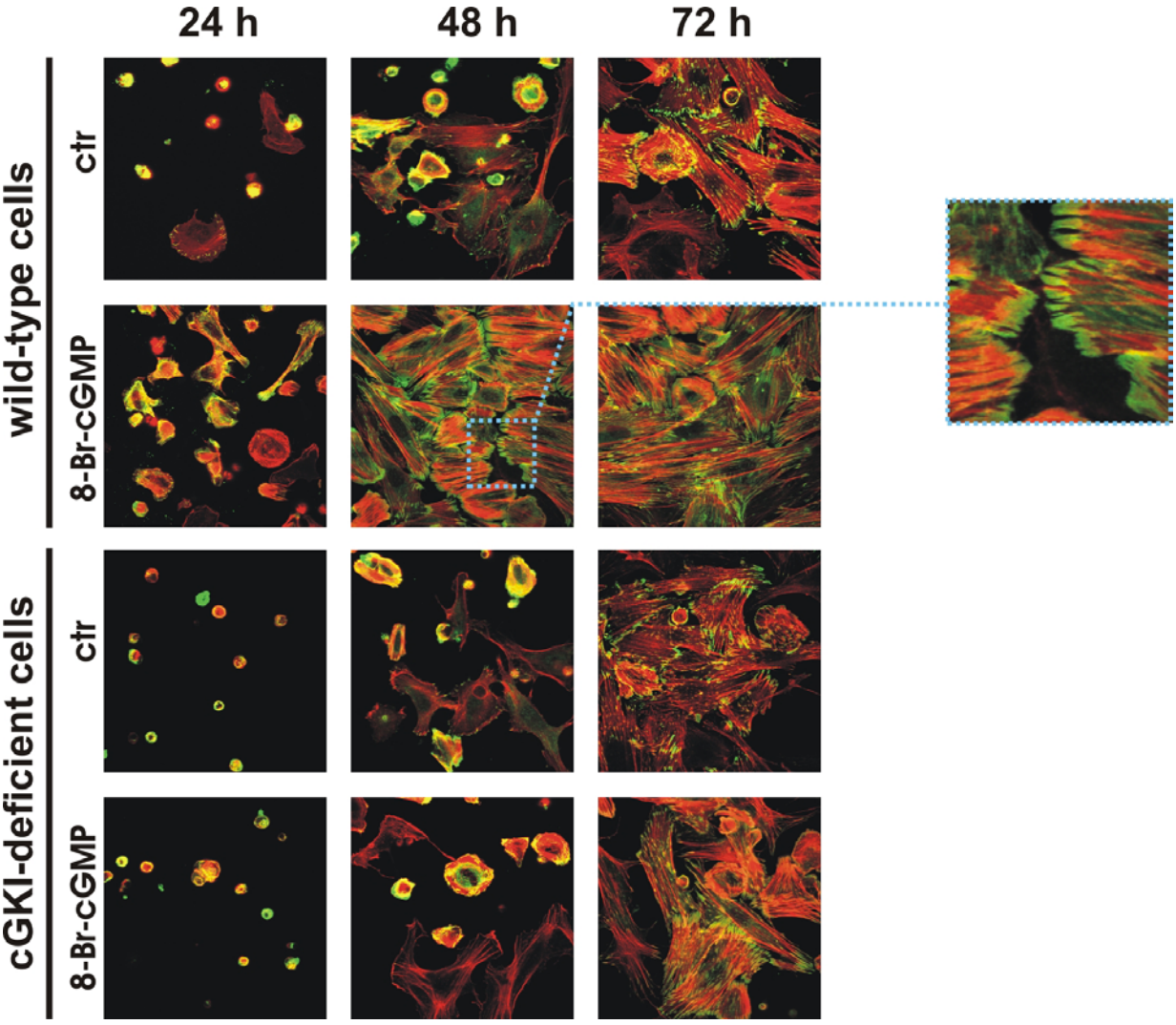


Fig. 20: Cytoskeletal staining of primary VSMCs. VSMCs were stained for F-Actin (red) and Vinculin (green). Vinculin staining was used as marker for focal adhesions. Focal adhesions (green) are shown in the enlarged section. Pictures were taken with a confocal microscope. Wild-type and cGKI-deficient cells were grown on glass coverslips for 24h, 48h, and 72h in the absence or presence of 1 mM 8-Br-cGMP. One representative of three experiments is shown.

In line with the time-lapse microscopy, 8-Br-cGMP treatment of wild-type cells (Fig. 20) increased the number of cells that attached during the first 72 hours after seeding in comarison to control conditions (Fig. 20). Furthermore, administration of 8-Br-cGMP to wild-type cells caused a strong increase in stress fiber formation and the generation of focal adhesions (Fig. 20). The difference between control and 8-Br-cGMP treatment in wild-type cells was absent in cGKI-deficient cells (Fig. 20). These results indicate that cGMP/cGKI signaling increases the attachment and spreading of cells in the culture dish and promotes the formation of stress fibers and focal adhesions.
Furthermore, as revealed by western blot analysis using protein extract of cells, which were grown for three days in the absence and presence of 8-Br-cGMP, vinculin expression appeared to be increased in a cGKI-dependent manner (data not shown).

3.3.2 Phosphorylation of MLC

The formation of stress fibers – which is indicative for increased contractility - should be linked to increased MLC phosphorylation. A recent work of Totsukawa et al. (Totsukawa et al., 2000) showed that MLC phosphorylation is both necessary and sufficient for the assembly of stress fibers and focal adhesions in 3T3 fibroblasts. As revealed by immunocytochemical staining for p-MLC on primary VSMCs, the signal for p-MLC was increased in response to 8-Br-cGMP as compared to control (Fig. 21a, b). Furthermore, in 8-Br-cGMP treated VSMCs p-MLC seemed to colocalize with the stress fibers. Western blot analysis confirmed a cGKI-mediated increase of the phosphorylation level of MLC. The p-MLC signal was increased in response to 8-Br-cGMP in wild-type cells as compared to control, whereas the level of p-MLC was unaltered in cGKI-deficient cells (Fig. 21c). The increased level of phosphorylated MLC fits well with the strong formation of stress fibers (see Fig. 20).

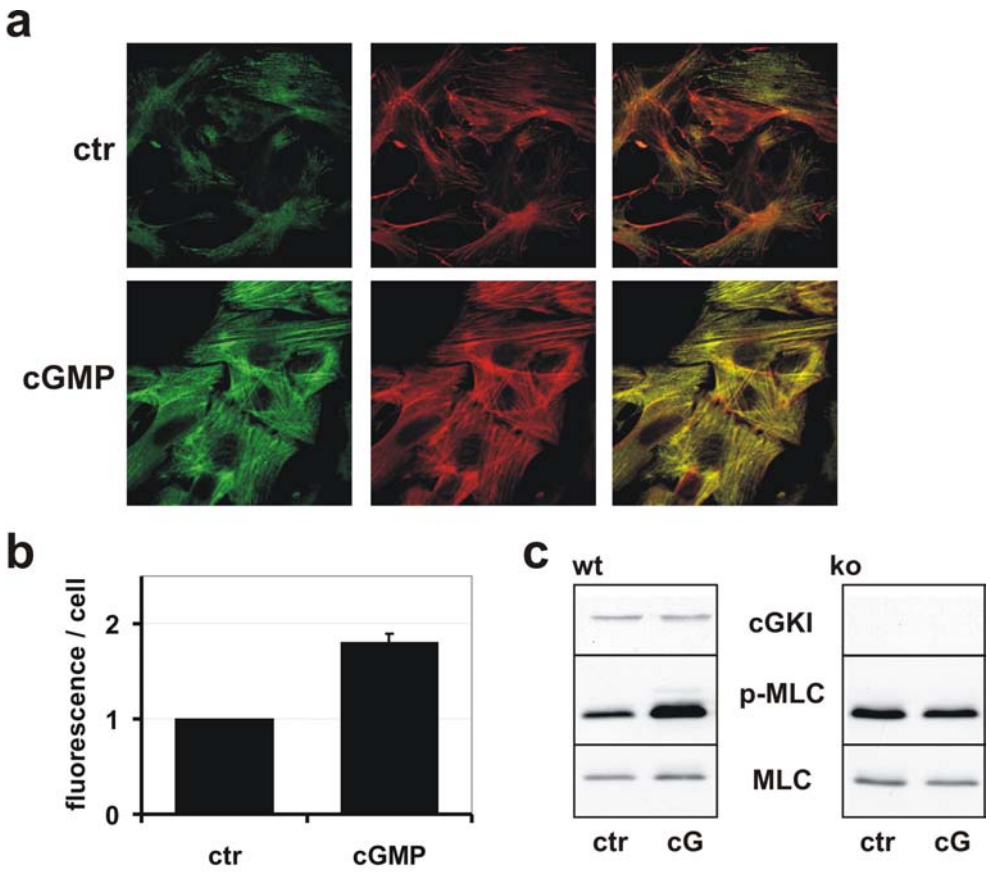
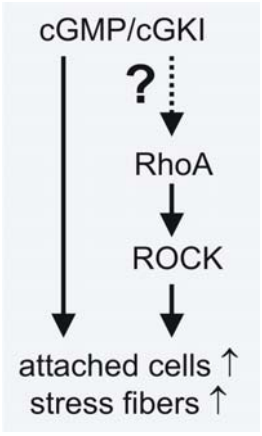


*Fig. 21: Detection of p-MLC level in primary VSMCs after three days of growth. (a) Immuncytochemistry for p-MLC (green) and F-actin (red) - merge (yellow). Cells were grown in the absence and presence of 1 mM 8-Br-cGMP (cGMP). (b) Quantification of total p-MLC (green) fluorescence per cell. Fluorescence was normalized to control. 8-Br-cGMP treated cells show a significantly higher total fluorescence per cell (***, p<0.001) as compared to control (ctr) (ctr 1.0 n=50 cells; cGMP 1.8±0.1 n=61 cells). Error bar represents SEM. (c) Western blot analysis of p-MLC. Cells were grown in the absence or presence of 0.1 mM 8-Br-cGMP (cG) for three days. One representative western blot of at least three is shown. Detection for cGKI determines wt and ko cells. MLC was used as loading control.*

The previous experiments indicate that activation of cGMP/cGKI signaling in primary VSMCs increases adhesion (Fig. 18, 20), thereby, generating increased stress fibers in the adherent cells. The following experiments were performed to investigate, whether RhoA/ROCK signaling is changed due to cGMP/cGKI signaling.

3.3.3 RhoA/ROCK Signaling



Proposed model for cGKI-mediated formation of stress fibers. Further explanation see text.

It is well known that RhoA/ROCK signaling is important for the formation of stress fibers (Ridley and Hall, 1992; Rottner et al., 1999; Worth et al., 2004). RhoA stimulates actomyosin-based contractility through its downstream target ROCK, and this kinase is required for stress fiber formation in cultured cells. Therefore, the activity of RhoA and ROCK was analyzed.

To see whether cGMP/cGKI signaling in primary VSMCs influences RhoA activity, a RhoA pulldown assay was conducted. To activate RhoA constitutively, cytotoxic necrotizing factor (CNF) was administrated. CNF causes deamination of RhoA, causing a shift in its electrophoretic mobility (Fig. 22 lane 3, 5) (Fiorentini et al., 1997; Richard et al., 1999). Because the amount of cells is critical for a successful pulldown, subcultured VSMCs were used as positive control. The amount of active RhoA (RhoA-GTP/RhoA*) in the cells represents about 0.5-5% of total RhoA (Ren et al., 1999).

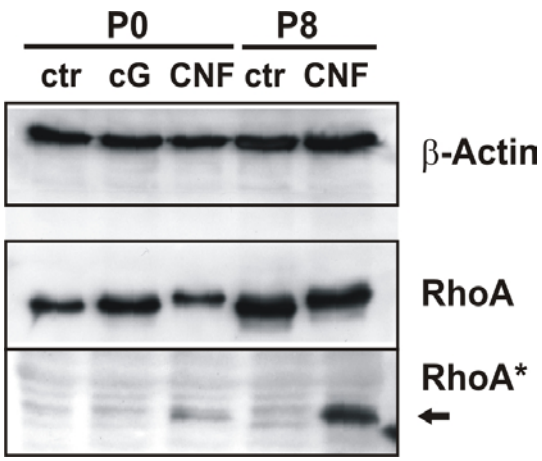


Fig. 22: RhoA pulldown assay. Lane 1-3: Primary VSMCs (P0) that were grown for three days in the absence or presence of 1 mM 8-Br-cGMP (cG) or 2.5 µg/ml CNF for three hours to activate RhoA constitutively (RhoA = RhoA-GTP) Lane 4-5: Subcultured cells – passage 8 (P8) – as positive control. β-Actin was used as loading control. RhoA indicates total RhoA level in the extracts. RhoA* indicates active RhoA in the extracts. No change of RhoA* can be detected in primary VSMCs in response to 8-Br-cGMP as compared to control. CNF causes a slight increase in RhoA* in primary VSMCs and a very strong increase in subcultured VSMCs as compared to control, demonstrating that the assay works. One representative example of three experiments is shown.*

To isolate RhoA-GTP from the cell extracts, the VSMC lysates were incubated with glutathione sepharose beads that have previously been loaded with the Rho-binding domain (RBD) of ROCK II, which was expressed as GST-RBD fusion construct. In comparison to untreated, passaged VSMCs, treatment with CNF led to a strong increase in RhoA-GTP (Fig. 22 lanes 4, 5). In primary VSMCs, CNF caused a slight increase in active RhoA (Fig. 22 lane 3). Active RhoA was hardly detectable in primary VSMCs under control and 8-Br-cGMP treated conditions (Fig. 22 lane 1, 2). Interestingly, 8-Br-cGMP treatment caused an increase

of total RhoA (Fig. 22 lane 1, 2) that is possibly mediated by cGKI, because it was absent in cGKI-deficient cells (data not shown). This increase might be due to phosphorylation at Ser188, which has been described to stabilize RhoA in the cytosol (Rolli-Derkinderen et al., 2005; Sauzeau et al., 2003). The expression on mRNA level was not changed as revealed by RT-PCR (data not shown), although this has previously been described (Sauzeau et al., 2003). Another pulldown experiment with cells in suspension (-/+ 1 mM 8-Br-cGMP for 2 hours) was performed because it has been described that RhoA is more active in cells in suspension (Ren et al., 1999). Nevertheless, RhoA-GTP could either not be detected in VSMCs in suspension (data not shown). To confirm these findings, another assay to detect RhoA-GTP was applied (G-Lisa; Cytoskeleton).

RhoA activity was tested again in response to 8-Br-cGMP in adherent cells (Fig. 23a) and in suspension (Fig. 23b). Under both conditions, no differences in RhoA activity could be observed in response to 8-Br-cGMP, although basal levels of RhoA-GTP could be detected (Fig. 23). To see whether RhoA activity could be increased at all, cells were treated with the thromboxane mimetic U-46619. As shown in Figure 23a, U-46619 was able to activate RhoA. The activation of RhoA with U-46619 could be suppressed with 8-Br-cGMP. The inhibitory action of cGMP signaling on RhoA activity has also been described by others (Sauzeau et al., 2000; Seko et al., 2003).

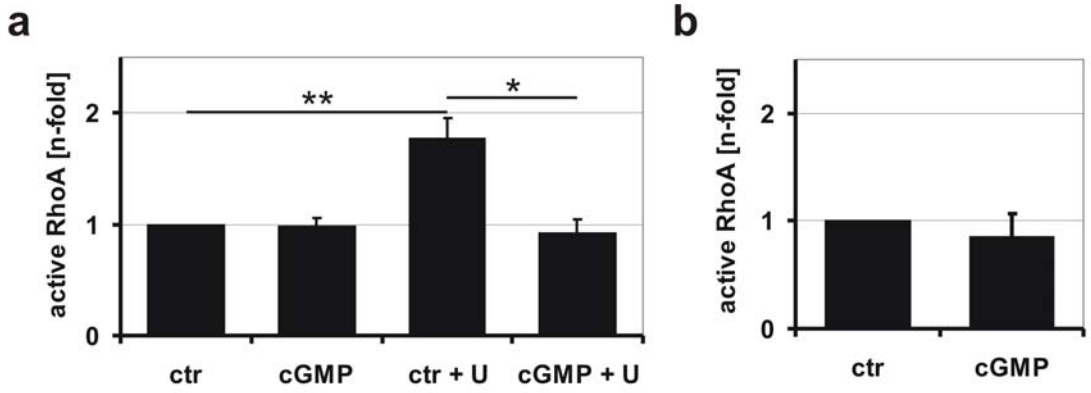
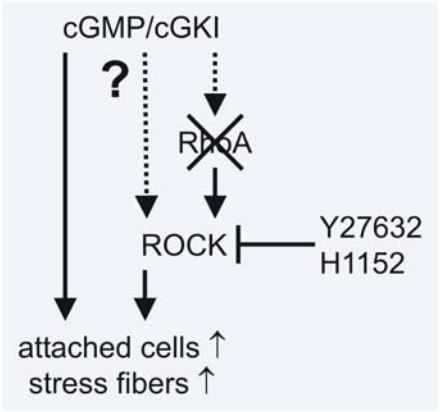


*Fig. 23: G-Lisa RhoA assay. (a) RhoA activity of primary VSMCs, grown for three days in the absence or presence of 0.1 mM 8-Br-cGMP (cGMP). Cells were treated with 2 µM U-46619 (U) for 5 min to activate RhoA. cGMP does not influence RhoA activity in comparison to control. Treatment with U causes a significant increase in RhoA activity (**, p<0.01) in comparison to control. 8-Br-cGMP suppresses the stimulatory effect of U-46619 on RhoA activity significantly (*, p<0.05) (ctr 1.0 n=3 wells; cGMP 1.0±0.06 n=3 wells; ctr + U 1.8±0.19 n=2 wells; cGMP + U 0.9±0.13 n=2 wells). One representative of two experiments is shown. (b) Primary VSMCs in suspension that were stimulated for 30 min with 0.1 mM 8-Br-cGMP after isolation. 8-Br-cGMP does not influence RhoA activity in comparison to control (ctr 1.0 n=2 wells; cGMP 0.9±0.22 n=2). RhoA activity was normalized to control. Error bars represent SEM.*

Taken together, a change in RhoA activity in response to 8-Br-cGMP could neither be detected with a RhoA pulldown experiment nor with the G-Lisa RhoA assay. Nevertheless,

RhoA activity could be increased as demonstrated by treating the primary VSMCs with the thromboxane mimetic U-46619. These findings implicate that cGMP/cGKI does not signal via a change of RhoA activity in the present work.



Proposed model for cGKI-mediated formation of stress fibers. Further explanation see text.

Another target that has been described to be essential for the formation of stress fibers is ROCK. It has often been described that inhibition of ROCK leads to a disruption of stress fibers (Katoh et al., 2001; Kaunas et al., 2005; Tsuji et al., 2002). Therefore, the effects of two ROCK inhibitors on the growth of primary VSMCs were tested. First, Y27632 was applied (Fig. 25c), a frequently used inhibitor, and secondly H1152 (Fig 25d), a more specific inhibitor of ROCK. Interestingly, both drugs increased the number of cells that were attached after two to three days of growth (Fig. 24a, 25) and also increased the number of stress fibers in comparison to control (Fig. 25c, d). In addition, treatment of the cells with the ROCK inhibitors caused a similar morphology and growth progression like 8-Br-cGMP treatment (Fig. 24, 25). The effects caused by blocking ROCK should be downstream or independent of cGKI, because Y27632 as well as H1152 increased the growth of cGKI-deficient VSMCs (Fig. 24a, and data not shown). Increased adhesion in response to inhibition of ROCK has also been observed by others (Koga et al., 2006).

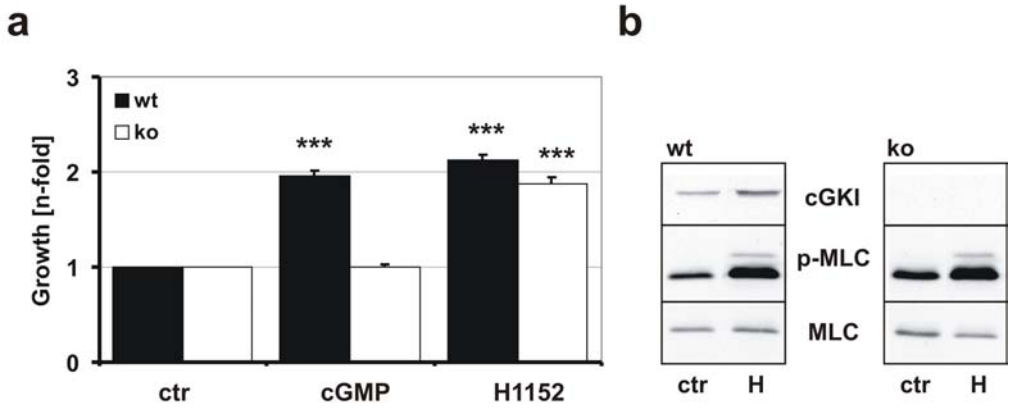


*Fig. 24: (a) Growth assays (MTS) of primary VSMCs in response to 8-Br-cGMP (cGMP) and H1152. In wt cells 8-Br-cGMP (0.1 mM) and H1152 (0.3 µM) significantly (***, p<0.001) increased growth of VSMCs in comparison to control (1.0 n=8 wells; cGMP 2.0±0.06 n=8 wells; H1152 2.1±0.05 n=7 wells). In ko cells 8-Br-cGMP has no effect on VSMC growth. In contrast H1152 significantly promotes growth of primary VSMCs in comparison to control (1.0 n=8 wells; cGMP 1.0±0.03 n=8 wells; H1152 1.9±0.07 n=6 wells). Growth was normalized to control. Error bars represent SEM. (b) Western blot analysis of p-MLC. Cells were grown in the absence or presence of 0.3 µM H1152 (H) for three days. One representative western blot of at least three is shown. MLC was used as loading control. Detection for cGKI determines wt and ko cells.*

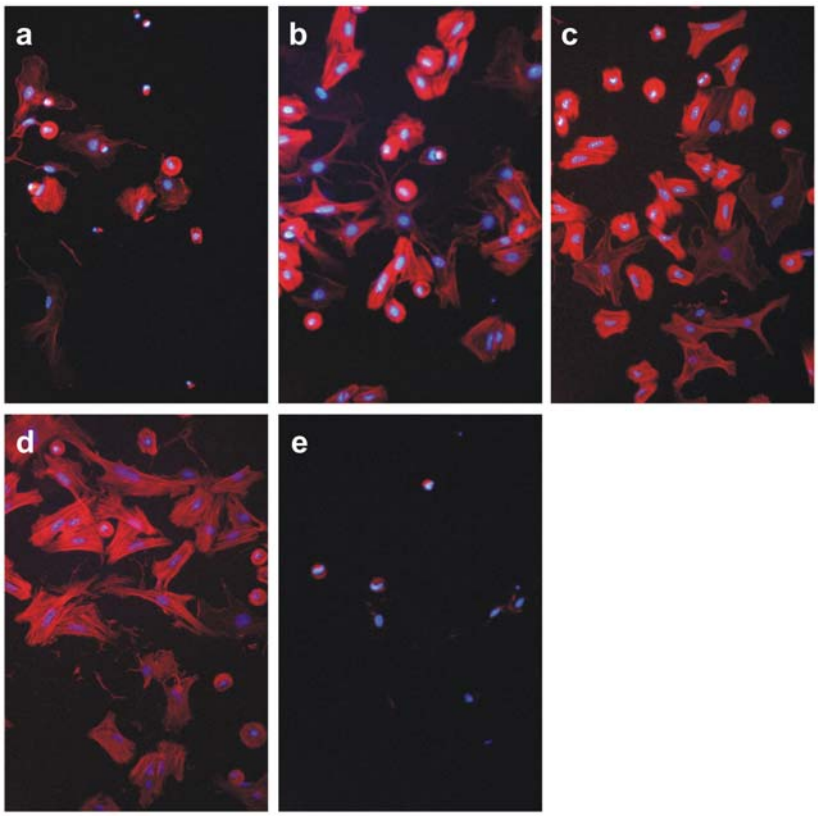


Fig. 25: Growth of primary VSMCs grown for 48 hours and stained for F-actin (red) and nuclei (blue/Hoechst dye). (a) control – untreated cells, (b) 0.1 mM 8-Br-cGMP, (c) 10 µM Y27632, (d) 0.3 µM H1152, and (e) 2 µM U-46619 treated cells. 8-Br-cGMP treatment as well as inhibition of ROCK with Y27632 or H1152 causes a strong increase in the number of attached cells and the formation of stress fibers. Treating primary VSMCs with U-46619 inhibits adhesion and the generation of stress fibers. One of at least three experiments is shown.

Nevertheless, the strong formation of stress fibers induced by these ROCK inhibitors was unexpected. In line with the strong formation of stress fibers is an increase of phosphorylated MLC in response to H1152 in wild-type and cGKI-deficient cells (Fig. 24b, compare to Fig. 21c).

To elucidate the effects of activation of RhoA/ROCK signaling on VSMC growth, cells were treated with U-46619 to increase RhoA activity. As shown in Figure 25e, only a few VSMCs attached and no spreading or formation of stress fibers occurred. These findings suggest that for the attachment of primary VSMCs RhoA/ROCK signaling has to be suppressed (Arthur and Burridge, 2001; Arthur et al., 2000). This is in line with the findings from the RhoA pulldown experiments, which revealed that basal RhoA activity was hardly detectable.

3.3.4 Rac Activity

Another member of the Rho family, namely Rac is implicated in cell adhesion and the generation of focal complexes, which mature to focal contacts (= focal adhesions). Commonly, Rac is described to induce the formation of lamellipodia (Rottner et al., 1999; Sander et al., 1999). Interestingly, as revealed by a recent study, Rac1 is supposed to play a critical role in actin stress fiber formation (Guo et al., 2006).

To investigate whether Rac1 is involved in our system, a Rac pulldown was performed with primary VSMCs that were grown for three days (Fig. 26). To isolate Rac-GTP from the cells, the lysate was incubated with glutathione sepharose beads that have previously been loaded with the CRIB domain of p21-activated kinase. To stimulate Rac activity in passaged cells, CNF was used. Figure 26 shows that Rac activity (Rac*/Rac-GTP) is increased in response to CNF in subcultured cells. In contrast, 8-Br-cGMP did not influence Rac activity in primary VSMCs (Fig. 26 lane 1+2).

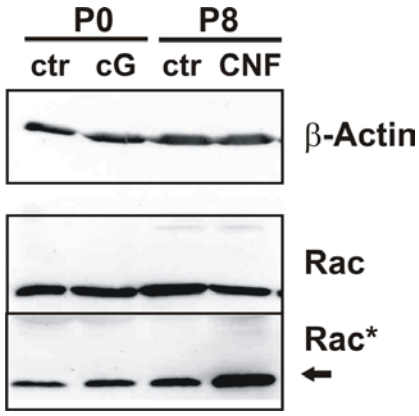


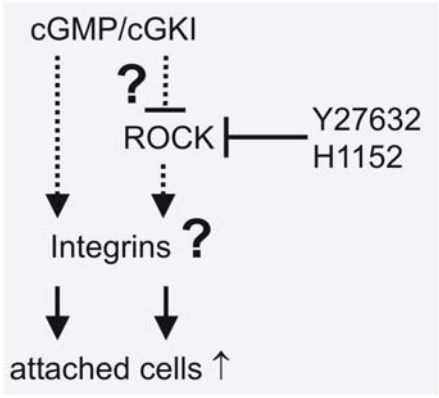
Fig. 26: Rac pulldown assay. Lane 1-2: Primary VSMCs (P0) that were grown without (ctr) or with 1 mM 8-Br-cGMP (cG) for three days. Lane 3-4: Subcultured cells – passage 8 (P8). Untreated cells (ctr) and CNF treated cells (2.5 µg/ml for 3 hours) are shown. β-Actin was used as loading control. Rac indicates total Rac level in the extracts. Rac indicates active Rac in the extracts. No change of Rac* can be detected in primary VSMCs in response to 8-Br-cGMP as compared to control. CNF causes an increase in Rac* in subcultured VSMCs as compared to control demonstrating that the assay works. One representative of two similar eriments is shown.*

Taken together, it can be concluded that the small GTPases RhoA and Rac1 are probably not the major mediators of stress fiber formation in primary VSMCs. No change of activity of RhoA or Rac1 was detectable in response to 8-Br-cGMP after 24 hours in suspension (only done for RhoA) or three days of growth, indicating that RhoA or Rac1 are not involved in cGMP/cGKI-mediated adhesion and the formation of stress fibers.

The previous experiments demonstrated that activation of cGMP/cGKI signaling in primary VSMCs increases adhesion (Fig. 18, 20, 25), thereby generating increased stress fibers in adherent cells probably as a secondary effect. A comparison of primary wild-type and cGKI-deficient VSMCs after 72 hours in the absence and presence of 8-Br-cGMP revealed that wild-type VSMCs as well as cGKI-deficient VSMCs form stress fibers (Fig. 20). According to these findings, cGMP/cGKI signaling mediates increased adhesion of primary VSMCs rather than a direct involvement in the formation of stress fibers.

3.3.5 Integrin-Mediated Adhesion

To analyze whether integrins are involved in cGKI-mediated adhesion, primary VSMCs were labeled for β_1 and β_3 integrins, two integrins that are known to be important for adhesion and attachment to the ECM. Primary VSMCs were kept in suspension for 24 hours in the absence or presence of 8-Br-cGMP. Previous to the analysis by flow cytometry, the cells were labeled for β_1 and β_3 integrins using specific antibodies. The fluorescence signals for both tested integrins, β_1 and β_3, were increased after 8-Br-cGMP stimulation in wild-type cells as compared to control, whereas no difference was observed in cGKI-deficient cells (Fig. 27). These results clearly demonstrate that the increase in integrins is mediated via cGKI.



Proposed model for cGKI-mediated increased adhesion. Further explanation see text.

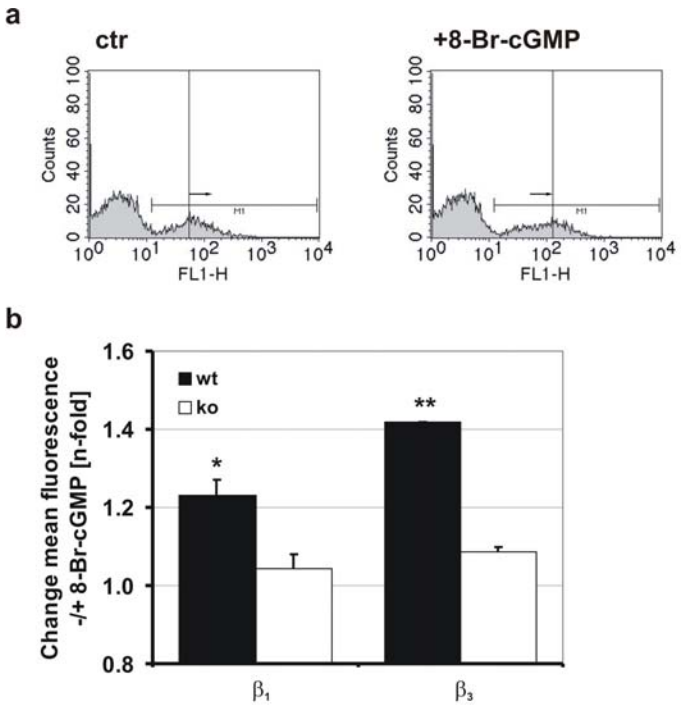


Fig. 27: Integrin analysis of primary VSMCs 24 h -/+ 8-Br-cGMP (1mM) in suspension by flow cytometry. (a) Wt VSMCs show an increased mean fluorescence signal for β_3 integrins in response to 8-Br-cGMP as compared to control. (b) Wt cells show a significantly elevated mean fluorescence signal for β_1 (, p<0.05) and β_3 (**, p<0.01) integrins in response to 8-Br-cGMP as compared to control (ctr 1.0 n=3 probes; β_1 1.23±0.04 n=2 probes; β_3 1.42±0.00 n=2 probes). In contrast, in ko cells β integrins are not increased in response to 8-Br-cGMP (ctr 1.0 n=3 probes; β_1 1.04±0.04 n=3 probes; β_3 1.09±0.01 n=3 probes). One representative of three experiments is shown. Error bars represent SEM.*

To elucidate whether the difference in signal intensity is mediated via a change in the expression level, RT-PCR was performed. No change on mRNA level for β_1 (Fig. 28) and β_3 (data not shown) could be detected, as revealed by the analysis of mRNA isolated from primary VSMCs that were grown in the absence and presence of 8-Br-cGMP for three days.

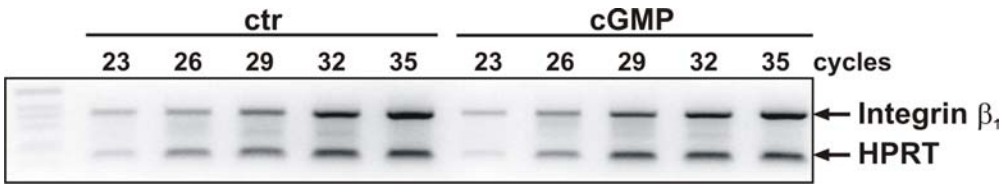


Fig. 28: RT-PCR for β_1 integrin. HPRT was co-amplified as an internal standard. The primer for β_1 integrin were used at 6.25 pmol/µl and the primer for HPRT were used at 50 pmol/µl. The mRNA level of β_1 integrin on mRNA level is not changed in response to 8-Br-cGMP after three days of growth as compared to control.

To assess the functional significance of β integrins for adhesion and to validate the results obtained by flow cytometry, a blocking experiment was performed using specific blocking antibodies for either β_1 or β_3. To block adhesion, a growth assay was conducted with primary VSMCs that were kept in culture for three days. The blocking effect on growth was investigated in the absence or presence of 8-Br-cGMP and integrin blocking antibodies. Attachment under control conditions was not impaired by the use of blocking antibodies. Neither the anti-β_1 nor the anti-β_3 antibody affected growth at the indicated concentration (Fig. 29). Interestingly, the growth-promoting effect of 8-Br-cGMP was blocked by the use of anti-β_1 in combination with anti-β_3 integrin antibody. Each blocking antibody alone partially suppressed the 8-Br-cGMP induced adhesion (Fig. 29). Thus, β_1 as well as β_3 integrins seem to be important for cGKI-mediated adhesion.

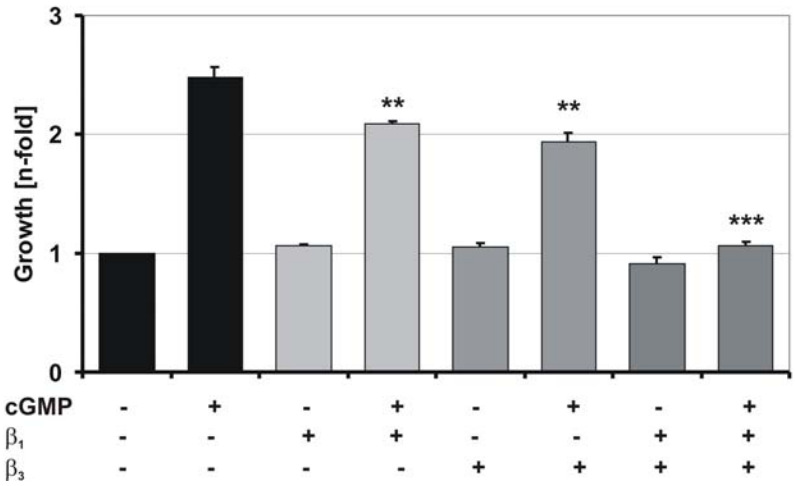


*Fig. 29: Integrin-mediated adhesion of primary VSMCs in response to 8-Br-cGMP. The assay (MTS) was performed with primary VSMCs that were grown for 72 h in the presence or absence of 1 mM 8-Br-cGMP and 25 µg/ml integrin blocking antibodies. Adhesion under control conditions is not affected by the integrin blocking antibodies (ctr 1.0 n=8 wells; ctr + β_1 1.1±0.02 n=4 wells; ctr + β_3 1.1±0.04 n=4 wells; ctr + β_{1+3} 0.9±0.05 n=4 wells). Increased adhesion in response to 8-Br-cGMP (cGMP) (0.1mM) is significantly reduced upon treatment with either β integrin blocking antibody alone (**, p<0.01) or even stronger by using a combination of both antibodies (each 25 µg/ml) (***, p=0.001) (cG 2.5±0.09 n=7 wells; cG + β_1 2.1±0.03 n=4 wells; cG + β_3 1.9±0.08 n=4 wells; cG + β_{1+3} 1.1±0.03 n=4 wells). Growth was normalized to control. Error bars represent SEM.*

Blocking of ROCK - as activation of cGKI - caused a strong increase in growth and leads to the same phenotypic change as compared to control (Fig. 24, 25). Next, it was assessed whether integrins are involved in the increased adhesion mediated via inhibition of ROCK. Therefore another blocking assay was performed and the effects of 8-Br-cGMP and H1152 on VSMC growth were compared (Fig. 30).

H1152 as well as 8-Br-cGMP induced growth was strongly reduced upon addition of β_1 and β_3 blocking antibodies. In addition, the increase in growth caused by cGKI or H1152 was not additive. This implicates that both drugs might signal via the same downstream pathways.

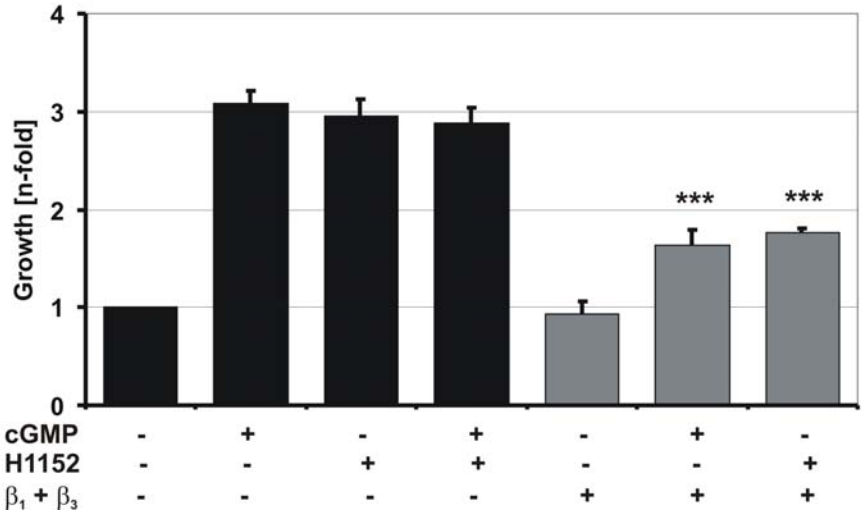


*Fig. 30: Comparison of integrin-mediated adhesion of primary VSMCs in response to 8-Br-cGMP and H1152. The assay (TB) was performed with primary VSMCs that were grown for 72 h in the presence or absence of 1 mM 8-Br-cGMP (cGMP), 0.3 µM H1152, and 25 µg/ml integrin blocking antibodies. Growth is strongly promoted in response to 8-Br-cGMP and H1152 in comparison to untreated cells (control). The growth effects of 8-Br-cGMP and H1152 are not additive (black bars). A combination of β_1 and β_3 integrin blocking antibodies does not influence adhesion under control conditions, but significantly (**; p<0.001) suppresses growth induced by 8-Br-cGMP or H1152 (ctr 1.0 n=16 wells; cGMP 3.1±0.14 n=7 wells; H1152 3.0±0.17 n=8 wells; cGMP + H1152 2.9±0.16 n=5 wells; ctr + β_{1+3} 0.9±0.13 n=4 wells; cGMP + β_{1+3} 1.6±0.16 n=4 wells; H1152 + β_{1+3} 1.8±0.04). Growth was normalized to control. Error bars represent SEM.*

As shown in Figures 29 and 30, growth of primary VSMCs under control conditions was not influenced by blocking β_1 and β_3 integrins with the used concentrations of blocking antibodies. In contrast, 8-Br-cGMP and H1152 induced growth was reduced by the use of β_1 and β_3 integrin blocking antibodies. A possible explanation for this finding is that 8-Br-cGMP and H1152 increase the number of integrins – as demonstrated by flow cytometry (Fig. 27) – and activate β_1 and β_3 integrins. Activation of integrins enables the primary VSMCs to adhere with a reduced number of integrins as compared to control conditions. As a consequence, the used blocking antibody concentration of 25 µg/ml is sufficient to block 8-Br-cGMP or

H1152 induced adhesion, whereas adhesion under control conditions – where more (unactivated) integrins are needed - is not affected.

After examining adhesion of primary VSMCs, it was investigated whether β integrins are also involved in adhesion of subcultured cells. To this end, a growth assay was performed with passaged cells. Adhesion of subcultured VSMCs depends rather on β_1 integrins, because blocking of β_3 integrins had little effect (Fig. 31). According to the literature β_3 integrins are linked to cell migration (Blaschke et al., 2002; Sajid et al., 2003; Slepian et al., 1998). Therefore, the rather little involvement of β_3 integrins in adhesion was not unexpected.

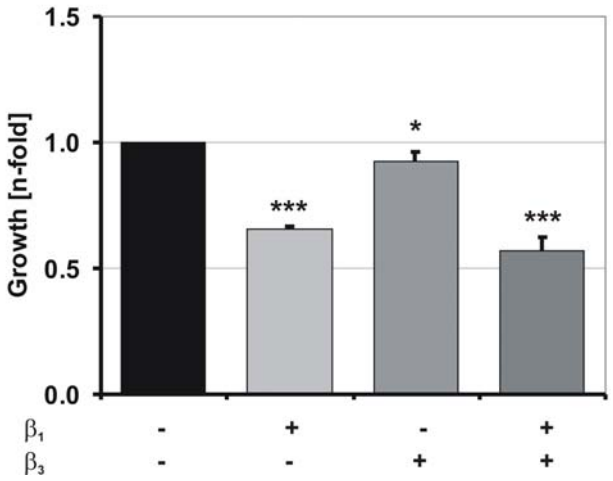


*Fig. 31: Integrin-mediated adhesion of subcultured VSMCs (P7). The assay (MTS) was performed with cells that were grown for 72h in the presence or absence of 25 µg/ml integrin blocking antibodies. Growth is significantly (***, p<0.001) inhibited with the β_1 blocking antibody as compared to control (ctr). Blocking of β_3 integrins reveals only a slight growth suppressing effect (*, p<0.05) (ctr 1.0 n=8 wells; β_1 0.7±0.01 n=3 wells; β_3 0.9±0.04 n=4 wells; β_{1+3} 0.6±0.05 n=4 wells). Growth was normalized to control. Error bars represent SEM.*

Interestingly, with the used 25 µg/ml of β_1 blocking antibody basal growth of subcultured VSMCs was significantly reduced as compared to control (Fig. 31). In contrast, in primary VSMCs basal growth was not influenced in the presence 25 µg/ml blocking (Fig. 29). This indicates that growth of primary VSMCs is different to growth of subcultured VSMCs.

In summary, the increased growth of primary VSMCs upon activation of cGMP/cGKI signaling as well as upon blockade of ROCK is probably mediated through β_1 and β_3 integrins, leading to increased adhesion.

3.3.6 FAK Phosphorylation

Focal adhesion kinase (FAK) is a major mediator of signal transduction by integrins and has been implicated in the regulation of cell spreading, migration, survival and proliferation (Schwartz et al., 1995). One marker for increased integrin activity is the phosphorylation of focal adhesion kinase (FAK) (Giancotti and Ruoslahti, 1999). In addition, it has been described that stress fibers are associated with increased levels of phosphorylated FAK (Chrzanowska-Wodnicka and Burridge, 1994). To assess whether the increased integrin activation influences FAK signaling, phosphorylation of FAK was investigated.

As revealed by western blot, activation of cGKI caused an increase in phosphorylation of FAK (Fig. 32). Furthermore, inhibition of ROCK led to an increase in FAK phosphorylation in wild-type VSMCs and in cGKI-deficient cells (Fig. 32). These results suggest that cGMP/cGKI signaling and inhibition of ROCK lead to increased integrin signaling causing increased p-FAK levels. This might be one possible signaling pathway, transferring the signal into the cell.

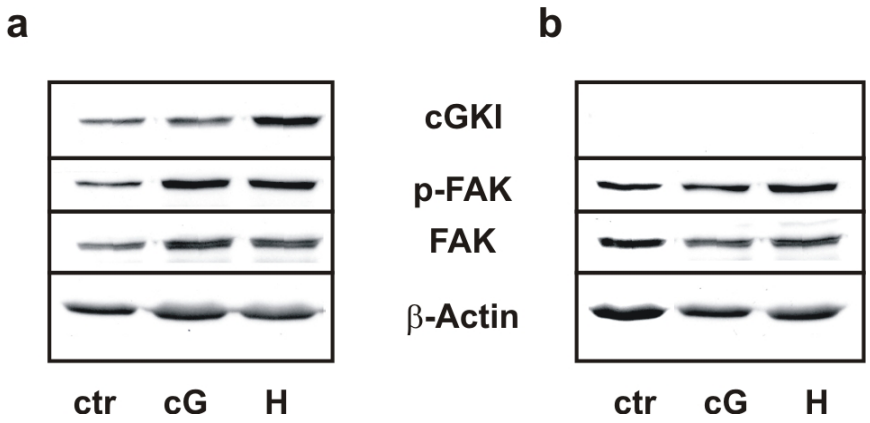
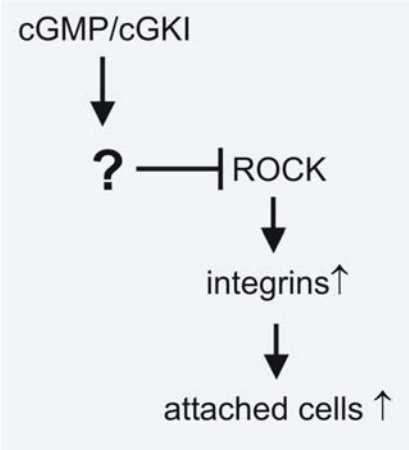


Fig. 32: Phosphorylation of FAK in primary VSMCs. Western blot analysis of primary VSMCs that were grown for 72 hours. Blot of (a) wt cells (b) ko cells are shown. Cells were kept under control conditions (ctr) or were treated with 0.1 mM 8-Br-cGMP (cG) or 0.3 µM H1152 (H). One representative blot of at least three is shown. β-Actin was used as loading control. An antibody against cGKI was used to differentiate wt from ko cells. Phosphorylation of FAK in wt cells is increased in response to 8-Br-cGMP and H1152 as compared to control. In ko cells FAK is only phosphorylated in response to H1152 as compared to control.

3.3.7 cGKI Signaling via Inhibition of ROCK

The previous findings suggest that cGMP/cGKI-mediated growth stimulation might, at least in part, proceed via inhibition of ROCK. A known intracellular inhibitor of ROCK is RhoE (Rnd3). RhoE belongs to a subset of the Rho family that binds GTP but has no or very low intrinsic hydrolytic activity. Binding of RhoE to ROCK I inhibits its kinase activity (Riento et al., 2005b). Examination of the mRNA level of RhoE after three days of growth did not reveal a significant difference in expression of RhoE between 8-Br-cGMP treated VSMCs in comparison to untreated cells (Fig. 33a).



Proposed model for cGKI-mediated increased adhesion. For further explanation see text.

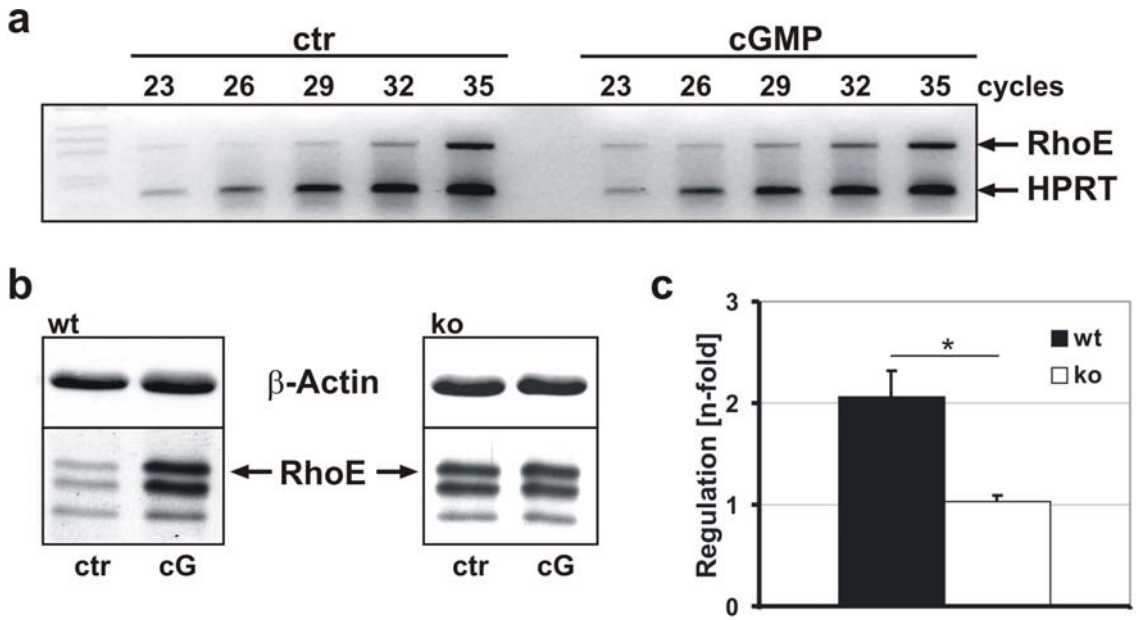


Fig. 33: Expression of RhoE in primary VSMCs that were grown for three days in the absence or presence of 1 mM 8-Br-cGMP. (a) RT-PCR for RhoE. HPRT was co-amplified as internal standard. Expression is not changed in response to 8-Br-cGMP (cGMP) in comparison to control (ctr). (b) Western blot analysis of RhoE protein expression (Rnd3, ~29kDa). The antibody cross reacts with Rnd1 (~27kDa). RhoE is upregulated in response to 8-Br-cGMP in wt cells as compared to control. No significant difference of RhoE expression can be observed in ko cells in response to 8-Br-cGMP. (c) Semi quantitative analysis of RhoE western blots. For densitometric analysis of RhoE expression the AIDA software, version 2.11 was used (Raytest Isotopenmessgeräte GmbH). Compared to ko cells RhoE expression is significantly increased in wt cells in response to 8-Br-cGMP (, $p<0.05$) (wt n=11 cell extracts; ko n=5 cells extracts). Error bars represent SEM.*

In contrast, the protein level was significantly changed in response to 8-Br-cGMP treatment (Fig. 33b, c). This increase in protein level was mediated by cGKI and could be possibly caused by phosphorylation of RhoE, thereby stabilizing the protein (Riento et al., 2005a).

In summary, several results indicate that the cGMP/cGKI-mediated effects on growth of primary VSMCs are, at least in part, mediated via inhibition of ROCK: First, treatment of primary VSMCs with 8-Br-cGMP as well as H1152 (or Y27632) caused a similar phenotype of primary VSMCs (Fig. 25) and led to an increase in stress fiber formation (Fig. 25) and p-MLC levels (Fig. 21, 24b). Moreover, H1152 might act on a signaling component downstream of cGKI, because its growth-promoting effect could also be observed in cGKI-deficient cells (Fig. 24a). Second, the effects of 8-Br-cGMP and H1152 were not additive (Fig. 30). Third, 8-Br-cGMP and H1152 led to increased integrin-mediated adhesion (Fig. 30), which led to an increase in adherent cells after three days of growth (Fig. 30).

D. Discussion

1. Growth of VSMCs

1.1 VASP as a "Biomarker"

VASP was originally purified in 1989 by Halbrügge and Walter (Halbrugge and Walter, 1989) and is a substrate for both cGK and cAK. Three phosphorylation sites on VASP have been identified, Ser157, Ser239 and Thr278. It has been reported that Ser239 is the preferential phosphorylation site for cGK, whereas Ser157 is the preferential phosphorylation site for cAK (Butt et al., 1994a). Recently, it was shown that Ser157 is also phosphorylated in response to growth factors by PKC activity (Chitaley et al., 2004). VASP has been discovered as a monitor for cGKI and cAK activity (Lohmann and Walter, 2005). Furthermore, Chen et al. suggest that VASP could play a critical role in cGKI-dependent control of VSMC growth and differentiation (Chen et al., 2004).

In the present work an antibody that recognizes total VASP was used for the detection of cGKI activity as well as cAK activity. In our experimental setup, 8-Br-cGMP induced a stronger phosphorylation signal on Ser157 in comparison to 8-Br-cAMP. This was unexpected since Ser157 is described to be the preferential phosphorylation site for cAK. This discrepancy might be due to our experimental setup for the phosphorylation of VASP. Signal intensity for Ser157 in response to 8-Br-cGMP and 8-Br-cAMP varies with the time of drug treatment and the medium used to perform the phosphorylation assay (Lukowski, 2006). With high doses of 8-Br-cGMP (1mM), a slight phosphorylation was detected in cGKI-deficient cells, which could be due to cross-activation of cAK (Fig. 8). A work by Li et al. (Li et al., 2003) proposed a predominant role for cAK in cGMP-induced phosphorylation of VASP in human platelets. We can exclude this for our system, because phosphorylation of VASP is absent in cGKI-deficient cells upon stimulation with 0.1 mM 8-Br-cGMP.

Several lines of evidence indicate that phosphorylation of VASP is not directly linked to VSMC growth: (1) In primary VSMCs growth is increased upon stimulation with 8-Br-cGMP whereas it is decreased upon stimulation with 8-Br-cAMP, although VASP is phosphorylated in response to 8-Br-cGMP and 8-Br-cAMP. (2) High doses of 8-Br-cGMP induce VASP phosphorylation in cGKI-deficient cells (Fig. 8) without affecting growth. (3) The cGKI-inhibitor Rp-8-Br-PET-cGMPs stimulates growth (Fig. 10), although phosphorylation of VASP could not be detected (Fig. 11). (4) Activation of cGKI causes phosphorylation of VASP at Ser157 in primary VSMCs, coincidencing with increased growth, and subcultured VSMCs associated with a growth reduction or no effect on growth respectively (Fig. 12, 16, 17).

1.2 cGMP/cGKI Signaling

There are inconsistent results concerning the growth effects mediated by cGKI in VSMCs. Several animal studies, using models of vascular injury that induce modulation of VSMCs, showed that NO/cGMP signaling suppresses VSMC proliferation and increases apoptosis (Anderson et al., 2000; Sinnaeve et al., 2002). In addition, a loss of cGKI expression was reported when VSMCs change to the proliferative phenotype. In line with these findings, immunoreactive cGKI staining was strongly reduced in neointimal VSMCs as compared to normal medial VSMCs of autopsy tissues of atherosclerotic human coronary artery (Anderson et al., 2000). The reported inhibitory effect of NO/cGMP signaling and the strong reduction of cGKI expression in proliferating VSMCs suggests an anti-proliferative effect for NO/cGMP/cGKI signaling. Furthermore, several studies, working with animal models of hypercholesterolemia-induced atherosclerosis (Boger et al., 1997; Cayatte et al., 1994; Napoli et al., 2002), suggest that NO has antiatherosclerotic effects in the arterial wall of hypercholesterolemic animals. Nevertheless, the above-mentioned studies failed to demonstrate an involvement of cGKI, the expected downstream target for NO/cGMP signaling. In contrast to the common view of cGMP/cGKI signaling as anti-proliferative and antiatherosclerotic, the analysis of endogenous cGKI function in a model of hyperlipidemia-induced atherosclerosis suggests a proatherogenic function of cGMP/cGKI signaling (Wolfsgruber et al., 2003). The opposing results concerning cGKI function on VSMC growth might be attributable to several reasons: (1) different species and (2) models for vascular remodeling were used, (3) constitutively active cGKI was delivered exogenously by gene transfer (Sinnaeve et al., 2002), whereas in the atherosclerosis model endogenous cGKI function was analyzed.

Supporting the view that NO/cGMP/cGKI signaling causes growth inhibition, several *in vitro* studies demonstrated an anti-proliferative effect for cGMP and cGKI (Garg and Hassid, 1989; Hassid et al., 1994; Li and Sun, 2005). Many of these studies used subcultured VSMCs (Garg and Hassid, 1989; Hassid et al., 1994), VSMC-derived cell lines (e.g. A7r5) (Capey et al., 2006) or VSMCs which have been transfected with cGKI (Boerth et al., 1997; Browner et al., 2004a; Dey et al., 2005). These systems are often highly artificial and probably do not represent the *in vivo* situation. In the present study, primary wild-type VSMCs were compared to cGKI-deficient VSMCs isolated from the murine aorta. Activation of cGKI in primary VSMCs causes a strong increase in growth (Fig. 12, 15) that is probably mainly mediated via increased cell adhesion (see below). Moreover, by activation of cGMP/cGKI and cAMP/cAK signaling in subcultured cells we could confirm the described anti-proliferative effects on VSMC growth (Fig. 13). These findings are in line with Hassid and co-workers who demonstrated that in freshly isolated contractile rat aortic VSMCs, NO-donors and cGMP

analogs do not inhibit cell proliferation but indeed enhance fibroblast growth factor-induced VSMC proliferation (Hassid et al., 1994). Once passaged, however, the cells respond to NO and cGMP treatment with inhibition of growth.

In contrast to several other studies which reported that cGKI expression is lost through passages (Boerth et al., 1997; Cornwell and Lincoln, 1989; Cornwell et al., 1994b; Dey et al., 1998), cGKI was expressed in VSMCs up to passage 11 in our system. These findings are in line with Lin et al. (Lin et al., 2004), who conducted a systematic investigation on the stability of cGKI expression in cultured VSMCs. This study indicates that cGKI expression is stably maintained in repetitively propagated VSMCs and is hardly affected by cell density. Furthermore, these results do not support the view that the phenotypic modulation of VSMCs is linked to a loss of cGKI expression. As shown in this work (Fig. 6), VSMCs modulate with the beginning of passaging, independent of cGKI expression. Wild-type VSMCs and cGKI-knockout VSMCs have a similar phenotype under control conditions.

1.3 Cross-Activation of cGMP and cAMP Signaling

Another kinase that has major impact on VSMCs growth is the cAK. It is well established that cAK has an inhibitory effect on VSMCs growth (Bonisch et al., 1998; Bornfeldt and Krebs, 1999; Chen et al., 2004; Osinski et al., 2001). The analysis of VSMC growth is further complicated by increasing evidence that some cGMP-mediated effects might be caused by direct cross-activation of cAK (Chen et al., 2004; Cornwell et al., 1994a; Komalavilas et al., 1999; Lin et al., 2001; Osinski et al., 2001; Wu et al., 2006) or indirectly via a cGMP-mediated inhibition of PDE3 and a subsequent increase in the cAMP level (Aizawa et al., 2003). Conversely, some cAMP-mediated effects might be caused by activation of cGKI (Barman et al., 2003; Cornwell et al., 1994a; Lin et al., 2001).

By investigating the effects of 8-Br-cGMP and 8-Br-cAMP on the growth of primary VSMCs in comparison to passaged VSMCs from the mouse aorta, we could detect no cross-activation in either way (Fig. 13). This implies that the molecular pathways regulated by 8-Br-cGMP and 8-Br-cAMP are distinct (Koyama et al., 2001). In addition, it was found that 8-Br-cAMP is more potent in inhibiting VSMC growth in comparison to 8-Br-cGMP (Fig. 13). This was also found by others (Fukumoto et al., 1999; Kariya et al., 1989) and might be an explanation for the finding that 8-Br-cGMP had no effect on the tested subcultured rat and human VSMCs, whereas cAMP had a strong growth suppressing effect. Nevertheless, cross-activation cannot be excluded *in vivo*. The primary VSMCs used in this work were stimulated with 8-Br-cGMP, which is a membrane-permeable cGMP analog. Herbert et al. showed that 8-Br-cGMP binds with lower affinity to the noncatalytic cGMP-binding sites of frog PDE than

endogenous cGMP (Hebert et al., 1998). This suggests that endogenously generated cGMP may also have a higher affinity for cAK, leading to its activation. The concentrations of cGMP that activate cAK are about 20-fold higher than those existing in cells under basal conditions, but could be reached under pathophysiological conditions in the presence of inflammatory cytokines that induce iNOS expression (Cornwell et al., 1994a).

1.4 Effect of NO-Donors and NPs on VSMC Growth

DETA/NO may mediate bivalent effects in wild-type and cGKI-deficient cells as well as cGMP-independent effects. In primary wild-type VSMCs NO stimulated growth via activation of cGKI, whereas deletion of cGKI may uncover a direct interaction of endogenous cGMP with cAK, resulting in growth suppression (Fig. 15). Feil et al. (Feil et al., 2002) showed in a previous work that DEA/NO increased the cGMP level in wild-type and cGKI-deficient VSMCs (P1 cells), whereas the cAMP level remained unaltered. Although DEA/NO as well as NPs increased the endogenous cGMP level (Lukowski, 2006) only NO caused a robust increase in growth (Fig. 15). Taken together, these results indicate that NO can exert a growth-promoting effect by activating cGKI, and that the anti-proliferative effect of NO is not mediated by cGKI (Ignarro et al., 2001). Two recent studies describe that cGMP-mediated effects depend on the cellular compartment where cGMP is generated (Castro et al., 2006; Piggott et al., 2006). These findings could explain the different results obtained with NPs and NO-donors. In summary, under physiologic or pathophysiologic conditions, effects on growth of VSMCs might be initiated by NO and activation of sGC rather than by ANP or CNP and activation of pGC (Fig. 15).

1.5 cGKI Agonists and “Specific Inhibitors“

The use of cGKI activators and inhibitors is widely accepted and many substances are in use. In this work, the effect of several membrane-permeable cGMP analogs, which have been described as either activators or inhibitors of cGKI, were tested. All tested cGKI agonists had a growth-promoting effect on primary VSMCs (Fig. 9). Of the tested cGKI antagonists, only Rp-8-pCPT-cGMPs revealed a slight growth suppressing effect on 8-Br-cGMP stimulated growth of VSMCs (Fig. 10). In contrast, Rp-8-Br-PET-cGMPs and DT-2, two other inhibitors of cGKI, failed to inhibit cGKI activity, as revealed by growth assays (Fig. 10) and phosphorylation of VASP (Fig. 11 and data not shown). Indeed, Rp-8-Br-PET-cGMPs was not effective as an inhibitor, but rather promoted the growth of VSMCs. These

data and findings from others (Taylor et al., 2004) indicate that Rp-8-Br-PET-cGMPs might be a partial agonist rather than a cGKI antagonist. Perhaps the inhibitors failed to inhibit cGKI in the present study because of the experimental setup. The inhibitors were given chronically for 72 hours, which might cause a degradation of the drugs. Furthermore, the concentrations used were possibly not high enough to suppress the growth-promoting effect mediated by cGKI in the presence of 8-Br-cGMP.

In summary, it can be concluded that the tested cGKI inhibitors should be used carefully. Smolenski et al. (Smolenski et al., 1998) have already suggested that cGK inhibitors should only be used in combination with other experimental approaches. A lack of efficiency has already been described for another cGKI inhibitor, KT5823. KT5823 blocks cGKI activity *in vitro* but was not effective in intact human platelets or rat mesangial cells (Burkhardt et al., 2000).

2. Mechanism of cGMP/cGKI-Mediated Growth of Primary VSMCs

2.1 cGKI-Mediated Adhesion - Rho/ROCK Signaling

As revealed by time-lapse microscopy, the strong “growth-promoting” effect of cGMP/cGKI signaling in the initial phase of primary VSMC culture (first 72 h) is not caused by increased proliferation. After enzymatic digestion, the freshly isolated cells need up to 72 hours for attachment to the culture dish and spreading (Fig. 18). This process is promoted through activation of cGKI. Furthermore, staining the cytoskeleton for F-actin showed that activation of cGKI leads to a homogenous phenotype with almost every cell having stress fibers after two to three days of growth as compared to untreated cells. Stress fiber formation is classically linked to increased RhoA activity. Rho stimulates actomyosin-based contractility through its downstream targets ROCK I/ROKβ and ROCK II/Rho-kinase/ROKα. ROCKs control the formation of stress fibers by inactivating MLCP, thus, maintaining MLC in the phosphorylated form (Kimura et al., 1996).

cGKI is known to relax smooth muscle, among other mechanisms, by inhibiting Ca^{2+}-sensitization of contraction via Rho/ROCK signaling (Sauzeau et al., 2000). cGKI has been described to phosphorylate and, thereby, to stabilize the inactivated RhoA protein, which should cause a decrease in stress fibers (Rolli-Derkinderen et al., 2005; Sauzeau et al., 2000; Sawada et al., 2001). Stabilization leads to an accumulation of total RhoA protein. This increase could also be observed in this work (Fig. 22). Moreover, although the RhoA-GTP level was increased in response to U-46619, no difference in activity could be detected in

response to 8-Br-cGMP as compared to control. According to the above mentioned findings, we suggest that RhoA-GTP is not critical for the formation of stress fibers in our system. Nevertheless, ROCK, a well-characterized Rho effector, might be involved. Blocking ROCK activity has been described to cause a breakdown of stress fibers (Katoh et al., 2001; Kaunas et al., 2005; Tsuji et al., 2002). Treatment of primary VSMCs with Y27632 and H1152, two commonly used ROCK inhibitors, caused the same phenotype as 8-Br-cGMP treatment, namely increased adhesion and the formation of stress fibers. Conversely, activating RhoA/ROCK signaling by treatment with U-46619 abolished adhesion almost completely and prevented the formation of stress fibers after two to three days of growth. In line with the increased amount of stress fibers after 8-Br-cGMP treatment or inhibition of ROCK by H1152 is the increase in p-MLC level (Fig. 21, 23) (Totsukawa et al., 2000). According to the experiments using activators or inhibitors of Rho/ROCK signaling (Fig. 25), it seems that Rho/ROCK pathway has to be suppressed for the adhesion of primary VSMCs. Studies by Arthur et al. (Arthur and Burridge, 2001; Arthur et al., 2000) show that integrin engagement initially inactivates RhoA. It was assumed that transient suppression of RhoA by integrins might reduce contractile forces, which would otherwise delay protrusion at the leading edge of migrating cells. Furthermore, one could speculate that cGMP/cGKI signaling as well as inhibition of ROCK cause increased adhesion, which subsequently triggers the formation of stress fibers, possibly through stimulation with serum (Giuliano et al., 1992). Formation of stress fibers also occurs in unstimulated wild-type cells as well as cGKI-deficient cells, which implicates that stress fiber formation is not a direct cGKI-mediated effect. Increased adhesion through inhibition of ROCK has also been observed by other groups (Kim et al., 2005; Koga et al., 2006). In addition, the previous results suggest that the increased adhesion is mediated by inhibition of ROCK, but is independent of RhoA. Several publications confirm these findings, demonstrating an activation of ROCK independent of RhoA (Castellani et al., 2006; Deroanne et al., 2003; Feng et al., 1999).

In summary, we could show that cGMP/cGKI signaling in primary VSMCs mediates an increase in growth via increased cell adhesion. In contrast, cAMP/cAK signaling is known to mediate growth inhibition. Elevated cAMP levels and subsequent activation of cAK affects cell morphology, including loss of actin stress fibers and focal adhesions, rounding of cells and detachment from the underlying substratum. One proposed mechanism for the described cAMP effects is a cAK-dependent phosphorylation of RhoA at Ser188 (Glass and Kreisberg, 1993; Lang et al., 1996; Laudanna et al., 1997). These findings fit well with our own. As revealed by growth assays and light microscopy (Fig. 17e), a growth inhibiting effect of cAMP in primary as well as in subcultured cells could be observed with similar morphological changes.

2.2 Integrin-Mediated Adhesion

Cell contacts with the ECM are important determinants of cell growth, differentiation, and migration. These contacts, also termed focal adhesions, are mediated by the integrin family of cell surface receptors. Integrins are $\alpha\beta$ heterodimeric transmembrane receptors that recognize and bind many components of the ECM as well as some cell surface adhesion molecules. Even though integrins are present on the cell surface, they may require activation in order to bind their ligand and, thus, to anchor the cell to the ECM or to another cell. Integrin ligand binding is tightly regulated via conformational changes of integrins by cell signaling. Resting, inactive integrins have low affinity for their ligands. Integrins in an active state bind to their ligands with high affinity (Moiseeva, 2001). Integrins can signal through the cell membrane in either direction: The extracellular binding activity of integrins is regulated from the inside of the cell (inside-out signaling), while the binding of the ECM elicits signals that are transmitted into the cells (outside-in signaling) (Giancotti and Ruoslahti, 1999).

β_1 integrins are predominant in vascular smooth muscle *in vivo* and in cultured VSMCs (Moiseeva, 2001). In the present work, analysis of primary VSMCs by flow cytometry revealed that activation of cGKI leads to an increased presentation of β_1 and β_3 integrins at the cell surface (Fig. 27). Furthermore, performing a functional blocking assay with primary VSMCs revealed that the growth-promoting effect of cGMP/cGKI signaling is probably caused by increased adhesion via β_1 and β_3 integrins (Fig. 29). In addition, examination of adhesion of subcultured VSMCs revealed that β_1 integrins are more important compared to β_3 integrins (Fig. 31). This is in line with the literature. β_1 is described to play a major role in adhesion (Clyman et al., 1992), whereas β_3 integrins are described to be essential for migration (Blaschke et al., 2002; Sajid et al., 2003; Slepian et al., 1998). Interestingly, adhesion of primary VSMCs under control conditions was not affected in the presence of blocking antibodies (Fig. 29). As described in the results (p. 61), this might be due to the activation status of the integrins and the chosen concentration of the blocking antibodies. Moreover, the increased adhesion of primary VSMCs caused by inhibition of ROCK is probably also mediated via β_1 and β_3 integrins as determined by a functional blocking assay (Fig. 30) indicating that cGKI activation and ROCK inhibition might have the same downstream signaling pathway. This view is supported by the growth assay, which shows that treatment with 8-Br-cGMP and H1152 does not result in additive growth (Fig. 30). Based on the findings described above, we suggest that activation of cGKI and subsequent increased adhesion could be in part mediated via inhibition of ROCK. Supporting these findings, a recent work by Worthylake et al. (Worthylake and Burridge, 2003) describes that ROCK negatively regulates integrin-mediated adhesion and phosphotyrosine signaling.

2.2.1 Inside-Out Signaling

Inhibition of ROCK seems to cause the same effects on growth as activation of cGMP/cGKI signaling. One known intracellular inhibitor for ROCK is RhoE. RhoE belongs to the family of Rnd proteins, which are a subset of Rho family proteins that are unusual in that they bind but do not hydrolyze GTP. RhoE acts antagonistically to RhoA by binding to ROCK I, thereby preventing it from phosphorylating its targets (Chardin, 2006; Riento et al., 2005b). The analysis of RhoE expression revealed that the protein level is increased in a cGKI-dependent manner (Fig. 33) suggesting that cGMP/cGKI might signal via inhibiton of ROCK.

Interactions between the actin cytoskeleton and integrins regulate integrin activity. The link between the actin cytoskeleton and integrins can either promote or restrain integrin adhesiveness depending on cell type and environmental context. Early investigations of integrin-actin linkages in fibroblasts demonstrated that actomyosin-dependent integrin clustering was required for strong integrin adhesions. Upon activation, the restraining integrin-actin linkage is broken resulting in increased integrin mobility to allow clustering and the formation of new integrin-actin interactions that promote adhesion and signaling (Lub et al., 1997). One known target for ROCK that signals to the cytoskeleton is LIM-kinase (LIMK), which signals via phosphorylation of cofilin (Maekawa et al., 1999). Cofilin both depolymerizes and generates cortical F-actin filaments, thereby facilitating actin remodeling (Bamburg, 1999). Western blot analysis of the two isoforms LIMK1 and LIMK2, revealed that both isoforms are expressed in primary VSMCs (data not shown). Cofilin is the only known physiological substrate of LIMK1 (Okano et al., 1995). Inhibition of ROCK with H1152 leads to a strong de-phosphorylation of cofilin (data not shown). A reduction of the p-cofilin level and, thus, an increase in cofilin activity in response to ROCK inhibition resulting in increased adhesion has also been described by other groups (Bongalon et al., 2004; Koga et al., 2006; Worthylake and Burridge, 2003). This implicates that increased cofilin activity leads to a facilitation of integrin clustering due to increased F-actin remodeling and subsequent increased adhesion of primary VSMCs. Moreover, preliminary results (data not shown) indicate that activation of cGKI leads to a reduction of p-cofilin.

2.2.2 Outside-In Signaling

Cell attachment to the ECM results in integrin clustering, causing the activation of various protein tyrosine kinases, including focal adhesion kinase (FAK). Several groups found that increased phosphorylation of FAK correlates with the formation of stress fibers (Chrzanowska-Wodnicka and Burridge, 1994; Retta et al., 1996). Moreover, a recent work by Wu et al. describes a cGMP/cGKI-mediated increase of p-FAK in VSMC at high serum, which correlates with increased proliferation (Wu et al., 2006).

FAK activation and tyrosine phosphorylation have been shown in a variety of cell types to be dependent on integrin binding to their extracellular ligands (Schwartz et al., 1995). This is in line with our own finding that primary VSMCs in suspension show no signal for phosphorylated FAK (data not shown). Moreover, FAK activation probably depends on ECM proteins. It has been suggested that VSMCs cultured on fibronectin show robust activation of FAK in response to different growth factors, however, cells cultured on laminin show little-to-no activation of FAK in response to the growth factors (Morla and Mogford, 2000; Taylor et al., 2001). VSMCs in the media are quiescent because they are surrounded by basement membranes which contain laminin but lack fibronectin, whereas cells in the intima of atherosclerotic plaques are surrounded by a matrix that is rich in fibronectin indicating proliferation (Morla and Mogford, 2000). Furthermore, FAK appears to play a major role in conveying survival signals from the ECM. Because FAK binds to PI3-kinase, its protective effect against anoïkis may be the result of PI3-kinase-mediated activation of protein kinase B/Akt (Giancotti and Ruoslahti, 1999). Anoïkis is defined as programmed cell death induced by the loss of cell/matrix interactions. Adhesion to structural glycoproteins of the extracellular matrix is necessary for survival of the differentiated adherent cells in the cardiovascular system, including endothelial cells, smooth muscle cells, fibroblasts and cardiac myocytes (Michel, 2003).

Based on the results of the present study, we can draw the following model for cGKI-mediated adhesion and growth of primary VSMCs (Fig. 34). We propose that cGMP/cGKI signaling leads to inhibition of ROCK via upregulation of RhoE, an endogenous inhibitor of ROCK. Inhibition of ROCK causes increased adhesion due to facilitated integrin clustering. Increased adhesion leads to phosphorylation of FAK and, by unknown mechanisms, to the formation of stress fibers secondary to adhesion. Whether or not signaling from ROCK to integrins is mediated via cofilin needs further analysis. Moreover, we propose that adhesion and the formation of stress fibers are two distinct processes. This view is supported by a recent work of Kee et al. who suggest that in keratinocytes the process of cell adhesion can occur separate from stress fiber formation (Kee et al., 2002).

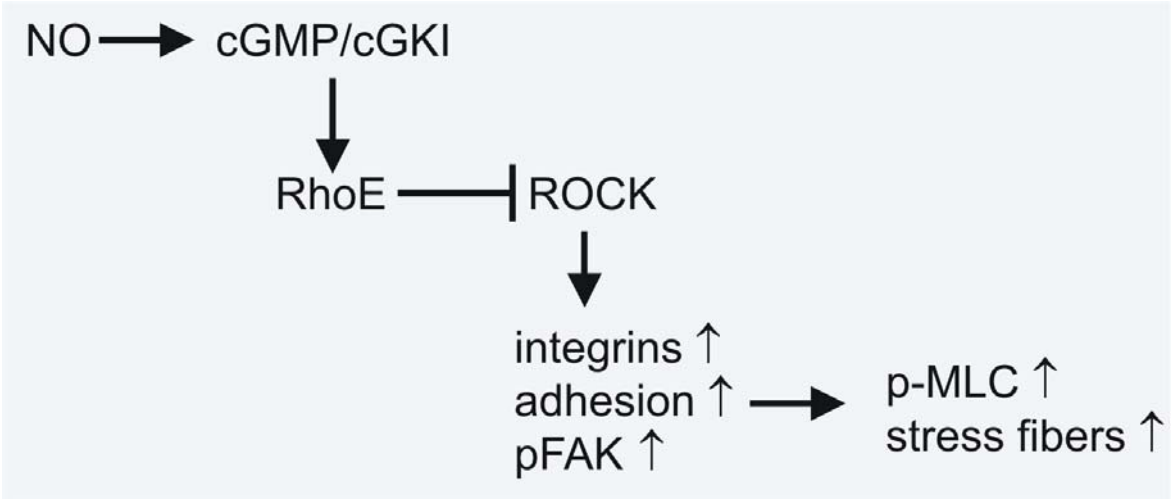


Fig. 34: Model for cGMP/cGKI-mediated adhesion and growth of primary VSMCs. Explanation see text.

2.3 Possible *In Vivo* Impact

Whether the fact that cGMP/cGKI signaling influences integrin signaling *in vitro* has an impact *in vivo* remains to be determined. A recent study of von Wnuck Lipinski et al. (von Wnuck Lipinski et al., 2006) suggests that VSMCs exposed to degraded collagen are protected against apoptosis by a mechanism involving $\alpha_v\beta_3$-dependent NF-κB activation with subsequent activation of the inhibitor of apoptosis protein. This may constitute a novel anti-apoptotic pathway ensuring VSMC survival in settings of enhanced ECM degradation such as cell migration, vascular remodeling, and atherosclerotic plaque rupture (von Wnuck Lipinski et al., 2006). These findings correlate with our own results, since cGKI promotes survival in non-adherent cells and, probably more important, also in adherent cells by stimulating integrin-mediated adhesion to prevent anoïkis. According to the results presented in this study, NO/cGMP/cGKI signaling may protect primary VSMC from apoptosis due to inhibition of ROCK and subsequent increased integrin-mediated adhesion. These findings suggest a deleterious effect for cGMP/cGKI signaling in vascular disease rather than a protective effect. This is in line with a recent work of Wolfsgruber et al. (Wolfsgruber et al., 2003) showing that cGKI has a pro-atherogenic effect in an *in vivo* mouse model.

3. Future Aims

Further work has to be done to disect the mechanism of cGKI-mediated regulation of RhoE expression. As shown in this work, RhoE is not regulated at the mRNA level (Fig. 33) indicating that cGKI-mediated upregulation of RhoE is not mediated via a change in gene expression. It has been described that RhoE is stabilized through phosphorylation by ROCK (Riento et al., 2005a), thereby causing an increase of the protein level in the cytosol. It is tempting to speculate that cGKI upregulates the protein level of RhoE via direct phosphorylation and, thus, stabilization of RhoE. In addition, further research has to be done on RhoE downstream signaling. As mentioned before, there are preliminary results, which indicate that inhibition of ROCK may lead to an activation of the LIMK/cofilin pathway causing facilitated integrin clustering. In summary, most of the upcoming experiments will focus on protein analysis by western blot with specific antibodies and phospho-antibodies against RhoE and its possible downstream targets.

E. Abstract

The aim of this work was to elucidate the effect of cyclic nucleotide signaling on the growth of vascular smooth muscle cells (VSMCs). In particular, the role of cGMP-dependent protein kinase type I (cGKI) as a mediator of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway was studied in primary VSMCs. Recent results of the analysis of atherosclerosis *in vivo* in transgenic mice strongly suggest that activation of cGKI in VSMCs promotes the phenotypic modulation of medial VSMCs and, thus, vascular lesion formation. In contrast, numerous *in vitro* studies suggested an anti-proliferative effect for cGKI. In the present work, the role of cGKI in VSMC growth was analysed in primary and subcultured VSMCs derived from wild-type and cGKI-deficient mice. In primary VSMCs, activation of cGMP/cGKI signaling led to a strong increase in growth. In contrast, in repeatedly passaged VSMCs derived from mouse, rat and human, cGMP/cGKI had either no effect on growth or had a weak growth suppressing effect. Thus, cGKI signaling differs in primary *vs.* subcultured VSMCs. The further analysis of proliferation, apoptosis, cytoskeletal dynamics, and various signaling pathways indicated that an increase in cell adhesion is the major mechanism for cGKI-mediated growth in primary VSMCs. The pro-adhesive effect of cGKI might be mediated via (1) an increase in the level of RhoE, an endogenous inhibitor of Rho kinase (ROCK), (2) inhibition of ROCK and (3) enhanced integrin signaling. Thereby, cGMP/cGKI signaling in primary VSMCs might inhibit anoïkis, the programmed cell death induced by the loss of cell/matrix interactions.

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