DIFFERENTIAL MODULATION OF $\mu$- AND $\delta$-OPIOID RECEPTOR AGONISTS BY ENDOGENOUS RGS4 PROTEIN IN SH-SY5Y CELLS

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Running head: Modulation of opioid signaling by endogenous RGS4

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Regulator of G-protein signaling (RGS) proteins are a family of molecules that control the duration of G protein signaling. A variety of RGS proteins have been reported to modulate opioid receptor signaling. Here we show that RGS4 is abundantly expressed in human neuroblastoma SH-SY5Y cells that endogenously express $\mu$- and $\delta$-opioid receptors and test the hypothesis that the activity of opioids in these cells is modulated by RGS4. Endogenous RGS4 protein was reduced by approximately 90% in SH-SY5Y cells stably expressing short hairpin RNA (shRNA) specifically targeted to RGS4. In these cells, the potency and maximal effect of $\delta$-opioid receptor agonist (SNC80)-mediated inhibition of forskolin-stimulated cAMP accumulation was increased compared to control cells. This effect was reversed by transient transfection of a stable RGS4 mutant (HA-RGS4C2S). Furthermore, MAP kinase activation by SNC80 was increased in cells with knockdown of RGS4. In contrast, there was no change in the $\mu$-opioid (morphine) response at adenylyl cyclase or MAP kinase. Flag-tagged opioid receptors and HA-RGS4C2S were transiently expressed in HEK293T cells and co-immunoprecipitation experiments showed that the $\delta$-opioid receptor, but not the $\mu$-opioid receptor could be precipitated together with the stable RGS4. Using chimeras of the $\delta$- and $\mu$-opioid receptors, the C-tail and third intracellular domain of $\delta$-opioid receptor were suggested to be the sites of interaction with RGS4. The findings demonstrate a role for endogenous RGS4 protein in modulating $\delta$-opioid receptor signaling in SH-SY5Y cells and provide evidence for a receptor specific effect of RGS4.
Much work has been performed with RGS4 because it is a smaller RGS protein with a structure consisting of the RGS consensus (box) sequence and a small N-terminus (19, 20). It also has a wide distribution in brain, especially in brain regions important for opioid actions including the striatum, locus coeruleus, dorsal horn of the spinal cord and cerebral cortex (21).

In vitro RGS4 has been shown to reverse δ-opioid receptor agonist-induced inhibition of cAMP synthesis in membranes prepared from NG108-15 cells (6). Overexpression of RGS4 in HEK293 cells also attenuated morphine-, DAMGO- and DPDPE-induced inhibition of adenylyl cyclase (22, 23). Co-expression of RGS4 with GIRK1/GIRK2 channels in Xenopus oocytes reduced the basal K+ current and accelerated the deactivation of GIRK channels activated by κ-opioid receptor agonist U69593 (24). Even though these previous studies have provided evidence that RGS4 can negatively regulate opioid receptor signaling, they do not confirm a functional role for endogenous RGS4 in endogenous, non-transfected systems.

Human neuroblastoma SH-SY5Y cells endogenously express μ- and δ-opioid receptors and a variety of Gαi/o proteins (25-27). Here we show that RGS4 is abundantly found at both the mRNA and protein levels in these cells. Consequently, we used SH-SY5Y cells to examine the hypothesis that RGS4 negatively modulates opioid receptor signaling under physiological conditions. The endogenously expressed RGS4 level in SH-SY5Y cells was reduced using lentiviral delivery of short hairpin RNA (shRNA) targeting the RGS4 gene. This resulted in changes in δ- but not μ-opioid receptor-mediated signaling to adenylyl cyclase and the MAP kinase pathway. These findings argue for a selective interaction of RGS4 with the δ-opioid receptor. To test this, we expressed Flag-tagged μ- and δ-opioid receptors together with a construct for a stable, proteasome-resistant RGS4 protein in HEK293T cells. Co-immunoprecipitation indicated that the δ- but not the μ-opioid receptor was closely associated with RGS4 providing further evidence for a selective interaction between RGS4 and δ-opioid receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Materials** - [3H]DAMGO ([D-Ala, N-Me-Phe, Gly-ol]-Enkephalin) and [3H]DPDPE ([D-Pen2, D-Pen5]-enkephalin) were from PerkinElmer Life Sciences (Waltham, MA). Morphine, SNC80 and naloxone were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI), and DAMGO was from Sigma (St Louis, MO). Cyclic AMP radioimmunoassay kits were from GE Healthcare (Piscataway, NJ). Tissue culture medium, LipofectAMINE 2000 reagent, OPTI-MEM medium, fetal bovine serum, 100 x Penicillin-Streptomycin and trypsin were from Invitrogen (Carlsbad, CA). Antibodies were from the indicated sources: anti-phospho-p44/42 MAPK (ERK1/2) and anti-p44/42 MAPK (ERK1/2) (Cell Signaling Technology, Beverly, MA), anti-Flag M 1 and anti-β-actin (Sigma, St Louis, MO), anti-δ-opioid receptor, anti-mouse, anti-rabbit, anti-HA (hemagglutinin), anti-HA antibody conjugated agarose beads, Protein A/G plus agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-µ-opioid receptor antibody was as previously published (28) and U1079 RGS4-antisera was a kind gift from Dr. Stephen Gold (Merck, Rahway, NJ). SuperSignal West Pico chemiluminescent substrate was from Pierce (Rockford, IL). Protease inhibitor cocktail tablets (Complete Mini, EDTA-free) were purchased from Roche diagnostics (Indianapolis, IN). Immobilon™P transfer membrane (0.45 um pore size) was purchased from Millipore Corporation (Bedford, MA). Polybrene (Sequabrene) and all other chemicals were from Sigma (St Louis, MO) and were of analytical grade. N-glycosidase F (PNGase F) with 10 x G7 reaction buffer, 10 x denaturing buffer and 10% NP-40 were purchased from New England BioLabs (Beverly, MA).

**Cell culture** - SH-SY5Y, C6 rat glioma cells and HEK293T cells were purchased from ATCC (Manassas, VA). They were grown in DMEM medium containing 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 μg/ml) under 5% CO2 at 37 °C. SH-SY5Y cell lines expressing Gαo constructs with a PTX-insensitive mutation (GαoPTXi) or both PTX- and RGS-insensitive mutations (GαoRGSi/PTXi) were generated by transfecting the appropriate plasmid DNAs (see below) using Lipofectamine 2000. Stable cell lines were selected and maintained in the presence of 0.4 mg/ml Geneticin.
Plasmid constructs- Flag-tagged mouse μ-opioid receptor construct was a gift from Dr. Lakshmi Devi (Mount Sinai School of Medicine, NY). Flag-tagged human δ-opioid receptor construct (29) and the HA-RGS4C2S construct (30) were as previously described. A μ/δ-C chimeric receptor comprising amino-acids 1-339 of the rat μ-opioid receptor and 322-372 of the human δ-opioid receptor C-terminal domain and a δ/μ-C chimeric receptor comprising amino-acids 1-321 of human δ-opioid receptor and 340-398 of the rat μ-opioid receptor C-terminal domain have been described earlier (23). Both chimeras have the Flag epitope tag at the N-terminus. Human PTX-insensitive Ga<sub>o</sub> (PTXi, with a C to G mutation at amino acid 351) was from the Missouri S&T cDNA Resource Center (www.cDNA.org). The RGS-insensitive construct (Ga<sub>o</sub>RGSi/PTXi) was generated by a G to S mutation at amino acid 184 of PTXi Ga<sub>o</sub> (31).

Reverse transcription-PCR (RT-PCR)- Total RNA was prepared from SH-SY5Y or C6 cells using the VersaGENETM RNA purification system (Genta Systems, Minneapolis, MN) and then subjected to the RT-PCR with SuperScript<sup>™</sup> One-Step<sup>™</sup> RT-PCR System according to the supplier’s manual (Invitrogen, Carlsbad, CA). Primers for detection of RGS4 were designed from the RGS4 coding region as follows: sense primer 5’ GAAGTCAAGAAATGGGCTGAATC 3’; anti-sense primer 5’ CGGTTCTGAGATACGAGAC 3’. The primers were first checked by amplifying RGS4 plasmid DNA to make sure that the correct size of the PCR product was achieved with the expected size of 502 bp. Total RNA (200 ng) was used with primers (0.3 µM each) and MgSO<sub>4</sub> (1.2 mM) in a 25 µl volume. The reverse transcription was performed by heating RNA at 65 °C for 10 min, then at 45 °C for 30 min followed by PCR with 30 cycles at 95 °C for 30 s, 45 °C for 45 s, and 72 °C for 1 min. The RT-PCR products were separated by electrophoresis on a 1.8% agarose gel, stained with ethidium bromide and photographed using a Kodak Image Station 440.

Design and construction of lentivirus encoding shRNA against RGS4- The shRNA lentiviral delivery system developed by Dr. Didier Trono (32) was used. In brief, four targeting sites were designed based on the mouse RGS4 gene (33) as follows: site 1- 5’ AGCTTTCCTGAAGTCGGAA 3’; site 2- 5’ AAGAGGTGAACCTGGACTC 3’; site 3- 5’ CTTCCTCAAGTCTCGATTC 3’ and site 4- 5’ AATGCCAAGACTCTATGCT 3’. Sites 3 and 4 are identical between mouse and human; site 1 has two nucleotides different and site 2 has one nucleotide different. The four shRNA oligos against RGS4 were constructed into the plVTHM lentivector by direct cloning of annealed shRNA at Mlu1-Cla1 sites. The gene for Green Fluorescent Protein (GFP) is encoded by the vector plVTHM. Lentivirus was produced by co-transfecting each plVTHM-shRNA construct individually with the other components of the virus including pMDLg/pRRE, pRSV-Rev, pRRL and pMD2G into low passage number HEK293T cells using Lipofectamine 2000 in OPTI-MEM medium. Lentiviruses were harvested from the supernatant, concentrated by centrifugation (35,000 rpm), resuspended in phosphate buffered saline (pH 7.2), and flash-frozen in liquid N<sub>2</sub>. The concentrated lentiviruses were stored in aliquots at -80 °C. A titration for each lentivirus was obtained by infecting HEK293T cells with serially diluted virus in 24-well plates and the number of GFP-expressing cells was counted to calculate transducing units. There were approximately 3 x 10<sup>-10</sup> units (TU/ml) for each lentivirus.

Western Blot for RGS4 Protein- Lysates were prepared from SH-SY5Y or C6 cells using the VersaGENETM RNA purification system (Genta Systems, Minneapolis, MN) and then subjected to the RT-PCR with SuperScript<sup>™</sup> One-Step<sup>™</sup> RT-PCR System according to the supplier’s manual (Invitrogen, Carlsbad, CA). Primers for detection of RGS4 were designed from the RGS4 coding region as follows: sense primer 5’ GAAGTCAAGAAATGGGCTGAATC 3’; site 2- 5’ AAGAGGTGAACCTGGACTC 3’; site 3- 5’ CTTCCTCAAGTCTCGATTC 3’ and site 4- 5’ AATGCCAAGACTCTATGCT 3’. Sites 3 and 4 are identical between mouse and human; site 1 has two nucleotides different and site 2 has one nucleotide different. The four shRNA oligos against RGS4 were constructed into the plVTHM lentivector by direct cloning of annealed shRNA at Mlu1-Cla1 sites. The gene for Green Fluorescent Protein (GFP) is encoded by the vector plVTHM. Lentivirus was produced by co-transfecting each plVTHM-shRNA construct individually with the other components of the virus including pMDLg/pRRE, pRSV-Rev, pRRL and pMD2G into low passage number HEK293T cells using Lipofectamine 2000 in OPTI-MEM medium. Lentiviruses were harvested from the supernatant, concentrated by centrifugation (35,000 rpm), resuspended in phosphate buffered saline (pH 7.2), and flash-frozen in liquid N<sub>2</sub>. The concentrated lentiviruses were stored in aliquots at -80 °C. A titration for each lentivirus was obtained by infecting HEK293T cells with serially diluted virus in 24-well plates and the number of GFP-expressing cells was counted to calculate transducing units. There were approximately 3 x 10<sup>-10</sup> units (TU/ml) for each lentivirus.

Western Blot for RGS4 Protein- Lysates were prepared from SH-SY5Y cells, HEK293T cells or striatal tissue from wild-type or RGS4 knockout mouse brain (34) by suspension in ice-cold RIPA lysis buffer containing 1 x phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with fresh addition of 1 x protease inhibitor cocktail mix (0.15–0.3 TIU/ml aprotinin, 1 mM sodium orthovanadate, 0.1% SDS with fresh addition of 1 x protease inhibitor cocktail mix (0.15–0.3 TIU/ml aprotinin, 1 mM sodium orthovanadate, 1 mM benzamidine, 1 µg/ml pepstatin, 10 µg/ml phenylmethylsulfonyl fluoride (PMSF) and 2 µg/ml leupeptin). The suspension was passed 10 times through a 21-gauge needle, 10 µg/ml PMSF was added, and the mixture was incubated for another 1 h on ice and finally centrifuged at 10,000 x g for 10 min. The supernatant was saved as total cell lysate, aliquoted and stored at -20 °C. Protein (~ 50 µg) was subjected to SDS-PAGE on a 12% mini-gel and transferred to an Immobilon<sup>™</sup>-P transfer membrane. The membrane was blocked with 1% BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05%
Tween 20) for 1 h and incubated with U1079 RGS4-antis serum (34) at a 1:10,000 dilution for 1 h at room temperature or overnight in the cold room. After three consecutive washes with TBST (10 min for each), the membrane was incubated with 1:10,000 dilution of secondary antibody (goat anti- rabbit IgG-HRP) for 1 h at room temperature. Prestained SDS-PAGE protein standards (Bio-Rad, Precision Plus Protein Standards, Kaleidoscope™) were used to determine the size of detected proteins. The membranes were stripped and re-blotted with anti-β-actin antibody at 1:1,000 dilution as an internal control for protein loading. Proteins were visualized by chemiluminescence with SuperSignal West Pico (Pierce) and exposed to X-ray film.

Lentiviral infection of SH-SY5Y cells- Cells plated (at ~80% confluency) in 35 mm dishes were infected with a mixture of the four lentiviral stocks encoding shRNA against RGS4 with 6 μg/ml of polybrene in DMEM medium. A control cell line was obtained using lentivirus stock encoding shRNA against GFP (from Dr. Didier Trono). The stable cell lines were generated by passaging cells into larger dishes and GFP expression was used as an indicator for the presence of shRNA through out the cell culture maintenance.

cAMP Accumulation- Cells were plated to 80–90% confluency and treated with 10 μM retinoic acid for 6-8 days. On the day of assay, cells were washed once with fresh serum-free medium and the medium was replaced with 1 mM IBMX (3-isobutyl-1-methylxanthine) in serum-free medium for 15 min at 37 °C, and then replaced with medium containing 1 mM IBMX, 30 μM forskolin, and varying concentrations of SNC80 or morphine for 5 min at 37 °C. Reactions were stopped by replacing the medium with ice-cold 3% perchloric acid and samples were kept at 4 °C for at least 30 min. An aliquot (0.4 ml) from each sample was removed, neutralized with 0.08 ml of 2.5 M KHCO₃, vortexed, and centrifuged at 15,000 x g for 1 min to pellet the precipitates. Accumulated cAMP was measured by radioimmunoassay in a 10-15 μl aliquot of the supernatant from each sample following the manufacturer’s instructions and calculated as pmol/μg protein accumulation of cAMP.

MAP Kinase Analysis- Cells were plated in 24 well plates for 5-7 days and serum-starved for 24 h. Then they were washed once with fresh serum-free medium and stimulated with 100 nM morphine, 1 μM SCN80, or dH2O, respectively, for 5 min at 37 °C. The reaction was stopped by adding 0.1 ml of ice-cold SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue) and samples were removed from the wells, boiled for 5 min, and then subjected (10-15 μl each) to electrophoresis using a 12% SDS-PAGE mini gel, followed by transferring to an Immobilon™ membrane for Western blotting. The blot was probed with 1:6000 dilution of anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody and visualized using horseradish peroxidase-conjugated anti-rabbit IgG, followed by enhanced chemiluminescence detection to measure the activated phospho-ERK and then exposed to the Kodak X-ray film. To assure equal loading, the same membranes were stripped and re-blotted with a 1:4000 dilution of anti-p44/42 MAPK antibody to measure total ERK levels. MAP kinase activity was calculated as normalized arbitrary units (a.u.) of phosphorylated MAP kinase (ERK1/2) over total ERK 1/2 by densitometry analysis of films in the linear range of exposure using a Kodak Image Station 440.

Co-immunoprecipitation (Co-IP)- Flag-tagged μ- or δ-opioid receptor or chimeric μ/δ opioid receptor cDNA was transiently transfected together with mutant HA-RGS4C2S cDNA at 1:1 ratio of plasmid DNAs into HEK293T cells using Lipofectamine 2000. At 48 h post-transfection, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 0.5% Nonidet P-40, 0.1% Lubrol, 1 mM EDTA, 1 mM sodium orthovanadate and 1x protease inhibitor cocktail mix and then passed through a 21-gauge needle for 10 times. Equal amount of protein lysates (500 μg each) were subjected to immunoprecipitation. The lysates were depleted of nuclei and cell debris by centrifugation for 10 min at 14,000 rpm in an Eppendorf centrifuge and pre-cleared once with 20 μl of protein A/G plus agarose for 30 min at 4 °C on a rocking platform, then incubated with or without (protein A/G plus agarose only) anti-HA antibody conjugated agarose beads (10 μl each) for 4 h at 4 °C, with rocking. Subsequently, the immune complexes were washed four times in NET buffer (50 mM Tris-HCl, pH 7.6, 5 mM
EDTA, 150 mM NaCl, 0.5% Nonidet P-40). Finally, complexes were dissociated by heating for 5 min in SDS sample buffer, resolved on a 10% SDS-PAGE mini gel, transferred to an Immobilon™-P transfer membrane, and blotted with anti-Flag M1 antibody at a 1:2000 dilution. U11079 RGS4 specific antiserum at a 1:10,000 dilution was used to detect RGS4 protein. Secondary antibodies (anti-mouse or anti-rabbit) were used at 1:10,000 dilution. Proteins were visualized by enhanced chemiluminescence and exposed to the Kodak X-ray film.

Deglycosylation of co-immunoprecipitated Flag-tagged δ- and chimeric δ/μ-opioid receptor-

Immunoprecipitated Flag-tagged δ- and chimeric δ/μ-opioid receptors with RGS4 were eluted from the anti-HA antibody agarose conjugated beads with 1% (w/v) SDS, 1 x G7 reaction buffer, 1 x glycoprotein denaturing buffer in total volume of 10 μl, incubated for 15 min at room temperature (22 °C) and then denatured by boiling for 10 min at 100 °C. The elutants were diluted 5-fold with 1 x G7 reaction buffer and 1% NP40 in a total volume of 50 μl. Deglycosylation was performed with 3 μl of N-glycosidase F (500,000 units/ml) overnight at 30 °C before terminating the reaction by addition of 4 x SDS sample buffer and 50 mM DTT. Proteins were separated by SDS-PAGE and analyzed by Western blot with anti-Flag M1 antibody at 1:2000 dilution. Anti-mouse secondary antibody was used at 1:10,000 dilution. Proteins were visualized by enhanced chemiluminescence and exposed to the Kodak X-ray film.

**RESULTS**

**RGS4 expression in SH-SY5Y cells.** RGS4 expression in SH-SY5Y cells was confirmed by both RT-PCR (Fig. 1A) and Western blot (Fig. 1B). Using RGS4 specific primers for the coding region, a strong 502 bp band was detected in SH-SY5Y cells which was not present in C6 rat glioma cells, previously reported not to express RGS4 (12). Using the validated U1079 RGS4-specific antibody (34), a strong band was detected at 28 kDa in HEK293T cells overexpressing RGS4, striatal tissue from wild-type mouse brain and SH-SY5Y cells. This band was absent in striatal tissue from RGS4 knockout mice, C6 cells and untransfected HEK293T cells. In SH-SY5Y cells, there were two smaller bands detected which may indicate the presence of short forms of RGS4 or metabolism products. In order to confirm specificity, the RGS4 antibody was pre-absorbed with purified full length RGS4 protein (Fig. 1D). This completely blocked the 28 kDa band, and two smaller bands in SH-SY5Y cells, leaving some low intensity non-specific bands.

**Development of a SH-SY5Y cell line with reduced expression of RGS4.** To study the role of RGS4, we developed a SH-SY5Y cell line stably expressing shRNA against RGS4 or GFP were prepared essentially as previously described (25), suspended in 50 mM Tris-HCl (pH 7.4) to 0.5-1.0 mg/ml protein and frozen in aliquots at -80 °C. Membrane protein (50 μg for μ-receptor binding and 100 μg for δ-receptor binding) was incubated in 50 mM Tris-HCl (pH 7.4) with 2.5 nM [3H]DAMGO or 4 nM [3H]DPDPE, with or without 50 μM naloxone (to define nonspecific binding), in a total volume of 0.2 ml for 75 min in a shaking water bath at 25°C. Samples were filtered through glass fiber filters (No. 32, Schleicher & Schull) mounted in a cell harvester (Brandel Inc., Gaithersburg, MD) and rinsed three times with ice-cold 50 mM Tris-HCl (pH 7.4). Radioactivity retained was counted by liquid scintillation in 4 ml of EcoLume scintillation cocktail (ICN, Aurora, OH).

**Data analysis** - Data from at least three separate experiments, each carried out in duplicate (except where stated), are presented as means ± S.E. For analysis, each data point was treated as a separate value. Data were compared by two-way ANOVA with Bonferroni posttest analysis of variance and differences considered significant if p < 0.05. Concentration-response data were fitted and compared using the simultaneous non-linear regression curve fitting package in GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, CA).
expressing shRNA against GFP (Fig. 2B). In contrast, RGS4 protein was easily detectable in SH-SY5Y control cells stably expressing shRNA against GFP (Fig. 2B).

To determine if opioid receptor levels were changed in the stable SH-SY5Y cell lines expressing shRNA against RGS4 or GFP, receptor binding assays were performed with the selective μ-ligand [3H]DAMGO (2.5 nM) and selective δ-ligand [3H]DPDPE (4 nM) (25). As shown in Fig. 2C, the levels of expression were the same in both cell lines with μ- and δ-opioid receptors expressed in a 3.5 : 1 ratio, similar to previous reports (25, 35). This was also confirmed by Western blot analysis of separated proteins for the expressed μ- and δ-opioid receptors before and after retinoic acid differentiation (data not shown), similar to a previous report (36).

Differential effects of RGS4 on inhibition of forskolin-stimulated cAMP accumulation by SNC80 and morphine. RGS4-deficient and control SH-SY5Y cells were first differentiated with 10 μM retinoic acid treatment for 6-8 days, then adenylyl cyclase activity was measured by the accumulation of cAMP stimulated by forskolin (30 μM) in the presence of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine, 1 mM). Cells differentiated with 10 μM retinoic acid showed increased inhibition of cAMP accumulation by opioid agonists (~20%, data not shown) and so allowed for an improved agonist-to-noise ratio in this assay (37). The levels of forskolin-stimulated cAMP accumulation were similar in control and RGS4-deficient cells (5.3 ± 1.1 versus 5.9 ± 0.9 pmol/μg protein, respectively). The degree of inhibition of forskolin-stimulated cAMP accumulation by the μ-opioid agonist morphine at 100 nM was the same in both cell lines (35 ± 6% in RGS4-deficient cells and 33 ± 7% in control cells), but the effect of the selective δ-opioid agonist SNC80 at 1 μM was significantly increased to 47 ± 4% in the RGS4-deficient cell line compared to 30 ± 4% in control cells expressing shRNA against GFP (p = 0.005; Fig. 3A).

The above concentrations of morphine and SNC80 are submaximal. The difference observed with SNC80 could be explained by a change in potency or a change in maximal effect of the agonist. Concentration-effect curves for both morphine and SNC80 to inhibit forskolin-stimulated cAMP accumulation were determined as shown in Fig. 3B and Fig. 3C respectively. The forskolin-stimulated values in control cells or RGS4-deficient cells in the absence of either SNC80 or morphine were not different (2.42 ± 0.05 pmol/μg protein in control cells versus 2.34 ± 0.03 pmol/μg protein in RGS4-deficient cells). Morphine was consistently more potent and gave higher maximal effects (85% and 87% inhibition, respectively) than SNC80 in control cells expressing endogenous levels of RGS4 and in RGS4 deficient cells. There was no difference in the concentration-response curves for morphine between the two cell lines (Fig. 3B). In contrast, the maximum effect and EC50 values for SNC80, were significantly different ([F(2, 80) = 35.48, p = 0.0001]) when compared by simultaneous curve-fitting (GraphPad prism, 5.0) after constraining the forskolin-only values (Fig. 3C). In the RGS4-deficient cells, there was a significant increase in the maximal inhibition of forskolin-stimulated cAMP accumulation to 62 ± 3.5% compared to 41 ± 5% in control cells with a 4-fold leftward shift of the EC50 value (from 348 nM in control cells to 84.7 nM in RGS4-deficient cells).

Reversal of the SNC80 gain of function by transient transfection of mutant HA-RGS4C2S in SH-SY5Y cells with reduced expression of RGS4. In order to determine that the increased inhibition of forskolin-stimulated cAMP accumulation in the RGS4-deficient SH-SY5Y cells by SNC80 was indeed due to the loss of RGS4 protein, we replenished RGS4 in these cells. It is known that RGS4 is unstable due to high proteasomal degradation (37). Consequently, we used a stable RGS4 construct with a mutation (cysteine to serine) at the second amino acid of the N-terminus at the second amino acid of the N-terminus and with a C-terminal HA tag (HA-RGS4C2S) (30). Following transient transfection of the mutant HA-RGS4C2S into the RGS4-deficient SH-SY5Y cells, the expressed HA-RGS4C2S was easily detectable using anti-HA antibody (Fig. 4A) with no bands detected in control cells transfected with the pcDNA3.1 vector alone. The inhibitory effect of SNC80 in the RGS4-deficient SH-SY5Y cells on forskolin-stimulated cAMP accumulation was reversed significantly from 68 ± 7% to 57 ± 7.9%...
(p = 0.03) following transfection of the stable RGS4 (Fig. 4B). The effect of HA-RGS4C2S transfection was not greater due to the low transfection efficiency of SH-SY5Y cells using lipofectamine 2000 (~15%) as monitored by transfection of pEGFP plasmid DNA under the same conditions. In contrast, transient transfection of HA-RGS4C2S had no effect on the degree of inhibition of forskolin-stimulated cAMP by morphine.

Effect of μ-opioid receptor agonists (morphine and DAMGO) on inhibition of forskolin-stimulated cAMP in SH-SY5Y cells expressing RGS-insensitive Gαo. Since the level of inhibition of forskolin-stimulated cAMP accumulation by morphine was similar in both RGS4-deficient and control SH-SY5Y cells, it was necessary to determine if μ-opioid receptor coupling to adenyl cyclase was generally sensitive to other RGS proteins expressed in these cells. Accordingly, SH-SY5Y cells stably expressing Gαo with a PTX insensitive mutation (GαoPTXi) or with both PTX and RGS insensitive mutations (GαoRGSi/PTXi) were used to study the inhibition of forskolin-stimulated cAMP accumulation by a submaximal concentration of the μ-opioid receptor agonists morphine and DAMGO (Fig. 5). After overnight treatment with PTX (100 ng/ml) to uncouple endogenous Gα proteins, both morphine and DAMGO showed increased inhibition of forskolin-stimulated cAMP accumulation in cells expressing RGS-insensitive Gαo compared to cells expressing RGS-sensitive Gαo. The effect was only significant with morphine (p < 0.05), possibly because of the higher efficacy of DAMGO (31). These results indicated that μ-opioid receptor agonists are sensitive to global inhibition of RGS protein activity in SH-SY5Y cells.

Differential effects of RGS4 on stimulation of MAP kinase by SNC80 and morphine. To determine if the differential effect of RGS4 on μ- and δ-agonist signaling is specific to inhibition of adenyl cyclase, we examined whether MAP kinase regulation by opioid receptor agonists was also modulated by endogenous RGS4 protein. Stimulation of ERK phosphorylation by morphine (100 nM) and SNC80 (1 μM) was measured in both RGS4-deficient and control SH-SY5Y cells (Fig. 6). The basal level of ERK phosphorylation, measured as normalized arbitrary units (a.u.) of phosphorylated ERK over total ERK, was similar in both cell lines as was the level of total ERK. Morphine stimulated a similar, approximately 50%, increase in ERK phosphorylation in both control and RGS4-deficient cells. SNC80 was less effective in stimulating ERK phosphorylation than morphine giving only a 12 ± 9% increase in control cells. However, the level of ERK phosphorylation by SNC80 in the RGS4-deficient cells was markedly increased to 89 ± 20% (Fig. 6; p < 0.05).

Co-immunoprecipitation of δ- but not μ-opioid receptors with RGS4 in HEK293T cells. We could not identify opioid receptor antibodies suitable for immunoprecipitation of endogenous receptors in SH-SY5Y cells. Therefore, to study the possible interaction of μ- or δ-opioid receptors with RGS4, HEK293T cells were co-transfected with Flag-tagged μ- or δ-opioid receptor together with the mutant HA-tagged RGS4C2S construct. Subsequently, HA-RGS4C2S protein was immunoprecipitated with anti-HA antibody conjugated agarose beads and the immune complexes were resolved and blotted with an anti-Flag M1 antibody to detect any Flag-tagged μ- or δ-opioid receptors.

The expression of the Flag-tagged μ- and δ-opioid receptors in HEK293T cells was easily detectable on Western blots by the presence of multiple bands following SDS-PAGE of whole cell lysates using anti-Flag M1 antibody (Fig. 7A) compared to lysates from cells transfected with vector alone (pcDNA3.1). The two main bands detected in cells expressing the δ-opioid receptor were at approximately 75-85 kDa and 37-42 kDa with a weak band at 50-55 kDa, observed only after longer exposure. In the μ-opioid receptor expressing cells, there were three main bands at approximately 42-45 kDa, 55 kDa and 90-95 kDa. The lower MW bands likely represent non-glycosylated receptors. In preparations from both μ- and δ-expressing cells, there were higher MW bands identified, possibly representing receptors in protein aggregates. Similar complex Western blot patterns have been previously observed for Flag-tagged opioid receptors expressed in HEK293 cells (38).

Following immunoprecipitation of lysates from HEK293T cells expressing the δ-opioid receptor with anti-HA antibody conjugated beads, the δ-opioid receptor band at approximately 75-85 kDa
was identified by SDS-PAGE using anti-Flag M1 antibody. This band was not observed following immunoprecipitation with agarose beads alone or when the immunoprecipitation experiment was performed in mock transfected cells (Fig. 7A). In contrast, there was no evidence for co-immunoprecipitation of any μ-opioid receptor protein by anti-HA antibody from HEK293T cells co-expressing HA-RGS4C2S and the μ-opioid receptor. This was true even with a much longer exposure time of the blot and with double the amount of μ-opioid receptor plasmid DNA in the transfection. Agonist stimulation of the δ-opioid receptor (SNC80, 1.0 μM) or μ-opioid receptor (morphine, 1.0 μM) for 15 min did not enhance the degree of the co-immunoprecipitation (data not shown).

We were unable to perform the reverse experiment to study RGS4 in immune complexes precipitated with Flag M1 antibody against the receptors due to the presence of an IgG light chain band at the same MW as RGS4 (approximately 27 kDa). However, the presence of expressed HA-RGS4C2S in the immune complex following immunoprecipitation with anti-HA antibody was confirmed by re-blotting with the RGS4-specific antibody (U1079), after stripping the transfer membrane (Fig. 7B).

Co-immunoprecipitation of RGS4 and chimeric δ/μ-opioid receptor. A previous report has suggested that the C-tail of μ- and δ-opioid receptors is an interacting site for RGS4 protein (39). To further confirm the C-tail as a site for interaction of the δ-opioid receptor with RGS4, we used chimeric μ/δC- and δ/μC-opioid receptors both with an N-terminal Flag epitope (23). The chimeric opioid receptor constructs were co-expressed with the stable RGS4 mutant HA-RGS4C2S in HEK293T cells. SDS-PAGE of lysates from cells expressing the Flag-tagged opioid receptor chimeras showed very similar patterns (Fig. 8A) as Flag-tagged δ and μ-opioid receptors (Fig. 7A). In lysates from cells transfected with the μ/δC opioid receptor, two main bands at approximately 90-95 kDa and 42-45 kDa were observed with some higher molecular weight bands. Treatment of cell lysates with anti-HA antibody conjugated agarose beads co-immunoprecipitated a protein of approximately 90-95 kDa in the transfected chimeric μ/δC opioid receptor cells that was not present in the control HEK293T cells transfected with vector alone (pcDNA3.1; Fig. 8A). RGS4C2S was present in the immune complex (Fig. 8B). These findings indicate that the C-tail of the δ-opioid receptor contributes to the site of interaction with RGS4. Western blotting following electrophoretic separation of lysates from HEK293T cells expressing the δ/μC chimeric receptor construct gave two main bands at approximately 75-85 kDa and 37-42 kDa. The protein at 75-85 kDa was co-immunoprecipitated by anti-HA antibody (Fig. 8A) together with RGS4C2S (Fig. 8B). Since we found no biochemical evidence for interaction of the μ-opioid receptor and RGS4, this would suggest a role for other regions of the δ-opioid receptor, possibly the third intracellular loop (39), as well as the C-terminus as interacting sites for RGS4 protein.

Deglycosylation of Flag-tagged δ and chimeric δ/μ-opioid receptor immunoprecipitated with RGS4. Experiments depicted in Figs. 7A and 8A showed that we were able to immunoprecipitate the larger species (approximately 75-85 kDa), but not smaller species (approximately 37-42 kDa) of the δ-opioid receptor with RGS4C2S, even though the smaller species was abundantly detected in whole cell lysates. This could be because the higher apparent MW species is the mature glycosylated plasma membrane form. N-glycosylation is necessary to maintain expression of fully functional and stable δ-opioid receptor molecules at the cell surface while lower MW forms of the receptor are likely intracellular precursors (40). To support this idea, precipitated immune complexes were subjected to N-glycosidase F (PNGase F) digestion (Fig. 9). This resulted in a shift in the apparent MW of both immunoprecipitated Flag-δ and chimeric Flag-δ/μ receptors compared to the immune complexes without PNGase F digestion.

DISCUSSION

In this study, we used lentiviral delivery of shRNA to specifically suppress the level of endogenously expressed RGS4 protein in human neuroblastoma SH-SY5Y cells. This suppression of RGS4 message and protein enhanced the inhibition of cAMP accumulation and especially
the stimulation of MAP kinase activity by the δ-opioid receptor agonist SNC80, but not by the μ-opioid receptor agonists morphine or DAMGO. The effect on SNC80 was reversed by overexpression of a stable RGS4 mutant (HA-RGS4C2S). An interaction between RGS4 and an N-glycosylated mature form of the δ-opioid receptor was confirmed by co-immunoprecipitation of HA-tagged RGS4 with Flag-tagged δ- but not Flag-tagged μ-opioid receptor when the proteins were transiently co-expressed in HEK293T cells. These results provide novel evidence for opioid receptor type specificity of the action of RGS4 in modulating G protein signaling in SH-SY5Y cells and demonstrate for the first time that endogenous RGS4 differentially modulates μ- and δ-opioid receptor signaling in an endogenous system.

The effect of RGS4 knockdown on δ-opioid signaling to adenylyl cyclase was only modest. A similar small effect on opioid inhibition of adenylyl cyclase has been reported in cells overexpressing RGS4 (22, 23) and we have confirmed in HEK293T cells that overexpressed HA-RGS4C2S caused only a 4-fold rightward shift in the concentration-effect curve for SNC80 (data not shown). This could indicate that RGS proteins other than RGS4 are involved in modulating δ-opioid receptor signaling. On the other hand, RGS proteins act to decrease the lifetime of active Gα-GTP, but do not stop the G protein cycle. Indeed, even in SH-SY5Y cells after elimination of all RGS activity by the use of RGS-insensitive Gα subunits, there was only a limited effect on μ-opioid signaling, in agreement with previous data in other cell systems (31). In contrast to adenylyl cyclase, the effect on δ-opioid receptor signaling to MAP kinase was much more robust, possibly suggesting a preferential action of RGS4 for this signaling pathway. We have previously shown a differential ability of RGS protein activity to modulate cell signaling pathways (31).

The finding of a negative modulatory role for RGS4 on δ-opioid receptor signaling in SH-SY5Y cells agrees with the results of Hepler and colleagues (6) who reported that exogenously added RGS4 successfully reversed inhibition of cAMP accumulation in NG108-15 cells by the δ-opioid agonist [Leu⁵]enkephalin, and with findings that GST-fusion proteins of the C-tail and third intracellular loop peptides of the δ-opioid receptor, can interact with RGS4 (39). Conversely, these results are in contrast to the observation that transiently expressed RGS4 in HEK293T cells did not modulate the ability of the δ-opioid agonist DPDPE to inhibit adenylyl cyclase via a transiently expressed δ-opioid receptor (23). Indeed, that study found a lack of activity of RGS proteins in modulating δ-opioid signaling to adenylyl cyclase with the exception of RGS9. However, we could not identify the expression of RGS9 protein in SH-SY5Y cells by Western blot using a highly selective anti-RGS9 antibody (41, data not shown).

Our finding that μ-opioid receptor signaling to adenylyl cyclase or to the MAP kinase pathway is not modulated by RGS4 in SH-SY5Y cells is consistent with our finding that RGS4 and the μ-opioid receptor expressed in HEK293T cells did not co-immunoprecipitate. However, our findings are inconsistent with previous reports using heterologous expression systems. For example, overexpression of RGS4 in HEK293 cells caused a significantly attenuation of μ-opioid receptor-mediated inhibition of adenylyl cyclase (22, 23). In addition, RGS4 accelerates the deactivation of G protein-coupled inwardly rectifying K+ channel (GIRK) after termination of μ-opioid receptor signaling in Xenopus oocytes (42). There is also evidence for an interaction of RGS4 with GST-fusion C-terminal tail peptides of the μ-opioid receptor (39). Then again, a major difference between the current study and previous work is that we have knocked down endogenously expressed RGS4 in the neuroblastoma SH-SY5Y cell line that endogenously expresses both μ- and δ-opioid receptors. This provides a model much closer to the endogenous situation compared with cells heterologously overexpressed with RGS4 and/or receptors. Our finding that μ-opioid signaling is altered in SH-SY5Y cells stably expressing RGS-insensitive Gαo compared to cells expressing wild-type Gαo demonstrates that μ-opioid receptor signaling is sensitive to the GAP activity of RGS proteins expressed in SH-SY5Y cells. Consequently, it is possible that in SH-SY5Y cells μ-opioid receptor signaling is insensitive to, or in some way protected from, RGS4.
An alternative scenario for our observed selectivity of RGS4 and one that explains previous results in heterologous expression systems, is that RGS4 does modulate both δ- and μ-opioid signaling in SH-SY5Y cells, but that other RGS proteins expressed in these cells can substitute for RGS4 at the μ- but not at the δ-opioid receptor. That is, there is a functional redundancy at the μ- but not at the δ-opioid receptor. This could be due to the lack of expression of alternative RGS partners for the δ-opioid receptor in SH-SY5Y cells, for example RGS9 is not expressed as discussed above. Alternatively, there may be a higher stringency of interaction between RGS4 and the δ-opioid receptor. Certainly, it is known that the δ-receptor is coupled tightly to Gα proteins (43) and also has a high level of agonist independent activity, indicative of tight coupling (44, 45). RGS4 could thus be an integral part of a δ-opioid receptor-Gα-Gβγ-RGS4 signaling complex (39).

Indeed, our immunoprecipitation results do indicate that the δ-opioid receptor has a more robust interaction with RGS4 than the μ-opioid receptor in the heterologously expressed HEK293T cells. This is likely due to interaction with the third intracellular loop and/or C-tail of the δ-opioid receptor in agreement with a report that RGS4 interacts directly, probably through its N-terminal region (46), with GST-fusion proteins of the C-tail and third intracellular loop of the δ-opioid receptor, but only interacts with GST-fusion C-terminal tail peptides of the μ-opioid receptor (39). The presence of two interacting sites between RGS4 and the δ-opioid receptor, C-terminus and third intracellular loop, could explain why we observe co-immunoprecipitation of the δ-, but not the μ-opioid receptor, even though RGS4 has been reported to act as a GAP for μ-opioid signaling in HEK293 cells (22, 23). Certainly the presence of multiple contact sites between the δ-opioid receptor and RGS4 could be responsible for the observed interaction and/or the high stringency in the SH-SY5Y cell system.

We have previously shown that μ- and δ-opioid receptors share the same G proteins in SH-SY5Y cells (25), but the present results suggest that these G proteins are differentially regulated by RGS4. This conundrum can be explained by postulating that RGS4 is closely associated in a complex with the δ-opioid receptor as discussed above, rather than the Gα protein, in accordance with the immunoprecipitation experiments and previous studies with purified proteins (39). Indeed, several studies have identified receptor-specific effects of RGS protein action. For example, only RGS3 expression suppressed gonadotropin-releasing hormone-induced IP3 responses (47) and in pancreatic acinar cells, microinjected RGS4 selectively inhibited calcium signals induced by muscarinic but not cholecystokinin receptors (11). In addition, Wang et al. (9) showed, using ribozyme-mediated gene knockdown that RGS3 is a negative modulator of ERK activation by muscarinic m3 receptors but that endogenous RGS5 is a negative modulator of AT1a receptor-mediated activation of ERK in both A-10 cells and primary culture of aorta smooth muscle cells. However, since we were unable to show a direct interaction between endogenous δ-opioid receptors and endogenous RGS4 in SH-SY5Y cells, we cannot rule out the possibility that the RGS4-δ-opioid receptor interaction is indirect (48), perhaps mediated by an intermediate scaffold such as spinophilin. Spinophilin is known to bind to several GPCRs at the third intracellular loop and to RGS4 (49).

In conclusion, the current findings demonstrate that endogenous RGS4 in SH-SY5Y cells differentially modulates the ability of μ- and δ-opioid receptor agonists to inhibit adenylyl cyclase and stimulate the MAP kinase pathway, possibly due to a high stringency of interaction between RGS4 and the δ-opioid receptor. This is in contrast to studies in heterologous overexpression systems and shows the value of lentiviral delivery of shRNA targeting RGS genes to permit the analysis of RGS function and its receptor interactions in endogenous systems. Given that RGS4 is widely expressed in many brain regions (21) including the amygdala, nucleus accumbens, and caudate putamen where δ-opioid receptors are also highly expressed (50), the selective RGS4 modulation of δ- over μ-opioid receptor signaling may play a significant role in modulation of δ-opioid receptor-mediated behaviors.
REFERENCES

FOOTNOTES

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The abbreviations used are: GFP, green fluorescent protein; HA, hemagglutinin; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; MAP kinase, mitogen-activated protein kinase; MW, molecular weight; shRNA, short hairpin RNA; RGS, regulator of G protein signaling.
FIGURE LEGENDS

Fig. 1. RGS4 expression in SH-SY5Y cells. A. RGS4 mRNA expression by RT-PCR. Total RNA prepared from SH-SY5Y or C6 glioma cells was subjected to RT-PCR using RGS4-specific primers. The PCR product was separated on a 1.8% agarose gel, stained and photographed. The expected 502 bp PCR product was detected in SH-SY5Y (SY5Y) cells but not in C6 glioma cells (C6). B. RGS4 protein expression by Western blot. Lysates were prepared from HEK293T cells overexpressing RGS4 (OE), striatal brain tissue from wild type (WT) or RGS4 knockout mice (KO), SH-SY5Y cells (SY5Y), C6 glioma cells (C6), untransfected HEK293T cells (HEK), and resolved (50 μg each) on a 12% SDS-PAGE mini gel for Western blot analysis with U1079 anti-RGS4 antibody as described under “Experimental Procedures”. C. The same blot was stripped and blotted with anti β-actin antibody with similar intensity in each lane except in HEK293T cells overexpressing RGS4 which used 100-fold less protein. D. Purified full length RGS4 protein completely neutralized the anti-RGS4 antibody causing loss of the 28 kDa band and two smaller bands in the immunoblot of lysates from SH-SY5Y cells.

Fig. 2. Development of a SH-SY5Y cell line stably expressing shRNA against RGS4. A. Delivery of shRNA into SH-SY5Y cells. A mixture of four lentiviruses encoding four shRNA targeting four different sites on the RGS4 gene was used to infect SH-SY5Y cells. Photograph using a Leica inverted fluorescence microscope at 5 days post-infection shows GFP expression as an indication of expressed shRNA in the cells (left panel) compared to the phase contrast (right panel). B. RGS4 protein knockdown. Whole cell lysates were prepared from the SH-SY5Y cells stably expressing shRNA against RGS4 or against GFP as a negative control, respectively. Equal amounts of protein (50 μg each) were loaded onto a 12% SDS-PAGE mini gel and blotted with anti-RGS4 antibody (U1079) (1:10,000 dilution) as described under “Experimental Procedures”. There was almost complete knockdown of RGS4 expression in cells expressing shRNA against RGS4 compared to cells expressing shRNA against GFP. The β-actin loading control was unchanged. C. Expressed μ- and δ-opioid receptor numbers (n = 3 in duplicate, mean ± S.E.) were the same in membranes from SH-SY5Y cells expressing shRNA against RGS4 or GFP determined with [3H]DAMGO (μ) or [3H]DPDPE (δ), as described under “Experimental Procedures”.

Fig. 3. Increased inhibition of forskolin-stimulated cAMP accumulation by SNC80 but not by morphine in RGS4-deficient SH-SY5Y cells. A. The δ-agonist (SNC80) but not the μ-agonist (morphine) showed enhanced inhibition of forskolin-stimulated cAMP in RGS4-deficient SH-SY5Y cells. Cells were treated with 10 μM retinoic acid 6-8 days before the assay and pretreated with 1 mM IBMX in serum-free medium for 15 min. Cells were then incubated with 30 μM forskolin, 1 mM IBMX, and 100 nM morphine or 1 μM SNC80 for 5 min at 37°C and accumulation of cAMP (pmol/μg protein) was measured by radioimmunoassay described under “Experimental Procedures”. *p = 0.005 for SNC80 in RGS4-deficient cells compared to control cells. B. Concentration-response for morphine or C. SNC80 to inhibit forskolin-stimulated cAMP accumulation in control (solid line and open symbols) and RGS4-deficient cells (dashed line and solid symbols), expressed as pmol/μg protein accumulation of cAMP from 4 to 6 experiments each performed in duplicate (where error bars are not shown, they are smaller than the symbols). The procedure was carried out as described in A and under “Experimental Procedures” with varying concentrations of morphine (10^9 to 10^6 M) or SNC80 (10^8 to 10^5 M). The forskolin-stimulated values in control cells or RGS4-deficient cells in the absence of either SNC80 or morphine were not different (2.42 ± 0.05 pmol/μg protein in control cells versus 2.34 ± 0.03 pmol/μg protein in RGS4-deficient cells). Concentration-response curves were fitted, after constraining the forskolin only value, and compared by simultaneous curve-fitting using GraphPad Prism, version 5.0, treating each value as a separate point (n = 42). EC50 and maximal inhibition values were significantly different in the RGS4-
deficient cells compared to the control cells for SNC80 ([F(2, 80) = 35.48, p = 0.0001]), but not for morphine ([F(2, 80) = 0.41, p = 0.66]).

**Fig. 4.** Transfection of a stable mutant of RGS4 reverses increased inhibition of adenylyl cyclase by SNC80 in RGS4-deficient SH-SY5Y cells. SH-SY5Y cells stably expressing shRNA against RGS4 were treated with 10 μM retinoic acid for 6 days and then transfected with either pcDNA3.1 (vector) or a HA-tagged stable RGS4 construct (HA-RGS4C2S). A. At two days post-transfection, protein lysates were prepared and subjected to SDS-PAGE followed by Western blot analysis using anti-HA antibody. B. Forskolin-stimulated cAMP accumulation (pmol/μg protein, n = 4 in duplicate, mean ± S.E.) in the absence or presence of morphine (100 nM) or SNC80 (1 μM) was carried out at two days post-transfection as described in Figure 3A. * p < 0.05 compared to SNC80 effect in cells expressing vector alone.

**Fig. 5.** Sensitivity of μ-opioid agonists (morphine or DAMGO) to RGS protein activity. SH-SY5Y cells stably expressing PTX insensitive Gαo (GαoPTXi) or RGS- and PTX-insensitive Gαo (GαoRGSi/PTXi) were grown in the presence of 10 μM retinoic acid for 6-8 days. Cells were then treated overnight with 100 ng/ml PTX, before determination of the degree of inhibition of forskolin-stimulated cAMP accumulation by 100 nM morphine or 100 nM DAMGO, as described in Figure 3A. Data expressed as pmol/μg protein cAMP accumulation, are from three separate experiments in duplicate. * p < 0.05 compared to morphine effect in cells expressing GαoPTXi.

**Fig. 6.** Enhanced MAP kinase activation by SNC80 but not by morphine in RGS4 deficient SH-SY5Y cells. Cells stably expressing shRNA against RGS4 or GFP (control) were grown for 5-7 days and serum starved for 24 h before the assay. SNC80, or dH2O as a control, respectively, for 5 min at 37 °C. The samples were harvested in 1 x SDS sample buffer and subjected to a 12% SDS-PAGE mini gel for MAP kinase analysis by Western blot using anti-phospho-p44/42 MAPK (ERK1/2) antibody for the detection of phosphorylated ERK. Membranes were then stripped and re-blotted with anti-p44/42 MAPK (ERK1/2) antibody to measure total ERK levels. MAP kinase activity was expressed as normalized arbitrary units (a.u.) of phosphorylated ERK over total ERK by densitometry analysis. A. shows mean ± S.E. of 5 individual, independent, experiments and B. is a representative Western blot. * SNC80 produced a statistically significant Cells were then treated with 100 nM morphine, 1 μM (p < 0.05) enhancement of MAP kinase activity in RGS4-deficient cells compared to control cells.

**Fig. 7.** Co-immunoprecipitation of Flag δ- but not Flag μ-opioid receptor with RGS4 in HEK293T cells. Flag-tagged μ- or δ-opioid receptor was transiently transfected together with stable HA-tagged mutant RGS4 (HA-RGS4C2S) into HEK293T cells. At 48 h post-transfection, cells were lysed and treated with or without anti-HA antibody conjugated agarose beads (+ , + AB) to precipitate immune complexes as described under “Experimental Procedures”. A. Immune complexes were resolved on a 10% SDS-PAGE mini gel and blotted with anti-Flag M1 antibody. Proteins were visualized by enhanced chemiluminescence. Only the 75-85 kDa band of δ-opioid receptor (arrow, compared to the same band in whole cell lysates (WL) from cells expressing δ-opioid receptors) was immunoprecipitated (CO-IP) together with HA-RGS4C2S protein. This band was absent in cells transfected with pcDNA3.1 vector alone (V). There was no evidence for co-immunoprecipitation of the μ-opioid receptor with HA-RGS4C2S although the receptor was readily detected in whole cell lysates. B. Detection of RGS4 protein in the immune complex containing the 75-85 kDa band. The same blot in A. was stripped and re-blotted with anti-RGS4 antibody to show RGS4 was precipitated by the anti-HA antibody conjugated agarose beads.

**Fig. 8.** Co-immunoprecipitation of Flag-tagged chimeric μ/δ-opioid receptors with RGS4 in HEK293T cells. A. HEK293T cells were transiently transfected with Flag-tagged μ/δ-opioid receptor
chimera constructs with the C-tails exchanged (μ/δC or δ/μC) together with the HA-tagged mutant RGS4C2S, as described under “Experimental Procedures”. Cells were lysed and treated with or without anti-HA antibody conjugated agarose beads (-, + AB). Precipitated immune complexes were resolved on 10% SDS-PAGE and subjected to Western blot analysis with anti-Flag M1 antibody. Arrows indicate there was co-immunoprecipitation (CO-IP) of chimeric μ/δC and δ/μC opioid receptors with RGS4 identified by comparison with whole cell lysate (WL) of μ/δC or δ/μC opioid receptors. No receptors were observed in lysates from cells transfected with vector (pcDNA3.1) alone (V). B. Detection of RGS4 protein in the immune complex. The same blot in A. was stripped and re-blotted with anti-RGS4 antibody to show that RGS4 was precipitated with the anti-HA antibody conjugated agarose beads.

**Fig. 9.** Deglycosylation of the Flag-tagged δ- and chimeric δ/μ- opioid receptors immunoprecipitated by HA-RGS4C2S. Transfected Flag δ- or chimeric Flag δ/μ-opioid receptor in HEK293T cells were immunoprecipitated with HA-RGS4C2S using anti-HA antibody conjugated agarose beads as in Figs. 7A and 8A. The immune complexes were eluted from the anti-HA antibody agarose conjugated beads, denatured and subjected to the deglycosylation with (+) or without (-) N-glycosidase F (PNGase F) as described under “Experimental Procedures”. The reaction was terminated, proteins were resolved with SDS-PAGE, and Western blot analysis was performed using anti-Flag M1 antibody. Only the larger MW species (approximately 75-85 kDa as indicated by arrows) of both the Flag δ and the chimeric Flag δ/μ receptors were precipitated compared to the whole cell lysates (WL) and this migrated faster following PNGase F digestion (“+”) compared to the samples without PNGase F digestion (“-”). The vector alone control cells (V) transfected with pcDNA3.1 and treated similarly in the right panel showed smaller background bands indicating possible IgG heavy chain (IgG HC) and light chain (IgG LC) due to non-specific immunoprecipitation.
Figure 1

A. M SY5Y C6

B. OE WT KO SY5Y C6 HEK

C. β-actin

D. 25 kDa
Figure 2

A. GFP Phase

B. shRNA: GFP RGS

C. Receptor numbers (fmol/μg protein)

<table>
<thead>
<tr>
<th></th>
<th>GFP shRNA</th>
<th>RGS4 shRNA</th>
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<tr>
<td>μ</td>
<td>244 ± 9</td>
<td>230 ± 27</td>
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<tr>
<td>δ</td>
<td>70 ± 17</td>
<td>63 ± 7</td>
</tr>
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</table>
Figure 3

A. Forskolin-stimulated cAMP (pmol/μg protein)

B. Forskolin-stimulated cAMP (pmol/μg protein) vs Morphine log (M)

C. Forskolin-stimulated cAMP (pmol/μg protein) vs SNC80 log (M)
Figure 4

A.

B.

Forskolin-stimulated cAMP (pmol/μg protein)

- 25 kDa
Figure 5

Forskolin-stimulated cAMP (pmol/μg protein)

GαoPTXi

GαoRGsi/PTXi

0.0
0.5
1.0
1.5
2.0
0.0
0.5
1.0
1.5
2.0

Forsk
Morphine
DAMGO
Forsk
Morphine
DAMGO
Figure 6

A. Ratio of Phos-ERK/Total-ERK (a.u.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Morphine</th>
<th>SNC80</th>
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<tr>
<td>RGS4 shRNA</td>
<td>*</td>
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B. Western Blot Images

- Phos-ERK
- Total-ERK
Figure 7

A. CO-IP  WL

<table>
<thead>
<tr>
<th></th>
<th>v</th>
<th>μ</th>
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<tbody>
<tr>
<td>AB</td>
<td>-</td>
<td>+</td>
<td>-</td>
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100 kDa-  75 kDa-  50 kDa-  25 kDa-  37 kDa-

B. RGS4

50 kDa-  37 kDa-  25 kDa-

← RGS4
Figure 8

A. CO-IP

<table>
<thead>
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<td>-</td>
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</table>

WL

v μ/δC δ/μC

B. RGS4

50 kDa- 37 kDa- 25 kDa-

RGS4
Figure 9
Differential modulation of mu- and delta-opioid receptor agonists by endogenous RGS4 protein in SH-SY5Y cells
Qin Wang, Dr., Lee-Yuan Y Liu-Chen, Dr. and John R Traynor, Dr.

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