Published in final edited form as:

J Neurochem. 2010 February ; 112(4): 1026–1034. doi:10.1111/j.1471-4159.2009.06519.x.

Differential modulation of mu-opioid receptor signaling to adenylyl cyclase by RGS proteins 4 or 8 and 7 in permeabilised C6 cells is Gα subtype dependent

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Abstract

Regulators of G protein signaling (RGS) proteins act as GTPase accelerating proteins (GAPs) to negatively modulate G protein signaling and are defined by a conserved RGS domain with considerable amino acid diversity. To determine the effects of specific, purified RGS proteins on mu-opioid signaling, C6 cells stably expressing a mu-opioid receptor were rendered permeable to proteins by treatment with digitonin. Mu-opioid inhibition of forskolin-stimulated adenylyl cyclase (AC) by DAMGO, a mu-specific opioid peptide, remained fully intact in permeabilized cells. Purified RGS domain of RGS4 added to permeabilized cells resulted in a two-fold loss in DAMGO potency but had no effect in cells expressing RGS-insensitive G proteins. The inhibitory effect of DAMGO was reduced to the same extent by purified RGS4 and RGS8. In contrast, the RGS domain of RGS7 had no effect and inhibited the action of RGS8 due to weak physical association with Gαi2 and minimal GAP activity in C6 cell membranes. These data suggest that differences in conserved RGS domains of specific RGS proteins contribute to differential regulation of opioid signaling to AC and that a permeabilized cell model is useful for studying the effects of specific RGS proteins on aspects of G protein-coupled receptor signaling.

Keywords

RGS proteins; mu-opioid; adenylyl cyclase; permeabilization; Gα proteins

Regulators of G protein signaling (RGS) are key accessory proteins that dictate the timing of the G protein cycle of several types of heterotrimeric G proteins following activation of their cognate receptor. RGS proteins act as GTPase accelerating proteins or GAPs that directly

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interact with GTP-bound $G\alpha$ to stimulate the inherent GTPase activity of the $G\alpha$ subunit (Hollinger and Hepler 2002). Thus, RGS proteins control the lifetime of active $G\alpha$ and $G\beta\gamma$ signaling molecules and thereby regulate downstream effects of G-protein mediated signaling.

Although RGS proteins share a conserved 120 amino acid “RGS” domain (RGS box), they vary extensively in size and domain architecture and have been classified into six families based on sequence similarity (Ross and Wilkie 2000). For example, members of the B/R4 subfamily such as RGS4 and RGS8 are mostly small proteins of 20–30 kDa that consist almost entirely of the conserved RGS domain. In contrast, members of the C/R7 subfamily such as RGS7 and RGS9-2 are much larger possessing multiple domains, such as DEP (disheveled, Egl-10, pleckstrin) and GGL (G protein gamma subunit-like). These motifs participate in interactions with signaling and/or structural proteins and contribute to the stability, localization, and non-GAP related functions of RGS proteins (Siderovski and Willard 2005). However, there is considerable diversity in the conserved RGS domains themselves that contributes to $G\alpha$ protein binding selectivity (Soundararajan *et al.*, 2008) and as such are potential drug targets to disturb RGS- $G\alpha$ protein interactions (Roman *et al.* 2007;Roof *et al.* 2006). For example, the RGS domain of RGS4 shares 58% homology to the closely related R4 family member RGS8 and only 35% homology with RGS7. Yet, small molecules have been identified that selectively inhibit RGS4 but not RGS8, both in terms of $G\alpha$ binding and functional regulation of $G\alpha$ -mediated inhibition of adenylyl cyclase (AC) (Roman *et al.* 2007). In addition, RGS4 and RGS8 both bind to and functionally regulate $G\alpha_i$ and $G\alpha_q$ whereas RGS7 is selective for $G\alpha_o$ (Posner *et al.* 1999;Lan *et al.* 2000).

Here we compare the ability RGS4 and RGS8, which consist mostly of the RGS domain, with the RGS domain of RGS7 (RGS7-box) to regulate mu-opioid receptor signaling to AC via $G\alpha_i/o$ proteins since these RGS proteins are expressed in brain regions rich in mu-opioid receptors and participate in the manifestation of clinically-relevant opioid behaviors. For example, the thalamus, a mu-opioid receptor-rich relay station for transmitting pain information and the locus coeruleus, a noradrenergic center that influences opioid withdrawal behavior, are enriched in RGS4, RGS8, and RGS7 (Gold *et al.* 1997;Ingi and Aoki 2002;Chao and Nestler 2004). In addition, RGS4, RGS8, and the R7 family member, RGS9, are highly expressed in the nucleus accumbens, a region within the striatum thought to mediate the rewarding properties of addictive drugs such as morphine, and the caudate putamen, which is involved in the locomotor-stimulating properties of opioids (Gold *et al.* 1997;Ingi and Aoki 2002;Chao and Nestler 2004).

Most studies comparing selectivity of RGS proteins in whole cell systems have relied on manipulating RGS protein by heterologous overexpression in transfected cell lines or by genetic knockdown or deletion. Accurately quantifying levels of the manipulated protein in these systems is problematic, making it difficult to establish relationships between RGS protein concentration and effect. Moreover, little information is available regarding the direct comparison between these RGS proteins to regulate a given opioid receptor response. Consequently, we have used digitonin-permeabilized rat C6 glioma cells stably expressing the mu-opioid receptor (C6 μ) and $G\alpha_i2$ (Charpentier *et al.* 1993) to directly compare the effects of purified RGS4 and RGS8, with RGS7-box on mu-opioid inhibition of forskolin-stimulated AC activity. A similar permeabilized-cell system has been used to determine the relative effect of several RGS proteins, including RGS4, on Ca^{2+} responses stimulated by G q-coupled receptors (Xu *et al.* 1999;Hepler *et al.* 1997). Dramatic differences in the regulation of opioid receptor inhibition of AC were observed between the R4 family members and the RGS domain of RGS7 which depend on the cognate $G\alpha$ protein involved.

Materials and Methods

Chemicals and reagents

Tissue culture media, Geneticin, fetal bovine serum, trypsin, Zeocin and LipofectAMINE Plus were purchased from Invitrogen (Carlsbad, CA). Digitonin was obtained from Gallard-Schlesinger (Plainview, NY). cAMP kits were purchased from GE Healthcare (Piscataway, NJ). γ [32 P]GTP (6000 mCi/ml) and [35 S]GTP γ S was purchased from Perkin Elmer (Boston, MA). Anti-Gai2 was purchased from Calbiochem (San Diego, CA) and anti- Gao from Santa Cruz Biotechnology (La Jolla, CA). IBMX, forskolin, DAMGO and all other general laboratory reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Rat C6 glioma cells stably expressing the mu opioid receptor (C6 μ) were grown on 100 mm culture dishes under 5% CO₂ in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and in the presence of Geneticin (0.5 mg/ml) to maintain stable expression of the mu opioid receptor (Lee *et al.* 1999). Cells were also transfected with human PTX-insensitive Gai2^{C352I} or Gao^{C351I} without or with a FLAG-tag on the N-terminus (FLAG-Gai2^{C352I} or FLAG-Gao^{C351I}) or RGS-insensitive Gao^{CI/G184S} or Gai2^{CI/G184S} using LipfectAMINE Plus reagent and grown in the presence of zeocin (0.4 mg/ml) to promote stable expression (Clark *et al.* 2003; Clark *et al.* 2008). Clones were matched for equivalent levels of Ga expression by Western blot and for level of mu receptor expression (Clark *et al.* 2008).

Protein Purification

RGS proteins were expressed as native or GST fusion-proteins and purified as described previously (Lan *et al.* 1998; Roman *et al.* 2007). Gao and Gai2 were expressed as 6XHIS-fusion proteins and purified from transformed BL21-DE3 E. coli as previously described (Linder and Gilman 1991). Activity of purified Ga protein was determined by [35 S]GTP γ S binding (Traynor and Nahorski 1995).

[35 S]GTP γ S Binding Assay

Stimulation of [35 S]GTP γ S binding was measured as previously described (Clark *et al.* 2003). Briefly, membranes from cells treated with or without PTX (100 ng/ml) overnight were incubated for 60 min in a shaking water bath at 25 °C with 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 0.1 mM dithiothreitol (DTT; freshly prepared), 30 μ M GDP, 0.1 nM [35 S]GTP γ S, and 0.01–10 μ M DAMGO or dH₂O. Samples were filtered through glass-fiber filters mounted in a Brandel cell harvester and rinsed three times with ice-cold 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 100 mM NaCl. Radioactivity retained on filters was counted by liquid scintillation counting in 4 ml of EcoLume scintillation mixture (ICN, Aurora, OH).

Permeabilization

Cells were pelleted, washed once in ice-cold modified KGEH buffer (140 mM potassium glutamate, 10 mM EDTA, 3 mM HEPES, 4 mM MgCl₂, pH 7.4) and resuspended in KGEH buffer containing 20 μ M digitonin. The cell suspension was incubated at 37°C (5 min), pelleted, washed, and resuspended in assay buffer (128 mM NaCl, 2.4 mM KCl, 3 mM MgSO₄, 2 mM NaHCO₃, 10 mM Na₂HPO₄, and 10 mM Glucose, 10 μ M GTP, 100 μ M ATP, pH 7.4) + 2 mM IBMX. The degree of permeabilization was determined after each assay by trypan blue exclusion. The addition of digitonin routinely resulted in $\geq$ 90% trypan blue positive cells; $\leq$ 10% of cells were positive for trypan blue when digitonin was excluded.

Assay of Adenylyl Cyclase (AC) Activity

100 μ l of cells were added to an equal volume of assay buffer containing forskolin (Fsk) to stimulate AC activity, purified RGS proteins, and any additional agonists or antagonists. The reaction mixture was incubated for 15 minutes in a shaking water bath (37°C) and stopped by the addition of ice-cold perchloric acid. The activity of AC was then determined as the accumulation of cAMP measured by radioimmunoassay essentially as described previously.

RGS-Stimulated GTPase

Single turnover GTP hydrolysis measurements with and without RGS7 were based on (Roof *et al.* 2006). Briefly, 200 nM $G\alpha_o$ was loaded with a 3 fold molar excess of γ [32 P]GTP in 20 mM Hepes, 20 mM EDTA pH 8.0 for 5 minutes at room temperature and then cooled on ice. The loaded $G\alpha_o$ was then added to equal volumes of ice-cold initiation buffer (20 mM Hepes, 40 mM $MgCl_2$ pH 8.0 and 5–20 μ M unlabeled GTP) containing RGS7 such that the final concentration was 0 or 127 nM, with no more than 2% DMSO. After incubation for various times on ice, the reaction was quenched with 5% activated charcoal in buffer containing 20 mM sodium phosphate buffer (pH 2.0). After 20 minutes, the charcoal was centrifuged and the supernatant counted in a Perkin Elmer TopCount 96 well plate counter by Cerenkov counting. The amount of [32 P]Pi released at each time point was fit to an exponential function to calculate the rate constant:

$$[^{32}\text{P}] \text{ Pi counts}(t) = \text{counts}_{(t=0)} + \text{counts}_{(t=30 \text{ min})} * (1 - e^{-kt})$$

Fitting constraints were setting $\text{counts}_{(t=0)}$ for each curve to the average of the $\text{counts}_{(t=0)}$ for the experiment and setting $\text{counts}_{(t=30 \text{ min})}$ to the same value for all curves in an experiment.

Steady State GTPase Assay

C6 μ cells were treated overnight with 100 ng/ml PTX and membranes were prepared as described (Clark *et al.* 2003). Membranes (15–30 mg/tube) were preincubated for 5 min at 30°C with 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol (DTT; freshly prepared), an ATP-regenerating system (0.2 mM ATP, 0.2 mM AppNHp, 50 units/ml creatine phosphokinase, and 5 mM phosphocreatine), with or without 1 μ M RGS protein and with or without 10 μ M DAMGO. To start the assay, [γ - 32 P]GTP (diluted to 200,000–500,000 cpm/tube) was added to a final concentration of 0.1 μ M. After 2 min the assay was stopped by adding ice-cold 15% charcoal with 20 mM phosphoric acid in 0.1% gelatin. Samples were allowed to sit on ice for 30 min, centrifuged, and 32 Pi in the supernatant was measured by liquid scintillation counting as described (Clark *et al.* 2003).

Flow Cytometry Protein Interaction Assay

For flow cytometry interaction studies, RGS proteins were biotinylated with amine-reactive Biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt in a 2:1 stoichiometry (biotin:RGS). $G\alpha_o$ was chemically labeled with Alexa Fluor R 532 carboxylic acid succinimidyl ester (Invitrogen, Carlsbad, CA) at a stoichiometry of 3:1 (fluor:protein). Excess biotin/fluor labels were removed by purification on 1 ml Sephadex G25 spin columns. Activity and effective concentration of $G\alpha_o$ was determined post-labeling using [35 S]GTP γ S. Biotinylated RGS4 or RGS7 proteins were pre-incubated with LumAvidin microbeads, washed, and allowed to associate with AMF-activated AF532-labeled $G\alpha_o$ for 30 minutes in a 96-well plate. The RGS- $G\alpha_o$ interaction was then measured on a Luminex 100IS flow cytometer as the median fluorescence intensity. Increasing concentrations of unlabeled $G\alpha_i2$ were added to compete for RGS protein binding.

Data Analysis

All concentration response and rate data were analyzed using Graphpad Prism (San Diego, CA) to determine rate constants, potency (EC₅₀ values) and maximal effects.

Results

Adenylyl Cyclase Assays in Permeabilized Cells

Cell permeabilization has been used extensively to introduce macromolecules such as non-permeable ions, small molecules, and exogenous proteins to the cell interior without significantly disrupting cell architecture and signaling complexes (see Schulz 1990 for review). Rat C6 glioma cells expressing the mu opioid receptor (C6 μ) have been permeabilized by treatment with digitonin, a mild non-ionic detergent, while leaving receptor coupling to G proteins intact (Alt *et al.* 2001). In order to determine the contribution of individual RGS proteins to the regulation of opioid signaling to downstream effectors, conditions were optimized for assaying adenylyl cyclase activity in permeabilized cells in suspension.

Greater than 90% permeabilization of suspended C6 μ cells as measured by Trypan blue exclusion was obtained following treatment of cells with 20 μ M digitonin (5 min, 37°C; Table I). In contrast, treatment of cells in an identical manner but without digitonin resulted in less than 10% cells stained, presumably due to manipulation during the suspension procedure. The degree of permeabilization produced by digitonin treatment was sensitive to incubation temperature and cell concentration but not incubation time (data not shown). Treatment of intact, non-permeabilized, cells with forskolin (Fsk) produced a robust increase in AC activity measured as the accumulation of cAMP (Table 1). Fsk was ineffective in cells permeabilized with digitonin (Table I), likely the result of the depletion of intracellular ATP concentrations required for AC activity (Brooker and Pedone 1986), and was rescued by the addition of ATP to the assay buffer. Importantly, in the presence of ATP, Fsk stimulation of AC was equipotent in permeabilized vs. intact cells (Table I). D-Ala², MePhe⁴, Gly⁵enkephalin (DAMGO), a mu-opioid specific agonist, produced similar maximum inhibition in control and permeabilized cells but inhibited Fsk-stimulated AC activity with a slightly reduced potency in permeabilized cells (25 $\pm$ 13 nM) compared to control cells (4.9 $\pm$ 2.6 nM; Fig. 1). These data demonstrate that opioid signaling to AC through the mu receptor remains intact in digitonin-permeabilized C6 μ cells.

RGS Protein Regulation of Opioid Signaling to Adenylyl Cyclase

In order to determine whether exogenous RGS proteins modulate opioid coupling to AC in permeabilized cells we used Δ N-RGS4, a modified RGS4 protein lacking 18 amino acids at the N-terminus (Krumins *et al.* 2004; Roman *et al.* 2007). Increasing concentrations of Δ N-RGS4 (EC₅₀ 720 $\pm$ 280 nM) resulted in a profound loss of DAMGO inhibition of Fsk-stimulated AC (Fig. 2A). There was no effect of purified Δ N-RGS4 on basal levels of AC activity (data not shown) or on Fsk-stimulated cyclase in the absence of DAMGO (Fig. 2B). These data are consistent with the role of RGS4 as a GAP that attenuates G protein-mediated signaling to AC.

To confirm that the effect of Δ N-RGS4 was the result of GAP activity, a model was utilized in which the GAP effects of RGS proteins are silenced by a Gly – Ser mutation at position 184 in the switch I region of G α that disrupts its association with RGS proteins (Clark *et al.* 2003; Clark and Traynor 2004). Rat C6 μ cell clones were generated that stably express pertussis toxin (PTX)-insensitive G α i2 that are either sensitive (RGS-s) or insensitive (RGS-i) to RGS-mediated GAP activity. Clones were matched for equal G α i2 expression as assessed by Western blot (Fig 3A, inset) and equal activation of G α i2 as verified by

DAMGO stimulation of [³⁵S]GTPγS binding following treatment with PTX to block the effects of endogenous Gi/o proteins ((Clark *et al.* 2003); Fig 3A). In PTX-treated cells expressing RGS-i Gαi2, DAMGO was nearly twice as efficacious (61% maximum inhibition of Fsk-stimulated AC activity) and almost 20 times more potent (EC₅₀: 3.8 ± 0.9 nM) than cells expressing RGS-s Gαi2 (35%; EC₅₀: 147 ± 92 nM) (Fig 3B) due to the loss of endogenous RGS protein activity at Gαi2 through the RGS-insensitive mutation. ΔN-RGS4 (1 μM) attenuated inhibition of AC activity by near-maximal concentrations of DAMGO in cells expressing RGS-s but not RGS-i Gαi2 (Fig 3C).

RGS4 and RGS8 are two closely related members of the R4 family of RGS proteins that share 58% sequence identity and consist almost entirely of the canonical RGS domain (Hollinger and Hepler 2002). The inhibition of AC activity by a range of DAMGO concentrations in the presence of a maximal concentration of RGS protein showed that the potency of DAMGO (EC₅₀: 53 ± 13 nM) was reduced similarly by RGS4 (to 830 ± 730 nM) and RGS8 (to 400 ± 220 nM) (Fig 4A). Moreover, RGS4 and RGS8 decreased the maximal effect of DAMGO by ~50%, indicating that even maximal concentrations of DAMGO were unable to overcome the negative regulation imposed by the RGS proteins. Increasing concentrations of purified RGS4 and RGS8 attenuated the inhibition of AC by DAMGO (100 nM) with similar potency (Fig 4B; EC₅₀: 710 ± 380 nM versus 240 ± 100 μM, respectively).

In contrast to RGS4 and RGS8, addition of purified RGS7-box, comprising mainly the 120 amino-acid RGS domain (aa 156 – 341; (Lan *et al.* 1998)) had no effect on opioid inhibition of AC activity, even at concentrations up to 10 μM (Fig 4B). However, the same preparation of RGS7-box exhibited GAP activity at Gαo in the single turnover GTP hydrolysis assay, demonstrating that this protein does possess viable GAP activity (Fig 4C). The predominate Gαi/o protein expressed in rat C6 glioma cells is Gαi2 (Charpentier *et al.* 1993), and data suggest that opioid receptors preferentially couple to AC via Gαi2 relative to other Gαi/o family members (Moon *et al.* 2001). In vitro, RGS7 is a less potent GAP at Gαi2 (Lan *et al.* 2000; Hooks *et al.* 2003), therefore we hypothesized that RGS7-box did not regulate mu-opioid inhibition of AC due to inefficient regulation of opioid-stimulated Gαi2. To address this the GAP activity of RGS7-box and RGS8 was directly compared via DAMGO-stimulated steady state GTPase activity in membranes prepared from PTX-treated C6μ cells stably expressing PTX-insensitive Gαo (C351I) or Gαi2 (C351I) (Clark *et al.* 2006). Robust GAP activity was induced at both Gαi2 and Gαo by RGS8 (1 μM), which increased DAMGO-stimulated GTP hydrolysis by more than 3-fold showing no Gα selectivity (Fig 5A, B). In contrast, RGS7-box at a higher concentration (5 μM) increased DAMGO-stimulated GTPase activity of Gαo by 2-fold (Fig 5A) but had no effect on Gαi2 (Fig 5B). Indeed, RGS7-box antagonized the ability of RGS8 to inhibit DAMGO-mediated inhibition of AC (Fig 5C). These findings confirm those of Hooks *et al.* (2003) demonstrating that more efficacious RGS proteins can be antagonized by those with weak activity towards a particular Gα subunit.

To determine if this antagonism was due to the ability of RGS7 box to bind Gαi2 without promoting significant GAP activity the direct interaction of RGS proteins at Gαo and Gαi2 was tested using a flow cytometry-based protein interaction assay (FCPIA). This assay makes use of purified RGS protein that is immobilized to Avidin-coated microspheres through a biotin linkage. The immobilized RGS protein is then allowed to interact with AMF-activated Alexa532-labeled Gα protein. Fluorescence resulting from the RGS/Gα association is monitored and quantified by flow cytometry. This method has been used previously to quantify the binding affinity of RGS4 with Gαo subunits (Roman *et al.* 2007). Gαi2 competed with fluorescently labeled AF532-Gαo for binding to RGS4 resulting in the loss of fluorescence (Fig 6). In contrast, fluorescence resulting from the RGS7-box/AF532-

G α o association was weakly disrupted by the presence of unlabeled G α i2, and only at high concentrations. These data confirm that RGS7-box fails to act as a GAP for opioid signaling in C6 μ cells due to its low binding affinity for G α i2 (Lan *et al.* 2000). However, the binding is sufficient that at high concentrations the RGS domain of RGS7 can antagonize the actions of RGS proteins with higher binding affinity, without promoting GTPase activity itself.

Discussion

The purpose of this study was to quantitatively compare the ability of individual RGS proteins to regulate opioid signaling to downstream effectors. The ability to establish concentration-effect relationships for RGS proteins using permeabilized cells allows for the activity of individual RGS proteins to be determined and compared. Using an AC-coupled system, the RGS domains of RGS4 and RGS8 were seen to be equally effective whereas, RGS7 (RGS7-box) lacked GAP activity towards G α i2. Indeed, RGS7-box antagonized the GAP activity of the more efficacious RGS4 and RGS8. These findings are consistent with results using purified in vitro systems wherein both RGS7-box (Lan *et al.* 2000) and the full length RGS7 (Hooks *et al.* 2003) are particularly poor GAPs at G α i and G α o relative to members of the R4 family RGS4 or RGS8 and where the less efficacious RGS7 antagonizes the GAP activity of more robust RGS proteins (Hooks *et al.* 2003).

Comparison of residues within the RGS domains of RGS4, RGS8 and RGS7 points to structural determinants that may underlie their differences in binding to G α proteins. Based on the structure of the RGS4-G α i1 complex (Tesmer *et al.* 1997), specific contact sites between the RGS domain and switch regions I, II, and III of G α i1 have been resolved. Sequence analysis of these regions shows that RGS4 and RGS8 share identical homology in those residues projected to contact G α i; however, RGS7 differs in at least eight residues within the same regions, including several residues directly implicated in forming the proposed G α i-binding contact site. Of these, the most likely to account for differences in G α i binding are those residues immediately surrounding Asn¹²⁸ given its involvement in forming the conserved RGS domain (Tesmer *et al.* 1997). For example, there is an interaction between the conserved Arg¹³⁴ of RGS4 and a highly conserved Glu in Switch III of G α . In RGS7 this Arg is substituted with Tyr and so there are likely compensatory interactions, as occurs with RGS9 in which Arg¹³⁴ is replaced by Met (Slep *et al.* 2001). This differential interaction between G α proteins and the RGS domains of R4 and 7 family members, especially in key residues forming the RGS/G α i interface imply that the RGS domain itself contributes to selectivity exhibited by RGS proteins by dictating the affinity of G α binding and therefore the strength of GAP regulation (Soundararajan *et al.* 2008).

The physiologic effects of the opioid system are mediated almost exclusively by the G α o/i family of G proteins, and are therefore subject to RGS-mediated GAP regulation. Both RGS4 and RGS8 are enriched in the thalamus (Gold *et al.* 1997), a region dense in mu opioid receptor expression that functions as a relay station for transmitting pain information, and there is considerable in vitro evidence that RGS4 and RGS8 act as GAPs for opioid-mediated signaling (Clark *et al.* 2003; Saitoh *et al.* 1997; Ikeda *et al.* 2002). This may be of particular interest given that RGS4^{-/-} mice show no observable opioid-related phenotype (Grillet *et al.* 2005) pointing to a functional redundancy between RGS4 and RGS8 in vivo. In addition, gene deletion and/or knockdown studies have also implicated members of the R7 family, including RGS7 and RGS9-2, in the regulation of actions of morphine and other mu-opioids drugs in vivo (Garzon *et al.* 2003; Zachariou *et al.* 2003). However, the present results suggest these effects may be specific for opioid actions mediated by G α o rather than G α i2. Data from our study and others (Lan *et al.* 2000; Hooks *et al.* 2003) indicate poor GAP regulation of G α i2 by both the RGS domain of RGS7 (RGS7-box) and full length RGS7 co-expressed with its binding partner G β 5 to promote stability. Although mu-opioid receptors

couple to Gai2 in cloned cell lines (Clark *et al.* 2003) the contribution of this G α protein to the in vivo actions of opioids is unknown. However, several studies suggest Gai2 may contribute to opioid antinociception in certain rodent models of pain (Raffa *et al.* 1994; Sanchez-Blazquez *et al.* 1995). Also, in spite of the fact that RGS7 does not act as a GAP towards Gai2 in the present experiments, it is able to antagonize the actions of RGS8, suggesting it does bind Gai2 to some extent as indicated by the FCPIA assay. The low affinity of RGS7 box for Gai2, is in line with the reported low catalytic activity of RGS7 box towards Gai2, which is 100-times less than that of RGS4 (Lan *et al.* 2000). This raises the possibility that, depending on the relative levels of expression, RGS7 may serve to inhibit the GAP activity of more efficient RGS protein in cells where they are co-expressed.

Finally, our data demonstrate that RGS protein regulation of opioid signaling to downstream effector enzymes such as AC can be characterized in a permeabilized whole-cell model. Such models have been used to study protein function, including the characterization of RGS protein regulation of G α q-coupled receptor coupling to increases in Ca²⁺ release (Xu *et al.* 1999). Unlike reconstituted lipid vesicle systems, permeabilized cells allow for study of RGS protein function in a more native signaling environment with endogenously expressed signal transducers and downstream effectors. These methods also allow for precise quantities of purified RGS proteins to be introduced into cells with minimal disruption of the signaling environment relative to cell homogenization/membrane preparations. Due to their relative simplicity, these methods can be extended to other Gai/o-coupled receptor systems and potentially to additional signaling pathways (Hepler *et al.* 1997; Xu *et al.* 1999).

Abbreviations Used

AC	Adenylyl cyclase
DAMGO	[D-Ala ² ,N-Me-Phe ⁴ ,Gly-ol]-enkephalin
Fsk	Forskolin
GAP	GTPase accelerating protein
GPCR	G protein-coupled receptor
IBMX	3-isobutyl-1-methylxanthine
RGS	Regulators of G protein Signaling

Acknowledgments

This work was supported by the National Institutes of Health grants DA04087 (JRT), T32-DA007268 (JRT, JNT), GM039561 (RRN), F32-GM076821 (DLR), and HL071818 (JJT).

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Fig. 1. Inhibition of forskolin-stimulated AC activity by the mu opioid receptor agonist DAMGO in digitonin-permeabilized cells. C6 μ cells incubated with (closed circles) or without (open circles) 20 μ M digitonin were incubated with increasing concentrations of DAMGO. Adenylyl cyclase activity was measured as pmol cAMP per mg protein*min in the presence of 1 μ M IBMX. EC₅₀ values were calculated by non-linear regression analysis of three independent experiments. Data presented are the mean $\pm$ SEM (n=3).

Fig. 2. Δ N-RGS4 (RGS4-box) alters mu-opioid inhibition of forskolin-stimulated AC activity. **A)** Purified Δ N-RGS4 (lacking N-terminal 18 amino-acids) decreased the inhibitory effect of DAMGO (100 nM) in a concentration-dependent manner. Cells were stimulated with 10 μ M forskolin (Fsk) $\pm$ DAMGO and/or purified RGS protein. cAMP was measured as described. Data are expressed as the percentage of the Fsk response and are the mean $\pm$ SEM (n=5–8). **B)** Increasing concentrations of forskolin (Fsk) were used to stimulate AC activity in the presence (filled circles) or absence (open circles) of a maximal concentration of Δ N-RGS4 (1 μ M). Data are expressed as the pmol of cAMP per mg protein*min and are the mean $\pm$ SEM from at least four independent experiments (n=4).

Fig. 3.

Activity of Δ N-RGS4 in cells expressing RGS-insensitive $G\alpha i2$. **(A)** [35 S]GTP γ S binding was determined in membranes from cells stably expressing either RGS-sensitive (RGS-s) or RGS-insensitive (RGS-i) $G\alpha i2$. Overnight treatment with 100 ng/ml pertussis toxin (PTX) was used to block endogenous Gi/o protein activity. Data are derived from four assays, each carried out in duplicate, and are expressed as a percentage of basal binding. *Inset*: levels of RGS-i and RGS-s $G\alpha i2$ expression were verified by SDS-PAGE (20 μ g of membranes or 20 ng of $G\alpha i2$ standard ($G\alpha i2$)). Proteins were transferred to a nitrocellulose membrane and probed with anti- $G\alpha i2$ antibody. Shown is a representative blot from three separate blots. **(B)** Increasing concentrations of DAMGO were also used to inhibit AC activity stimulated by 10

μM forskolin (Fsk) in RGS-s and RGS-i Gαi2-expressing cells that were permeabilized with digitonin. Again cells were treated overnight with PTX (100 ng/ml) to block coupling to endogenous Gα. Data are the percentage of the Fsk response and are the mean ± SEM from at least three independent experiments. (C) Near maximal concentrations of DAMGO were used to inhibit Fsk-stimulated AC activity in digitonin-permeabilized RGS-s and RGS-i Gαi2-expressing cells (1 μM and 0.1 μM, respectively). Addition of ΔN-RGS4 (1 μM) reduced the inhibitory effect of DAMGO in cells expressing RGS-sensitive Gαi2 but not RGS-insensitive Gαi2. Data are expressed as the percentage of the Fsk response and are the mean ± SEM from at least four independent experiments; *p < 0.05 compared to DAMGO-treated RGS-s Gαi2 expressing cells as indicated (determined by one-way ANOVA with Tukey's post-hoc test).

Fig. 4.

Purified RGS4 and RGS8 negatively regulate opioid coupling to AC. **(A)** DAMGO inhibition of forskolin-stimulated AC was determined in the presence of maximal concentrations (1 μ M) of RGS4 (circles) or RGS8 (squares). Each RGS protein decreased DAMGO potency and efficacy to a similar extent. Data are expressed as the percentage of the Fsk response and are the mean $\pm$ SEM from at least four independent experiments. EC_{50} values were calculated by non-linear regression (GraphPad Prism). **(B)** Inhibition of forskolin-stimulated AC by DAMGO (100 nM) was measured in the presence of increasing concentrations of RGS4 (circles), RGS8 (squares), or RGS7-box (triangles). Data are expressed as the percentage of the Fsk response and are the mean $\pm$ SEM from four to six

independent experiments. (C) Single turnover GTP hydrolysis of Gαo was measured with (filled circles) and without RGS7-box (open circles) to confirm GAP activity of the RGS7-box preparation. Measurements were taken in the presence of 200 nM Gαo ± 125 nM RGS7 with a 3-fold molar excess of γ[³²P]GTP. [³²P]P_i released at each time point was fit to an exponential function: [³²P] P_i counts(t) = counts_(t=0) + counts_(t=30 min)*(1-e^{-kt}), to calculate the rate constant (k). Fitting constraints included setting counts_(t=0) for each curve to the average of the counts_(t=0) for the experiment, and setting counts_(t=30 min) to the same value for all curves in an experiment. Data are the mean ± SEM of four independent experiments performed in triplicate.

Fig. 5.

Direct Regulation of G α i and G α o by RGS Proteins. GAP activity of RGS8 and RGS7-box was measured by DAMGO-stimulated 32 Pi release in C6 μ cells stably expressing similar levels of mu-opioid receptor and G α o (**A**) or G α i2 (**B**). RGS proteins were added at a concentration of 1 μ M with 10 μ M DAMGO prior to the addition of [γ - 32 P]GTP. DAMGO-stimulated Pi released is shown as the mean $\pm$ SEM from at least 3 assays measured in duplicate. (**C**) RGS7-box binds to and GAPs G α o but has reduced affinity for G α i2 compared to RGS4. The binding of fluorescently labeled G α o (Alexa Fluor 532®) to LumAvidin-conjugated RGS4 was measured by flow cytometry. Activity and effective concentration of G α o was determined post-labeling using [35 S]GTP γ S. The RGS-G α o

interaction was measured on a Luminex 100IS flow cytometer as the median fluorescence intensity. Increasing concentrations of unlabeled G α i2 were added to compete for RGS protein binding. Data are the mean $\pm$ SEM of three separate experiments each performed in duplicate.

Fig. 6. Antagonism of RGS8 activity by RGS7-box. Inhibition of forskolin-stimulated AC by DAMGO (100 nM) was measured in the presence of either RGS7-box (5 μ M), RGS8 (1 μ M) or both (RGS7 + RGS8). Data are expressed as the percentage of the Fsk response and are the mean $\pm$ SEM (n=3–4).

Table 1Forskolin-stimulated cAMP in rat C6 μ glioma cells

Treatment	cAMP (pmol/mg*min) ¹	% Permeabilization ²	Fsk EC ₅₀ (μ M) ³
-	96 $\pm$ 13	8 $\pm$ 1	1.3 $\pm$ 0.4
digitonin	5 $\pm$ 2	92 $\pm$ 1	ND
ATP + digitonin	95 $\pm$ 8	90 $\pm$ 1	1.6 $\pm$ 0.4
ATP	110 $\pm$ 6	ND	ND

¹ cAMP accumulation was measured in response to 10 μ M forskolin. Data are the mean $\pm$ SEM of three to four independent experiments.

² Data are the mean percentage of cells permeabilized $\pm$ SEM determined by Trypan blue exclusion.

³ EC₅₀ values were determined from three independent curves and are the mean $\pm$ SEM. Optimal concentrations of ATP (100 μ M) and digitonin (20 μ M) were empirically determined (data not shown); ND - not determined.