Endogenous RGS Protein Action Modulates μ-Opioid Signaling Through Gαo: Effects on Adenylyl Cyclase, Extracellular Signal-Regulated Kinases and Intracellular Ca²⁺ Pathways*

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Running Title:  RGS Proteins and Opioid Signaling
Regulators of G protein signaling (RGS) proteins are GTPase activating proteins (GAPs) for the Gα subunits of heterotrimeric G proteins and act to regulate signaling by rapidly cycling of G protein. RGS proteins may integrate receptors and signaling pathways by physical or kinetic scaffolding mechanisms. To ask whether this results in enhancement and/or selectivity of agonist signaling we have prepared C6 cells stably expressing the µ-opioid receptor and either pertussis toxin insensitive, or RGS and pertussis toxin insensitive Gαo. We have compared the activation of G protein, inhibition of adenylyl cyclase, stimulation of intracellular calcium release and activation of the extracellular signal-regulated kinase (ERK1/2) mitogen-activated protein kinase (MAP kinase) pathway between cells expressing mutant Gαo that is either RGS insensitive or RGS sensitive. The µ-receptor agonist DAMGO and partial agonist morphine were much more potent and/or had an increased maximal effect in inhibiting adenylyl cyclase and in activating MAP kinase in cells expressing the RGS insensitive Gαo. In contrast µ-opioid agonist increases in intracellular calcium were less affected. The results are consistent with the hypothesis that the GAP activity of RGS proteins provides a control that limits agonist action through effector pathways and may contribute to selectivity of activation of intracellular signaling pathways.
Opioid receptors are a typical 7-transmembrane domain receptor family that signal through inhibitory G proteins to a multitude of second messengers and cellular effectors including adenylyl cyclase, voltage operated calcium channels, inwardly rectifying K+ channels (1), intracellular calcium stores (2), and the extracellular signal-regulated (ERK)mitogen-activated protein kinase (MAP) pathway (3,4). There are three principle types of opioid receptors, µ, δ and κ, with approximately 60% homology. However, the µ-opioid receptor has generated the most interest as the principle site of action for clinical analgesics and abused opiate drugs. The µ-opioid receptor can couple to all members of the Gαi/o family with little selectivity for particular Gα subunits (5). Selectivity of intracellular µ-opioid signaling would, therefore, appear to depend on cell-specific expression of G protein subunits coupled with the selectivity of G proteins to interact with particular effectors. However, it has been suggested that other factors besides G protein and effectors may contribute to signaling specificity (6,7,8).

Agonist activation of G protein coupled receptors leads to exchange of GDP for GTP on Gα and dissociation of the Gα-GTP and Gβγ subunits. Deactivation is brought about by the intrinsic GTPase activity of Gα, causing GTP to be hydrolyzed to GDP and subsequent re-association of the Gα-GDP and Gβγ subunits. G protein signaling in this fashion is negatively controlled by a family of proteins known as regulators of G protein signaling (RGS) proteins (6). These proteins act as GAPs (GTPase activating proteins) for Gα and speed up the hydrolysis of the Gα bound GTP, thus reducing the steady-state levels of Gα-GTP and inhibiting signaling. Therefore, it has been suggested that, as with other G protein coupled receptors, RGS proteins act to inhibit µ-opioid signaling and may play a controlling role in the effectiveness of opioid receptor ligands. In support of this idea, over expression of RGS2 shifts the dose-effect curve for morphine stimulated pigment aggregation to the right to a small (2-fold) degree (9) in cultured dermal melanophores from Xenopus laevis transiently expressing a murine µ-opioid receptor. Furthermore, a reduction in RGS9 levels in mice using antisense oligonucleotide leads to an
increase in the antinociceptive potency of morphine (10). Although these changes are small, they are suggestive of a role for RGS proteins in opioid coupling efficiency.

An important question is whether RGS proteins alter the efficiency of all intracellular signaling pathways equally or whether there is a variable effect that would provide for selectivity. Selectivity for particular pathways may be obtained by several mechanisms. RGS containing proteins have a wide variety of non-RGS domains (11-13) that, when RGS binds to Gα, can link other proteins and signaling pathways to provide for diversity of signaling. In addition the interaction of RGS protein with receptors may contribute to selectivity, for example RGS12 binds with the carboxy terminus of the interleukin-8 receptor (14) and inhibition of Ca²⁺ signaling in rat pancreatic acinar cells by RGS4 is selective for muscarinic receptors relative to bombesin and cholecystokinin receptors (15) possibly through interaction of the N-terminal domain of RGS4 with the receptors (16). Recently, Wang and colleagues (17) have demonstrated, using ribozyme technology, that RGS3 is a negative modulator of muscarinic m3 receptor signaling while RGS5 is a negative modulator of angiotensin AT1a receptor signaling through Gq/11.

In addition to RGS proteins selectively modulating the coupling of different receptors to a single effector, it is possible that RGS proteins could selectively modulate the coupling of a single receptor to different effectors. Indeed, we have recently proposed a "kinetic scaffolding" model for G protein signaling in which RGS proteins confer selectivity for signaling pathways by their ability to shorten the lifetime of Gα-GTP (18). In this model, RGS accelerates hydrolysis of the Gα-bound GTP, permitting recombination of Gα-GDP and Gβγ and re-coupling of the heterotrimer and receptor, allowing rapid reactivation by agonist bound receptors. This maintains active Gα-GTP and Gβγ proteins in the close vicinity of the receptor, but spillover of Gα-GTP and Gβγ to more distant effectors is prevented by the GAP activity of RGS. This effect can be mediated by the RGS domain alone and does not depend on other protein interaction modules.
Here we test the hypothesis that RGS proteins differentially regulate µ-opioid receptor coupling to signaling pathways thus contributing to selectivity of receptor activation of second messenger pathways. Since 30 mammalian proteins with RGS activity have been identified to date (12,13) the choice of which RGS to study is a difficult one. We have, therefore, made use of a point mutation in G\(\alpha_o\) (G184S) that is known to block interaction with all members of the RGS family without affecting GTPase activity (RGSi) (19), together with a mutation (C351G) to confer pertussis toxin (PTX) insensitivity (PTXi) (20). In this way, when the RGS and pertussis toxin resistant G\(\alpha_o\) (G\(\alpha_o\)RGS/PTXi) is expressed in a cellular system, coupling to endogenously expressed G proteins can be inactivated by PTX treatment and the system must then signal through the expressed mutated G\(\alpha_o\) (21).

Our findings demonstrate that the µ-opioid agonists DAMGO and morphine show increased potency and/or efficacy of signaling to adenylyl cyclase in cells expressing RGS insensitive G\(\alpha_o\) compared with RGS sensitive G\(\alpha_o\). Signaling through the MAP kinase pathway also shows an increased potency with the full agonist DAMGO, but not an increased maximal effect, though the maximal effect of the partial agonist morphine is significantly enhanced. In contrast, the ability of DAMGO or morphine to stimulate the release of calcium from intracellular stores is altered to a much lesser extent in cells expressing RGS insensitive G\(\alpha_o\) compared with RGS sensitive G\(\alpha_o\). These results confirm that RGS proteins can modulate effector signaling by a single G protein and may play an important role in directing effector responses to µ-opioid receptor signaling.
EXPERIMENTAL PROCEDURES

Materials—[^3]H]DAMGO, [γ-32P]GTP, and [35S]GTPγS were from Perkin Elmer Life Sciences (Boston, MA) and cyclic AMP radioimmunoassay kits from Diagnostic Products Corp. (Los Angeles, CA). Tissue culture media, Lipofectamine Plus Reagent, Geneticin, Zeocin, fetal bovine serum, and trypsin were from Invitrogen Life Technologies (Carlsbad, CA). Morphine and naloxone were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI) and DAMGO was obtained from Sigma (St. Louis, MO). Trizma base, GDP, ATP, and other biochemicals were purchased from Sigma (St. Louis, MO) and were of analytical grade. Anti-phospho-p44/42 MAP kinase (ERK1, ERK2) antibody and anti-p44/42 MAP kinase (ERK1, ERK2) antibody were from Cell Signaling Technology, Inc. (Beverly, MA), anti-Gαo antibody (K-20) and secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and SuperSignal West Pico chemiluminescent substrate was from Pierce (Rockford, IL). Pertussis toxin insensitive Gαo (C351G; PTXi) and RGS and pertussis toxin insensitive Gαo (G184S/C351G; RGS/PTXi) DNA in pCI vector were obtained from Steve Ikeda (Guthrie Research Institute, Sayre, PA). GST fusion protein containing RGS8 (GST-RGS8) and His6-tagged Gαo were prepared as previously described (22).

Expression of PTXi or RGS/PTXi Gαo in C6μ Cells and Cell Culture—PTXi or RGS/PTXi Gαo DNA was excised from the plasmid vector (pCI Vector) with Not I and Nhe I restriction enzymes and inserted into Zeocin resistance vector (pcDNA3.1zeo-). Plasmid DNA was transfected into C6 glioma cells stably expressing the rat μ-opioid receptor (C6μ) (23) using Lipofectamine Plus Reagent. Colonies were isolated from transfected cells grown in Dulbecco’s
modified Eagles medium (DMEM) containing 10% fetal bovine serum under 5% CO₂ in the presence of 0.25 mg/ml geneticin (to maintain expression of the µ-opioid receptor in a geneticin resistant plasmid) and 0.4 mg/ml Zeocin. Clones were maintained under the same conditions and typically subcultured at a ratio of 1:20 to 1:30 with partial replacement of the media on Day 4, and the day before subculturing or harvesting at Day 7.

Membrane Preparation—Cells were treated with or without pertussis toxin (100 ng/ml) overnight before collection. Cells were washed two times with ice-cold phosphate buffered saline (0.9% NaCl, 0.61 mM Na₂HPO₄, 0.38 mM KH₂PO₄, pH 7.4), then detached from the plates by incubation in harvesting buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.68 mM EDTA) at room temperature and pelleted by centrifugation at 200 x g for 3 min. The cell pellet was suspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4, and homogenized with a Tissue Tearor (Biospec Products) for 20 sec at setting 4. The homogenate was centrifuged at 20,000xg for 20 min at 4°C and the pellet resuspended in 50 mM Tris-HCl, pH 7.4, with a Tissue Tearor for 10 sec at setting 2, followed by re-centrifugation. The final pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4 to 0.5-1.0 mg protein/ml and frozen in aliquots at -80°C. To determine protein concentration, membrane samples were dissolved with 1 N NaOH for 30 min at room temperature, neutralized with 1 M acetic acid, and assayed by the method of Bradford (1976) using a bovine serum albumin standard.

Determination of Gαo expression—Membrane proteins (20 µg) or 10-40 ng His₆-Gαo standards were separated by SDS PAGE on 12% gels (Protogel, National Diagnostics, Atlanta, GA). Proteins were transferred to a nitrocellulose membrane (45 micron, Osmonics, Minnetonka, MN), probed with a 1:200 dilution of anti-Gαo antibody treated with goat anti-rabbit IgG-HRP.
and visualized by enhanced chemiluminescence. Quantification was done using a Kodak Image Station 440.

Receptor binding assay—Membranes (10-20 µg) were incubated in 50 mM Tris-HCl, pH 7.4, with 0.2-28 nM [³H]DAMGO with or without 50 µM naloxone (to determine nonspecific binding) in a total volume of 0.2 ml for 60 min in a shaking water bath at 25°C. Samples were filtered through glass fiber filters (Schleicher and Schuell no. 32, Keene, NH) mounted in a Brandel cell harvester (Gaithersburg, MD), and rinsed 3 times with ice-cold 50 mM Tris-HCl, pH 7.4. Radioactivity retained on the filters was counted by liquid scintillation counting in 4 ml EcoLume Scintillation Cocktail (ICN, Aurora, OH).

[^35]S[GTPγS Binding Assay—Membranes (14-20 µg) 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 (freshly prepared), 30 µM GDP, 0.1 nM[^35]S[GTPγS, and 0.01-10 µM DAMGO, 0.01-10 µM morphine or dH₂O. Samples were filtered through glass fiber filters (Schleicher and Schuell no. 32, Keene, NH) mounted in a Brandel cell harvester (Gaithersburg, MD) and rinsed 3 times with ice-cold 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 100 mM NaCl. Radioactivity retained was determined as above. For kinetic studies membranes (approximately 20 µg) were incubated for 10 min at 25°C in 20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, 0.1 mM dithiothreitol, 1 mM EDTA, pH 7.4, containing 30 µM GDP. DAMGO (10 µM) was added and the mixture was further incubated for 10 min before the addition of 0.1 nM[^35]S[GTPγS to start the reaction. At various times (3 min to 2 h) bound and free radioactivity were separated and quantified as above.

GTPase Assay—Membranes (14-20 µg) were prewarmed for 5-20 min at 30°C with 10 mM Tris, pH 7.6, 2 mM MgCl₂, 20 mM NaCl, 0.2 mM EDTA, 0.1 mM dithiothreitol (fresh solution), ATP regenerating system (0.2 mM ATP, 0.2 mM AppNHp, 50 units/ml creatine phosphokinase, and 5 mM phosphocreatine) and 0.01-10 µM DAMGO, 10 µM morphine or dH₂O with or without 1 µM GST-RGS8. The reaction was initiated by the addition of 0.1 µM [γ-³²P]GTP, prewarmed to 30°C, for a final volume of 0.1 ml. The reaction was stopped after 15-120 sec by
the addition of ice-cold 15% charcoal with 20 mM phosphoric acid in 0.1% gelatin. After at least 30 min on ice, samples were centrifuged at 4000 x g for 20 min at 4°C, and 0.3 ml was taken from the supernatant for liquid scintillation counting with 4 ml EcoLume Scintillation Cocktail (ICN, Aurora, OH). Blank values for each time point (without membranes) were subtracted from each value.

*Inhibition of cyclic AMP Accumulation*—Cells were plated to confluency in 24 well plates the day before the assay and treated overnight with 100 ng/ml pertussis toxin. To start the assay, cells were rinsed with serum free media, then incubated with serum free media containing 30 µM forskolin, 1 mM IBMX, and 0.001-10 µM DAMGO, 0.01-10 µM morphine or dH₂O for 30 min at 37°C. The reaction was stopped by replacing the media with ice-cold 3% perchloric acid. After at least 30 min at 4°C, 0.4 ml was removed from each sample, neutralized with 0.08 ml 2.5 M KHCO₃, vortexed and centrifuged at 15,000xg for 1 min. A radioimmunoassay kit was used to quantify accumulated cyclic AMP in a 10 µl aliquot of the supernatant from each sample. Inhibition of cyclic AMP formation was determined as a percent of forskolin-stimulated cyclic AMP accumulation in the absence of opioid agonist.

*Stimulation of p44/42 MAP Kinase Phosphorylation*—Cells were plated into 6 well plates the day before the assay to reach 70-90% confluency on the day of the assay and treated overnight with 100 ng/ml pertussis toxin. Media was replaced with serum free media for 2 h before the addition of 0.001-10 µM DAMGO, 10 µM morphine or dH₂O. The assay was stopped after 1-20 min by rinsing the cells twice with ice-cold PBS and adding 0.1 ml ice-cold SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue). Samples were removed from the wells, sonicated for 10-15 sec, and boiled for 5 min then subjected (120 µg) to 12% SDS PAGE, followed by transfer to nitrocellulose membranes (45 micron, Osmonics, Minnetonka, MN) for Western blotting. The blot was probed with a 1:2000 dilution of anti-phospho p44/42 MAP kinase antibody (ERK 1/2) and visualized using anti-mouse IgG-HRP, followed by enhanced chemiluminescence detection and quantification using a
Kodak Image Station 440. To assure equal loading, membranes were stripped and re-blotted with a 1:1000 dilution of anti-p44/42 MAP kinase antibody (ERK 1/2) to measure total ERK levels.

**Release of Intracellular Calcium**—After overnight treatment with 100 ng/mL pertussis toxin and 5 µM forskolin, confluent cells were harvested with 10 mM HEPES buffered 0.9% saline containing 0.05% EDTA, pH7.4, washed twice with, and then re-suspended in, Krebs-HEPES buffer of the following composition (in mM):- NaCl 143.3, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, Glucose 11.7, HEPES 10, pH7.4 with 10 M NaOH. Cell suspensions were loaded with 3 µM fura 2 acetoxymethylester for 30 min at 37°C, washed and incubated at 20°C for 20 min, then re-washed. Intracellular calcium concentrations were measured in 1 ml volumes at 37°C using a Shimazdu RF5000 spectrofluorophotometer (Shimazdu, Columbia, MD) at 340/380 nm excitation and 510 nm emission. In certain experiments, nominally Ca$^{2+}$ free buffer containing 0.1 mM EGTA was used, and was included in the final re-suspension only. Data are presented as Δ340/380 ratio, mean ± SEM.

**Data Analysis**—Concentration response data from GTPase, [$^{35}$S]GTP$\gamma$S binding, adenylyl cyclase, MAP kinase phosphorylation and [Ca$^{2+}$], assays were fitted to sigmoidal concentration response curves using GraphPad Prism (GraphPad, San Diego, CA) to determine EC$_{50}$ and maximal effect. Specific binding data were fitted to a one-site binding hyperbola using GraphPad Prism (GraphPad, San Diego, CA) to determine $K_D$ and $B_{max}$ values. Data are presented as mean ± standard error of the mean from at least 3 separate experiments and compared using 2-tailed Students t-test unless stated otherwise.
RESULTS

Expression of PTXi and RGS/PTXi Gαo Mutants in C6µ Glioma Cells—C6µ cells were stably transfected with pertussis toxin insensitive GαoA (C6µ GαoPTXi) or pertussis toxin and RGS protein insensitive Gαo (C6µ GαoRGS/PTXi). A C6µ GαoPTXi clone (C1) and a C6µ GαoRGS/PTXi clone (M1) chosen as expressing similar levels of Gαo (Fig. 1A) were used for most studies. In membrane preparations, the binding affinity of [3H]DAMGO in Tris-HCl buffer was similar with a value of 2.0 ± 0.1 nM for C6µ GαoPTXi (clone C1) and 3.5 ± 0.9 nM for C6µ GαoRGS/PTXi (clone M1). The B\text{max} for [3H]DAMGO was 7.6 ± 1.3 pmol/mg in C6µ GαoPTXi (clone C1) and 14.4 ± 2.0 fmol/mg in C6µ GαoRGS/PTXi (clone M1). Pertussis toxin treatment (100 ng/ml overnight) of wild-type C6µ cells abolished opioid agonist-mediated signaling, as assessed by inhibition of cyclic AMP accumulation, stimulation of [35S]GTPγS binding, stimulation of MAP kinase phosphorylation, and increases in [Ca\text{2+}]\text{i} through endogenous G proteins (data not shown). Pertussis toxin treatment of C6µ cells expressing GαoPTXi or GαoRGS/PTXi allowed signaling through the transfected pertussis toxin resistant Go proteins to be measured.

Stimulation of [35S]GTPγS Binding—Basal levels of [35S]GTPγS binding were not different in membranes from PTX-treated GαoPTXi (0.026 ± 0.003 pmol/mg) and GαoRGS/PTXi (0.031 ± 0.002 pmol/mg) expressing cells. The maximum stimulation of [35S]GTPγS binding produced by DAMGO over basal binding in membranes (Fig. 1B) was lower in C6µ GαoRGS/PTXi membranes (157 ± 26% over control), versus C6µ GαoPTXi membranes (250 ± 67% over control), but the difference was not statistically significant. A similar pattern was seen for maximum stimulation by morphine (C6µ GαoRGS/PTXi membranes 48 ± 7% over control; C6µ GαoPTXi membranes 66 ± 17% over control). The potencies of the two mu agonists for the stimulation of [35S]GTPγS binding was also the same (DAMGO: C6µ GαoPTXi EC\text{50} = 296 ± 30 nM; C6µ GαoRGS/PTXi EC\text{50} = 316 ± 30 nM; morphine: C6µ GαoPTXi EC\text{50} = 124 ± 24 nM; C6µ GαoRGS/PTXi EC\text{50} = 159 ± 41 nM). The rate of stimulation of [35S]GTPγS binding by 10
µM DAMGO (Fig. 1C) was the same in C6µ GαoPTXi membranes (k = 0.17 ± 0.05 hr⁻¹) as C6µ GαoRGS/PTXi membranes (k = 0.17 ± 0.09 hr⁻¹).

Stimulation of GTPase Activity—Basal GTP hydrolysis in membranes from C6µ GαoPTXi was 10.3 ± 0.3 pmol/mg/min and 13.2 ± 0.3 pmol/mg/min in membranes from C6µ GαoRGS/PTXi PTX-treated cells. DAMGO stimulated GTP hydrolysis in membranes from both C6µ GαoPTXi and C6µ GαoRGS/PTXi pertussis toxin treated cells (Fig. 2A). The DAMGO stimulation of GTP hydrolysis in C6µ GαoPTXi (5.70 ± 0.50 pmol/mg/min) was greater (p < 0.05) than in C6µ GαoRGS/PTXi (2.64 ± 0.75 pmol/mg/min) consistent with reduced GTPase activity. Furthermore, addition of 1 µM GST-RGS8 increased DAMGO stimulation of GTP hydrolysis by a maximal concentration (1µΜ) of DAMGO in C6µ GαoPTXi, but not in C6µ GαoRGS/PTXi membranes, indicating the effectiveness of the RGS-insensitive mutation in preventing the GAP activity of RGS8. GTPase stimulation in C6µ GαoPTXi membranes by DAMGO at 2 min was concentration dependent (Fig. 2B). GST-RGS8 increased the maximal stimulation over basal by DAMGO from 60 ± 7 % to 151 ± 19 % (p < 0.05), with a shift in the EC₅₀ value from 34 ± 12 nM to 92 ± 31 nM, though this did not reach significance (p = 0.16). Maximal stimulation of GTP hydrolysis by 10 µM morphine at 2 min was 45 ± 5 % in the C6µ GαoPTXi membranes and increased significantly (p < 0.05) to 94 ± 17 % in the presence of 1 µM GST-RGS8, though relative to DAMGO, morphine was significantly less efficacious (p < 0.05) in the presence of GST-RGS8 (61 ± 3 %) than in its absence (75 ± 1 %). RGS8 was chosen for these studies because it is structurally a simpler RGS protein and is known to be a GAP for Gαo (22).

Inhibition of Adenylyl Cyclase—Adenylyl cyclase activity was measured by the accumulation of cyclic AMP stimulated by forskolin in the presence of the phosphodiesterase inhibitor IBMX in whole, PTX-treated cells. The level of accumulated forskolin-stimulated cyclic AMP was the same in both the GαoPTXi and GαoRGS/PTXi expressing cells. The maximal inhibition of cyclic AMP accumulation by DAMGO was significantly greater (p < 0.05) in C6µ GαoRGS/PTXi cells (58 ± 5 % inhibition) than in C6µ GαoPTXi cells (35 ± 6 %
inhibition; Fig. 3). More impressive was the fact that DAMGO was approximately 35-fold more potent in C6µ GoRGS/PTXi (EC50 = 12 ± 1 nM) than in C6µ GoPTXi (EC50 = 404 ± 112 nM) (p < 0.05). Morphine inhibition of forskolin-stimulated cyclic AMP accumulation (Fig. 3) increased significantly (p < 0.01) from a maximum of 10 ± 5 % in C6µ GoPTXi cells to 54 ± 7 % in C6µ GoRGS/PTXi cells and showed an 8-fold increase in potency (C6µ GoRGS/PTXi EC50 = 21.7 ± 11.2 nM; C6µ GoPTXi, EC50 = 170 ± 53 nM), though this did not quite reach significance at the 0.05 level (p = 0.053). To ensure that the striking difference between the GαoPTXi and GαoRGS/PTXi expressing cells was not caused by differences in receptor and Gαo expression levels, inhibition of cyclic AMP accumulation was measured in two additional C6µ GαoPTXi clones (C2 and C3) and an additional C6µ GαoRGS/PTXi clone (M2). DAMGO and morphine were consistently more potent and gave higher maximal effects in the C6µ GαoRGS/PTXi clones (Table 1).

In C6µ wild-type, GαoRGS/PTXi (Clone M1) and GαoPTXi (clone C1) cells not treated overnight with PTX, DAMGO and morphine robustly inhibited cyclic AMP accumulation with similar EC50 values and maximum effect (Table 2). In the GαoRGS/PTXi cells DAMGO had somewhat increased potency compared with the GαoPTXi cells > C6µ wild-type cells. Morphine was more potent in the cells expressing the GαoRGS/PTXi Gαo. When cells were treated with pertussis toxin overnight the effect of DAMGO and morphine was completely lost in the C6µ cells expressing wild-type Gα proteins, however, inhibition of cyclic AMP accumulation was retained in the cells expressing PTXi Gαo.

Stimulation of p44/42 ERK Phosphorylation—To determine whether MAP kinase regulation was also under control of endogenous RGS proteins, stimulation of ERK phosphorylation by DAMGO (100 nM) was measured in PTX-treated C6µ cells expressing either GαoPTXi or GαoRGS/PTXi cells at intervals from 0 to 20 min (Fig 4A,B). The stimulation of phosphorylation by 100 nM DAMGO followed a similar time course in both cell clones, but the percentage stimulation over basal was consistently higher in the C6µ GαoRGS/PTXi cells. To determine if the increased phosphorylation by 100 nM DAMGO in the GαoRGS/PTXi cells...
expressing cells was due to a change in potency or a change in maximal effect, a concentration-effect curve for DAMGO was determined at the 5 min time point. DAMGO stimulated the phosphorylation of p44/42 MAP kinase (Fig. 5A,B) with an 18-fold greater potency (p < 0.05) in C6µ GαoRGS/PTXi cells (clone C1, EC50 = 48 ± 11 nM) than in C6µ GαoPTXi cells (clone M1, EC50 = 839 ± 187 nM), a shift of 18-fold. Although the basal phosphorylation level was consistently lower in C6µ GαoRGS/PTXi expressing cells the maximal percent increase over basal was similar (C6µ GαoPTXi, 530 ± 115 %; C6µ GαoRGS/PTXi, 590 ± 104 % stimulation). Thus, the enhanced ERK phosphorylation seen in the initial time course study was due to a change in the EC50 for DAMGO and not in the maximum response. In contrast morphine, even at 10 µM gave a small stimulation of p44/42 MAP kinase phosphorylation in the C6µ GαoPTXi cells, representing just 14% of the stimulation seen with DAMGO. However, this concentration of morphine was significantly (p < 0.05) more efficacious in C6µ GαoRGS/PTXi cells (527 ± 174% stimulation over basal) than in C6µ GαoPTXi cells (68 ± 41% stimulation over basal; Fig 5C).

Increases in Intracellular Calcium—To obtain a measurable increase in the intracellular calcium signal in response to opioid agonists cells were grown for 24 h in the presence of 5 µM forskolin (24,25). In these cells DAMGO produced a stimulation of [Ca2+]i in PTX-treated C6µ GαoRGS/PTXi (EC50 = 80 ± 34 nM) and C6µ GαoPTXi (EC50 = 89 ± 17 nM) cells. There was no significant different in the EC50 values or maximal stimulation (Fig. 6A). Compared with DAMGO the relative maximal effect of morphine was somewhat higher in C6µ GαoRGS/PTXi cells (71.9 ± 9.1 %) than in C6µ GαoPTXi cells (35.3 ± 14.5 %; Fig. 6B), but this did not reach statistical significance (p = 0.11, Wilcoxon matched pairs). At 1 µM DAMGO, the induced rise in [Ca2+]i was from intracellular stores since the increase was not significantly different (p > 0.05, Wilcoxon matched pairs) in the presence of extracellular Ca2+ (Δ340/380 ratio: C6µ GαoRGS/PTXi = 0.11 ± 0.02; GαoPTXi = 0.06 ± 0.01) or in its absence (Δ340/380 ratio: C6µ GαoRGS/PTXi = 0.07 ± 0.01; C6µ GαoPTXi = 0.07 ± 0.01).
To confirm that the G\(\alpha\)oPTXi and G\(\alpha\)oRGS/PTXi cells treated overnight with forskolin still showed different sensitivities to \(\mu\)-opioid agonist inhibition of adenylyl cyclase, cells were examined for DAMGO inhibition of cyclic AMP accumulation. Forskolin treatment increased the maximal effect and the potency of the DAMGO in the G\(\alpha\)oPTXi cells. However, the differential response between the G\(\alpha\)oPTXi and G\(\alpha\)oRGS/PTXi cells was retained. The degree of maximal inhibition of cyclic AMP accumulation was increased in the presence of the RGSi mutant G\(\alpha\)o (G\(\alpha\)oPTXi: 68.5 ± 5.4 %; G\(\alpha\)oRGS/PTXi: 83.8 ± 1.2 %; \(p = 0.05\)) as was the enhanced potency of DAMGO (G\(\alpha\)oRGS/PTXi EC50 = 4.24 ± 1.44 nM; G\(\alpha\)oPTXi EC50 = 157 ± 35 nM; \(p < 0.05\)).
DISCUSSION

In this study we have expressed Gαo that is insensitive to RGS protein action in C6 cells expressing a µ-opioid receptor to demonstrate to a role for endogenous RGS proteins in the control of opioid receptor signaling. Two main conclusions can be drawn from these studies. Firstly, that endogenous RGS proteins reduce the effectiveness of Gαo signaling to adenyl cyclase and MAP kinase pathways, suggesting that endogenous RGS protein action has substantial regulatory effects on agonist potency and maximal response. Secondly, since only minor differences were seen between the RGS insensitive Gαo mutant compared with its RGS sensitive counterpart in coupling to intracellular calcium release, but significant differences were measured with the adenyl cyclase and MAP kinase pathways, we conclude that endogenous RGS proteins contribute to the control of effector selectivity of Gαo signaling.

One of the most direct measures of receptor activation is stimulation of [35S]GTPγS binding seen upon addition of agonist. The potency and maximal effect of DAMGO and morphine to stimulate [35S]GTPγS binding was similar in membranes from C6µ cells expressing GαoPTXi or GαoRGS/PTXi, or, if anything, was greater for the RGS sensitive Gαo. One would not expect the GAP activity of RGS to play a role in the binding of the GTP analog to Gαo subunit, because the assay is done in the presence of a large excess of GDP and the rate limiting step is the dissociation of GDP from the Gα subunit (26).

In contrast to agonist-stimulated [35S]GTPγS binding, DAMGO-stimulated GTPase activity in membranes from the cells expressing GαoRGS/PTXi was significantly less than in membranes from cells expressing Gαo/PTXi. This indicates that endogenous RGS GAP activity in the C6µ cell membranes is significant but this cannot function to stimulate GTP hydrolysis by the GαoRGS/PTXi mutant. Furthermore, DAMGO-stimulated GTP hydrolysis in membranes from the PTXi Gαo-expressing cells was markedly enhanced in the presence of added RGS8, while no stimulation by exogenously added RGS8 was observed in the membranes expressing GαoRGS/PTXi, confirming the insensitivity in the RGS-insensitive Gαo. As a percent of the maximal DAMGO stimulation, morphine produced a significantly smaller increase in GTPase
activity in the presence of GST-RGS8 than in the absence of GST-RGS8 demonstrating that
RGS is able to produce a greater enhancement of steady-state GTPase in the presence of a full
agonist; this could relate to the greater rate of GDP release caused by the full agonist (26).

The potency and maximal effect of DAMGO and morphine to inhibit adenylyl cyclase
through activation of the transfected RGS sensitive and PTXi Gαo in PTX-treated cells were
poor compared with both wild-type C6μ cells and cells expressing PTXi Gαo before treatment
with PTX. C6 cells do not endogenously express Gαo, and Gαi2 is the predominant Gα subunit
expressed (27). The robust inhibition of adenylyl cyclase in the C6μ cells expressing exogenous
Gαo before PTX treatment confirms that endogenous Gαi2 couples very efficiently to adenylyl
cyclase. In contrast, it is probable that the transfected PTXi Gαo, although activated efficiently
by the μ-opioid receptor, couples poorly to adenylyl cyclase such that this response can be
dramatically improved by inhibition of RGS activity. In support of this, Gαo does not couple
to adenylyl cyclase in NG108-15 cells or HEK cells compared with Gαi2 (28,29), though is
reported to have a role in this regard in SHSY5Y cells (30). The inhibition of cyclic AMP
accumulation was very much improved in the GαoRGS/PTXi expressing cells, giving a large
increase in both the maximal inhibition and agonist potency in the different clones examined.  It
was also noticeable that without PTX treatment cells expressing the PTX/RGSi mutant Gαo
were more efficiently inhibited by morphine and DAMGO than cells expressing only the PTX
insensitive Gαo. In a series of experiments using the opposite approach, expression of RGS4 or
GAIP in HEK293 cells reduced the level of somatostatin receptor-induced inhibition of cyclic
AMP accumulation (31), demonstrating the ability of RGS proteins to control the magnitude of
inhibitory G protein signaling. Our data extend this by demonstrating a role for endogenous RGS
proteins in this effect.

This effect of the GαoRGSi mutant on agonist-mediated inhibition of adenylyl cyclase was
particularly marked for the partial μ agonist morphine that became almost (90%) as efficacious
as the full agonist DAMGO in the C6μ GαoRGS/PTXi expressing cells. This indicates that RGS
proteins may be more effective when the receptor-G protein-effector system is signaling at sub-
maximal level. In agreement with this several authors have shown that at high agonist concentrations, RGS proteins are less effective (32, 33). In addition, RGS5-mediated reduction in intracellular calcium release by the angiotensin 1a receptor (Gq linked) is less effective when receptors are expressed at high levels (34). Since the relative efficacy of an agonist is tissue specific, it may be possible that differential expression of RGS proteins in tissues is one factor in determining agonist efficacy.

μ-Opioids strongly activate phosphorylation of p42/44 ERK in C6µ cells (3). This effect was retained in C6µ cells expressing either GαoRGS/PTXi or GαoPTXi after PTX treatment. However, similar to the μ-agonist effect on the cyclic AMP system, μ-agonist activation of the MAP kinase pathway was increased in the C6µ cells expressing the RGS/PTXi mutant Gαo compared with the PTX insensitive, but RGS sensitive, Gαo. Thus, when the μ-receptor was coupled through GαoRGS/PTXi a greater than 10-fold increase in potency of DAMGO and an increase in the maximal effect of morphine was observed, compared with coupling through the GαoPTXi. These results are consistent with findings that the 5HT1β receptor activation of ERK (p42/44 MAP) is reduced by overexpression of RGS4 in neuroblastoma cells (32), as is stimulation of MAP kinase through interleukin-8 (35) and dopamine D2 (33) receptor activation. In contrast to the effects on adenylyl cyclase and MAP kinase, the μ-opioid mediated increase in [Ca²⁺] showed a much smaller increase in effect in the GαoRGS/PTXi expressing cells such that the different these and the GαoPTXi expressing cells did not quite reach significance. This differential effect causes a shift in the most potent response to the μ-opioid agonist DAMGO. Thus, in the presence of endogenous RGS activity the rise in intracellular calcium is the most potent response (EC₅₀ = 89 nM) and the inhibition of adenylyl cyclase the weaker response (EC₅₀ = 400 nM), but in the absence of endogenous RGS activity the order is reversed and adenyly cyclase is the most potent response (EC₅₀ = 12 nM) while the calcium response is the weakest response (EC₅₀ = 80 nM).

The present data show that endogenous RGS proteins may differentially affect signaling by a single G protein depending on the effector pathway to which the G protein couples. Several
mechanisms could account for this specificity. One is that the GAP activity of endogenous RGS proteins controls signaling by a kinetic scaffolding mechanism (18). The kinetic scaffolding model predicts that RGS action reduces depletion of local Gα-GTP levels and so permits rapid recycling of G protein, rapid re-coupling of the receptor and maintains local G protein activation. The adenylyl cyclase and MAP kinase pathways are poorly signaled to in the presence of RGS activity, but when this activity is blocked, as in cells expressing the GαRGSi mutant, then signaling can occur because spatial control is lost, allowing spill over of Gα-GTP and Gβγ subunits to more distant effectors. In contrast, coupling to intracellular calcium stores is the more similar in cells expressing GαPTXi or GαRGS/PTXi. Thus, for the kinetic scaffolding model to account for this effect the G proteins involved in coupling to this pathway must be organized closely with receptor and effector such that they show a reduced RGS-dependent effect.

The differential effect of RGS on the three pathways examined is consistent with, but does not provide direct proof for this theory. Other mechanisms may explain the findings. The increased opioid effect at adenylyl cyclase and MAP kinase may simply be due to an increased life-time of Gα-GTP in the absence of RGS GAP activity, but if so then the question arises as to why the intracellular calcium signal is not enhanced to a similar extent. There may be differential location or compartmentalization of effectors within the cell (36), such that the [Ca\(^{2+}\)] signaling complex is protected from RGS action. One possibility is that RGS proteins provide a protein scaffold that allows signaling to the [Ca\(^{2+}\)] pathway but in the absence of this restraining scaffold other pathways become available to the Gα-GTP or Gβγ subunits. Certainly, C6 cells contain message for a variety of RGS proteins (RGS2, 3, 8, 10, 12 and 14) (37), several of which have regions outside of the RGS box that could be involved in protein-protein interactions. Alternatively, RGS proteins may not have a controlling function in modulation of the [Ca\(^{2+}\)] signal; the rate of GTP hydrolysis and lifetime of α-GTP may not be the rate limiting step in this signal pathway. The increase in [Ca\(^{2+}\)] response is transient in nature. Opioid stimulation of [Ca\(^{2+}\)] is thought to be mediated through Gβγ subunit activation of phospholipase Cβ, to
break down phosphatidylinositol(4,5)biphosphate (PIP$_2$) and provide Ins(1,4,5)P$_3$, that binds to
the Ins(1,4,5)P$_3$ receptor on the intracellular store, causing an increase in [Ca$^{2+}$], (23). The nature
of the [Ca$^{2+}$] signal may be controlled by other factors such as Ins(1,4,5)P$_3$ receptor
desensitization or the fullness of the Ca$^{2+}$ store; indeed there may not be a direct temporal
relationship between Ins(1,4,5)P$_3$ and Ca$^{2+}$ signaling (38).

In summary, we have shown in a transfected C6 cell that RGS proteins differentially
regulate µ-opioid receptor mediated signaling to different effectors through G$\alpha_0$, consistent with
a kinetic scaffolding mechanism. Coupling to adenylyl cyclase and the MAP kinase pathway
appears to be efficiently limited by endogenous RGS proteins, whereas coupling to intracellular
calcium stores is less susceptible to RGS protein action. Since the potency and maximal effect of
agonist is altered it is possible that differential expression of RGS proteins in tissues plays a role
in tissue-specific differences in agonist selectivity and efficiency. Finally, since cyclic AMP
(39) and MAP kinase (40, 41) have been implicated in contributing to the tolerance associated
with long-term opioid administration the effect of endogenous RGS proteins on these cellular
adaptations merits further investigation.
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Footnotes

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1 The abbreviations used are: ERK, extracellular signal-regulated kinase (p42/p44 MAP kinase); [Ca\textsuperscript{2+}]i, intracellular calcium; DAMGO, [D-Ala\textsuperscript{2}, MePhe\textsuperscript{4}Glyol\textsuperscript{1}]enkephalin; DMEM, Dulbecco’s modified Eagle’s medium; GAP, GTPase-activating proteins; \([^{35}\text{S}]\text{GTP}\gamma\text{S}\), guanosine 5’-O-(3-\([^{35}\text{S}]\text{thio})\text{triphosphate}; IBMX, 3-isobutyl-1-methyl-zanthine; MAP kinase, mitogen-activated protein kinase; PTX, pertussis toxin; RGS, regulator of G protein signaling.
FIGURE LEGENDS

FIG. 1. Expression and activation of $G_\alpha$PTXi or $G_\alpha$RGS/PTXi in C6µ cells. A. Membranes were prepared from C6µ or C6µ cells stably transfected with $G_\alpha$PTXi or $G_\alpha$PTXi/RGSi, as described in Experimental Procedures, and subjected to SDS PAGE (20 µg membranes or 20 ng $G_\alpha$ standard). Proteins were transferred to a nitrocellulose membrane, incubated with anti-$G_\alpha$ antibody, followed by anti-rabbit IgG-HRP and visualized by chemiluminescence, as described in Experimental Procedures. Shown is a representative blot from 3 separate blots. B. Membranes (14-20 µg) from pertussis toxin treated C6µ $G_\alpha$PTXi (closed symbols) or C6µ $G_\alpha$RGS/PTXi (open symbols) were incubated for 60 min at 25°C with 50 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 30 µM GDP, 0.1 nM [³⁵S]GTP$\gamma$S, and DAMGO (squares), morphine (circles) or dH$_2$O. Data are derived from 4 assays each in duplicate and expressed as a percent of basal binding. C. Membranes (14-20 µg) from pertussis toxin treated C6µ $G_\alpha$PTXi (closed symbols) or C6µ $G_\alpha$RGS/PTXi (open symbols) were incubated for 10 min at 25°C in 20 mM HEPES, 10 mM MgCl$_2$, 100 mM NaCl, 0.1 mM dithiothreitol, 1 mM EDTA, pH 7.4, containing 30 µM GDP followed by 10 min incubation with or without 10 µM DAMGO before the addition of 0.1 nM [³⁵S]GTP$\gamma$S to start the assay which was allowed to proceed for 3-100 min. Samples were then harvested and counted as described in Experimental Procedures. Data are presented as percent of the mean maximal DAMGO response from 5 assays each performed in duplicate.

FIG. 2. A. Stimulation of GTPase activity by 10 µM DAMGO in C6µ $G_\alpha$PTXi or C6µ $G_\alpha$RGS/PTXi cells in the presence or absence of RGS8 A. Membranes (14-20 µg) from PTX-treated C6µ $G_\alpha$PTXi (closed symbols) or PTX-treated C6µ $G_\alpha$RGS/PTXi (open symbols) were prewarmed for 5 min at 30°C with 10 µM DAMGO in the absence (squares) or presence (triangles) of 1 µM RGS8, in a buffer system of 50 mM Tris, pH 7.4, 1 mM EDTA, 10 mM MgCl$_2$, 100 mM NaCl, 1 mM DTT and ATP regenerating system. The reaction was initiated by the addition of prewarmed [³²P]GTP to a final concentration of 0.1 µM and stopped at varying
times between 15 and 120 s by the addition of ice-cold 15% charcoal in 20 mM H₃PO₄ plus 0.1% gelatin. Data are expressed as the stimulation of Pi released by DAMGO after subtraction of basal release. Shown are the combined data from three assays. B. **Dose-effect curve for stimulation of GTPase activity by DAMGO in C6µ GαoPTXi cells in the presence and absence of RGS8.** Membranes (14-20 µg) from PTX-treated C6µ GαoPTXi cells were prewarmed for 5 min at 30°C with varying concentrations of DAMGO in the absence (squares) or presence (triangles) of 1 µM RGS8 in a buffer system of 50 mM Tris, pH 7.4, 1 mM EDTA, 10 mM MgCl₂, 100 mM NaCl, 1mM DTT and ATP regenerating system. The reaction was initiated by the addition of prewarmed [³²P]GTP to a final concentration of 0.1 µM and stopped after 120 s by the addition of ice-cold 15% charcoal in 20 mM H₃PO₄ plus 0.1% gelatin. Data are given as Pi released and are the combined data from three assays.

**FIG. 3. Inhibition of cyclic AMP accumulation.** Cells were plated to confluency in 24 well plates the day before the assay and treated overnight with 100 ng/ml pertussis toxin. C6µ GαoPTXi (closed symbols) or C6µ GαoRGS/PTXi (open symbols) cells were rinsed with serum free media, then incubated with serum free media containing 30 µM forskolin, 1 mM IBMX, and DAMGO (squares), morphine (circles) or dH₂O for 30 min at 37°C. The reaction was stopped by replacing the media with ice cold 3% perchloric acid. After keeping the samples at 4°C for at least 30 min, the samples were neutralized and cyclic AMP was quantified using a radioligand binding assay kit as described in Experimental Procedures. Values are expressed as a percent of the values with forskolin only (in the absence of ligand), which were the same for C6µ GαoPTXi (6.6 ± 0.5 pmol/mg) or C6µ GαoPTXi/RGSi (6.8 ± 0.9 pmol/mg). Shown are the combined data from 3 assays, each measured in duplicate.

**FIG. 4. Time dependence of DAMGO stimulated p44/42 MAP kinase phosphorylation.** C6µ GαoPTXi (closed squares) or C6µ GαoRGS/PTXi (open squares) cells were treated overnight with 100 ng/ml pertussis toxin. Media was replaced with serum free media for 2 h and
the assay started with the addition of 100 nM DAMGO. The assay was stopped after 0-20 min and Western blotting was performed as described in Experimental Procedures. Shown is a representative blot of phosphorylated and total MAP kinase (A). Bands were quantitated as sum intensity (pixels) and plotted as percent of basal (without ligand). Shown in B are the combined data from 3-4 assays.

FIG. 5. Dose dependence of agonist stimulated p44/42 MAP kinase phosphorylation. C6µ GαoPTXi (closed squares) or C6µ GαoRGS/PTXi (open squares) cells were treated overnight with 100 ng/ml pertussis toxin. Media was replaced with serum free media for 2 h and the assay started with the addition of 0-10 µM DAMGO (B) or 10 µM morphine (C). The assay was stopped after 5 min and Western blotting was performed as described in Experimental Procedures. Shown is a representative blot of phosphorylated and total MAP kinase (A). Bands were quantified as sum intensity (pixels) and plotted as percent of basal (without ligand). The basal sum intensity was lower in C6µ GαoPTXi (157,000 ± 17,000 pixels) than in C6µ GαoRGS/PTXi (67,000 ± 12,000 pixels). Shown is the combined data from 3-4 assays.

FIG. 6. Agonist stimulated increase in [Ca²⁺]. [Ca²⁺] was measured in the presence of 0-10 µM DAMGO (A) or 10 µM morphine (B) in fura-2 loaded whole cell suspensions from C6µ GαoPTXi (closed squares) and C6µ GαoRGS/PTXi (open squares) cells treated for 24 h with forskolin and overnight with pertussis toxin as described in Experimental Procedures. Morphine stimulation was expressed as a percent of the 10 µM DAMGO response. Shown are the combined data from 6-7 assays.
### TABLE 1

**Inhibition of cyclic AMP accumulation in C6µ GαoPTXi and C6µ GαoRGS/PTXi cells by DAMGO and Morphine after treatment with PTX**

| Clone                | Damgo $^{EC_{50}}$ (nM) | Maximum (%) | Morphine $^{EC_{50}}$ (nM) | Maximum (%) | Morphine/Damgo$^b$ |
|----------------------|--------------------------|-------------|-----------------------------|-------------|--------------------|
| C6µ GαoPTXi          |                          |             |                             |             |                    |
| C1                   | 404 ± 112                | 35.2 ± 5.6  | 170 ± 53                    | 10.1 ± 5.2  | 0.29               |
| C2                   | 100 ± 11                 | 59.1 ± 6.5  | 368 ± 127                   | 29.0 ± 6.6  | 0.49               |
| C3                   | 94.0 ± 30                | 27.3 ± 5.3  | 223 ± 47                    | 10.2 ± 1.3  | 0.37               |
| Mean (n = 3)         | 199 ± 102                | 40.5 ± 9.5  | 254 ± 59                    | 16.4 ± 6.3  | 0.38 ± 0.06        |
| C6µ GαoRGS/PTXi      |                          |             |                             |             |                    |
| M1                   | 11.5 ± 1.0               | 57.5 ± 4.9  | 21.7 ± 11.2                 | 54.2 ± 7.0  | 0.94               |
| M2                   | 12.9 ± 8.3               | 59.4 ± 2.2  | 44.5 ± 12.0                 | 47.4 ± 9.1  | 0.80               |
| Mean (n = 2)         | 12.2 ± 0.7$^*$           | 58.5 ± 1.0  | 33.1 ± 11.4$^{**}$          | 50.8 ± 3.4$^{**}$ | 0.87 ± 0.07$^*$   |

Potency and maximal inhibition of cyclic AMP accumulation by DAMGO and morphine were measured in different clones expressing GαoPTXi (C1-C3) or GαoRGS/PTXi (M1-M2) after PTX treatment as described in Experimental Procedures. Data are expressed as means ± S.E.M. of 3-4 experiments each performed in duplicate. The group means are derived from data for the separate clones. $^b$ Morphine/DAMGO gives the efficacy of morphine relative to DAMGO as the fraction of the maximum inhibition by morphine compared to the maximum inhibition by DAMGO. * p < 0.05 or ** p < 0.01 compared to GαoPTXi.
TABLE 2

Inhibition of cyclic AMP accumulation in C6µ, C6µ GαoPTXi and C6µ GαoRGS/PTXi cells by DAMGO and Morphine without PTX treatment

| Clone           | DAMGO' | Morphine' |
|-----------------|--------|-----------|
|                 | EC_{50} (nM) | Maximum (%) | EC_{50} (nM) | Maximum (%) |
| C6µ             | 11.5 (8.6-15.5) | 83.6 (79.2-87.9) | 19.6 (14.6-26.2) | 76.9 (72.8-81.0) |
| C6µ GαoPTXi     | 6.7 (5.3-8.5) | 92.7 (89.4-96.0) | 21.2 (15.5-28.9) | 89.7 (85.2-94.2) |
| C6µ GαoRGS/PTXi | 3.4 (2.3-4.9) | 90.3 (85.9-94.7) | 10.2 (7.1-14.6) | 88.4 (83.4-93.4) |

'Potency and maximal inhibition of cyclic AMP accumulation by DAMGO and morphine were measured in C6µ cells or C6µ cells expressing GαoPTXi (clone C1) or GαoRGS/PTXi (clone M1) as described in Experimental Procedures. Data are expressed as means (95 % Confidence Interval) of 3 experiments (C6µ cells) or two experiments (clones C1 and M1) each performed in duplicate. Data from individual experiments were pooled and analyzed using GraphPad prism.
A.

wt PTxi RGSi Go

B.

\[^{35}\text{S}]\text{GTP-S binding} \] (% basal)

\[
\begin{array}{c}
\text{log [ligand] M} \\
-8 \\
-7 \\
-6 \\
-5
\end{array}
\]

C.

\[^{35}\text{S}]\text{GTP-S binding} \] (% maximum)

time (min)

0 20 40 60 80 100
cyclic AMP accumulation
(fraction of control)

log [ligand] M

M
A.

| [DAMGO] M | PTXi | RGS/PTXi |
|-----------|------|----------|
| -9        | -9   | -9       |
| -8        | -8   | -8       |
| -7        | -7   | -7       |
| -6        | -6   | -6       |
| -5        | -5   | -5       |

B.

C.
Endogenous RGS protein action modulates \( \mu \)-opioid signaling through \( \alpha \)-effects on adenylyl cyclase, extracellular signal-regulated kinases and intracellular Ca\(^{2+}\) pathways

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