Endogenous RGS proteins facilitate dopamine D_{2S} receptor coupling to $G_{\alpha o}$ proteins and Ca^{2+} responses in CHO-K1 cells

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Abstract The role of RGS proteins on dopaminergic D_{2S} receptor ($D_{2S}R$) signalling was investigated in Chinese hamster ovary (CHO)-K1 cells, using recombinant RGS protein- and PTX-insensitive $G_{\alpha o}$ proteins. Dopamine-mediated [^{35}S]GTPγS binding was attenuated by more than 60% in CHO-K1 $D_{2S}R$ cells coexpressing a RGS protein- and PTX-insensitive $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein versus cells coexpressing a similar amount of PTX-insensitive $G_{\alpha o}Cys^{351}Ile$ protein. Dopamine-agonist-mediated Ca^{2+} responses were dependent on the coexpression with a $G_{\alpha o}Cys^{351}Ile$ protein and were fully abolished upon coexpression with a $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein. These results suggest that interactions between the $G_{\alpha o}$ protein and RGS proteins are involved in efficient $D_{2S}R$ signalling.

Key words: RGS; Dopamine D_2 receptor; G protein coupling; GTPγS; Ca^{2+} response

1. Introduction

Regulators of G protein signalling (RGS) proteins are GTPase activating proteins (GAP) for G_α protein subunits of heterotrimeric G proteins [1,2]. First functionally identified as GAP for the $G_{\alpha i}$ subclass [3], RGS proteins have now been described as GAP for three other G_α protein subclasses $G_{\alpha q}$, $G_{\alpha 12/13}$ and $G_{\alpha s}$ also [4–6]. RGS proteins, besides their role as GAP, can play additional roles in cell signalling [7–9]. For example RGS4 can behave as an effector shield for $G_{\alpha q}$ [4], and p115RhoGEF as an effector for $G_{\alpha 13}$ [10]. Most previous studies on RGS proteins emphasise their role as negative regulators in G protein signalling pathways. However, positive effects of overexpressed RGS proteins on G protein coupled receptor (GPCR) signalling have also been reported. For example RGS proteins have been shown to enhance the activation of K^+ channels [11–13], suggested to be caused by an increase in availability of free $G_{\beta\gamma}$ subunits [14]. A positive effect of endogenous RGS proteins on signalling can be explained by considering that RGS proteins enhance the overall efficacy of the GDP/GTP binding cycle of the G_α protein in a receptor/G_α protein/RGS complex [1]. Few studies on the function of endogenous RGS proteins have emerged. Specific RGS knockouts in mice have defined an important role for RGS9-1 in vision [15], and similar studies implicated RGS2 in phenomena as diverse as T cell activation, anxiety and aggressive behaviour of mice [16], perhaps because of the diversity in multiple possible G_α protein partners for RGS2. Recently, a ribozyme approach was used successfully to define receptor-selective roles of endogenous RGS3 and RGS5 in smooth muscle cells for muscarinic M_3 receptor and angiotensin AT_{1A} receptor, respectively [17]. An alternative way to study the role of RGS proteins in specific G protein signalling pathways is to disrupt the G_α/RGS interaction by mutation of the G_α subunit. Such a point mutation in $G_{\alpha o}$ protein ($G_{\alpha o}Gly^{184}Ser$) renders the mutant $G_{\alpha o}$ protein insensitive to RGS proteins, without a change in its GDP release, GTPγS binding and intrinsic GTP hydrolysis parameters [18]. The introduction of this mutation in $G_{\alpha o}$ protein, in combination with the C-terminal $Cys^{351}Ile$ (or $Cys^{351}Gly$) mutation that confers pertussis toxin (PTX) insensitivity [19], was shown very useful to study the role of endogenous RGS proteins in $G_{\alpha i/o}$ coupled signalling pathways [20,21]. The dopamine D_2 receptor (D_2R) has been shown to couple via $G_{i/o}$ to diverse effectors in different cell lines [22,23], and in Chinese hamster ovary (CHO)-K1 cells specifically D_2R activation leads to an increase in Ca^{2+} levels [24]. By abolishing the $G_{\alpha o}$ protein/RGS proteins interactions using the $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein, we observed a decrease of $D_{2S}R$ signalling at the level of G_α protein activation ([^{35}S]GTPγS binding) and second messenger (Ca^{2+} response). This suggests that endogenous RGS proteins play a positive role for efficient signalling in a receptor/G protein/RGS/effector complex.

2. Materials and methods

2.1. Cloning of human dopamine D_2 receptor

The short splice variant of the human D_2R (RC: 2.1.DA.02) was cloned as previously described [25] by PCR using oligonucleotide primers designed according to the sequence deposited in the GenBank database (accession number S69899).

2.2. Construction of rat $G_{\alpha o}$ insensitive to PTX and RGS

Rat $G_{\alpha o}Cys^{351}Ile$ protein (insensitive to PTX) was constructed as described previously [19]. An additional point mutation $Gly^{184}Ser$, conferring insensitivity of $G_{\alpha o}$ subunits to RGS proteins, was introduced by using a Quick Change site-directed mutagenesis kit (Stratagene) according to the supplier's instructions. Mutation was con-

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Abbreviations: AFU, arbitrary fluorescence units; D_{2S}, D_{2short}; CHO, Chinese hamster ovary; DA, dopamine; GAP, GTPase activating protein; GPCR, G protein coupled receptor; PTX, pertussis toxin; D_2R, dopamine D_2 receptor; RGS, regulator of G protein signalling; TBS–T, Tris buffered saline–Tween; (−)-NPA, R(−)-propylnorapomorphine; (+)-NPA, S(+)-propylnorapomorphine

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firmed by sequencing on ABI Prism 310 Genetic analyser using a Big Dye Terminator Cycle Sequencing reaction kit.

2.3. Cell culture

CHO-K1 cell line stably expressing human $D_{2S}R$ (CHO-K1 $D_{2S}R$) was generated upon dilution of transfected cells (10- to 1000-fold) and selection in Ham's F12 plus 10% heat-inactivated foetal calf serum, penicillin (65 μg/ml), streptomycine (100 μg/ml) and geneticin (1.25 mg/ml).

2.4. Membrane preparation

CHO-K1 $D_{2S}R$ cells were transfected with either $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ in pCR3.1 plasmid using Lipofectamine (Gibco BRL) [26]. Cells were harvested 48 h after transfection. Treatment with PTX (20 ng/ml) was performed during 16 h. Membrane preparations were performed as follows: cells were washed with phosphate buffered saline, stored at −80°C, collected mechanically in Tris–HCl 10 mM/EDTA 0.1 mM (pH 7.5), homogenised and centrifuged twice for 10 min at $45\,000 \times g$. The final pellet was resuspended in the same buffer and stored at −80°C until further use.

2.5. [^{35}S]GTPγS binding response

[^{35}S]GTPγS binding on membrane preparations from CHO-K1 cells was performed as described previously [27]. Briefly, basal and agonist-dependent [^{35}S]GTPγS binding was performed with membranes incubated at 25°C with or without compound for 30 min in 20 mM HEPES (pH 7.4) supplemented with 30 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ascorbic acid followed by addition of 0.5 nM of [^{35}S]GTPγS and another 30 min incubation. Basal [^{35}S]GTPγS binding was defined as [^{35}S]GTPγS binding obtained in absence of compound. Activation of [^{35}S]GTPγS binding was determined as the percentage of increased basal [^{35}S]GTPγS binding after stimulation with compound. EC_{50} values were defined as the concentration of ligand yielding 50% of its own maximal [^{35}S]GTPγS binding response. Protein levels were quantified with a dye-binding assay kit (Bio-Rad), using bovine serum albumin as a standard [28].

2.6. [^{3}H]Nemonapride binding

Scatchard analysis was performed as described [25] using concentrations of radioligand [^{3}H]nemonapride ranging from 3 pM to 3 nM. Membrane preparations were diluted in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, (pH 7.4). 10 μM of (+)-butaclamol was used to determine non-specific binding. The reactions were stopped after 1 h incubation at 25°C by addition of 3.0 ml of ice-cold 50 mM Tris–HCl (pH 7.7) and rapid filtration over Whatman GF/B glass fibre filters using a Brandel harvester, washed and radioactivity was counted.

2.7. Immunological detection

Total proteins (25 μg) from CHO-K1 $D_{2S}R$ membranes transfected with either $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein were separated in Tris–glycine SDS gels (12% w/v polyacrylamide) and electrotransfered onto polyvinylidene difluoride membranes. After blocking in TBS–T [10 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)]+5% of non-fat milk, the membranes were probed with a polyclonal antibody (1:1000) raised against the whole rat $G_{\alpha o}$ protein (Calbiochem) in TBS–T+1% non-fat milk. Secondary antibody (anti-rabbit immunoglobulin horseradish peroxidase conjugate, Amersham) incubations and all washes were performed in TBS–T+1% non-fat milk. Detection was performed by enhanced chemiluminescence (Pierce) and exposure to Biomax ML film (Kodak). Densitometric analysis was performed using a computer-based image analysis system (AIS, Imaging Research).

2.8. Measurement of Ca^{2+} responses

CHO-K1 $D_{2S}R$ cells were transfected by electroporation [26] with 10 μg of either empty pCR3.1 vector, $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ (in pCR3.1). Treatment with PTX (20 ng/ml) was performed during 16 h before Ca^{2+} measurement. Cells were assayed 48 h post-transfection for Ca^{2+} responses after 1 h incubation with 2 μM fluo-3 fluorescent calcium indicator dye as described [26]. Fluorescent readings were made every 2 s for 3 min using a fluorometric imaging plate reader (FLIPR, Molecular Devices). Data for Ca^{2+} responses were expressed in arbitrary fluorescence units (AFU) and were not translated into Ca^{2+} concentrations.

3. Results

The high-efficacy dopaminergic agonists dopamine (DA) and R(−)-propylnorapomorphine [(−)-NPA] produced an increase of respectively 50 ± 8% and 65 ± 13% over basal [^{35}S]GTPγS binding on CHO-K1 $D_{2S}R$ membranes expressing the $G_{\alpha o}Cys^{351}Ile$ protein and pretreated with PTX; the partial

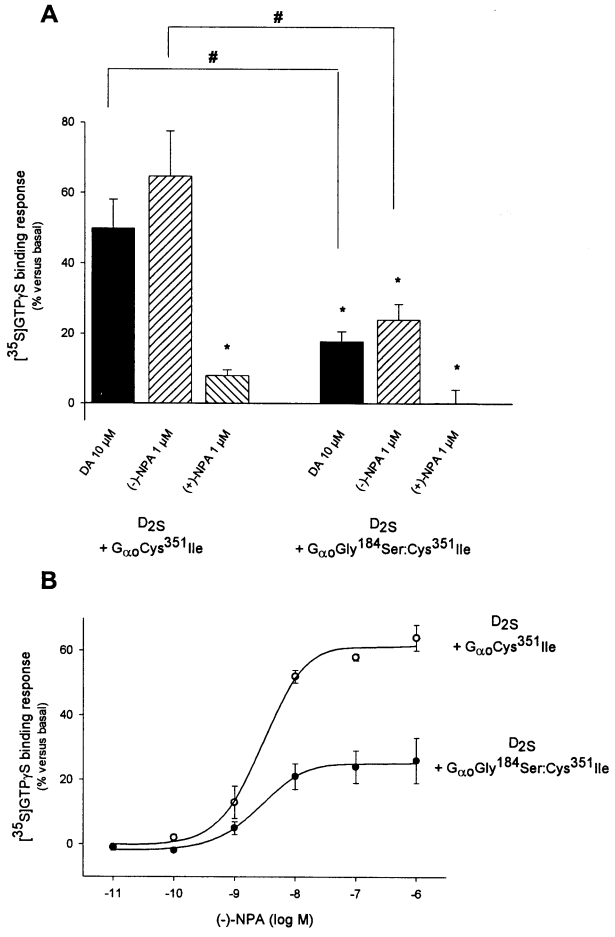

Fig. 1. A: [^{35}S]GTPγS binding response after stimulation with either partial or high-efficacy dopaminergic ligands on PTX-pretreated CHO-K1 $D_{2S}R$ membranes coexpressing either $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein. Results obtained after stimulation with either DA 10 μM, (−)-NPA 1 μM or (+)-NPA 1 μM. The agonists did not stimulate [^{35}S]GTPγS binding on mock-transfected, PTX-pretreated CHO-K1 $D_{2S}R$ membranes, whereas 10 μM of DA stimulated basal [^{35}S]GTPγS binding by 117 ± 10% on mock-transfected CHO-K1 $D_{2S}R$ membrane in the absence of PTX-pretreatment (data not shown). Statistical analysis was performed with one-way analysis of variance followed by an all pairwise multiple comparison (Student–Newman–Keuls procedure). *Difference statistically significant ($P < 0.05$) versus stimulation of DA on CHO-K1 $D_{2S}R$ membranes expressing $G_{\alpha o}Cys^{351}Ile$ protein. #Difference statistically significant ($P < 0.05$) for CHO-K1 $D_{2S}R$ membranes expressing $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein versus stimulation obtained with the same compound on CHO-K1 $D_{2S}R$ membranes expressing $G_{\alpha o}Cys^{351}Ile$ protein. B: Concentration-dependent [^{35}S]GTPγS binding response of high-efficacy agonist (−)-NPA on PTX-pretreated CHO-K1 $D_{2S}R$ membranes coexpressing either $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein. Data are expressed as percent of stimulation of basal [^{35}S]GTPγS binding (basal [^{35}S]GTPγS binding was 81 ± 9 fmol/mg of protein and 78 ± 4 fmol/mg of protein when either $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein was coexpressed, respectively). Data are mean ± S.E.M. of three to four independent experiments, each performed in triplicate.

agonist S(+)-propylnorapomorphine [(+)-NPA] displayed a weak stimulation of 8 ± 2% of basal [^{35}S]GTPγS binding on the same membranes (Fig. 1A). When CHO-K1 $D_{2S}R$ cells expressed the $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein, instead of the $G_{\alpha o}Cys^{351}Ile$ protein, stimulation of [^{35}S]GTPγS binding with dopaminergic ligands was reduced by 64% and 62% for DA and (−)-NPA respectively (Fig. 1A). In the same conditions, (+)-NPA was unable to stimulate [^{35}S]GTPγS binding (Fig. 1A). Concentration–response curves of the ligand (−)-NPA for [^{35}S]GTPγS binding revealed that the difference observed could be attributed to reduction in the efficacy of $D_{2S}R$ and $G_{\alpha o}$ protein coupling, the ligand potency remaining unchanged (EC_{50} was 2.7 ± 0.4 nM and 3.3 ± 0.9 nM for CHO-K1 $D_{2S}R$ cells expressing the $G_{\alpha o}Cys^{351}Ile$ protein and the $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein, respectively) (Fig. 1B). The above results suggest that endogenous RGS proteins are necessary for efficient activation of recombinant $G_{\alpha o}$ protein via $D_{2S}R$. Binding analyses of $D_{2S}R$ using [^{3}H]nemonapride as a radioligand revealed similar affinities and receptor expression levels: the K_d and B_{max} were 56 ± 12 pM and 1.03 ± 0.10 pmol/mg of protein respectively for CHO-K1 $D_{2S}R$ cells expressing the $G_{\alpha o}Cys^{351}Ile$ protein compared to 64 ± 21 pM and 1.10 ± 0.11 pmol/mg of protein respectively for CHO-K1 $D_{2S}R$ cells expressing the $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein. Western blot analysis showed equal expression levels of both $G_{\alpha o}Cys^{351}Ile$ and $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein (at approximately 40 kDa, Fig. 2; in agreement with its theorical molecular weight calculated from its published sequence, GenBank database accession number M17526). $D_{2S}R$ signalling was also monitored measuring Ca^{2+} responses. The agonists DA and (−)-NPA induced a strong increase of Ca^{2+} response (2698 ± 439 AFU and 2908 ± 303 AFU respectively, Fig. 3A) in CHO-K1 $D_{2S}R$ cells transfected with the empty pCR3.1 plasmid. This response was totally abolished by PTX pretreatment (Fig. 3A), confirming that this is a $G_{i/o}$ and not a $G_{q/11}$ protein-mediated event in this experimental system. Expression of $G_{\alpha o}Cys^{351}Ile$ protein in CHO-K1 $D_{2S}R$ cells restored a PTX-insensitive, DA-mediated Ca^{2+} response (1620 ± 314 and 1763 ± 217 AFU after DA and (−)-NPA stimulation, respectively) (Fig. 3B). By contrast, expression of $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein in CHO-K1 $D_{2S}R$ cells was unable to restore PTX-insensitive, DA-mediated Ca^{2+} response (Fig. 3B).

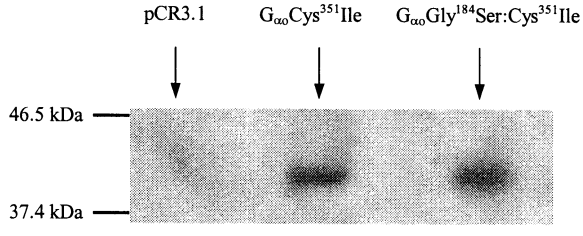

Fig. 2. Immunological detection of $G_{\alpha o}$ proteins in membrane preparations of CHO-K1 $D_{2S}R$ cells transfected with either empty pCR3.1 plasmid (mock), $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ plasmid, PTX pretreated. Molecular weights are indicated in the left margin. Quantification (percentage versus $G_{\alpha o}Cys^{351}Ile$ protein upon subtraction of the background), was 105% for the $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein. A rectangle covering the signal of $G_{\alpha o}Cys^{351}Ile$ protein was identically reproduced as surface template for the quantification of the $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein.

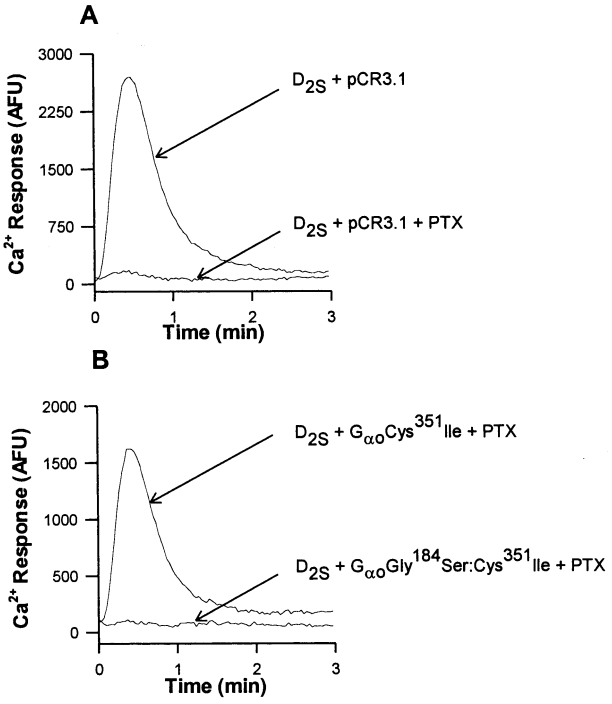

Fig. 3. A: Ca^{2+} response after 10 μM DA stimulation of CHO-K1 $D_{2S}R$ cells transfected with the empty pCR3.1 plasmid, cells non-pretreated or pretreated with PTX. B: 10 μM DA modulation of Ca^{2+} response of CHO-K1 $D_{2S}R$ cells expressing either $G_{\alpha o}Cys^{351}Ile$ or $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein, cells were pretreated with PTX. Results are expressed in AFU. One representative experiment out of four independent experiments, each experimental point performed in quadruplicate, is shown. For each condition Ca^{2+} response obtained with 1 μM (−)-NPA was similar to response observed with 10 μM DA (data not shown).

4. Discussion

The role of endogenous RGS proteins on the signalling efficacy of the $G_{\alpha o}$-coupled $D_{2S}R$ was investigated using PTX-insensitive $G_{\alpha o}$ subunits unable to interact with RGS proteins. In CHO-K1 cells, D_2R occupation by dopaminergic agonists activates $G_{i/o}$ signalling pathways, typically leading to inhibition of adenylyl cyclase activity and Ca^{2+} response [24], increase of arachidonic acid release [29], stimulation of the Na^+/H^+ exchanger and mitogenesis [30]. By abolishing the $G_{\alpha o}$ protein/RGS proteins interactions and inactivating endogenous $G_{\alpha i/o}$ subunits, we observed a significant drop in the magnitude of agonist-dependent D_2R signalling at the level of G_α protein activation ([^{35}S]GTPγS binding) as well as at the level of a second messenger production (Ca^{2+} response) in CHO-K1 cells. We did not observe a change in the potency for the agonist (−)-NPA (Fig. 1B). Also the affinity of (−)-NPA for the D_2R (measured by displacement of [^{3}H]nemonapride) was similar in the case of $G_{\alpha o}Cys^{351}Ile$ protein coexpression and $G_{\alpha o}Gly^{184}Ser{:}Cys^{351}Ile$ protein coexpression (data not shown). Neither D_2R nor G protein expression levels displayed a difference, and the observed decrease in coupling is unlikely to result from a difference in [^{35}S]GTPγS binding due to the $Gly^{184}Ser$ mutation as compared to the wild type $G_{\alpha o}$ subunit. In fact, Lan and co-work-

ers [18] have described that this mutation does modify neither GDP release, GTPγS binding nor GTP hydrolysis. It is unlikely that the $Gly^{184}Ser$ mutation directly influences receptor–G protein coupling; the position of Gly^{184} in the first switch region of the G_α subunit has never been described for making receptor contacts, but rather for being stabilised by RGS domains [31]. Furthermore, since GTPγS is considered as a non-hydrolysable analogue of GTP, we presume that the present observation is independent from the GAP activity of RGS proteins that may interact with $G_{\alpha o}$ proteins. From the present results it appears that RGS proteins facilitate $D_{2S}R{:}G_{\alpha o}$ protein coupling, probably because RGS proteins increase the pool of $G_{\alpha o}$ proteins available for activation.

Reasoning along lines of RGS-GAP activity does not explain our observations. Indeed, if the $G_{\alpha o}$ subunit can no more interact with RGS proteins, it should stay active (GTP-bound) a longer time and thus show enhanced signalling towards effectors. The surprising fact that we observed decreased signalling efficacy can be explained in the context of a multiprotein complex. This complex comprises receptor, G protein and RGS protein, in which the G protein/RGS protein interaction is implicated in the activation of the G protein by the receptor. In a reconstituted phospholipid system (containing $G_{\alpha i1}$ protein and muscarinic M_2 receptor) at steady state, the addition of RGS4 increases the rate of receptor-catalysed GDP/GTP exchange (measured by an increase of GTPγS binding) [1]. According to the theory of Ross and Wilkie [1] the presence of a RGS protein in the cycle of activation/deactivation of GPCR signalling could also allow the G protein to activate and deactivate without receptor dissociation and thereby favour the overall cycle activation. Thus, the absence of G protein/RGS interaction in a receptor/G protein/RGS protein complex may lead to an alteration of receptor-catalysed GTPγS loading and a decrease in signalisation mediated by GPCR, which is what we observed.

Although many RGS proteins show affinity and in vitro GAP activity towards $G_{\alpha o}$ subunits, the functional RGS partner for $G_{\alpha o}$ protein in our CHO-K1 system remains unknown. By reverse transcription polymerase chain reaction RGS1, RGS2, RGS3, RGS16 and RGS-GAIP mRNAs were shown to be present in CHO-K1 cells [32] and we additionally detected RGS10 mRNA (data not shown). From in vitro studies, RGS12 [33] and RGS14 [34] also seem good candidates; especially RGS12 with its multiple protein binding modules [35] would lend itself well as a scaffolding protein to increase signalling efficacy at the plasma membrane. Similarly it was shown for RGS4 and for RGS-GAIP that domains outside their RGS domain may confer receptor specificity and thus contribute to the efficacy of Ca^{2+} signalling [36,37].

In conclusion, positive effects of RGS proteins on receptor signalling have until now been observed in the frame of enhanced kinetics of K^+-channel activation [38]. The present study expands this notion and suggests a positive role for RGS proteins in efficacy of GPCR signalling.

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