Journal of Biochemistry and Molecular Biology, Vol. 40, No. 6, November 2007, pp. 899-910

JOURNAL OF Biochemistry and Molecular Biology

RGS Protein Specificity Towards G_q- and $G_{i/o}$-Mediated ERK 1/2 and Akt Activation, *in vitro*

Thomas Anger[1,*], Nils Klintworth[1], Christian Stumpf[1], Werner G. Daniel[1], Ulrike Mende[2] and Christoph D. Garlichs[1]

[1]Department for Cardiology, Friedrich-Alexander University Erlangen, 91054 Erlangen, Germany

[2]Cardiovascular Research Center at Brown Medical School, Providence, USA

Received 1 May 2007, Accepted 26 June 2007

Extracellular Regulated Kinases (ERK) and Protein Kinase B (Akt) are intermediaries in relaying extracellular growth signals to intracellular targets. Each pathway can become activated upon stimulation of G protein-coupled receptors mediated by G_q and $G_{i/o}$ proteins subjected to regulation by RGS proteins. The goal of the study was to delineate the specificity in which cardiac RGS proteins modulate G_q- and $G_{i/o}$-induced ERK and Akt phosphorylation. To isolate G_q- and $G_{i/o}$-mediated effects, we exclusively expressed muscarinic M_2 or M_3 receptors in COS-7 cells. Western blot analyses demonstrated increase of phosphorylation of ERK 1.7-/3.3-fold and Akt 2.4-/6-fold in M_2-/M_3- expressing cells through carbachol stimulation. In co-expressions, M_3/G_q-induced activation of Akt was exclusively blunted through RGS3s/RGS3, whereas activation of ERK was inhibited additionally through RGS2/RGS5. M_2/$G_{i/o}$ induced Akt activation was inhibited by all RGS proteins tested. RGS2 had no effect on M_2/$G_{i/o}$-induced ERK activation. The high degree of specificity in RGS proteins-depending modulation of G_q- and $G_{i/o}$-mediated ERK and Akt activation in the muscarinic network cannot merely be attributed exclusively to RGS protein selectivity towards G_q or $G_{i/o}$ proteins. Counter-regulatory mechanisms and inter-signaling cross-talk may alter the sensitivity of GPCR-induced ERK and Akt activation to RGS protein regulation.

Keywords: Akt, ERK-1/2, Muscarinic GPCR specifity, R4 RGS proteins

Introduction

Best known for G protein coupled receptor signal transduction is the signaling pathway of Mitogen-Activated Protein Kinases (MAPK) and Protein Kinase B (Akt). These kinases are regulating signal information for apoptosis (Franke *et al.*, 2003), cell stress (Tibbles and Woodgett, 1999), cell growth and proliferation (Chang *et al.*, 2003c), cell survival, migration and differentiation (Neri *et al.*, 2002; Matsui *et al.*, 2003; Steelman *et al.*, 2004), neoplastic transformation (Smalley, 2003; Chang *et al.*, 2003b), are point of interest in the discussion for human cancer treatment (Lee and McCubrey, 2002; Reddy *et al.*, 2003), are presumably responsible for angiogenesis (Shiojima and Walsh, 2002) and gene expression changes in acute and/or chronic inflammatory responses (Gerthoffer and Singer, 2003). On cells, there are two different types of receptors transferring regulatory information from the surface to the MAP kinases or Akt to transmit information to the nucleus: (i) G-protein coupled receptors and (ii) receptor-tyrosine kinases (Bommakanti *et al.*, 2000; Chang *et al.*, 2003b). Ligand-dependant activation of these receptors ultimately leads to phosphorylation of MAP Kinases and thereby stimulation of downstream effectors in the nucleus for further activations (Vazquez-Prado *et al.*, 2003). Stimulation of MAP kinases divers into three separately different signaling downstream pathways: activation of Extracellular-Regulated Kinases subtype 1 and 2 (ERK 1/2) which are subject of this investigation, c-Jun NH(2)-Terminal kinases regulating cytokine expression and p38 effecting mostly apoptosis (Chang *et al.*, 2003c).

Many extracellular stimuli elicit intracellular responses by activating seven-transmembrane receptors that are coupled to

Abbreviations: ERK 1/2, Extracellular Regulated Kinases 1 and 2; AKT, Protein Kinase B; GPCR, G protein-coupled receptors; RGS, Regulators of G protein Signaling; PLCβ, phospholipase C beta; HA , hemagglutinin; PBS, phosphate-buffered saline; GAP, GTPase-activating protein; MAPK, Mitogen-Activated Protein Kinases; BSA, bovine serum albumine; PTX, pertussis toxin.

*To whom correspondence should be addressed.
Tel: 49 9131 85 35301; Fax: 49 9131 85 35460
E-mail: th_anger@arcor.de

heterotrimeric G-proteins comprised of α and βγ subunits (Gilman, 1995; Neer, 1995). Focusing on these G-Protein coupled receptors (GPCR) bound to either $G_{i/o}$ or G_q signaling, both G-protein mediated signaling lead to further activation of MAP Kinases and or Akt (Yan *et al*., 1997; Murga *et al*., 1998; Vazquez-Prado *et al*., 2003).

Regulators of G protein Signaling (RGS) proteins belong to a family of more than 20 proteins with a conserved RGS core domain of ~120 amino acids that is necessary and sufficient for binding to Gα subunits (Hollinger and Hepler, 2002). RGS proteins exert an inhibitory effect on both Gα- and Gβγ-mediated downstream effects by either diminishing signal production generated by GPCR defined as effector antagonistic function of RGS proteins (Hepler *et al*., 1997; Yan *et al*., 1997; Anger *et al*., 2004) or by terminating of GPCR coupled signals through activation of the Gα-GTPase: GTPase-activating protein function of RGS (GAPs) (Ross and Wilkie, 2000; Wieland and Mittmann, 2003; Anger *et al*., 2004).

Activation of ERK 1/2 and Akt subjected to GPCR-mediated signaling is regulated through RGS proteins, i.e. (Leone *et al*., 2000). Many efforts were taken to characterize specific susceptibilities of diverse RGS proteins towards phosphorylation of ERK 1/2 and Akt in different cell systems in regard to G-protein coupled receptors and endogenously expressed G_q and/or $G_{i/o}$, i.e. in rat smooth muscle cells (Blanc *et al*., 2003), in neuroblastoma cells (Leone *et al*., 2000), in rat cardiomyocytes (Nishida *et al*., 2005), in human cancer cells (Ogier-Denis *et al*., 2000) and in baby hamster kidney cells (Chatterjee *et al*., 1997).

Specifically cardiac expressed RGS proteins (subfamily R4: RGS2, RGS3s, RGS3, RGS4, RGS5 and RGS16) known as potent inhibitors of G_q-mediated PLCβ activation *in vitro* as well as *in vivo* (Hepler *et al*., 1997; Yan *et al*., 1997; Kardestuncer *et al*., 1998; Shi *et al*., 2001) are subject in G-protein mediated cardiac hypertrophy (Zhang *et al*., 2006). Activation of ERK 1/2 and Akt is part of the signaling pathway leading from GPCR stimulation via activation of protein kinase C to G_q-mediated cardiac hypertrophy (Dorn and Brown, 1999; Dorn *et al*., 1999).

This study was designed to further characterize specific effects of cardiac expressed RGS proteins towards G_q- or $G_{i/o}$-mediated activation of ERK 1/2 and Akt in an *in vitro* system in where specific effects of RGS proteins were previously described towards activation of G_q-mediated PLCβ (Anger *et al*., 2004; Zhang *et al*., 2006).

The goal of the study was to further define specific effects on G-Protein signaling targets (ERK 1/2 and AKT) of cardiac expressed RGS proteins (R4 subfamily: RGS2, RGS3 [truncated short and untruncated long isoform], RGS4, RGS5 and RGS16) in respect to their ability towards activation of PLCβ. Thereby, we looked forward to characterise further RGS protein effects despite their ability to inhibit activation of PLCβ using PLCβ subjected as further downstream activator for ERK 1/2 and/or AKT phosphorylation. COS-7 cells lacking any muscarinic receptors were used as previously described system and transiently transfections were carried out to expresses either muscarinic receptor M_3 coupling to endogenous G_q or M_2 coupling to $G_{i/o}$ respectively. Proper endogenous coupling was observed through pertussis toxin. Monitoring of (i) receptor expression through receptor-binding assays and protein expression of (ii) co-transfected RGS Proteins as well as endogenously expressed (iii) G proteins through western blot analysis were assessed (see Fig. 1). Western blot and immunoblot analysis using specific antibodies recognizing phoshorylated fraction of ERK 1/2 and Akt was used to define effects of RGS protein in co-expression experiments.

Together with the recently tested inhibitory effects of cardiac expressed R4 RGS proteins as GTPase activating proteins and/or effector antagonists on the G_q-mediated activation of PLCβ in COS-7 cells (Anger *et al*., 2004) and together with the important role of RGS2 expression as potent *in vivo* inhibitor of G_q-mediated activation of PLCβ supporting cardiac hypertrophy (Zhang *et al*., 2006) we generated a system to distinguish specific effects on G_q- and $G_{i/o}$- activation of ERK 1/2 and Akt subjected to RGS proteins where we know, that investigated R4 RGS proteins were able to inhibit differentially the subjects of ERK 1/2 or AKT activation: signaling of PLCβ. This study explores further specific views insight possible RGS signaling upon G-protein receptor activation (G_q-/$G_{i/o}$-differentiation) and opens a field of further specifity towards different G-protein signaling targets (PLCβ, ERK 1/2 and AKT) upon receptor activation.

Materials and Methods

Generation of cDNA constructs. Each RGS protein was tagged at its N-terminus with the FLAG epitope using PCR as previously described (Anger *et al*., 2004). RGS2 (Heximer *et al*., 1997), RGS3s (a truncated/short isoform of RGS3) (Dulin *et al*., 2000; Reif and Cyster, 2000), RGS3 and RGS4 (Druey *et al*., 1996), RGS5 and RGS16 (Kardestuncer *et al*., 1998) were used as templates. The PCR products were subcloned into pcDNA3 (Invitrogen) using convenient restriction sites. All sequences were confirmed by DNA sequencing. The cDNAs encoding human HA tagged muscarinic M_3 and M_2 receptor were purchased from Guthrie Institute. The cDNA for the β-adrenergic receptor kinase 1 carboxyl- terminal peptide encoding minigene ($\beta ARK1_{CT}$) was a kind gift from W. Koch (Duke University) (Koch *et al*., 1994).

Cell culture and transfections. COS-7 cells were maintained in complete growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 U/ml penicillin and 50 μg/ml streptomycin]. Cells in 12-well plates were transiently transfected for 48 h using FuGENE6 (Roche Applied Science) and cDNA at a 3 : 1 ratio (w/w) and in total 0.5 μg cDNA/well. The amount of plasmid(s) transfected in each experiment varied according to described approach. In all experiments, 165 ng or 40 ng cDNA plasmid carrying either muscarinic receptor M_2 or M_3 respectively was co-transfected in presence or absence of 335 ng plasmid DNA carrying either specific RGS proteins, βARK_{CT}

or an empty pcDNA3 vector (Invitrogen) as control to establish equal DNA amount per transfection.

Western Blot Analysis. COS-7 cells transiently differentially transfected and stimulated were rinsed twice in ice-cold PBS and directly lysed in cell lysis buffer (Cell Signaling Biotech) containing phosphatase inhibitors and additional proteinase inhibitors (Complete Mini) for 30 min on a shaker at 4ºC. Equal amounts of total cell lysates [30 μg per line, determined in front using BioRad's DC Protein Assay according manufacturer's instructions] were size-fractionated on Tris-glycine SDS-PAGE (10%) and transferred to nitrocellulose membrane (Schleicher-Schuell). Ponceau-S staining was used to confirm equal loading. Membranes were blocked in PBS containing 5% bovine serum albumine and probed with antibodies against FLAG (M2, 1 : 3000, Sigma), HA (12CA5, 1 : 1000; BabCo), β-Actin as loading control, ERK-1/2, phosphorylated ERK-1/2 [Thr202/Tyr204], Akt and phosphorylated Akt [Ser 437] (all 1 : 1000, from Cell Signaling Tech.). After 3 washes in PBS containing 0.1% Tween-20 and incubation with peroxidase-coupled secondary antibody, proteins of interest were digitally visualized using SuperSignal West Pico chemiluminescent substrate (Pierce) in BioRads XRS gel documentation system. Semi-quantitative densitometric measurements were obtained using BioRads Quantity One software for investigated target proteins (ERK 1/2 or AKT). All experiments were performed at least three times in duplicates using always 30 μg protein (total cell lysate) per line, monitored through protein determination prior to western blot analysis using BioRad's DC Protein Assay as above mentioned.

Pertussis toxin treatment. Thirtysix hours after transfections with muscarinic receptors M_2 or M_3, COS-7 cells were treated with 50 ng/ml (accordingly 1 ml/well) pertussis toxin (PTX) or vehicle for 12 h. *In vitro* back ADP ribosylation assays for the amount of PTX used in this approach were established and effectiveness of that dose was demonstrated elsewhere (Zhang and Neer, 2001). Western Blot analyses for phosphorylated ERK 1/2 or AKT were carried out as described before.

Muscarinic receptor binding assay. The receptor-binding assay to monitor expression of muscarinic receptors M_2 and M_3 was described previously (Anger *et al*., 2004). Briefly, 48 h after transient transfection with cDNA encoding either M_2 or M_3 muscarinic receptor, COS-7 cells were rinsed with DMEM containing 0.1% BSA and incubated for 90 min at room temperature with 7.5 to 4,000 pmol/l N-Methyl [^{3}H]Scopalamine ([^{3}H]NMS, 84 Ci/mmol; Amersham Pharmacia Biotech) in the presence or absence of atropine (1 mM) to determine non-specific binding (background monitoring). The binding reaction was stopped by removing the labeling medium and washing the wells twice with ice-cold PBS, followed by cell lysis in 0.2 M NaOH and 0.1% SDS. For each lysate, the amount of radioactivity was determined by scintillation counting and normalized to the amount of protein present (DC Protein Assay, BioRad). Saturation binding assays were fitted by non-linear regression (one-site binding model) using GraphPad Prism 4 (GraphPad Software, San Diego, USA) to determine the maximal number of binding sites (B_{max} [in fmol/mg protein]) and radioligand binding affinity (K_d [in nM]) as demonstrated previously for muscarinic receptor M_3 (Anger *et al*., 2004).

Measurement of [^{3}H]Inositol phosphate formation. PLC activity was assessed by measuring total inositol phosphate formation in 12-well plates, as described previously (Zhang and Neer, 2001). Briefly, 24 h after transient transfection, the cells were labeled in inositol-free medium supplemented with myo[^{3}H]inositol (2 μCi/well; Amersham Biosciences) overnight. The next day, LiCl (final concentration, 10 mM) was added prior to the addition of muscarinic receptor agonist carbachol (final 10^{-4} M). After 30 min at 37ºC, the inositol phosphates were extracted in 20 mM formic acid, neutralized, separated by anion exchange chromatography (Dowex AG1-X8), and quantitated in a scintillation counter. Cell density and protein amount were monitored for each of the different transfection conditions. Normalization to the protein amount in each well yielded similar results (data not shown).

Statistical analysis. Data represent mean ± SEM for at least three independent experiments in dupilcates. Where appropriate, statistical differences were assessed by Student's unpaired t-test and were demonstrated using SigmaPlot 9.0, SYSTAT Software Inc., USA. A p value < 0.05 was considered statistically significant and market through*.

Results

Experimental model. To assess the specific distribution of endogenously G_q- or $G_{i/o}$-driven activation of ERK1/2 or Akt, we used COS-7 cells lacking muscarinic receptors (Jakubik and Wess, 1999; Joseph *et al*., 2002; Anger *et al*., 2004) and transiently transfection assays to establish a specific G_q-driven, muscarinic receptor M_3-coupled or $G_{i/o}$-driven, muscarinic receptor M_2 coupled system (see Fig. 1).

Initially, transiently transfections were performed using M_2 or M_3 coupling to endogenously expressed $G_{i/o}$ or G_q, respectively and carbachol (an unselective muscarinic receptor agonist) stimulation was used to define maximum of activation of endogenously expressed ERK 1/2 and Akt (see Fig. 2). Under baseline conditions, carbachol independently to G_q or $G_{i/o}$ stimulates time-dependent ERK 1/2 activation where as Akt phosphorylation upon muscarinic receptor activation of both, M_2 (G_i) and M_3 (G_q) was observed dose-dependent as well as time-dependent. Basal samples did not reveal any further phosphorylation by carbachol stimulation demonstrating lack of endogenously expressed muscarinic receptors on used COS-7 cells. In reflection to published data (Igarashi *et al*., 2001) it turned out, (i) that COS-7 cells express a valid amount of ERK 1/2 and Akt as well as (ii) that 10^{-4} M carbachol stimulation for 7 min is sufficient to exert maximum of phosphorylation of endogenous ERK 1/2 and Akt.

Pertussis toxin sensitivity is considered exclusively for $G_{i/o}$-Proteins and was used to demonstrate proper endogenously coupling. 50 ng/ml pertussis toxin 12 h prior carbachol (10^{-4} M for 7 min) stimulation was sufficient to completely blunt phosphorylation of ERK 1/2 as well as Akt when M_2 muscarinic receptor was transiently expressed (see Fig. 3). No effect was seen on muscarinic receptor M_3-driven ERK 1/2 and Akt activation demonstrating proper endogenously coupling of

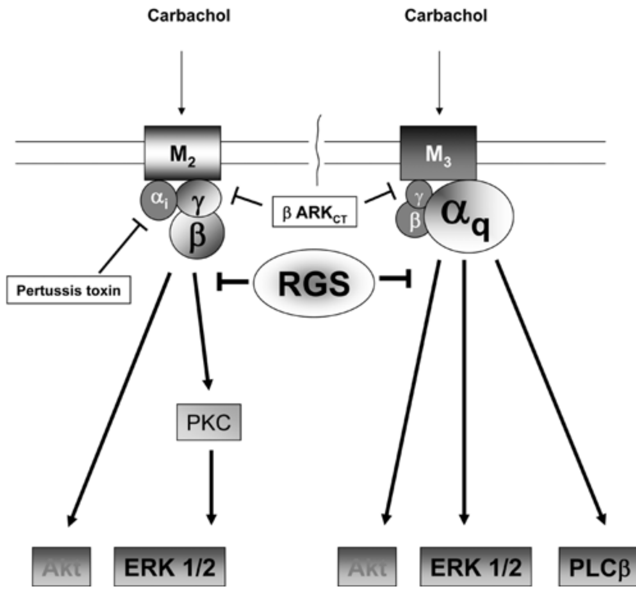

Fig. 1. Experimental System for muscarinic M_2 and M_3 receptor in COS-7 cells. COS-7 cells were transiently transfected with either muscarinic M_2 or M_3 receptor in presence or absence of transiently expressed RGS proteins. Endogenously coupling of transiently expressed muscarinic receptor to the G-Protein, here $G_{i/o}$ or G_q respectively was confirmed through Pertussis toxin-sensitivity of the receptor. Activation of Akt or ERK 1/2 - determined via western blot analysis, revealed potent downstream signaling of the βγ-subunit with only little effect of the α-subunit in respect to transiently co-expression of βAKT_{CT}, when M_2 was transiently co-expressed. Main down streaming of muscarinic receptor M_3 was established through Gα and similarly activation of Proteinkinase C (PKC) mediated through PLCβ.

transiently expressed receptors to their complementary G protein: M_2-$G_{i/o}$ and M_3-G_q, respectively.

The protein expression of all endogenous G-proteins (G_q or $G_{i/o}$) of used COS-7 cells was constantly monitored through western blot analyses and revealed no change of protein expression upon co-transfections and/or upon carbachol stimulations (data not shown).

The C-terminus of the β-adrenergic receptor kinase 1 minigene (βARK_{CT}) a known Gβγ-scavenger blocks further Gβγ-mediated downstream signalling (Koch *et al*., 1994; Murga *et al*., 1998). Transient co-expression of βARK_{CT} was used to distinguish either Gα- (not blunted through βARK_{CT}) or Gβγ-driven phosphorylation of ERK 1/2 and Akt (see Fig. 4). No effect was established through co-expressed βARK_{CT} on carbachol stimulated phosphorylation of ERK 1/2 and Akt when compiled to M_3/G_q demonstrating dominantly signaling through Gα. Interestingly, in presence of βARK_{CT} over-all basal activation of ERK 1/2 and Akt was - to date unexplainable - reproducible relevantly increased with no further raise using carbachol when M_2 was transiently expressed.

Co-expression of βARK_{CT} itself was monitored by western/

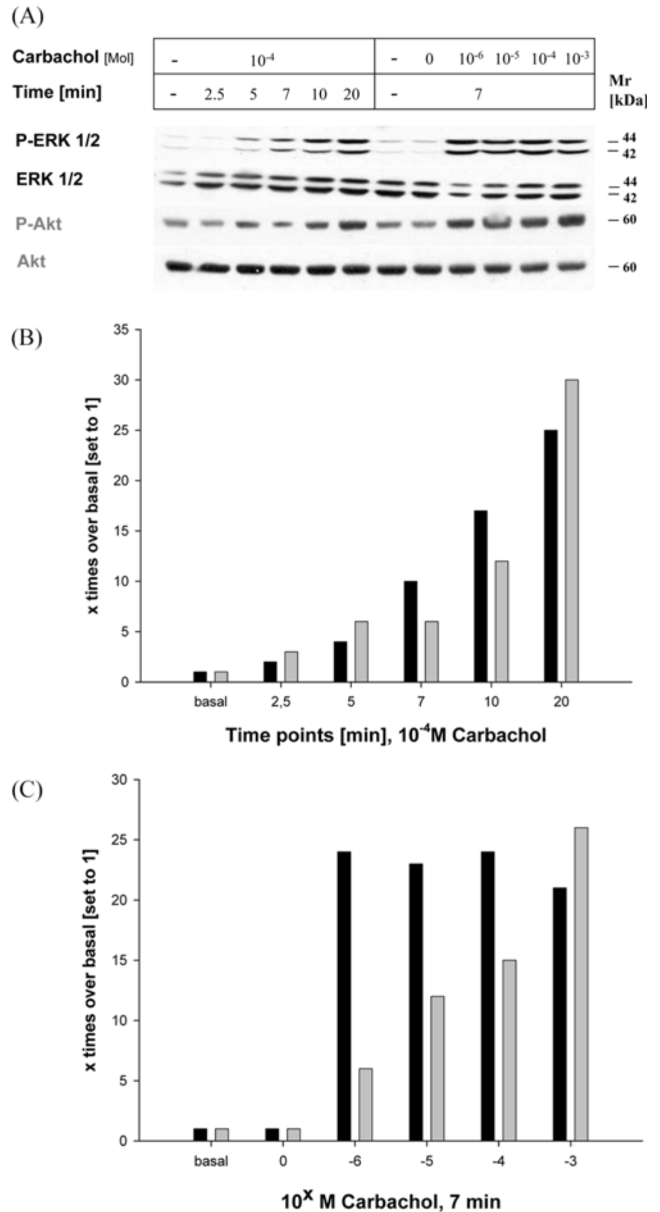

Fig. 2. Timecourse and dose-dependency of carbachol stimulated, muscarinic M_3 receptor coupled activation of ERK 1/2 and Akt. (A) Representative immunoblots for phosphorylated and total ERK-1/2 (upper panels) and Akt (lower panels) demonstrating COS-7 cells, transiently transfected with muscarinic receptor M_3 (40 ng/12 well) for 48 h and stimulated for different time points as indicated (0, 2.5, 5, 7, 10 and 30 min) with final 10^{-4} M of non-selective muscarinic receptor agonist carbachol (left panel) or stimulated for 7 min in different final concentrations of carbachol as indicated (0, 10^{-6} to 10^{-3} M) (see right panel). In regard to the literature (Igarashi *et al.,* 2001), finally 10^{-4} M carbachol stimulation of the muscarinic receptor M_3 for 7 min established a significant phosphorylation of ERK 1/2 and AKT in COS-7 cells. Below the graph in the upper panel: (B) Time-Dependency and (C) Dose-Dependency as mean without SEM due to limited numbers (n = 2 for *B* or *C*) set in x-times over basal (= 1) as indicated: Black bars for ERK 1/2 phosphorylation, and grey bars for AKT activation.

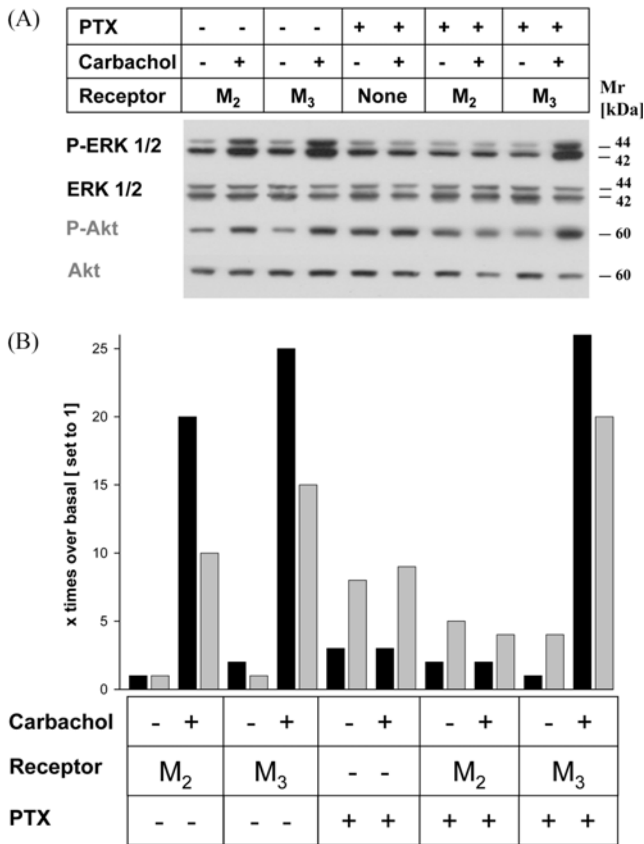

Fig. 3. Confirmation of coupling of endogenously expressed G_i or G_q to M_2 or M_3, respectively and βARK_{CT} effects. (A) Representative immunoblots for phosphorylated and total ERK-1/2 (upper panels) and Akt (lower panels) demonstrating COS-7 cells, transiently transfected with either M_2 (165 ng/12 well), M_3 receptor (40 ng/12 well) or vehicle (basal), and stimulated 48 h post-transfections with carbachol (final 10^{-4} M, 7 min) as indicated. Shown is the effect on ERK-1/2 and AKT activation of pre-treatment with pertussis toxin (50 ng/ml) for 12 h prior stimulation as indicated (PTX). (B) Semiquantitative densitometric data demonstrated as mean without SEM due to limited numbers (n = 3) as x times over basal, set to 1 as indicated: Black bars for ERK 1/2 phosphorylation, and grey bars for AKT activation.

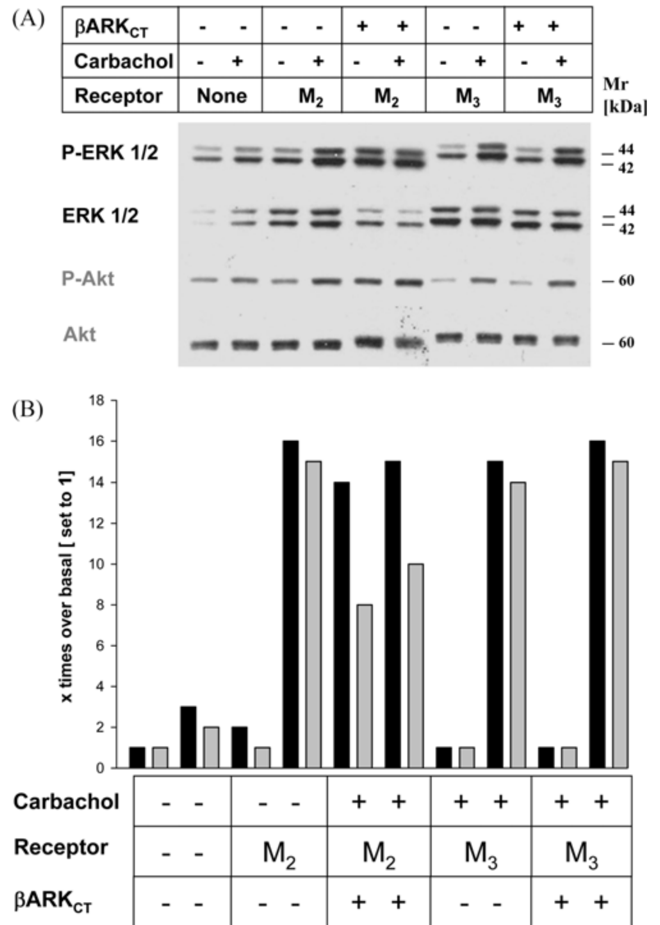

Fig. 4. βARK_{CT} effect on ERK 1/2- /AKT activation upon $G_{i/o}$ (M_2) or G_q (M_3) signaling, respectively. (A) COS-7 cells were transiently co-transfected with either muscarinic M_2 (165 ng/12 well), M_3 receptor (40 ng/12 well) or vehicle (basal) in presence or absence of βARK_{CT} (335 ng/12 well) and stimulated 48 h post-transfections with carbachol (final 10^{-4} M, 7 min) as indicated. Shown are representative immunoblots for phosphorylated and total ERK-1/2 (upper panels) and Akt (lower panels). Reproducible increased phosphorylation of both, ERK 1/2 and AKT in presence of βARK_{CT} without carbachol stimulation of muscarinic receptor M_2 and negotiable increase of phosphorylation when carbachol stimulation was achieved suggested carbachol induced M_2 signaling mainly through Gβγ when βARK_{CT} as Gβγ- scavenger was co-expressed. (B) Semiquantitative densitometric data demonstrated as mean without SEM due to limited numbers (n = 3) as x times over basal, set to 1 as indicated: Black bars for ERK 1/2 phosphorylation, and grey bars for AKT activation.

immuno blot analyses demonstrating comparable expression in all transiently βARK_{CT} co-transfected samples (data not shown).

Monitoring of transiently muscarinic receptor expression was established via receptor-binding assay and western blot analyses due to the fact, that we observed much intense phosphorylation of ERK 1/2 and Akt under carbachol stimulation of muscarinic receptor M_3 than M_2. This was highly attributed to different transient expression levels of the used receptors. We failed to demonstrate M_2 expression using western blot analysis. Importantly, overall transfection conditions were halted comparable among the two different signaling pathways implicating the use of same total cDNA amount per transfection (500 ng/well) as reason not to increase total cDNA for co-transfections of muscarinic receptor M_2 with investigated RGS proteins. In contrast, monitoring of M_3 expression was possible in comparison to the protein expression seen for transiently co-expressed FLAG tagged RGS proteins (Berman *et al*., 1996; Chen *et al*., 1997) (see Fig. 5, panel A).

Overall, the non-selective muscarinic receptor agonist carbachol itself did not show any effect on the protein expression for either muscarinic receptor M_3 (assessed through

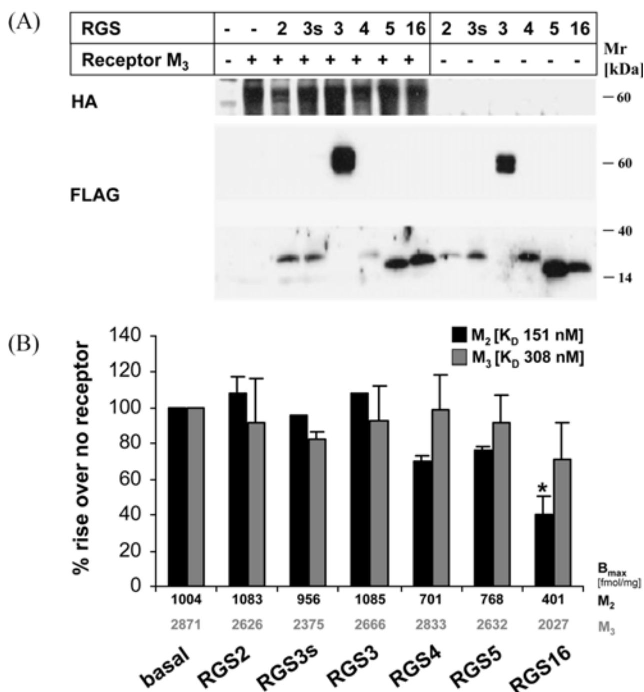

Fig. 5. Confirmation of equal protein expression in co-transfections using western blot analysis and receptor-binding assay. (A) Western/Immunoblots of total cell lysates from COS-7 cells transiently co-transfected with HA tagged-muscarinic M_3 receptor (40 ng/12 well) in absence/presence of different FLAG-tagged RGS proteins (each 234 ng/12 well) for 48 h. Demonstrated are representative blots with immunoblotting for HA (upper panel) to detect M_3 and for FLAG to detect specific RGS proteins (lower panel). Over-all, investigated R4 RGS protein expression was unchanged upon M_3 co-expression which was previously demonstrated (Anger *et al.,* 2004). Adequate protein expression could not be demonstrated for muscarinic receptor M_2 as we were forced to establish receptor binding assays to compare equal receptor co-expression: (B) Receptor-binding assay for both muscarinic receptors M_2 (165 ng/12 well, black bars) and M_3 (40 ng/12 well, grey bars). Maximum [^{3}H] NMS-binding in presence or absence of atropine to determine background binding on transiently transfected muscarinic receptors was determined and set to 100% (basal) in absence of co-transfected RGS proteins (335 ng/12 well). The rise over basal [in %] is demonstrated for each co-transfected RGS protein specifically for either M_2 or M_3. In addition, the number B_{max} for the maximal binding capacitiy of the investigated muscarinic receptor is given below the bars in black for M_2 and grey for M_3 [in fmol/mg protein] as well as the calculated maximal binding affinity K_D of the radioligand [^{3}H] NMS [in nM]. * indicates statistically significant reduced [^{3}H] NMS binding to M_2 in regard to significant down-regulation of expressed M_2-receptor in presence of transiently co-transfected RGS16.

western blot analysis and receptor binding assay), M_2 (assessed through receptor binding assay) or differentially co-expressed RGS proteins (assessed through western blot analysis).

All investigated R4 RGS proteins are differentially expressed upon transiently transfections in COS-7 cells with potent inhibitory influence in G_q- or $G_{i/o}$ mediated signaling (Anger *et al*., 2004). Immunoprecipitation of lysates from ^{35}S-labeled COS-7 cells and western blotting yielded bands of the expected molecular weights and demonstrated a dose-dependent increase in RGS protein expression with increasing amounts of RGS cDNA transfected (data not shown). The overall expression level varied among the different RGS proteins. Importantly, expression of each RGS protein was comparable between cells that were transfected with the M_3 receptor and those transfected with M_2 receptor, so that any potential functional differences between M_3- and M_2-expressing cells cannot be attributed to a difference in their amount of cellular RGS protein. Comparing the inhibitory effects of RGS protein in these setting could therefore provide insight into the functional importance of GTPase activation and effector antagonism for the inhibitory effect of R4 RGS proteins on G_q as well as in $G_{i/o}$ signaling in mammalian cells upon activation of ERK or AKT (Anger *et al*., 2004).

To further monitor equal protein expression for both muscarinic receptors in each experiment, we performed receptor-binding assays using non-selective binding of [^{3}H] NMS to any muscarinic receptor expressed. In presence and absence of atropine (muscarinic receptor antagonist) to monitor background binding and adjusted to total protein amount expressed in the COS-7 cells, it turned out, that we initially had to reduce dramatically amount of cDNA used for M_3 transfections to 40 ng/12 well being in at least comparable transient receptor expression levels of M_3 to M_2 under same transfection conditions (500 ng cDNA/12well using FuGENE6). However, in presence of co-transfected investigated R4 RGS-proteins, it secondly turned out, that the muscarinic receptor M_2 will be lower expressed in co-transfection with RGS16 (see Fig. 5, panel B). For all other RGS proteins, muscarinic receptor M_2 had comparable receptor expression levels (see Fig. 5B).

Effects of R4 RGS proteins on G_q- and $G_{i/o}$-mediated ERK 1/2 and AKT activation. COS-7 cells were transiently transfected with muscarinic M_2 or M_3 receptors in the absence or presence of five different R4 RGS proteins: RGS2, RGS3 (short and untruncated long isoform), RGS4, RGS5 and RGS16. Figure 6 shows representative Western blots for M_2 and M_3 expressing COS-7 cells (see Fig. 6, panel A and B, respectively) illustrating the effect of carbachol stimulated ERK 1/2 and Akt phosphorylation (upper panels) in COS-7 cell lysates. Total ERK 1/2 and Akt expression are shown for control (lower panels). Figure 7 shows for each respective RGS protein, quantitative analysis from in total five independent experiments performed in duplicates. The amount of phosphorylated ERK 1/2 and Akt was normalized to the total amount of ERK 1/2 or Akt expressed. The increase in phosphorylation in M_2- or M_3-expressing cells in the absence of co-transfected RGS protein was set as 100%. In M_2-receptor expressing cells, carbachol increased ERK 1/2 and Akt 1.7 ± 0.2 and 2.4 ± 0.3 fold over basal, respectively. Despite adjusted expression level of M_3-receptor expressing

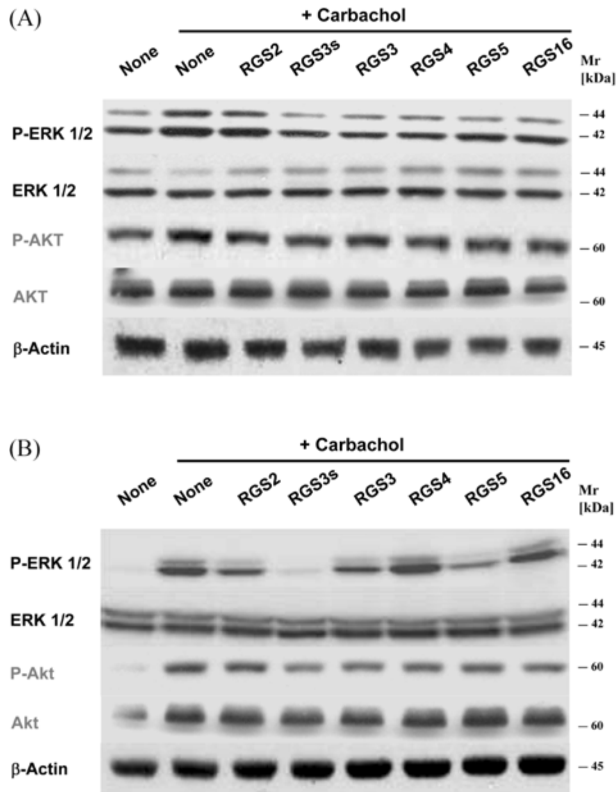

Fig. 6. Effect of RGS proteins on $M_2/G_{i/o}$- and M_3/G_q-coupled ERK 1/2 and Akt activation, protein analysis. Representative western blots of total cell lysates from COS-7 cells transiently co-transfected for 48 h with muscarinic M_2 (165 ng/12 well, panel A) or M_3 receptor (40 ng/12 well, panel B) in absence (None)/presence of investigated RGS proteins (each 335 ng/12 well) as indicated and stimulated with carbachol (10^{-4} M for 7 min) were probed with antibodies recognizing specifically phosphorylated (P-) or total fraction of ERK 1/2 [42 and 44 kDa] and Akt [60 kDa] as indicated. β-Actin [45 kDa] was used to demonstrate equal loading (30 μg protein per line).

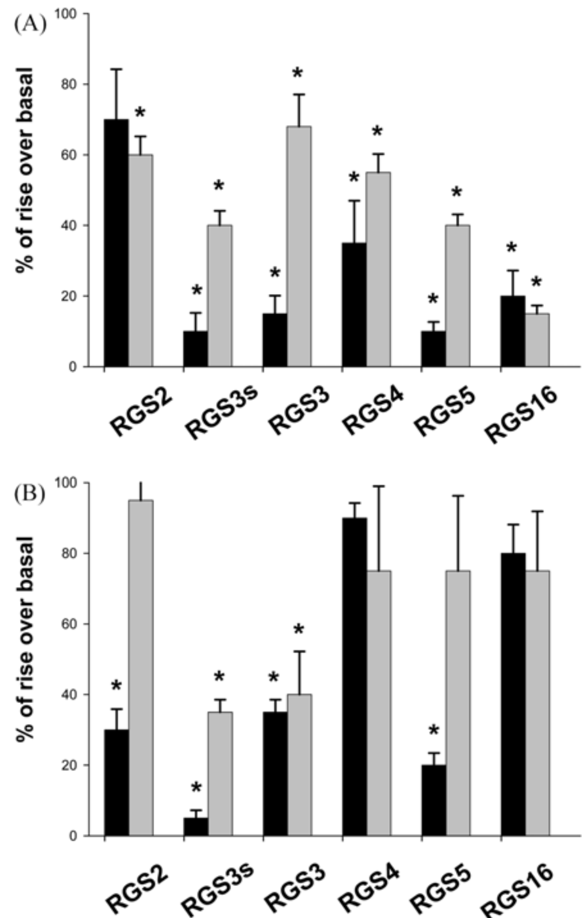

Fig. 7. Effect of RGS proteins on $M_2/G_{i/o}$- and M_3/G_q-coupled ERK 1/2 and Akt activation, statistically analysis. Semi quantitative densitometric analysis. The rise in the ratio of phosphorylated over total protein fraction for ERK 1/2 (black bars) and Akt (grey bars) in M_2 (panel A) or M_3 (panel B) receptor transiently transfected cells (in the absence of co-expressed RGS proteins set as basal, data not shown) was set to 100%. The effect of investigated transiently co-transfected RGS proteins is shown as rise over basal [in %] and as indicated. * indicates a p-value < 0.05 vs. basal. Bars are indicating mean + SEM for at least $n = 5$ different experiments in duplicates.

cells, ERK 1/2 and Akt phosphorylation was elevated to a higher intense: 3.3 ± 0.4 and 6.0 ± 0.4 fold, respectively. COS-7 cells, co-expressed with investigated RGS proteins and muscarinic receptor M_2 or M_3 not stimulated through carbachol, showed unaffected and unchanged basal phophorylation as well as total ERK 1/2 or AKT expression (data not shown).

In presence of R4 RGS proteins a highly reproducible carbachol stimulated pattern of effects on the phosphorylation of ERK 1/2 and Akt was observed. Differential RGS co-expression monitored through Western Blot analysis caused reduction in ERK 1/2 and Akt phosphorylation, which varied specifically depending on the respective G protein pathway involved. M_3/G_q-induced activation of Akt was exclusively blunted through RGS3s and RGS3, whereas activation of ERK 1/2 was inhibited additionally through RGS2 and RGS5. In contrast, $M_2/G_{i/o}$ induced Akt activation was inhibited by all RGS proteins tested, including RGS2. However, RGS2 had no effect on $M_2/G_{i/o}$-induced ERK 1/2 activation. Surprisingly, RGS4, one of the best investigated inhibitor for G_q-mediated cardiac PLCβ activation *in vitro* (Hepler *et al*., 1997; Heximer *et al*., 1999), revealed in contrat to $M_2/G_{i/o}$- no effects on the M_3/G_q-induced ERK 1/2 or AKT phosphorylation.

Effects of R4 RGS proteins on M_3-coupled G_q-mediated activation of PLCβ. Three independent experiments were performed in duplicates confirming previously published findings using here FuGENE6 as transfection reagent (see Fig. 8) (Anger *et al*., 2004).

Briefly, carbachol stimulation of muscarinic receptor M_3 in absence of RGS proteins lead to significant activation of

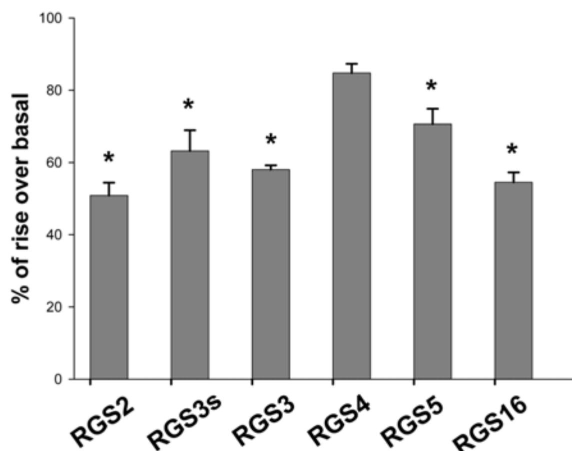

Fig. 8. Effect of RGS proteins on M_3/G_q coupled PLCβ activation. Measurement of [^{3}H] labeled total inositol formation in COS-7 cells transiently transfected with muscarinic M_3 receptor (40 ng/12 well) in absence or presence of RGS proteins (335 ng/12 well) and stimulated with carbachol (10^{-4} M for 30 min) directly correlates with the activity of phospholipase cb (PLCβ). The rise in the ratio of PLCβ activation in M_3 receptor transiently transfected COS-7 cells in the absence of co-expressed RGS proteins was set as basal to 100% (data not shown). The effect of each investigated RGS protein co-expressed in the same cells is shown in grey bars. * indicates p-value < 0.05 vs. basal. Bars are indicating mean + SEM for n = 3 experiments in duplicates.

PLCβ. Except for RGS4 - which *in vivo* is known as inhibitor for G_q-mediated PLCβ activation, all investigated cardiac R4 RGS proteins decreased significantly stimulated activity of PLCβ (see Fig. 8) (Anger *et al*., 2004).

Discussion

Experimental design. This study was designed (i) to generate a system where effects of R4 RGS proteins could easily attributed to either endogenously expressed G_q or $G_{i/o}$ proteins and (ii) to establish receptor - specificity as well as (iii) effector - specificity of R4 RGS proteins towards ERK-1/2 and Akt activation in the context, where R4 RGS Proteins except for RGS4 exert inhibition towards G_q-mediated PLCβ activation (Anger *et al*., 2004).

The lack of endogenous G_q-coupled muscarinic receptors (such as M_1, M_3 and M_5 receptors) and endogenous $G_{i/o}$-coupled muscarinic receptor (such as M_2) in COS-7 cells (Jakubik and Wess, 1999; Joseph *et al*., 2002; Anger *et al*., 2004) was confirmed by negotiable [^{3}H] NMS binding in vector-transfected cells (data not shown) and in basal controls of experiments where carbachol stimulation offered negotiable increased basal activation of phosphorylated ERK 1/2, Akt or PLCβ. Characterization of ERK 1/2 and Akt activation in COS-7 cells established endogenously coupling of transiently expressed muscarinic receptor M_3 to G_q and M_2 to $G_{i/o}$ respectively, seen by pertussis toxin pre-treatment (Rhee, 2001).

Expression of involved components was accurately monitored by western blot analysis and receptor-binding assays revealing exclusively the lack of reproducibly for M_2 expression coupling to $G_{i/o}$ when RGS16 was co-expressed. Further, co-expression of minigene $βARK_{CT}$, a Gβγ-scavenger demonstrated coupling of down stream signaling towards $Gα_q$, when M_3 and Gβγ, when M_2 was transiently expressed (Koch *et al*., 1994). Several further intermediaries between ligand-depending G-protein coupled receptor activation and phosphorylation of ERK 1/2 and Akt were not part of the interest and not investigated in this study. Never-the less, these several intermediaries have to be of interest for further studies.

Carbachol stimulation of transiently expressed muscarinic receptor M_3 lead to PLCβ activation mainly via $Gα_q$ since the endogenously expressed PLCβ1 and 3 are insensitive to the Gβγ subunit (Rebecchi and Pentyala, 2000). PLCβ stimulates activation of protein kinase C, which follows in downstream phosphorylation of Mitogen - Activated Protein Kinases (MAPK) and in part of extracellular signal - regulated kinases 1 and 2 (ERK 1/2). In addition, the activated Gβγ subunit exerts the signaling via activation of protein kinase B (Akt) (Koch *et al*., 1994; Murga *et al*., 1998; Bommakanti *et al*., 2000). Thereby, the amount of coupled endogenous G_q or $G_{i/o}$ protein was sufficient to demonstrate activation of ERK 1/2 and Akt.

RGS proteins inhibit ERK 1/2 or Akt phosphorylation. Since discovery of RGS proteins as GTPase activating proteins and effector antagonist of effectors subject to G protein-mediated signaling, i.e. (Hepler *et al*., 1997; Yan *et al*., 1997; Ross and Wilkie, 2000; Hollinger and Hepler, 2002; Wieland and Mittmann, 2003), lots of efforts were taken to characterize specification of RGS protein on equivalent effectors.

Transiently co-expressed RGS proteins are able to contribute to the activated signaling by enhancing the endogenous GAP activity. Thus, RGS proteins inhibit Gα as well as Gβγ signaling effectors leading to inhibited phosphorylation of ERK 1/2 and Akt (Crespo *et al*., 1994; Koch *et al*., 1994; Della Rocca *et al*., 1997). Additionally, co-expressed RGS proteins exert effector antagonistic inhibition towards G-protein coupled activation of ERK 1/2 and Akt. Thereby, for each RGS protein specific inhibitory effects on activation of ERK 1/2 or Akt was observed.

Despite the initial assumption that RGS2 is not able to explore activity towards $Gα_i$ *in vitro* (Heximer *et al*., 1997) different cell models were chosen to demonstrate function of RGS2 towards $Gα_I$, i.e. Sf9 cells (Cladman and Chidiac, 2002), yeast (Druey *et al*., 1996), HEK293 cells (Melliti *et al*., 2001), rat aorta smooth muscle cells (Wang *et al*., 2002). RGS2 inhibition of G_q-mediated signaling is well characterized. More effective GTP hydrolysis in an *in vitro* system in co-expression of RGS2 then RGS4 was described previously (Heximer *et al*., 1999). In conclusion with our findings in COS-7 cells, the specificity of RGS2 towards different G proteins: $G_{i/o}$ and G_q as well as signaling targets upon one G

protein (G_q): PLCβ and ERK 1/2 but not Akt explores new further questions.

RGS3s, the short, truncated isoform and RGS3 (untruncated, long form) were characterized as well in different cells, i.e. COS-7 cells (Shi *et al*., 2001; Anger *et al*., 2004), CHO cells (Dulin *et al*., 2000), rat aorta smooth muscle cells (Wang *et al*., 2002). Consistent with the previous published data, both, RGS3s and RGS3 inhibit G protein- mediated effector activation to the same extend. In our system RGS3s and RGS3 exert their inhibitory effect as GAP's as well as effector antagonists upon unselective ERK-1/2-, Akt- and PLCβ-activation mediated through endogenously expressed G_q or $G_{i/o}$ proteins under carbachol stimulation of muscarinic receptors M_3 or M_2 respectively (Wang *et al*., 2002; Anger *et al*., 2004).

The best characterized R4 RGS protein is RGS4 known as potent inhibitor of G_q-mediated PLCβ activation (Hepler *et al*., 1997; Heximer *et al*., 1999). In COS-7 cells, receptor-specific activation of ERK 1/2 by a $G_{q/11}$-coupled receptor, the bombesin receptor (BR) and a $G_{i/o}$-coupled receptor, the D2 dopamine receptor, transiently co-expressed in presence or absence of recombinant RGS4 was compared causing inhibited activation of ERK 1/2 by both receptors upon receptor agonist stimulation. Additionally, RGS4 inhibited BR-stimulated synthesis of inositol phosphates by PLCβ (Yan *et al*., 1997). However, in our COS-7 cell system, RGS4 exerts only significant inhibitory effects on muscarinic receptor M_2 stimulated investigated effector activation (ERK 1/2 and AKT) indicating regulatory effects of RGS4 for both: G_q as well as $G_{i/o}$ depending on the corresponding signaling receptor. This observation was previously done and confirmed in transfected BE(2)-C human neuroblastoma cells expressing human 5-HT(1B) receptor (Leone *et al*., 2000). Adjustement of increasing cDNA for more transient expression of RGS4 did not reveal significant inhibition on M_3/G_q-driven ERK 1/2 or AKT activation.

Only little is known about RGS5 and RGS16 and activation of ERK 1/2 and Akt. In CHO cells, RGS16 attenuates activation of ERK 1/2 significantly whereas RGS5 does not show any effect on platelet-activating factor stimulated G protein-coupled PAF receptor (Zhang *et al*., 1999). In contrast, endogenously expressed RGS5 inhibits G_q-mediated ERK 1/2 activation (Wang *et al*., 2002). In COS-7 cells, RGS5 was able to establish inhibitory effects towards activation of ERK 1/2 upon G_q- and $G_{i/o}$-signaling. In contrast, Akt phosphorylation got inhibited specifically upon muscarinic receptor M_2 signaling. RGS16 showed no effect on investigated G_q- and $G_{i/o}$-mediated effector activation.

RGS specificity towards $G_{i/o}/G_q$ signaling network. Remarkably, all investigated RGS proteins were able to develop both, their ability as GTPase activating protein (GAP) to accelerate endogenously expressed Gα-intrinsic GTPase activity and to block competitive binding of activated Gα to signaling targets as effector antagonists as we have used endogenously expressed G proteins.

In our COS-7 cells system, we were able to confirm cell-type selectivity of R4 RGS proteins towards G_q- or $G_{i/o}$-mediated ERK 1/2 and Akt activation. It appears that full activation of the ERK 1/2 or Akt pathway in different cell systems via GPCR requires the activation of distinct families of heterotrimeric G proteins.

Furthermore, activation of G_q-mediated ERK 1/2 or AKT phosphorylation is maintained through production of inositol phosphates, driven directly by activation of PLCβ, which in turn, was dose-dependent inhibited through investigated R4 RGS proteins: RGS2, RGS3s, RGS3 and RGS5 (Anger *et al*., 2004). Surprisingly, down streaming effectors of PLCβ activation (mainly ERK 1/2 and AKT) were themselves differentially influenced through investigated co-expressed R4 RGS proteins! Further components are needed to clarify inhibitory/non-inhibitory RGS effects on effector signaling upon similar receptor activation, here $M_2/G_{i/o}$ or M_3/G_q-mediated ERK 1/2-, AKT- or PLCβ-activation. This exclusively new observation defines our study and raises new questions concerning the specificity of RGS function. It seems that cardiac expressed RGS proteins (R4 Subfamily) develop a pattern of specificity towards different receptors as well as towards different signaling effectors assuming a signaling network in the boundaries of cell growth, proliferation, apoptosis and even specific cardiac signal transduction of contractility and heart rate. G-protein related intracellular network combines extracellular signal transduction to nuclear responds. The ability of RGS proteins to work within this network was established widely in all different cells and mechanisms of function, but the cell-specific pattern of specificity of RGS protein and the effect on the specifically investigated signaling pathway has to get compiled together. More clarity about RGS protein-Protein Binding is needed, more investigations are needed to develop new regulatory mechanisms for RGS proteins and more studies are needed to develop specifically the third function of RGS proteins: Binding to yet unknown intermediaries of G-protein mediated signaling components/effectors.

RGS-$G_{i/o}/G_q$ signaling network in cardiac cells: ventricular cardiomyocytes. In comparison to our findings of inhibitory effects of transiently expressed different RGS proteins in muscarinic receptor $M_2/G_{i/o}$ or M_3/G_q-mediated ERK 1/2-, AKT- or PLCβ-signaling in COS-7 cells, in cardiomyocytes different functionally effects were demonstrated: RGS2 selectively inhibits G_q-signaling, where as RGS3, RGS3s, RGS4 and RGS5 remain inhibitors for both, G_q- and even for $G_{i/o}$-signaling. None of the investigated RGS proteins (2-5) was subjected to regulate G_s-signaling (Hao *et al*., 2006). Using adenoviral gene transfer to induce RGS2 or RGS 4 expression in adult rat ventricular myocytes suppression of activated ERK under G_q-signaling (exclusively RGS2) or both G_q- and $G_{i/o}$-signaling (RGS4) was observed (Snabaitis *et al*., 2005). Under muscarinic receptor activation of AKT-/ERK-phosphorylation, we defined inhibitory effects of all investigated RGS proteins (2-5, [16]) under $G_{i/o}$-signaling (M_2), but only of

RGS2 and 3s/3 under G_q-signaling (M_3), which in contrast to the published findings, demonstrates for RGS2 newly interestingly regulating effects even on $G_{i/o}$-driven muscarinic signaling with a lack of RGS4 and 5 (Snabaitis *et al*., 2005; Hao *et al*., 2006).

Concluding Remarks

In COS-7 cells, transiently transfections liberate the ability to specifically investigate receptor depending effects of R4 RGS proteins towards endogenously expressed G-protein coupled signaling target effectors: ERK 1/2, Akt and PLCβ. Transiently expressed RGS proteins establish their function as GAP's as well as effector antagonists. Muscarinic receptors M_2 and M_3 are coupling to appropriate endogenously expressed G-proteins: $G_{i/o}$ or G_q, respectively and are subject of carbachol stimulation leading to activation of ERK 1/2, Akt and PLCβ. Co-expression of transiently transfected specific R4 RGS proteins revealed a pattern of specific effects of each single investigated RGS protein upon activation of above mentioned signal targets. Thereby, we demonstrate the ability of RGS proteins to inhibit Gα- as well as Gβγ-signaling indirectly. Additionally, Inhibitory effect of specific investigated R4 RGS proteins on PLCβ activation haven't automatically revealed same inhibitory extend on PLCβ down streaming effectors: ERK 1/2 and AKT. This observation has to get compiled within the signaling network of G-protein coupled receptors, regulatory mechanisms of RGS proteins and specific RGS protein-protein bindings in terms of RGS protein cross-talk to yet unknown signaling components. Since we used an over-expression model, further investigations (i.e. silencing RNA assays) for the role of endogenously expressed R4 RGS proteins and their specific functions are necessary.

Acknowledgments We thank Dr. Xiaomei Xu, Dr. Ming Zhu and Heinrich Baehr for their help in performing Western blot analyses as well as we thank Dr. Wei Zhang for preparing some of the N-terminal FLAG tagged RGS cDNA constructs.

References

Anger, T., Zhang, W. and Mende, U. (2004) Differential contribution of GTPase activation and effector antagonism to the inhibitory Effect of RGS Proteins on Gq-mediated Signaling *in vivo*. *J. Biol. Chem.* **279**, 3906-3915.

Berman, D. M., Kozasa, T. and Gilman, A. G. (1996) The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J. Biol. Chem.* **271**, 27209-27212.

Blanc, A., Pandey, N. R. and Srivastava, A. K. (2003) Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H_2O_2 in vascular smooth muscle cells: potential involvement in vascular disease (review). *Int. J. Mol. Med.* **11**, 229-234.

Bommakanti, R. K., Vinayak, S. and Simonds, W. F. (2000) Dual regulation of Akt/protein kinase B by heterotrimeric G protein subunits. *J. Biol. Chem.* **275**, 38870-38876.

Chang, F., Steelman, L. S., Lee, J. T., Shelton, J. G., Navolanic, P. M., Blalock, W. L., Franklin, R. A. and McCubrey, J. A. (2003b) Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* **17**, 1263-1293.

Chang, F., Steelman, L. S., Shelton, J. G., Lee, J. T., Navolanic, P. M., Blalock, W. L., Franklin, R. and McCubrey, J. A. (2003c) Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). *Int. J. Oncol.* **22**, 469-480.

Chatterjee, T. K., Eapen, A. K. and Fisher, R. A. (1997) A truncated form of RGS3 negatively regulates G protein-coupled receptor stimulation of adenylyl cyclase and phosphoinositide phospholipase C. *J. Biol. Chem.* **272**, 15481-15487.

Chen, C., Zheng, B., Han, J. and Lin, S. C. (1997) Characterization of a novel mammalian RGS protein that binds to Galpha proteins and inhibits pheromone signaling in yeast. *J. Biol. Chem.* **272**, 8679-8685.

Cladman, W. and Chidiac, P. (2002) Characterization and comparison of RGS2 and RGS4 as GTPase-activating proteins for m2 muscarinic receptor-stimulated G(i). *Mol. Pharmacol.* **62**, 654-659.

Crespo, P., Xu, N., Simonds, W. F. and Gutkind, J. S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* **369**, 418-420.

Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M. and Lefkowitz, R. J. (1997) Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of G_i- and G_q-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J. Biol. Chem.* **272**, 19125-19132.

Dorn, G. W., 2nd and Brown J. H. (1999) Gq signaling in cardiac adaptation and maladaptation. *Trends Cardiovasc Med.* **9**, 26-34.

Dorn, G. W., 2nd, Tepe, N. M., Lorenz, J. N., Koch, W. J. and Liggett, S. B. (1999) Low- and high-level transgenic expression of beta2-adrenergic receptors differentially affect cardiac hypertrophy and function in Galphaq-overexpressing mice. *Proc. Natl. Acad. Sci. USA* **96**, 6400-6405.

Druey, K. M., Blumer, K. J., Kang, V. H. and Kehrl, J. H. (1996) Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. *Nature* **379**, 742-746.

Dulin, N. O., Pratt, P., Tiruppathi, C., Niu, J., Voyno-Yasenetskaya, T. and Dunn, M. J. (2000) Regulator of G protein signaling RGS3T is localized to the nucleus and induces apoptosis. *J. Biol. Chem.* **275**, 21317-21323.

Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A. and Sugimoto, C. (2003) PI3K/Akt and apoptosis: size matters. *Oncogene* **22**, 8983-8998.

Gerthoffer, W. T. and Singer, C. A. (2003) MAPK regulation of gene expression in airway smooth muscle. *Respir Physiol. Neurobiol.* **137**, 237-250.

Gilman, A. G. (1995) Nobel Lecture. G proteins and regulation of adenylyl cyclase. *Biosci. Rep.* **15**, 65-97.

Hao, J., Michalek, C., Zhang, W., Zhu, M., Xu, X. and Mende, U. (2006) Regulation of cardiomyocyte signaling by RGS proteins: differential selectivity towards G proteins and susceptibility to regulation. *J. Mol. Cell. Cardiol.* **41**, 51-61.

Hepler, J. R., Berman, D. M., Gilman, A. G. and Kozasa, T.

(1997) RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha. *Proc. Natl. Acad. Sci. USA* **94**, 428-432.

Heximer, S. P., Srinivasa, S. P., Bernstein, L. S., Bernard, J. L., Linder, M. E., Hepler, J. R. and Blumer, K. J. (1999) G protein selectivity is a determinant of RGS2 function. *J. Biol. Chem.* **274**, 34253-34259.

Heximer, S. P., Watson, N., Linder, M. E., Blumer, K. J. and Hepler, J. R. (1997) RGS2/G0S8 is a selective inhibitor of Gqalpha function. *Proc. Natl. Acad. Sci. USA* **94**, 14389-14393.

Hollinger, S. and Hepler, J. R. (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol. Rev.* **54**, 527-559.

Igarashi, J., Bernier, S. G. and Michel, T. (2001) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J. Biol. Chem.* **276**, 12420-12426.

Jakubik, J. and Wess, J. (1999) Use of a sandwich enzyme-linked immunosorbent assay strategy to study mechanisms of G protein-coupled receptor assembly. *J. Biol. Chem.* **274**, 1349-1358.

Joseph, J. A., Fisher, D. R. and Strain, J. (2002) Muscarinic receptor subtype determines vulnerability to oxidative stress in COS-7 cells. *Free Radic. Biol. Med.* **32**, 153-161.

Kardestuncer, T., Wu, H., Lim, A. L. and Neer, E. J. (1998) Cardiac myocytes express mRNA for ten RGS proteins: changes in RGS mRNA expression in ventricular myocytes and cultured atria. *FEBS Lett.* **438**, 285-288.

Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M. and Lefkowitz, R. J. (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. *J. Biol. Chem.* **269**, 6193-6197.

Lee, J. T., Jr. and McCubrey, J. A. (2002) The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* **16**, 486-507.

Leone, A. M., Errico, M., Lin, S. L. and Cowen, D. S. (2000) Activation of extracellular signal-regulated kinase (ERK) and Akt by human serotonin 5-HT(1B) receptors in transfected BE(2)-C neuroblastoma cells is inhibited by RGS4. *J. Neurochem.* **75**, 934-938.

Matsui, T., Nagoshi, T. and Rosenzweig, A. (2003) Akt and PI 3-kinase signaling in cardiomyocyte hypertrophy and survival. *Cell Cycle* **2**, 220-223.

Melliti, K., Meza, U. and Adams, B. A. (2001) RGS2 blocks slow muscarinic inhibition of N-type Ca(2+) channels reconstituted in a human cell line. *J. Physiol.* **532**, 337-347.

Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A. and Gutkind, J. S. (1998) Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. *J. Biol. Chem.* **273**, 19080-19085.

Neer, E. J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80**, 249-257.

Neri, L. M., Borgatti, P., Capitani, S. and Martelli, A. M. (2002) The nuclear phosphoinositide 3-kinase/AKT pathway: a new second messenger system. *Biochim. Biophys. Acta* **1584**, 73-80.

Nishida, M., Tanabe, S., Maruyama, Y., Mangmool, S., Urayama, K., Nagamatsu, Y., Takagahara, S., Turner, J. H., Kozasa, T., Kobayashi, H., Sato, Y., Kawanishi, T., Inoue, R., Nagao, T. and Kurose, H. (2005) G alpha 12/13- and reactive oxygen species-dependent activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase by angiotensin receptor stimulation in rat neonatal cardiomyocytes. *J. Biol. Chem.* **280**, 18434-18441.

Ogier-Denis, E., Pattingre, S., El Benna, J. and Codogno, P. (2000) Erk1/2-dependent phosphorylation of Galpha-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. *J. Biol. Chem.* **275**, 39090-39095.

Rebecchi, M. J. and Pentyala, S. N. (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol. Rev.* **80**, 1291-1335.

Reddy, K. B., Nabha, S. M. and Atanaskova, N. (2003) Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev.* **22**, 395-403

Reif, K. and Cyster, J. G. (2000) RGS molecule expression in murine B lymphocytes and ability to down-regulate chemotaxis to lymphoid chemokines. *J. Immunol.* **164**, 4720-4729.

Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70**, 281-312.

Ross, E. M. and Wilkie, T. M. (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem.* **69**, 795-827.

Shi, C. S., Lee, S. B., Sinnarajah, S., Dessauer, C. W., Rhee, S. G. and Kehrl, J. H. (2001) Regulator of G-protein signaling 3 (RGS3) inhibits Gbeta1gamma 2-induced inositol phosphate production, mitogen-activated protein kinase activation, and Akt activation. *J. Biol. Chem.* **276**, 24293-24300.

Shiojima, I. and Walsh, K. (2002) Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ. Res.* **90**, 1243-1250.

Smalley, K. S. (2003) A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? *Int. J. Cancer* **104**, 527-532.

Snabaitis, A. K., Muntendorf, A., Wieland, T. and Avkiran, M. (2005) Regulation of the extracellular signal-regulated kinase pathway in adult myocardium: differential roles of G(q/11), Gi and G(12/13) proteins in signalling by alpha1-adrenergic, endothelin-1 and thrombin-sensitive protease-activated receptors. *Cell Signal.* **17**, 655-664.

Steelman, L. S., Pohnert, S. C., Shelton, J. G., Franklin, R. A., Bertrand, F. E. and McCubrey, J. A. (2004) JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* **18**, 189-218.

Tibbles, L. A. and Woodgett, J. R. (1999) The stress-activated protein kinase pathways. *Cell Mol. Life Sci.* **5**, 1230-1254.

Vazquez-Prado, J., Casas-Gonzalez, P. and Garcia-Sainz, J. A. (2003) G protein-coupled receptor cross-talk: pivotal roles of protein phosphorylation and protein-protein interactions. *Cell Signal* **15**, 549-557.

Wang, Q., Liu, M., Mullah, B., Siderovski, D. P. and Neubig, R. R. (2002) Receptor-selective effects of endogenous RGS3 and RGS5 to regulate mitogen-activated protein kinase activation in rat vascular smooth muscle cells. *J. Biol. Chem.* **277**, 24949-24958.

Wieland, T. and Mittmann, C. (2003) Regulators of G-protein signalling: multifunctional proteins with impact on signalling in

the cardiovascular system. *Pharmacol. Ther.* **97**, 95-115.

Yan, Y., Chi, P. P. and Bourne, H. R. (1997) RGS4 inhibits Gq-mediated activation of mitogen-activated protein kinase and phosphoinositide synthesis. *J. Biol. Chem.* **272**, 11924-11927.

Zhang, W., Anger, T., Su, J., Hao, J., Xu, X., Zhu, M., Gach, A., Cui, L., Liao, R. and Mende, U. (2006) Selective loss of fine tuning of Gq/11 signaling by RGS2 protein exacerbates cardiomyocyte hypertrophy. *J. Biol. Chem.* **281**, 5811-5820.

Zhang, W. and Neer, E. J. (2001) Reassembly of phospholipase C-beta2 from separated domains: analysis of basal and G protein-stimulated activities. *J. Biol. Chem.* **276**, 2503-2508.

Zhang, Y., Neo, S. Y., Han, J., Yaw, L. P. and Lin, S. C. (1999) RGS16 attenuates galphaq-dependent p38 mitogen-activated protein kinase activation by platelet-activating factor. *J. Biol. Chem.* **274**, 2851-2857.