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Differential modulation of mu-opioid receptor signaling to adenylyl cyclase by regulators of G protein signaling 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 protein 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 by [D-Ala^2,*N*-Me-Phe^4,Gly-ol]-enkephalin (DAMGO), a mu-specific opioid peptide, remained fully intact in permeabilized cells. Purified RGS domain of RGS4 added to permeabilized cells resulted in a twofold 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 as a result of weak physical association with Gαi2 and minimal GTPase-accelerating protein 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 adenylyl cyclase 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: adenylyl cyclase, Gα proteins, mu-opioid, permeabilization, regulators of G protein signaling proteins.

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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 (GAPs) that directly interact with GTP-bound Gα to stimulate the inherent GTPase activity of the Gα subunit (Hollinger and Hepler 2002). Thus, RGS proteins control the lifetime of active Gα and Gβγ 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

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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; PTX, pertussis toxin; RGS, regulators of G protein signaling.

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 Gα protein binding selectivity (Soundararajan *et al.* 2008) and as such these regions are potential drug targets to disturb RGS–Gα protein interactions (Roof *et al.* 2006; Roman *et al.* 2007). 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 Gαo binding and functional regulation of Gαo-mediated inhibition of adenylyl cyclase (AC) (Roman *et al.* 2007). In addition, RGS4 and RGS8 both bind to and functionally regulate Gαi and Gαq whereas RGS7 is selective for Gαo (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 Gαi/o proteins. 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 over-expression 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 (C6μ) and Gαi2 (Charpentier *et al.* 1993) to directly compare the effects of purified RGS4 and RGS8, with RGS7-box on mu-opioid inhibition of forskolin (Fsk)-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 Gq-coupled receptors (Hepler *et al.* 1997; Xu *et al.* 1999). 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 Gα 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, USA). Digitonin was obtained from Gallard-Schlesinger (Plainview, NY, USA). cAMP kits were purchased from GE Healthcare (Piscataway, NJ, USA). γ[^{32}P]GTP (6000 mCi/mL) and [^{35}S]GTPγS was purchased from Perkin Elmer (Boston, MA, USA). Anti-Gαi2 was purchased from Calbiochem (San Diego, CA, USA) and anti-Gαo from Santa Cruz Biotechnology (La Jolla, CA, USA). 3-isobutyl-1-methylxanthine, Fsk, [D-Ala^2,*N*-Me-Phe^4,Gly-ol]-enkephalin (DAMGO) and all other general laboratory reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Rat C6 glioma cells stably expressing the mu-opioid receptor (C6μ) were grown on 100 mm culture dishes under 5% CO_2 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 pertussis toxin (PTX)-insensitive Gαi2^{C352I} or Gαo^{C351I} without or with a FLAG-tag on the N-terminus (FLAG-Gαi2^{C352I} or FLAG-Gαo^{C351I}) or RGS-insensitive Gαo$^{CI/G184S}$ or Gαi2$^{CI/G184S}$ using LipfectAMINE Plus reagent and grown in the presence of zeocin (0.4 mg/mL) to promote stable expression (Clark *et al.* 2003, 2008). Clones were matched for equivalent levels of Gα expression by western blot and for level of mu receptor expression (Clark *et al.* 2008).

Protein purification

RGS proteins were expressed as native or glutathione-S-transferase fusion-proteins and purified as described previously (Lan *et al.* 1998; Roman *et al.* 2007). Gαo and Gαi2 were expressed as 6XHIS-fusion proteins and purified from transformed BL21-DE3 *Escherichia coli* as previously described (Linder and Gilman 1991). Activity of purified Gα protein was determined by [^{35}S]GTPγS binding (Traynor and Nahorski 1995).

[^{35}S]GTPγS binding assay

Stimulation of [^{35}S]GTPγS 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 $MgCl_2$, 100 mM NaCl, 0.1 mM dithiothreitol (freshly prepared), 30 μM GDP, 0.1 nM [^{35}S]GTPγS and 0.01–10 μM DAMGO or dH_2O. 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 $MgCl_2$ and 100 mM NaCl. Radioactivity retained on filters was counted by liquid scintillation counting in 4 ml of EcoLume scintillation mixture (ICN, Aurora, OH, USA).

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 the same buffer containing 20 μM 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 $MgSO_4$, 2 mM $NaHCO_3$, 10 mM Na_2HPO_4 and 10 mM Glucose, 10 uM GTP, 100 uM ATP, pH 7.4) + 2 mM 3-isobutyl-1-methylxanthine. The degree of permeabilization was determined after each assay by trypan blue exclusion. The addition of digitonin routinely resulted in ≥ 90% trypan blue positive cells; ≤ 10% of cells were positive for trypan blue when digitonin was excluded.

Assay of AC activity

One hundred microliters of cells were added to an equal volume of assay buffer containing Fsk to stimulate AC activity, purified RGS proteins and any additional agonists or antagonists. The reaction mixture was incubated for 15 min 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 radioimmunassay essentially as described previously (Clark *et al.* 2008).

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 threefold molar excess of $\gamma[^{32}P]$GTP in 20 mM HEPES, 20 mM EDTA pH 8.0 for 5 min 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 μM unlabeled GTP) containing RGS7 such that the final concentration was 0 or 127 nM, with no more then 2% dimethylsulfoxide. 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 min, the charcoal was centrifuged and the supernatant counted in a Perkin Elmer TopCount 96 well plate counter by Cerenkov counting. The amount of $[^{32}P]P_i$ released at each time point was fit to an exponential function to calculate the rate constant:

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

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

C6μ 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 pre-incubated 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 (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 μM RGS protein and with or without 10 μM DAMGO. To start the assay, $[\gamma^{32}P]$GTP (diluted to 200 000–500 000 cpm/tube) was added to a final concentration of 0.1 μM. 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). Gαo was chemically labeled with Alexa Fluor R 532 carboxylic acid succinimidyl ester (Invitrogen) 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 Gαo was determined post-labeling using $[^{35}S]$GTPγS. Biotinylated RGS4 or RGS7 proteins were pre-incubated with LumAvidin microbeads, washed and allowed to associate with AlF_4-activated AF532-labeled Gαo for 30 min in a 96-well plate. The RGS–Gαo interaction was then measured on a Luminex 100IS flow cytometer as the median fluorescence intensity. Increasing concentrations of unlabeled Gαi2 were added to compete for RGS protein binding.

Data analysis

All concentration response and rate data were analyzed using Graphpad Prism (San Diego, CA, USA) to determine rate constants, potency (EC_{50} values) and maximal effects.

Results

AC 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 (C6μ) have been permeabilized by treatment with digitonin, a mild non-ionic detergent, while leaving receptor coupling to G proteins intact (Alt *et al.* 2001). To determine the contribution of individual RGS proteins to the regulation of opioid signaling to downstream effectors, conditions were optimized for assaying AC activity in permeabilized cells in suspension.

Greater than 90% permeabilization of suspended C6μ cells as measured by Trypan blue exclusion was obtained following treatment of cells with 20 μM digitonin (5 min, 37°C; Table 1). In contrast, treatment of cells in an identical manner but without digitonin resulted in less than 10% cells stained, presumably because of 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,

Table 1 Forskolin-stimulated cAMP in rat C6μ glioma cells

Treatment	cAMP (pmol/mg min)[a]	% Permeabilization[b]	Fsk EC_{50} (μM)[c]
–	96 ± 13	8 ± 1	1.3 ± 0.4
Digitonin	5 ± 2	92 ± 1	ND
ATP + digitonin	95 ± 8	90 ± 1	1.6 ± 0.4
ATP	110 ± 6	ND	ND

ND, not determined.
[a]cAMP accumulation was measured in response to 10 μM forskolin. Data are the mean ± SEM of three to four independent experiments.
[b]Data are the mean percentage of cells permeabilized ± SEM determined by Trypan blue exclusion.
[c]EC_{50} values were determined from three independent curves and are the mean ± SEM. Optimal concentrations of ATP (100 μM) and digitonin (20 μM) were empirically determined (data not shown).

cells with 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 1), 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 versus intact cells (Table 1). 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 ± 13 nM) compared with control cells (4.9 ± 2.6 nM; Fig. 1). These data demonstrate that opioid signaling to AC through the mu receptor remains intact in digitonin-permeabilized C6μ cells.

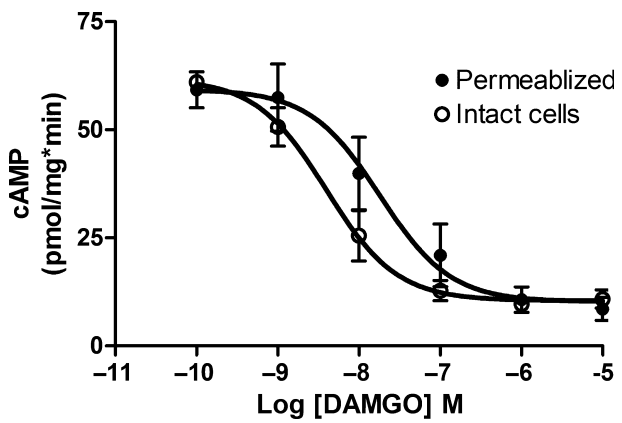

Fig. 1 Inhibition of forskolin-stimulated AC activity by the mu-opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly-ol]-enkephalin (DAMGO) in digitonin-permeabilized cells. C6μ cells incubated with (closed circles) or without (open circles) 20 μM digitonin were incubated with increasing concentrations of DAMGO. Adenylyl cyclase activity was measured as pmol cAMP per mg protein min in the presence of 1 μM 3-isobutyl-1-methylxanthine. EC_{50} values were calculated by non-linear regression analysis of three independent experiments. Data presented are the mean ± SEM (n = 3).

RGS protein regulation of opioid signaling to AC

To determine whether exogenous RGS proteins modulate opioid coupling to AC in permeabilized cells we used ΔN-RGS4, a modified RGS4 protein lacking 18 amino acids at the N-terminus (Krumins *et al.* 2004; Roman *et al.* 2007). Increasing concentrations of ΔN-RGS4 (EC_{50} 720 ± 280 nM) resulted in a profound loss of DAMGO inhibition of Fsk-stimulated AC (Fig. 2a). There was no effect of purified ΔN-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 ΔN-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 Gα that disrupts its

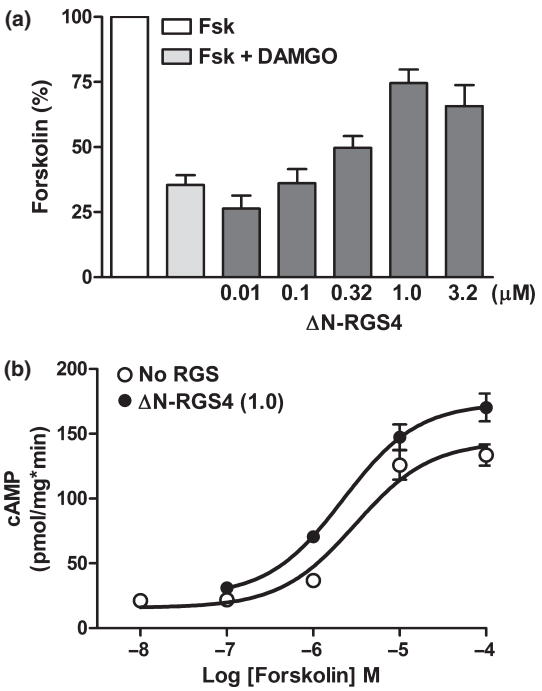

Fig. 2 ΔN-RGS4 (RGS4-box) alters mu-opioid inhibition of forskolin-stimulated AC activity. (a) Purified ΔN-RGS4 (lacking N-terminal 18 amino-acids) decreased the inhibitory effect of [D-Ala^2,*N*-Me-Phe^4, Gly-ol]-enkephalin (DAMGO) (100 nM) in a concentration-dependent manner. Cells were stimulated with 10 μM forskolin (Fsk) ± 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 ± 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 ΔN-RGS4 (1 μM). Data are expressed as the pmol of cAMP per mg protein min and are the mean ± SEM from at least four independent experiments (n = 4).

association with RGS proteins (Clark *et al.* 2003; Clark and Traynor 2004). Rat C6μ cell clones were generated that stably express PTX-insensitive Gαi2 that are either sensitive (RGS-s) or insensitive (RGS-i) to RGS-mediated GAP activity. Clones were matched for equal Gαi2 expression as assessed by western blot (Fig. 3a, inset) and equal activation of Gαi2 as verified by DAMGO stimulation of [^{35}S]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 (EC_{50}: 3.8 ± 0.9 nM) than cells expressing RGS-s Gαi2 (35%; EC_{50}: 147 ± 92 nM) (Fig. 3b) because of 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 (EC_{50}: 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; EC_{50}: 710 ± 380 nM vs. 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

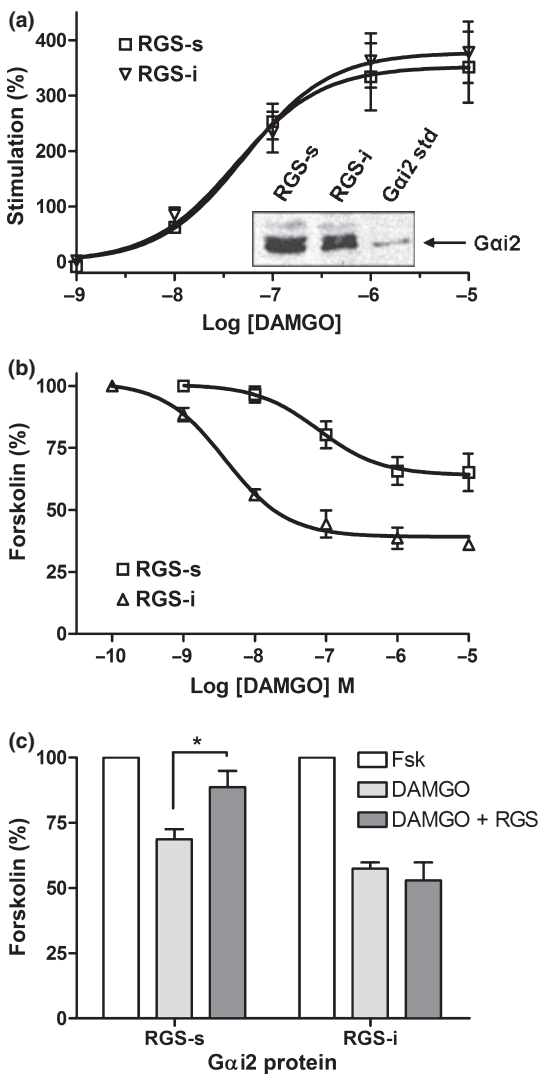

Fig. 3 Activity of ΔN-RGS4 in cells expressing RGS-insensitive Gαi2. (a) [^{35}S]GTPγS binding was determined in membranes from cells stably expressing either RGS-sensitive (RGS-s) or RGS-insensitive (RGS-i) Gαi2. Overnight treatment with 100 ng/mL PTX was used to block endogenous Gi/o protein activity. 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 Gαi2 expression were verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [20 μg of membranes or 20 ng of $G\alpha_{i2}$ standard (*Gαi2*)]. 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 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 with DAMGO-treated RGS-s Gαi2 expressing cells as indicated (determined by one-way ANOVA with Tukey's *post hoc* test).

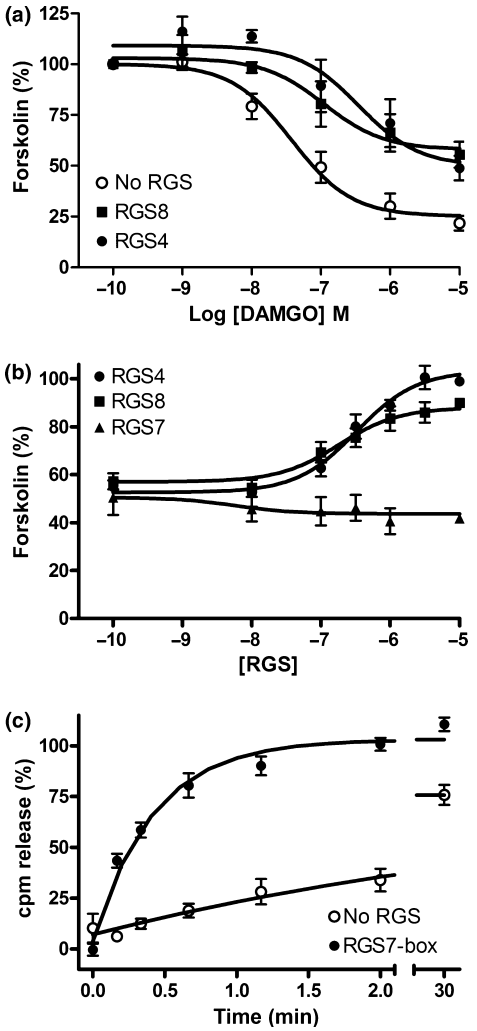

Fig. 4 Purified RGS4 and RGS8 negatively regulate opioid coupling to AC. (a) DAMGO inhibition of forskolin-stimulated AC was determined in the presence of maximal concentrations (1 μM) of RGS4 (circles) or RGS8 (squares). Each RGS protein decreased DAMGO potency and efficacy to a similar extent. Data are expressed as the percentage of the Fsk response and are the mean ± 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 ± 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 threefold molar excess of γ[^{32}P]GTP. [^{32}P]P_i released at each time point was fit to an exponential function: [^{32}P] Pi counts(t) = $\text{counts}_{(t=0)}$ + $\text{counts}_{(t=30\ \text{min})} \times (1 - e^{-kt})$, to calculate the rate constant (k). Fitting constraints included 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. Data are the mean ± SEM of four independent experiments performed in triplicate.

of AC because of 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 threefold showing no Gα selectivity (Fig. 5a and b). In contrast, RGS7-box at a higher concentration (5 μM) increased DAMGO-stimulated GTPase activity of Gαo by twofold (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 because of 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. 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 AlF_4-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-Gαo association was weakly disrupted by the presence of unlabeled Gαi2 and only at high concentrations. These data confirm that RGS7-box fails to act as a GAP for opioid signaling in C6 μ cells as a result of its low binding affinity for Gαi2 (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-box lacked GAP activity towards Gαi2. Indeed, RGS7-box antagonized the GAP activity of the more efficacious RGS8. These findings

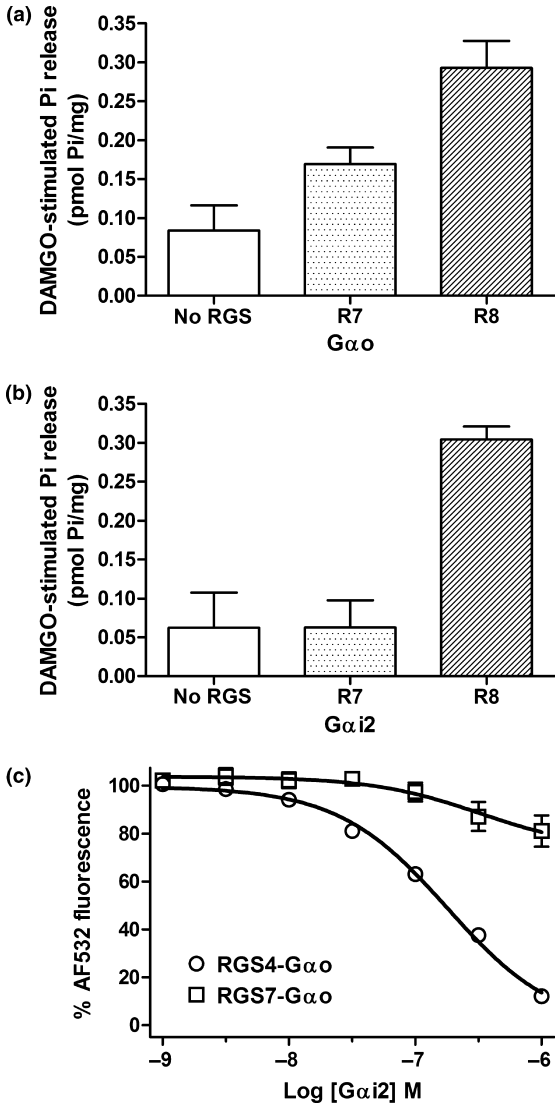

Fig. 5 Direct regulation of Gαi and Gαo by RGS proteins. GAP activity of RGS8 and RGS7-box was measured by DAMGO-stimulated ^{32}Pi release in C6μ cells stably expressing similar levels of mu-opioid receptor and Gαo (a) or Gαi2 (b). RGS proteins were added at a concentration of 1 μM with 10 μM DAMGO prior to the addition of [γ^{32}P]GTP. DAMGO-stimulated Pi released is shown as the mean ± SEM from at least three assays measured in duplicate. (c) RGS7-box binds to Gαo but has reduced affinity for Gαi2 compared with RGS4. The binding of fluorescently labeled Gαo (Alexa Fluor 532®) to Lum-Avidin-conjugated RGS4 or RGS7 was measured by flow cytometry. Activity and effective concentration of Gαo was determined post-labeling using [^{35}S]GTPγS. The RGS–Gαo interaction was measured on a Luminex 100IS flow cytometer as the median fluorescence intensity. Increasing concentrations of unlabeled Gαi2 were added to compete for RGS protein binding. Data are the mean ± SEM of three separate experiments each performed in duplicate.

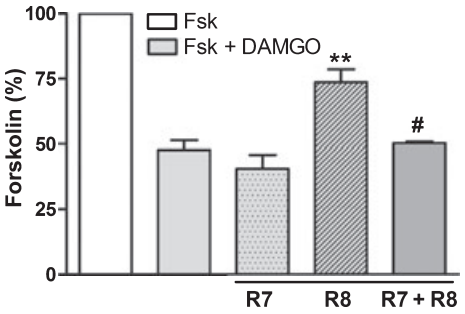

Fig. 6 Antagonism of RGS8 activity by RGS7-box. Inhibition of forskolin-stimulated AC by DAMGO (100 nM) was measured in the presence of either RGS7-box (5 μM), RGS8 (1 μM) or both (RGS7 + RGS8). Data are expressed as the percentage of the Fsk response and are the mean ± SEM (n = 3–4).

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 Gαi and Gαo 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 Gα proteins. Based on the structure of the RGS4-Gαi1 complex (Tesmer *et al.* 1997), specific contact sites between the RGS domain and switch regions I, II and III of Gαi1 have been resolved. Sequence analysis of these regions shows that RGS4 and RGS8 share identical homology in those residues projected to contact Gαi; however, RGS7 differs in at least eight residues within the same regions, including several residues directly implicated in forming the proposed Gαi-binding contact site. Of these, the most likely to account for differences in Gαi binding are those residues immediately surrounding Asn^{128} given its involvement in forming the conserved RGS domain (Tesmer *et al.* 1997). For example, there is an interaction between the conserved Arg^{134} of RGS4 and a highly conserved Glu in Switch III of Gα. In RGS7 this Arg is substituted with Tyr and so there are likely compensatory interactions, as occurs with RGS9 in which Arg^{134}is replaced by Met (Slep *et al.* 2001). This differential interaction between Gα proteins and the RGS domains of R4 and 7 family members, especially in key residues forming the RGS/Gαi interface imply that the RGS domain itself contributes to selectivity exhibited by RGS proteins by dictating the affinity of Gα 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 Gαo/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 (Saitoh

et al. 1997; Ikeda *et al.* 2002; Clark *et al.* 2003). 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 Gαo rather than Gαi2. Data from our study and others (Lan *et al.* 2000; Hooks *et al.* 2003) indicate poor GAP regulation of Gαi2 by both the RGS domain of RGS7 (RGS7-box) and full length RGS7 co-expressed with its binding partner Gβ5 to promote stability. Although mu-opioid receptors couple to Gαi2 in cloned cell lines (Clark *et al.* 2003) the contribution of this Gα protein to the *in vivo* actions of opioids is unknown. However, several studies suggest Gαi2 may contribute to opioid anti-nociception 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 Gαi2 in the present experiments, it is able to antagonize the actions of RGS8, suggesting it does bind Gαi2 to some extent as indicated by the flow cytometry-based protein interaction assay. The low affinity of RGS7 box for Gαi2, is in line with the reported low catalytic activity of RGS7 box towards Gαi2, 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 Gαq-coupled receptor coupling to increases in Ca^{2+} 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. Because of their relative simplicity, these methods can be extended to other Gαi/o-coupled receptor systems and potentially to additional signaling pathways (Hepler *et al.* 1997; Xu *et al.* 1999).

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