RGS Protein Specificity Towards Gq- and Gi/o-Mediated ERK 1/2 and Akt Activation, in vitro

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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 Gq and Gi proteins subjected to regulation by RGS proteins. The goal of the study was to delineate the specificity in which cardiac RGS proteins modulate Gq- and Gi/o-induced ERK and Akt phosphorylation. To isolate Gq- and Gi/o-mediated effects, we exclusively expressed muscarinic M2 or M3 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 M2/M3 expressing cells through carbachol stimulation. In co-expressions, M3/Gq-induced activation of Akt was exclusively blunted through RGS3/RGS3, whereas activation of ERK was inhibited additionally through RGS2/RGS5 M2/Gi/o-induced Akt activation was inhibited by all RGS proteins tested. RGS2 had no effect on M2/Gi/o-induced ERK activation.

The high degree of specificity in RGS proteins-depending modulation of Gq- and Gi/o-mediated ERK and Akt activation in the muscarinic network cannot merely be attributed exclusively to RGS protein selectivity towards Gq or Gi/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.

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.

Keywords: Akt, ERK-1/2, Muscarinic GPCR specificity, 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 (Bommalakii et al., 2000; Chang et al., 2003b). Ligand-dependent 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 N(H(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
heterotrimeric G-proteins comprised of α and βγ subunits (Gilman, 1995; Neer, 1995). Focusing on these G-Protein coupled receptors (GPCR) bound to either Gi or Gq signaling, both G-protein mediated signaling lead to further activation of MAP Kinases and or Akt (Yan et al., 1997; Munga et al., 1998; Vazquez-Pardo et al., 2003).

Regulators of G protein Signaling (RGS) proteins belong to a family of more than 20 proteins with a conserved G protein core domain of ~120 amino acids that is necessary and sufficient for binding to Go subunits (Hollinger and Hepler, 2002). RGS proteins exert an inhibitory effect on both Go- and Gβγ-mediated downstream effects by either diminishing signal production generated by GPCR defined as effector antagonistic function of G protein signaling, i.e. (Leone et al., 1997). Signals through activation of the G protein Gα subunits (Hollinger and Hepler, 2002; Anger et al., 2003) or by terminating of GPCR coupled signals through activation of the Gα-GTPase: GTPase-activating proteins (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 Gq and/or Gi, i.e. in rat smooth muscle cells (Blanc et al., 2003), in neuroblastoma cells (Nishida et al., 2005), in human cancer cells (Oger-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 Gq-mediated PLCβ activation in vitro as well as in vivo (Hepler et al., 1997; Yan et al., 1997; Kandestuncer 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 Gq-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 Gq or Gqα-mediated activation of ERK 1/2 and Akt in an in vitro system in which specific effects of RGS proteins were previously described towards activation of Gi-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 isofoms], 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 M3, coupling to endogenous Gi or M3 coupling to Gq, 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 phosphorylated 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 Gi-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 Gq-mediated activation of PLCβ supporting cardiac hypertrophy (Zhang et al., 2006) we generated a system to distinguish specific effects on Gq- and Gi-mediated 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 (Ci/Cq-Gi/Go-differentiation) and opens a field of further specificity 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 isofoms of RGS3) (Dulin et al., 2000; Reif and Cyster, 2000), RGS3 and RGS4 (Druey et al., 1996), RGS5 and RGS16 (Kandestuncer 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 M2 and M3 receptor were purchased from Guthrie Institute. The cDNA for the β-adrenergic receptor kinase 1 carboxy-terminal peptide encoding minigene (βARKCTβ) 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 M2 or M3 respectively was co-transfected in presence or absence of 335 ng plasmid DNA carrying either specific RGS proteins, βARKCTβ.
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 proteases inhibitors (Complete Mini) for 30 min on a shaker at 4°C. Equal amounts of total cell lysates [30 µg per line, determined in front of 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, BabCo), β-actin as loading control, ERK-1/2, phosphorylated ERK-1/2 [Thr202/Tyr204], Akt and phosphorylated Akt [Ser 437] 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 densiometric 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.** Thirty six hours after transfections with muscarinic receptors M3 or M2, COS-7 cells were treated with 30 ng/ml (accordingly 1 ml/well) pertussis toxin (PTX) or vehicle for 12 h. *In vitro* back ADP ribosylation assay was used 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 M2 and M3 was described previously (Anger et al., 2004). Briefly, 48 h after transient transfection with cDNA encoding either M2 or M3 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 N-Methyl [3H]Scopolamine ([3H]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 (Bmax [fmol/mg protein]) and radioligand binding affinity (Kd [nM]) as demonstrated previously for muscarinic receptor M2 (Anger et al., 2004).

**Measurement of [3H]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-[3H]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 37°C, the inositol phosphates were extracted in 20 mM formic acid, neutralized, separated by anion-exchange chromatography (Dionex, 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 duplicates. 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 Gq3 or Gi5-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 transient transfection assays to establish a specific Gq3-driven, muscarinic receptor M5-coupled or Gi5-driven, muscarinic receptor M1 coupled system (see Fig. 1).

Initially, transient transfections were performed using M2 or M3 coupling to endogenously expressed G5 or Gi5 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 basal conditions, carbachol independently to Gq or Gi5, stimulates time-dependent ERK1/2 activation where as Akt phosphorylation upon muscarinic receptor activation of both, M5 (G5) and M2 (Gi5) 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 endogenous 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 Gi5-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 M3 muscarinic receptor was transiently expressed (see Fig. 3). No effect was seen on muscarinic receptor M3-driven ERK 1/2 and Akt activation demonstrating proper endogenously coupling of
transiently expressed receptors to their complementary G protein: M2-Gi/o and M3-Gq, respectively.

The protein expression of all endogenous G-proteins (Gq or Gi/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 (βARKct) a known Gβγ-scavenger blocks further Gβγ-mediated downstream signalling (Koch et al., 1994; Murga et al., 1998). Transient co-expression of βARKct was used to distinguish either Gα- (not blunted through βARKct) or Gβγ-driven phosphorylation of ERK 1/2 and Akt (see Fig. 4). No effect was established through co-expressed βARKct on carbachol stimulated phosphorylation of ERK 1/2 and Akt when compiled to M3-Gq, demonstrating dominantly signaling through Go. Interestingly, in presence of βARKct overall basal activation of ERK 1/2 and Akt was - to due unexplainable-reproducible relevantly increased with no further raise using carbachol when M2 was transiently expressed.

Co-expression of βARKct itself was monitored by western

Fig. 1. Experimental System for muscarinic M2 and M3 receptor in COS-7 cells. COS-7 cells were transiently transfected with either muscarinic M2 or M3 receptor in presence or absence of transiently expressed RGS proteins. Endogenously coupling of transiently expressed muscarinic receptor to the G-Protein, here Gi/o or Gq 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 βARKct, when M2 was transiently co-expressed. Main down streaming of muscarinic receptor M3 was established through Go and similarly activation of Protein kinase C (PKC) mediated through PLCβ.

Fig. 2. Timecourse and dose-dependency of carbachol stimulated, muscarinic M3 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 M3 (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 M3 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.
immuno blot analyses demonstrating comparable expression in all transiently βARKCT 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 M2 than M3. This was highly attributed to different transient expression levels of the used receptors. We failed to demonstrate M3 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 M3 with investigated RGS proteins. In contrast, monitoring of M3 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 M2 (assessed through
effects of R4 RGS proteins on Gαi and Gαq-mediated ERK 1/2 and Akt activation. COS-7 cells were transiently transfected with muscarinic M2 or M3 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 M2 and M3 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 M2- or M3-expressing cells in the absence of co-transfected RGS protein was set as 100%. In M2-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 M2-receptor expressing
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 M₂ or M₃ not stimulated through carbachol, showed unaffected and unchanged basal phosphorylation 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. M₃/Gq-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, M₂/Gi/o-induced Akt activation was inhibited by all RGS proteins tested, including RGS2. However, RGS2 had no effect on M₂/Gi/o-induced ERK 1/2 activation. Surprisingly, RGS4, one of the best investigated inhibitor for Gq-mediated cardiac PLCβ activation in vitro (Hepler et al., 1997; Heximer et al., 1999), revealed in contrast to M₂/Gi/o-no effects on the M₃/Gq-induced ERK 1/2 or Akt phosphorylation.

**Effects of R4 RGS proteins on M₂-coupled Gq-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 M₂ in absence of RGS proteins lead to significant activation of
PLCβ. Except for RGS4 - which in vivo is known as inhibitor for Gβγ-mediated PLCβ activation, all investigated cardiac R4 GRS 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 GRS proteins could easily attributed to either endogenously expressed Gq or Gi, proteins and (ii) to establish receptor - specificity as well as (iii) effector - specificity of R4 GRS proteins towards ERK-1/2 and Akt activation in the context, where R4 GRS proteins except for RGS4 exert inhibition towards Gq-mediated PLCβ activation (Anger et al., 2004). The lack of endogenous Gq-coupled muscarinic receptors (such as M1, M3 and M5 receptors) and endogenous Gq-coupled muscarinic receptor (such as M3) in COS-7 cells (Jukubik and Wess, 1999; Joseph et al., 2002; Anger et al., 2004) was confirmed by negligible [3H]NMS binding in vector-transfected cells (data not shown) and in basal controls of experiments where carbachol stimulation offered negligible 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 M3 to Gq, and M2 to Giq, 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 reproducibility for M2 expression coupling to Giq when RGS16 was co-expressed. Further, co-expression of minigene βARKCT, a Giβγ-scavenger demonstrated coupling of downstream signaling towards Gai, when M2, and Giβγ, when M1 was transiently expressed (Koch et al. 1994). Several further intermediaries between ligand-dependent 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 M3 lead to PLCβ activation mainly via Gai, since the endogenously expressed PLCβ1 and 3 are insensitive to the Giβγ 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 Giβγ subunit exerts the signaling via activation of protein kinase B (Akt) (Koch et al., 1994; Murga et al., 1998; Bommakani et al., 2000). Therefore, the amount of coupled endogenous Giq or Gaiq 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 Mithmann, 2008), loss 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 Gai, as well as Giβγ 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 GRS protein specific inhibitory effect on activation of ERK 1/2 or Akt was observed. Despite the initial assumption that RGS2 is not able to explore activity towards Gai in vitro (Heximer et al., 1997) different cell models were chosen to demonstrate function of RGS2 towards Gai, i.e. S19 cells (Chadman and Chidiac, 2002), yeast (Dnuey et al., 1996), HEK293 cells (Melli et al., 2001), rat aorta smooth muscle cells (Wang et al., 2002). RGS2 inhibition of Giq-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: Gai and Giq as well as signaling targets upon one G
protein (G\textsubscript{q}): PLC\beta 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 extent. 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 PLC\beta-activation mediated through endogenously expressed G\textsubscript{q} or G\textsubscript{i/o} proteins under carbachol stimulation of muscarinic receptors M\textsubscript{2} or M\textsubscript{3} respectively (Wang et al., 2002; Anger et al., 2004).

The best characterized R4 RGS protein is RGS4 known as potent inhibitor of G\textsubscript{q}-mediated PLC\beta activation (Hepler et al., 1997; Heximer et al., 1999). In COS-7 cells, receptor-specific activation of ERK 1/2 by a G\textsubscript{q}/11 coupled receptor, the bombesin receptor (BR) and a G\textsubscript{11}-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 PLC\beta (Yan et al., 1997). However, in our COS-7 cell system, RGS4 exerts only significant inhibitory effects on muscarinic receptor M\textsubscript{2} stimulated investigated effector activation (ERK 1/2 and Akt) indicating regulatory effects of RGS4 for both: G\textsubscript{q} as well as G\textsubscript{i/o} dependent 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). Adjustment of increasing cDNA for more transient expression of RGS4 did not reveal significant inhibition on M\textsubscript{3}/G\textsubscript{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\textsubscript{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\textsubscript{q} and G\textsubscript{i/o}-signaling. In contrast, Akt phosphorylation got inhibited specifically upon muscarinic receptor M\textsubscript{2} signaling. RGS16 showed no effect on investigated G\textsubscript{q} and G\textsubscript{i/o}-mediated effector activation.

**RGS specificity towards G\textsubscript{i/o}/G\textsubscript{q} signaling network.** Remarkably, all investigated RGS proteins were able to develop both, their ability as GTPase activating protein (GAP) to accelerate endogenously expressed Go intrinsic GTPase activity and to block competitive binding of activated Go 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\textsubscript{q} or G\textsubscript{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\textsubscript{q}-mediated ERK 1/2 or Akt phosphorylation is maintained through production of inositol phosphates, driven directly by activation of PLC\beta, 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 PLC\beta 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\textsubscript{2}/G\textsubscript{q}, or M\textsubscript{3}/G\textsubscript{i/o}-mediated ERK 1/2-, AKT- or PLC\beta-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\textsubscript{i/o}/G\textsubscript{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\textsubscript{2}/G\textsubscript{q} or M\textsubscript{3}/G\textsubscript{i/o}-mediated ERK 1/2-, AKT- or PLC\beta-signaling in COS-7 cells, in cardiomyocytes different functionally effects were demonstrated: RGS2 selectively inhibits G\textsubscript{q}-signaling, where as RGS3, RGS3s, RGS4 and RGS5 remain inhibitors for both, G\textsubscript{q} and even for G\textsubscript{i/o}-signaling. None of the investigated RGS proteins (2-5) was subjected to regulate G\textsubscript{q}-signaling (Huo 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\textsubscript{q}-signaling (exclusively RGS2) or both G\textsubscript{q} and G\textsubscript{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\textsubscript{i/o}-signaling (M\textsubscript{3}), but only of
RGS2 and 3/3 under Gq-signaling (M3), which in contrast to the published findings, demonstrates for RGS2 newly interestingly regulating effects even on Goα-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 M1 and M3 are coupling to appropriate endogenously expressed G-proteins: Goα or Gq subunits 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 Goα 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.

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