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Mu opioid receptor activation enhances regulator of G protein signaling 4 association with the mu opioid receptor/G protein complex in a GTP-dependent manner

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Abstract

The interaction of Regulator of G protein Signaling 4 (RGS4) with the rat mu opioid receptor (MOR)/G protein complex was investigated. Solubilized MOR from rat brain membranes was immunoprecipitated in the presence of RGS4 with antibodies against the N-terminus of MOR (anti-MOR_{10–70}). Activation of MOR with [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO) during immunoprecipitation caused a 150% increase in Go α and a 50% increase in RGS4 in the pellet. When 10 μ M GTP was included with DAMGO, there was an additional 72% increase in RGS4 co-immunoprecipitating with MOR ($p = 0.003$). Guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) increased the amount of co-precipitating RGS4 by 93% (compared to DAMGO alone, $p = 0.008$), and the inclusion

of GTP γ S caused the ratio of MOR to RGS4 to be 1 : 1 (31 fmoles : 28 fmoles, respectively). GTP γ S also increased the association of endogenous RGS4 with MOR. In His₆RGS4/Ni²⁺-NTA agarose pull down experiments, 0.3 μ M GTP γ S tripled the binding of Go α to His₆RGS4, whereas the addition of 100 μ M GDP blocked this effect. Importantly, activation of solubilized MOR with DAMGO in the presence of 100 μ M GDP and 0.3 μ M GTP γ S increased Go α binding to His₆RGS4/Ni²⁺-NTA agarose ($p = 0.001$).

Keywords: adenylyl cyclase, cell signaling, G protein-coupled receptor, immunoprecipitation, opiate opioid, regulator of G protein signaling.

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Regulators of G protein signaling (RGS) accelerate the inactivation of G proteins that have been activated by their associated receptors by increasing the rate of GTP hydrolysis by the intrinsic GTPase of G proteins (Neubig and Siderovski 2002). Since the discovery of RGS proteins, attempts have been made to identify which RGS proteins modulate the signaling of known G protein-coupled receptors (GPCRs) and to determine the sequence of events that triggers the RGS

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Abbreviations used: anti-MOR_{10–70}, antiserum against the N-terminal region of MOR; anti-MOR_{349–398}, antigen-affinity purified antibody against the C terminal 50 amino acids of MOR; anti-RGS4_{170–205}, antigen-affinity purified antibody against the C terminal 36 amino acids of RGS4; buffer A, 50 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM Tris.EDTA, 1 mM Tris.EGTA, 5 mM MgCl₂, protease inhibitors, and phosphatase inhibitors (Sigma P0044); CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DAMGO, [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin; GST-RGS4_{170–205}, glutathione-S-transferase fused to the C terminal 36 amino acids of RGS4; MOR, mu opioid receptor; RGS4, regulator of G protein signaling 4; SHSY5Y cells, human neuroblastoma cell line expressing endogenous mu opioid receptors; TEM buffer, 50 mM Tris.HCl (pH 7.2), 2 mM EDTA, 1 mM EGTA, 5 mM MgCl₂.

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protein's association with its GPCR and its cognate G proteins (Dohlman *et al.* 1995; Dohlman and Thorner 1997). This study investigates the interaction of RGS4 with the endogenous rat brain mu opioid receptor (MOR) and Go, one of its cognate G proteins.

The MOR is expressed in many regions of the brain and spinal cord and is essential for natural opiate reward and analgesia (Matthes *et al.* 1996; Cui *et al.* 2014). Activation of MOR by endogenous enkephalins or exogenous opiates leads to suppression of neuronal activities. At the molecular level, MOR is associated with Gi/o-type G proteins that mediate inhibition of adenylyl cyclase activity, the opening of inwardly rectifying K⁺ channels and the closure of voltage-gated Ca²⁺ channels (North 1986; Gosse *et al.* 1989; Chen and Yu 1994; Chalecka-Franaszek *et al.* 2000). The activation of MOR results in the release of GDP from the α -subunits of G_i and G_o, and this allows GTP to bind to and activate these α subunits (Gilman 1987). The α -GTP and the $\beta\gamma$ subunits dissociate from the receptor and act on downstream effector systems. Signal termination is achieved by hydrolysis of GTP to GDP by an intrinsic GTPase of the α subunit of G_i and G_o, and the rate of GTP hydrolysis is accelerated ≥ 40 -fold by the interaction of RGS proteins with α -GTP (Berman *et al.* 1996a). Thus, the association of an RGS protein with a receptor shortens the time that the receptor's G proteins are active.

Multiple RGS proteins, including RGS2, RGS4, RGS6, RGS7, RGS8, RGS9-2, RGS11, RGS19, and RGS20 have been proposed to regulate MOR signaling (Neubig and Siderovski 2002; Xie *et al.* 2007; Talbot *et al.* 2010; Psigfogeorgou *et al.* 2011; Traynor 2012; Wang and Traynor 2013). RGS9-2, and to a lesser extent RGS4, appear to modulate the nociception pathway, whereas RGS4 modulates the reward pathway (Zachariou *et al.* 2003; Wang *et al.* 2009; Han *et al.* 2010). RGS4 also opposes morphine-induced physical dependence in the locus coeruleus. In addition, RGS4 and RGS8 are enriched in the thalamus, a region of the brain where MOR modulates the relay of pain signals to the sensory cortex (Gold *et al.* 1997). A number of studies have demonstrated that exogenous RGS4 can attenuate MOR signaling (Xie *et al.* 2007; Talbot *et al.* 2010). Understanding the processes leading to the association of RGS proteins with MOR, and determining which RGS proteins modulate MOR functioning in the nociception pathway versus the reward pathway, may reveal targets for drugs that can reduce the influence of opiates on the reward pathway and enhance the influence of opiates as anti-nociceptive agents.

It is not entirely clear if RGS4 can bind to endogenous MOR (and its cognate G proteins), if RGS4 is constitutively bound to MOR, or if activation of MOR can influence the binding of RGS4 to the MOR/G protein complex. Limited evidence suggests that GPCRs, like MOR, may play a role in attracting RGS proteins to their cognate G proteins

(Bernstein *et al.* 2004; Hague *et al.* 2005; Georgoussi *et al.* 2006). In a study performed in a heterologous system, it was found that HA-tagged RGS4 bound to myc-tagged MOR in the presence of AMF (100 μ M AlCl₃, 2 mM MgCl₂, 100 mM NaF) and that HA-tagged RGS4 could bind to a peptide identical to the 70 amino acids of the C-terminal region of MOR (MOR_{329–398}). These authors also demonstrated membrane translocation of RGS4 following MOR activation in HEK293 cells (Leontiadis *et al.* 2009).

In this study, we investigated, in depth, the interactions among endogenous rat brain MOR, endogenous G proteins and recombinant and endogenous RGS4. Our ability to solubilize MOR in a functionally active state allowed us to investigate the roles of [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO), a MOR agonist, and guanine nucleotides in attracting RGS4 to the MOR/G protein complex. By immunoprecipitating MOR with an antibody directed against the N-terminus of MOR, we were able to minimize any potential interference of the antibody with interactions among MOR, G proteins, and other proteins. This study demonstrates, for the first time, that GTP is required for maximal DAMGO-stimulated RGS4 binding to the MOR/G protein complex. Furthermore, we were able to demonstrate that DAMGO activation of soluble MOR can stimulate the binding of G α -GTP γ S to His₆RGS4. In functional assays, RGS4 was found to cause a concentration-dependent, non-competitive attenuation of MOR-mediated inhibition of adenylyl cyclase activity but did not affect MOR-stimulated binding of GTP γ S to G proteins.

Methods

Animals

See Supporting Information

Drugs and cell culture

[D-Ala², N-Me-Phe⁴, gly-ol] enkephalin (DAMGO) was purchased from Sigma, St. Louis, MO, USA; [³H]cAMP from NEN Life Sciences, and [³H]DAMGO from Amersham Pharmacia (Piscataway, NJ, USA). [³⁵S]GTP γ S was obtained from Perkin Elmer Life Sciences (Boston, MA, USA).

Preparation of recombinant RGS4

His₆RGS4 was expressed in JM109 *E. coli*, and His₆RGS4 was extracted (in the presence of protease inhibitors, Sigma P 8849) on Ni²⁺-NTA agarose resin. See Supporting Information Methods for details.

MOR and RGS4 Antibodies

See Supporting Information Methods for details.

Solubilization of rat brain membranes

Rat brain membranes were solubilized as previously described (Weems *et al.* 1996; Chalecka-Franaszek *et al.* 2000). See Supporting Information for details.

MOR immunoprecipitation

Three polyclonal anti-MOR antibodies were capable of immunoprecipitating MOR. Anti-MOR_{340–398}, anti-MOR_{10–70}, and anti-MOR_{349–398} were bound to Protein A Sepharose resin and covalently cross-linked to the resin with 20 mM dimethylpimelidate (Chalecka-Franaszek *et al.* 2000). A summary table of these antibodies and their potential uses is presented in Table S1.

Immunoblots

Routinely, immunoblots of material immunoprecipitated with anti-MOR_{10–70} were cut into three portions: the portion from 90 to 52 kDa was used to detect MOR (a smear running between ~54 and 70 kDa); the middle portion of the blot between 52 and 31 kDa was used to screen for Go α (~40 kDa); and the bottom third of the blot between 31 and 12 kDa was used to screen for RGS4 (~26 kDa). See Supporting Information for details.

Detection of [³H]DAMGO binding activity in immunoprecipitated material

See Supporting Information.

His₆RGS4/Ni²⁺-NTA agarose pull-down experiments

See Supporting Information for details. SHSY5Y cell cultures and adenylyl cyclase assays were performed as described in Supporting Information.

SHSY5Y cell cultures and adenylyl cyclase assays were performed

See Supporting Information for details.

Statistical analyses

The software ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997–2014) was used to quantify the densities of protein bands on film that had been exposed to immunoblots. In experiments involving the IP of MOR with anti-MOR_{10–70}, the density of each band of co-precipitated protein (Go α or RGS4) was divided by the density of the MOR band in the same lane (see Fig. 2). Also, the density of each Go α band was divided by the density of the RGS4 band in the same lane for comparisons in the Ni²⁺-NTA/His₆RGS4 pull down assay (see Fig. 4). Quantification of unknown amounts of RGS4 that co-immunoprecipitated with MOR was accomplished by interpolation of nonlinear regression curves generated from known standard amounts of RGS4 that were processed with the unknown samples (see Fig. 1b). All statistical and curve-fitting analyses were performed using PRISM v5.0 for Macintosh OS X (GraphPad Software, San Diego, CA, USA). Non-linear regression analysis was used to determine the best fit of full concentration-effect curves for adenylyl cyclase activity. The EC₅₀ values and maximal effects were determined from best-fit analyses. [³H]DAMGO receptor binding was analyzed using a one-site binding hyperbola model. All data are expressed as mean \pm standard error of the mean.

Results

Quantification of MOR and RGS4

Experiments were performed to determine if RGS4 associates with the MOR/G protein complex and, if it does, to

determine the stoichiometry of MOR and RGS4 under condition where no guanine nucleotide was present or where 0.3 μ M GTP γ S was present. Rat brain membranes were incubated for 10 min at 30°C with 1 μ M DAMGO (or 10 μ M morphine instead of DAMGO when [³H]DAMGO binding was to be determined, Fig. 1c) and 0.3 μ M His₆RGS4, a concentration of RGS4 that had a near maximal effect in attenuating MOR signaling (see Figure S5). The rat brain membranes were solubilized, and solubilized MOR was immunoprecipitated with anti-MOR_{10–70} as described in Methods. To determine the ratio of RGS4 to MOR, one portion of the immunoprecipitated material was used to quantify the amount of RGS4 by western blotting (Fig. 1b) and another portion was used to determine saturation [³H]DAMGO binding (Fig. 1c). GTP γ S (1 μ M) was included in some samples subjected to the immunoprecipitation procedure to determine if activation of the G proteins would affect His₆RGS4 binding to the MOR/G-protein complex (Fig. 1a and b). Interestingly, when 1 μ M GTP γ S was present, there was a 3-fold increase in the amount of His₆RGS4 co-immunoprecipitating with MOR in spite of the fact that the amount of Go α was reduced by 57% (paired, two-tailed *t*-test, *p* = 0.02). In the absence of GTP γ S, 9.0 \pm 1.1 fmoles of RGS4 were co-immunoprecipitated with MOR, whereas in the presence of 1 μ M GTP γ S, 28.4 \pm 5.4 fmoles of RGS4 were co-immunoprecipitated with MOR (Fig. 1b, bar graph). In four independent experiments, GTP γ S caused a 3-fold increase in the amount of RGS4 co-immunoprecipitated with MOR (unpaired *t*-test, *p* = 0.0128). The saturation [³H]DAMGO binding assay (Fig. 1c) indicated that the equivalent of 31 fmoles of MOR were present in the material that was loaded on each lane of the SDS gels shown in Fig. 1b. Thus, when G proteins were fully activated (with 1 μ M GTP γ S, the same concentration that maximally activates Go in inhibiting adenylyl cyclase activity, see Figure S6b), the ratio of MOR to RGS4 was approximately 1–1. The amounts of RGS4 co-immunoprecipitated with MOR were determined by interpolation of the densities of the unknown bands from non-linear regression curves generated from known His₆RGS4 standards run in the same gel as the co-immunoprecipitated His₆RGS4 samples. The densities of bands were determined using the software ImageJ, and the curves were generated using the program Prism[®] v5.0. Also, the inclusion of AMF (100 μ M AlCl₃, 2 mM MgCl₂ and 100 mM NaF) during the immunoprecipitation procedure resulted in an increase in His₆RGS4 (Fig. 1d).

DAMGO-stimulated binding of RGS4 to MOR

As activation of Go α with GTP γ S increased the amount of RGS4 bound to the MOR/Go α complex, the influence of DAMGO and guanine nucleotides on the association of Go α and RGS4 with the MOR/G protein complex was further investigated. As is evident in Figure S2c, DAMGO alone caused an approximate 2-fold increase in the association of

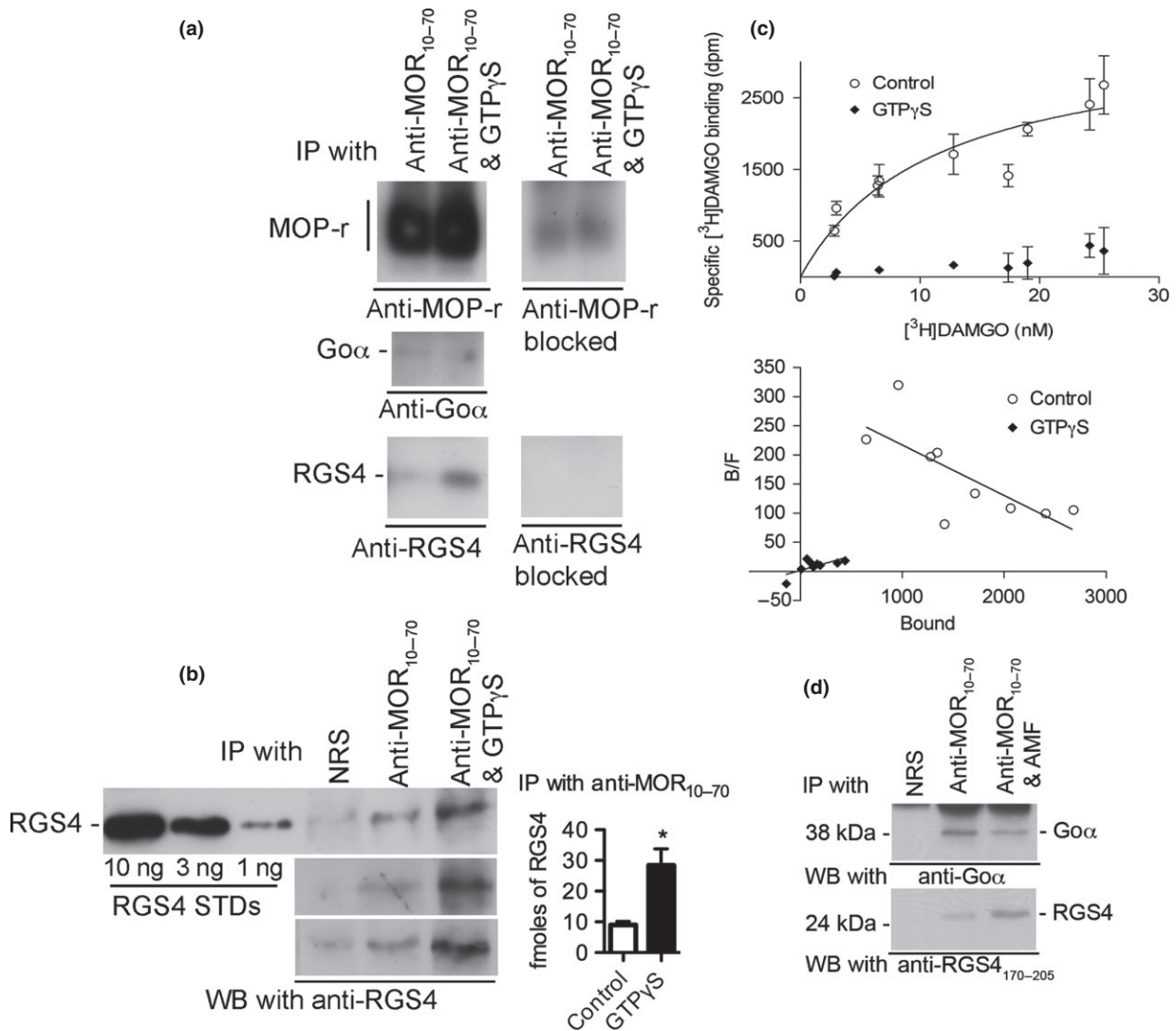


Fig. 1 Activation of G proteins with GTP γ S enhances the association of RGS4 with MOR. (a) MOR was immunoprecipitated with anti-MOR₁₀₋₇₀ in the presence of either 1 μ M [D-Ala², N-Me-Phe⁴, Gly⁵-O] enkephalin (DAMGO) and 0.3 μ M RGS4 or 1 μ M DAMGO, 0.3 μ M RGS4 and 1 μ M GTP γ S. The immunoprecipitate was subjected to SDS-PAGE (12% gel) and immunoblots were screened with the following antibodies: anti-MOR₃₄₉₋₃₉₈ (top panel), anti-Go α (second panel from the top), and anti-RGS4₁₇₀₋₂₀₅ (third panel from the top). The inclusion of the appropriate blocking peptides selectively blocked recognition of MOR and RGS4 [right side of (a)]. All westerns in A were performed on a single nitrocellulose blot that was cut into sections. (b) The experiment shown in (a) was repeated three additional times. Anti-RGS4₁₇₀₋₂₀₅ detected RGS4 that was co-immunoprecipitated with MOR by anti-MOR₁₀₋₇₀ in the absence or presence of 1 μ M GTP γ S in three independent experiments. RGS4 standards (10, 3, and 1 ng) were included in each experiment. Values of co-immunoprecipitated RGS4 were determined by extrapolation using nonlinear regression analysis of known RGS4 standards (Prism v5). In four independent experiments the amounts of RGS4 immunoprecipitated in the absence and in the presence of GTP γ S were 9 \pm 1.1 and 28.4 \pm 5.4 fmoles, respectively

[unpaired, two-tailed *t*-test, * indicates *p* = 0.013, see bar graph on right side of (b)]. (c) [³H]DAMGO saturation binding to MOR immunoprecipitated with anti-MOR₁₀₋₇₀ was used to quantify MOR. Aliquots of the immunoprecipitate identical to the aliquots that were loaded on to the gels depicted in (a) and (b), were used to measure [³H]DAMGO saturation binding (see Methods). Non-specific binding was subtracted from total binding to calculate specific binding (open circles). Specific binding ranged from 80% of total binding at the lowest concentration of [³H]DAMGO to 55% of total binding at the highest concentration of [³H]DAMGO. Samples were incubated in triplicate. Data shown were combined from two independent experiments. Analysis of the data by non-linear regression indicated that the B_{max} was 3400 dpm of [³H]DAMGO (31.0 fmoles); the 95% confidence interval was 2240 dpm to 4559 dpm (20.4 fmoles to 41 fmoles of MO). The K_D was determined to be 11 nM with a 95% confidence interval of 2.1–20 nM. The lower panel shows a Scatchard transformation of the data. (d) The inclusion of AMF (100 μ M AlCl₃, 2 mM MgCl₂ and 100 mM NaF) during the immunoprecipitation with anti-MOR₁₀₋₇₀ also caused an increase in the co-immunoprecipitated RGS4.

Go α with MOR. In five independent experiments, the inclusion of DAMGO during the IP process increased the amount of Go α by 2.5-fold (unpaired two-tailed *t*-test, $p = 0.03$). The inclusion of DAMGO also increased the amount of RGS4 co-immunoprecipitating with MOR by 53% (Fig. 2). Interestingly, the combination of DAMGO and GTP resulted in an additional 71.6% increase in the amount of RGS4 that was co-immunoprecipitated by anti-MOR₁₀₋₇₀ (unpaired two-tailed *t*-test, $p = 0.003$). As was shown in Fig. 1, the inclusion of GTP γ S along with DAMGO during the IP process also significantly increased the amount of RGS4 co-immunoprecipitated with the MOR/Go complex by 93% versus DAMGO alone (unpaired, two-tailed *t*-test, $p = 0.008$). The changes in Go α and RGS4 that resulted from the addition of DAMGO alone, or DAMGO in the presence of either GTP or GTP γ S are shown in Table 1. In statistical analyses, the density of each RGS4 band was divided by the density of the corresponding MOR band (detected from approximately 54–70 kDa) to correct for any differences in sample loading. This experiment demonstrates that activation of MOR increases the association of RGS4 with the MOR/G protein complex in a GTP-dependent manner.

As anti-RGS4₁₇₀₋₂₀₅ has not previously been characterized, we compared it to ABT17 (a.k.a. U1079, anti-RGS4₁₋₂₀₅ from Millipore), an antibody known to be effective for detecting endogenous RGS4 from brain membranes (Krumins *et al.* 2004; Schwendt and McGinty 2007). In this experiment, the concentrations of protease and phosphatase inhibitors were increased (doubled) to improve the chances that ABT17 would detect endogenous RGS4. In this experiment, solubilized membranes were incubated with His₆RGS4 and DAMGO and immunoprecipitated with anti-MOR₁₀₋₇₀ in the absence or presence of GTP γ S. Both RGS4 antibodies not only detected an increase in His₆RGS4 binding to MOR in the presence of GTP γ S, but both antibodies detected an increase in the binding of endogenous RGS4 (lower band) in the presence of GTP γ S (Fig. 3a). When the experiment was repeated with the addition of 10 μ M MG132 but without any exogenous His₆RGS4, only the lower molecular weight form of RGS4 (the endogenous form) was detected in the immunoprecipitate (Fig. 3b). An over-exposure of the same blot reveals that both RGS4 antibodies recognized endogenous RGS4 in brain membranes (Figure S3b).

Direct interaction of Go α with RGS4

The direct interaction of Go α with RGS4 was investigated using solubilized rat brain membranes (Weems *et al.* 1996) and His₆RGS4 attached to Ni²⁺-NTA agarose resin. In these experiments His₆RGS4, pre-bound to Ni²⁺-NTA agarose resin, was incubated with solubilized rat brain membranes in the absence of guanine nucleotides or in the presence of 100 μ M GDP, 10 μ M GTP or 1 μ M GTP γ S for 1 h at 4°C in Buffer A with 4 mM Chaps (Fig. 4a). His₆RGS4-Ni²⁺-NTA resin was separated from the soluble fraction by

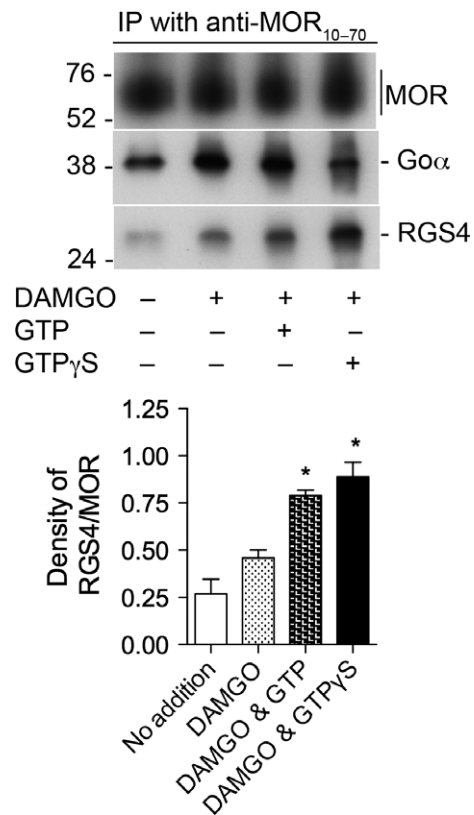


Fig. 2 Activation of MOR with [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO) stimulates the association of RGS4 with MOR in a GTP-dependent manner. Upper panel. Rat brain membranes were solubilized and processed as described in Methods (except in this experiment, intact membranes were not treated with DAMGO or guanine nucleotides prior to their solubilization). Soluble aliquots were incubated with 0.3 μ M RGS4 alone, 0.3 μ M RGS4, and 1 μ M DAMGO, 0.3 μ M RGS4, 1 μ M DAMGO and 10 μ M GTP or 0.3 μ M RGS4, 1 μ M DAMGO, and 1 μ M GTP γ S. Material immunoprecipitated by anti-MOR₁₀₋₇₀ was subjected to SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. The top section of the blot was incubated with anti-MOR₃₄₉₋₃₉₈, the middle section with anti-Go α , and the lower portion with anti-RGS4. The densities of all bands were determined with ImageJ. Lower Panel. The experiment depicted in the upper panel was repeated two additional times. In each experiment, the density of each RGS4 band was divided by the density of the corresponding MOR band. The values of RGS4/MOR for each test group are displayed in the bar graph as mean \pm SEM, $n = 3$. Of particular note, there was a 72% increase in the amount of RGS4 associated with MOR in the presence of DAMGO and GTP vs. DAMGO alone (unpaired, two-tailed *t*-test, * indicates $p < 0.008$). There was no difference in the densities of MOR among the four groups (two-way ANOVA, $p = 0.60$). There were differences in the levels of Go α among the groups: inclusion of DAMGO increased the amount of Go α (as was seen in Figure S2c) although the addition of GTP to DAMGO did not alter the amount of Go α associated with MOR (unpaired, two-tailed *t*-test, $p = 0.79$); and in six independent experiments, the addition of GTP γ S decreased the association of Go α with MOR by $57 \pm 7\%$ ($p = 0.02$). These data are summarized in Table 1.

Table 1 Relative changes in the levels of Go α and RGS4 that co-immunoprecipitated with MOR as a result of the presence of 1 μ M DAMGO alone or 1 μ M DAMGO in combination with either 10 μ M GTP or 1 μ M GTP γ S

	Go α , %	RGS4, %
No addition	100	100
DAMGO	256	150
DAMGO + GTP	225	256
DAMGO + GTP γ S	110	289

The relative amounts of Go α and RGS4 that were co-immunoprecipitated with MOR in the absence of agonist, or in the presence of DAMGO alone, or DAMGO in combination with either GTP or GTP γ S are shown. The values of Go α and RGS4 in the absence of either DAMGO or guanine nucleotides were set at 100%. Activation of MOR with DAMGO increases the association of Go α and RGS4 with MOR by 156% and 50%, respectively. Activation of MOR with DAMGO in the presence of 10 μ M GTP did not significantly affect the amount of Go α associated with MOR but increased the amount of RGS4 associated with MOR by an additional 72% (vs. DAMGO alone). The combination of DAMGO & GTP γ S caused a decrease in the level of Go α by 56% (vs. DAMGO alone) while causing an increase in the amount of RGS4 by 93% (vs. DAMGO alone). The data were compiled from 19 independent experiments where the densities of bands were quantified using ImageJ. Values of Go α and RGS4 were adjusted for the amount of MOR loaded in each lane of each gel.

centrifugation, and the pellet was washed 4 \times with Buffer A containing 4 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The relative amounts of Go α and RGS4 in the pull down were estimated by immunoblotting followed by band density measurements with ImageJ. To correct for possible gel loading discrepancies, the density of Go α in each lane was divided by the density of RGS4 in the same lane, and the ratios of Go α /RGS4 were compared among treatment groups. The inclusion of GDP or GTP failed to affect the association of Go α with His₆RGS4. However, the inclusion of GTP γ S caused a significant increase (3-fold) in the amount of Go α bound to His₆RGS4/Ni²⁺-NTA pull-down (Fig. 4a, unpaired *t*-test, *p* = 0.0001). In four independent experiments, the inclusion of GTP γ S during the His₆RGS4 pull-down caused a 2.8-fold increase in the amount of Go α in the pellet (*p* = 0.004). GTP γ S caused a concentration-dependent stimulation of Go α binding to RGS4 with an EC₅₀ of 0.12 μ M (Fig. 4b). The inclusion of 100 μ M GDP prevented 0.3 μ M GTP γ S from activating Go α and thus prevented GTP γ S from enhancing Go α binding to RGS4 (Fig. 4c). However, when 1 μ M DAMGO was included during the incubation, there was a 70% increase in the binding of Go α to RGS4 (*p* = 0.003, Fig. 4d). The addition of 1 μ M DAMGO alone (without guanine nucleotide) did not affect the binding of Go α to RGS4; nor did the addition of 1 μ M DAMGO further increase the amount of Go α binding to RGS4 caused by

0.3 μ M GTP γ S alone (data not shown). GTP γ S can also stimulate the binding of other G α subunits to RGS4, and studies are underway to determine if MOR activation can stimulate the binding of GTP γ S to these other G α subunits and cause them to associate with RGS4 (Figure S4).

RGS4 attenuates DAMGO-mediated inhibition of adenylyl cyclase activity RGS4 caused a concentration-dependent attenuation of DAMGO-mediated inhibition of adenylyl cyclase activity. Data from seven independent experiments (*n* = 28 for each data point) were combined to generate the curve shown in Figure S5. In the absence of RGS4, 1 μ M DAMGO plus 10 μ M GTP and 1 μ M DAMGO plus 1 μ M GTP γ S caused a 60.4 \pm 2.97 and 52.7% \pm 2.57% inhibition of adenylyl cyclase activity, respectively. The addition of increasing concentrations of RGS4 caused a concentration-dependent attenuation of DAMGO (plus GTP)-mediated inhibition of adenylyl cyclase (EC₅₀ of RGS4 = 83.6 \pm 1.5 nM with a 95% confidence interval (CI): 43.2–162 nM). In the presence of 3 μ M RGS4, DAMGO inhibited forskolin-stimulated adenylyl cyclase activity by only 21.8% \pm 2.27%. In contrast, RGS4 failed to attenuate GTP γ S-mediated inhibition of adenylyl cyclase activity.

Non-competitive nature of RGS4 in attenuating MOR-mediated inhibition of adenylyl cyclase activity DAMGO caused a concentration-dependent inhibition of adenylyl cyclase activity in homogenates of SHSY5Y cells (Fig. 5, left side). The EC₅₀s of DAMGO in the absence and in the presence of 1 μ M RGS4 were 40 nM (95% CI from 26.9 to 59.5 nM) and 66 nM (95% CI from 31.8 to 137 nM), respectively. In the absence and presence of 1 μ M RGS4, the maximal inhibitory effect of DAMGO was 53.7% \pm 1.72% inhibition of adenylyl cyclase activity (95% CI from 50.3% to 57.2%) and 28.9 \pm 1.83% inhibition of adenylyl cyclase activity (95% CI from 25.3% to 32.6% inhibition), respectively. Thus, RGS4 significantly diminished the maximal inhibitory effect (the efficacy) of DAMGO but did not significantly diminish the EC₅₀ (the potency) of DAMGO. Figure 5, left side, was generated from four independent experiments, *n* = 16 for each data point. Both His₆RGS4 and RGS4 (produced by cutting GST off GST-RGS4 with thrombin, see Figure S6a) were equally effective in attenuating the efficacy of DAMGO in the adenylyl cyclase assay. Therefore, the presence of the His₆ tag did not influence the effectiveness of RGS4. In contrast to the noncompetitive inhibition caused by RGS4, naltrexone (0.3 μ M), an opioid receptor antagonist, caused a rightward shift in the concentration–response curve to DAMGO that is typical of competitive inhibition (Fig. 5, right side). The EC₅₀s of DAMGO were 18.0 nM (95% CI from 10.5 to 30.8 nM) and 1.30 μ M (95% CI from 0.351 to 4.74 μ M) in the absence and presence of 0.3 μ M naltrexone, respectively. The maximal inhibition of adenylyl cyclase activity was unaffected by naltrexone. All data in Fig. 5 were analyzed by non-linear regression analysis using Prism[®] 5.

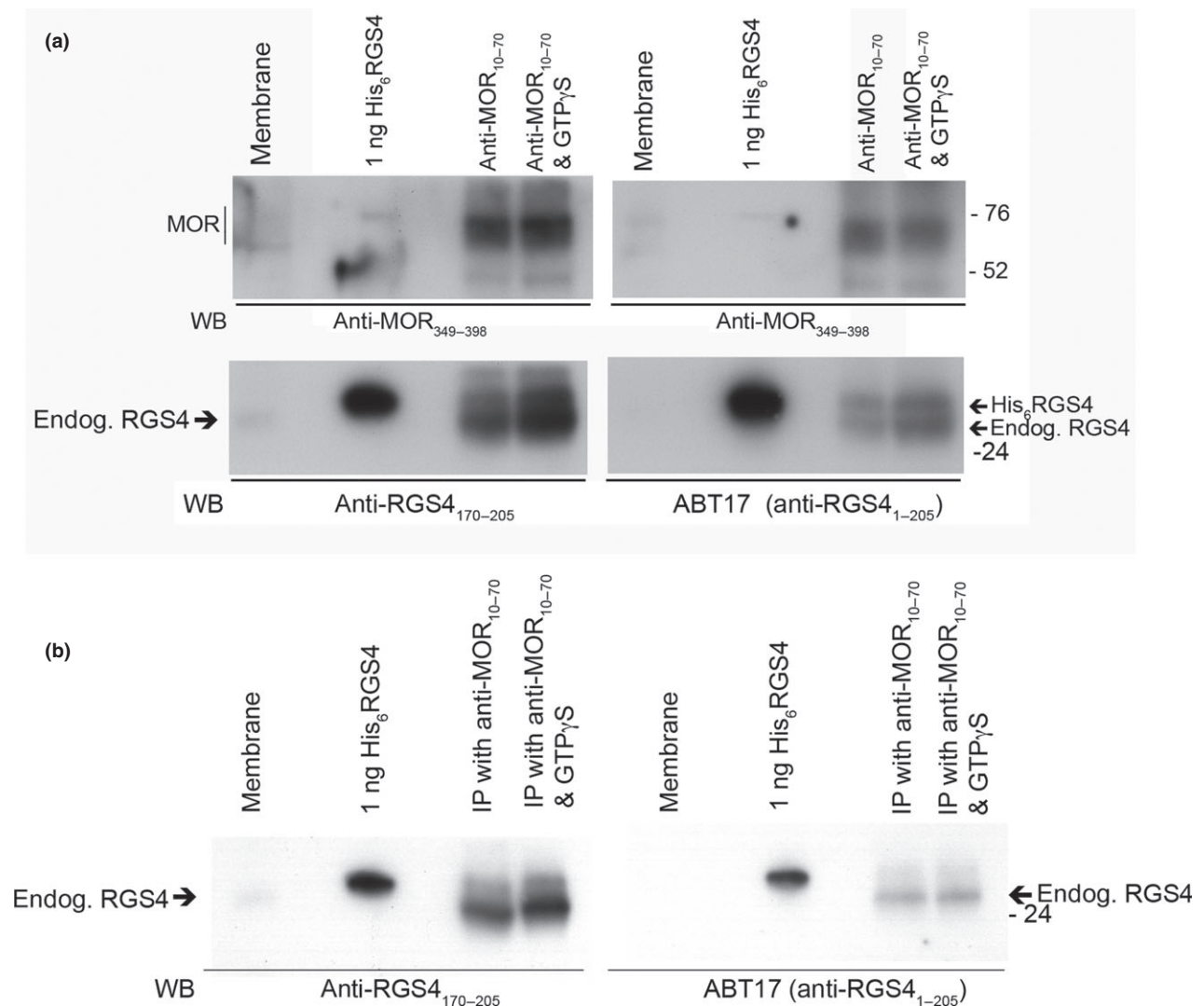


Fig. 3 Comparison of anti-RGS4₁₇₀₋₂₀₅ to anti-RGS4₁₋₂₀₅. A. In an experiment similar to the one depicted in Fig. 1a, solubilized MOR was immunoprecipitated either in the presence of [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO) and 1 μ M His₆RGS4 or in the presence of DAMGO, 1 μ M His₆RGS4 plus 0.3 μ M GTP γ S. However, in an attempt to minimize proteolysis of endogenous RGS4, the routine concentrations of protease and phosphatase inhibitors were doubled, and the material immunoprecipitated by anti-MOR₁₀₋₇₀ was screened with anti-RGS4₁₇₀₋₂₀₅ (a.k.a. 1554) and anti-RGS4₁₋₂₀₅ (a.k.a. ABT17 or U1079, an antibody capable of recognizing endogenous RGS4). The upper left and upper right panels were screened with 0.2 μ g/mL of anti-MOR₃₄₉₋₃₉₈ to ensure the presence of equal amounts of MOR. The lower left and lower right panels were screened for the presence of RGS4 with 2 μ g/mL of anti-RGS4₁₇₀₋₂₀₅ and a 1 : 2000 dilution of

Aspects of the MOR/G protein complex functioning not affected by RGS4. RGS4 failed to affect GTP γ S-mediated inhibition of adenylyl cyclase activity (Figure 6b). In concentration–response curves, the EC₅₀s of GTP γ S were 31.2 nM (95% CI from 20.5 to 47.5 nM) and 33.2 nM (95% CI from 21.9 to 50.1 nM) in the absence and

antiserum ABT17, respectively. Arrows to the right of lower panel mark the locations of His₆RGS4 and endogenous RGS4. The inclusion of GTP γ S appeared to increase the association of both His₆RGS4 and endogenous RGS4 (lower band) with MOR. This experiment was repeated one time with nearly identical results. Endogenous RGS4 was also detected in brain membranes by both anti-RGS4 antibodies. (b) When the same experiment depicted in A was repeated, but in the absence of exogenous RGS4, only the lower mol. weight band of RGS4 was detected in material co-immunoprecipitated with MOR. The inclusion of GTP γ S during the immunoprecipitation only modestly increased the association of RGS4 with MOR. When film was overexposed to this same blot, RGS4 was detected in brain membranes by both RGS4 antibodies (Figure S3b). Ten μ M of the proteasome inhibitor, MG132, was included in this experiment.

presence of 1 μ M RGS4, respectively. GTP γ S inhibited adenylyl cyclase activity to 43.7% of maximal activity (95% CI from 39.8 to 47.6% of maximal activity) and 39.4% of maximal activity (95% CI from 35.5 to 43.4% of maximal activity), in the absence and presence of 1 μ M RGS4, respectively.

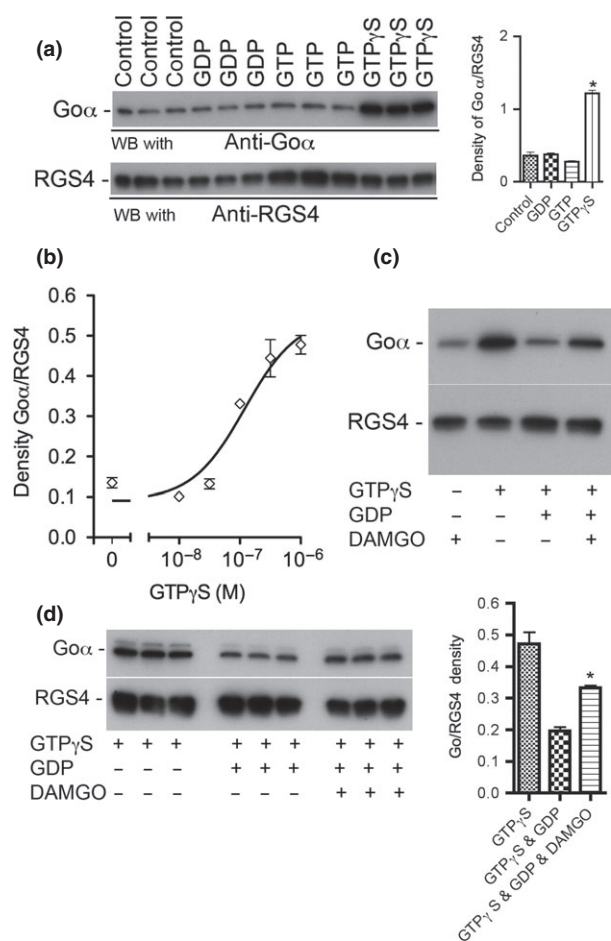


Fig. 4 [D-Ala², N-Me-Phe⁴, Gly⁵-o] enkephalin (DAMGO) stimulates GTP γ S binding to Go α in solubilized rat brain membranes and causes GTP γ S-activated Go α to bind to RGS4. Solubilized rat brain membranes were used in these experiments (see Methods). (a) Guanine nucleotides were tested for their ability to activate Go α and to cause activated Go α to bind to 0.3 μ M His₆RGS4 pre-bound to N²⁺-NTA agarose. Neither 100 μ M GDP nor 10 μ M GTP affected Go α binding to RGS4. However, 1 μ M GTP γ S caused a 3-fold increase in the density of the Go α band [right side of (a) unpaired, two-tailed *t*-test, *p* = 0.001 (the ratios of Go α /RGS4 in each lane were analyzed)]. In four independent experiments similar to that shown in (a), the addition of 1 μ M GTP γ S caused a 2.8-fold increase in the amount of Go α bound to RGS4 (unpaired, two-tailed *t*-test, * indicates *p* = 0.0044). (b) GTP γ S caused a concentration-dependent increase in the binding of Go α to RGS4 (*n* = 3). The EC₅₀ of GTP γ S was 1.2 \times 10⁻⁷ M. (c) The addition of 0.3 μ M GTP γ S caused an approximate 3-fold increase in Go α binding to RGS4 whereas the combination of 0.3 μ M GTP γ S and 100 μ M GDP caused Go α binding to RGS4 to return to the control level. However, when 1 μ M DAMGO was added along with 0.3 μ M GTP γ S and 100 μ M GDP, there was an increase in the binding of Go α to RGS4. (d) To confirm that activation of MOR with DAMGO could decrease the affinity of Go α for GDP and allow GTP γ S to activate Go α , the experiment depicted in (c) was performed again in triplicate. Activation of MOR with DAMGO caused a 70% increase in the amount of Go α binding to RGS4 [(d) right side bar graph: GTP γ S & GDP vs. GTP γ S, GDP & DAMGO, unpaired, two-tailed *t*-test, * indicates *p* = 0.001].

Also, RGS4 did not significantly alter DAMGO-stimulated binding of [³⁵S]GTP γ S to G proteins associated with SHSY5Y cell membranes (Figure S6c). This assay measures the ability of MOR activation by DAMGO to cause GDP to dissociate from cognate G proteins allowing [³⁵S]GTP γ S to bind to these G proteins. In this assay, the EC₅₀s of DAMGO were 39.0 nM (95% CI from 15.7 to 96.5 nM) and 62.0 nM (95% CI from 22.6 to 170 nM) in the absence and presence of 1 μ M RGS4, respectively. The maximal amounts of [³⁵S]GTP γ S bound in the absence and presence of 1 μ M RGS4 were 1432 \pm 58 dpm (95% CI from 1266 to 1598) and 1319 \pm 82 dpm (95% CI from 1149 to 1490), respectively. Finally, RGS4 did not affect the binding of [³H] DAMGO to MOR in SHSY5Y cell membranes (Figure S6d).

Discussion

RGS proteins have been established as negative regulators of all G protein-coupled receptors (Neubig and Siderovski 2002) including MOR (Zachariou *et al.* 2003; Rodriguez-Munoz *et al.* 2007; Leontiadis *et al.* 2009; Han *et al.* 2010; Talbot *et al.* 2010). This study focuses on the interaction of RGS4 with MOR and one of its cognate G proteins, Go, although Gi₃, when activated by GTP γ S, can also associate with RGS4 (Figure S4). We chose to study RGS4, because there is evidence that RGS4 acts as a GTPase activating protein for G_i/G_o-type G proteins, the type of G proteins that act as transducers for MOR and because RGS4 mRNA and protein are present in many brain regions that express MOR (Berman *et al.* 1996a,b; Gold *et al.* 1997; Hepler *et al.* 1997; Chalecka-Franaszek *et al.* 2000; Kruminis *et al.* 2004). Also, RGS4 has been implicated in the development of opiate physical dependence. RGS4 mRNA levels in the locus coeruleus doubled following 6 days of chronic morphine administration, and RGS4 knockout mice undergo a more severe withdrawal syndrome following acute withdrawal from morphine (Gold *et al.* 2003; Han *et al.* 2010). Surprisingly, RGS4^{-/-} mice do not show any major phenotype related to the MOR signaling system (Grillet *et al.* 2005); the co-expression of RGS8 and RGS4 in many regions of the brain suggests a functional redundancy between these two RGS proteins (Talbot *et al.* 2010). RGS4 is not the only RGS protein that modulates MOR activity, but RGS4 almost certainly modulates MOR activity in a number of brain regions. In this study, we show that endogenous RGS4 does indeed associate with MOR (Fig. 3). Future studies will focus on specific brain regions where RGS4 may regulate MOR signaling.

Association of RGS4 with the MOR/G protein complex

Earlier studies demonstrated that RGS4 binds directly to Gi/o-type proteins, particularly in the presence of aluminum, magnesium, and fluoride (AMF) (Berman *et al.* 1996a;

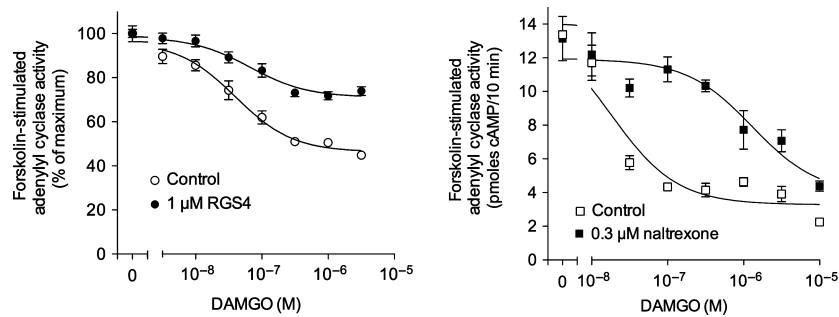


Fig. 5 RGS4 diminished the efficacy, but not the potency, of [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO) in inhibiting adenylyl cyclase activity (left panel); in contrast, naltrexone diminished the potency, but not the efficacy, of DAMGO (right panel). Left Panel. Adenylyl cyclase activity was measured in the presence of increasing concentrations of DAMGO in the absence (open circles) or the presence of 1 μM RGS4 (filled circles). The EC₅₀s of DAMGO in the absence and presence of RGS4 were 40.0 nM (95% confidence interval 27–59 nM) and 66.0 nM (95% confidence interval 32–137 nM), respectively (EC₅₀ values were not significantly different). In the absence and presence of 1 μM RGS4, DAMGO maximally inhibited adenylyl cyclase activity by 54% (95% confidence interval 25.3–32.6%), respectively (efficacies were significantly

different $p < 0.05$). Data from four independent experiments were combined, $n = 16$ for each point. Right Panel. Adenylyl cyclase activity was measured in the presence of increasing concentrations of DAMGO in the absence (open squares) or presence of 0.3 μM naltrexone (filled squares). The EC₅₀s of DAMGO in the absence and presence of naltrexone were 19 nM (95% confidence intervals 10.5–31 nM) and 1.30 μM (95% confidence intervals 0.351–4.74 μM), respectively ($p < 0.05$). The maximal inhibitions caused by DAMGO in the absence or presence of naltrexone were to 3.28 pmoles cAMP formed in 10 min (95% confidence intervals 2.44–4.01 pmoles) and 3.92 pmoles cAMP formed in 10 min (95% confidence intervals 1.28–6.56 pmoles), respectively (not significantly different). Data shown are from a single experiment ($n = 4$ for each point).

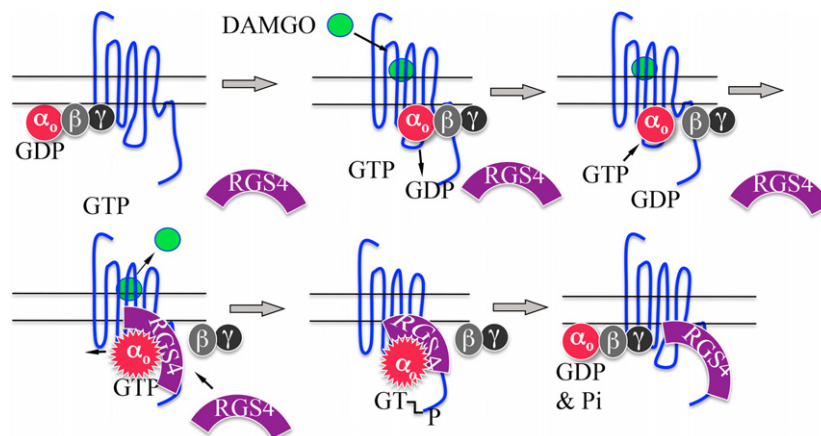


Fig. 6 Proposed model of the interaction of RGS4 with MOR and G_o. It is proposed that the unoccupied MOR (blue serpentine line) is weakly associated with Gi/Go-type G proteins (α_o-GDPβγ). Activation of MOR by [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO), a mu opioid agonist, causes α_o-GDPβγ to bind more tightly to MOR and causes GDP to dissociate from α_o. GTP now binds to and activates α_o (activated α_o shown as serrated red circle bound with GTP). RGS4 is

now attracted to 'activated' α_o-GTP. The interaction of RGS4 with α_o-GTP increases the intrinsic GTPase of α_o resulting the hydrolysis of GTP to GDP and Pi. α_o-GDP re-associates with βγ and, α_o-GDPβγ binds less tightly to MOR in the absence of agonist. We speculate the RGS4 may remain bound to the C terminal of MOR and remain situated in position to accelerate the inactivation of the next molecule of α_o-GTP.

Watson *et al.* 1996). As MOR is associated with Gi and Go-type G proteins, we investigated the interaction between RGS4 and the MOR/G protein complex from rat brain membranes. Previously, we developed a method to solubilize

and immunoprecipitate active MOR in association with Gi1, Gi3, and Go; the ratio of MOR to G protein was approximately one to one when the immunoprecipitation was performed in the presence of morphine (Weems *et al.* 1996;

Chalecka-Franaszek *et al.* 2000). In this study, we followed the co-immunoprecipitation of $G\alpha$ as a representative of the G proteins that interact with MOR because $G\alpha$ is the most abundant of G proteins in rat brain, and good $G\alpha$ antibodies are available for immunoblotting. MORs were immunoprecipitated with three different anti-MOR antibodies; each of these MOR antibodies caused the co-immunoprecipitation of RGS4 (shown for anti-MOR_{10–70} in Figs 1–3). The inclusion of the MOR agonist DAMGO during the immunoprecipitation caused a 150% increase in amount of $G\alpha$ and a 50% increase in RGS4, whereas the addition of GTP γ S (along with DAMGO) caused a three-fold increase in RGS4 and a 50% decrease in the amount of $G\alpha$ (Table 1). The concomitant increase in RGS4 and decrease in $G\alpha$ caused by GTP γ S suggests that activated G proteins attract RGS4 to the MOR/G protein complex and that a region of MOR may contribute to the continued binding of RGS4 to the complex following the dissociation of $G\alpha$. Leontiadis *et al.* (2009) have shown evidence that RGS4 binds directly to a portion of the C-terminal region of MOR (MOR_{329–355}), that is part of the 4th intracellular loop of MOR, without the assistance of G proteins. We speculate that G proteins that bind to the 3rd intracellular loop of MOR may occlude a portion of the 4th intracellular loop of MOR, the region of MOR that has been proposed to bind RGS4. When a GTP-activated G protein dissociates from MOR, RGS4 is able to bind to the 4th intracellular loop of MOR positioning RGS4 to rapidly inactivate the next GTP-activated G protein. Most importantly, in the current study, DAMGO stimulated the binding of RGS4 to the MOR/G protein complex in a GTP-dependent manner. This finding clearly shows that activation of MOR causes GTP to bind to its cognate G proteins (including Go) and that the activated G proteins attract RGS4 to the MOR/G protein complex. Interestingly, using the procedures described in Methods, 28 fmoles of RGS4 (determined by western blotting) were co-immunoprecipitated with 31 fmoles of MOR (determined by saturation [³H]DAMGO binding) in the presence of GTP γ S. Thus, when G proteins (like Go) are fully activated, one molecule of RGS4 becomes associated with one molecule of MOR.

Association of endogenous RGS4 with the MOR/G proteins complex When the concentrations of protease inhibitors were double and when the proteasome inhibitor was included during the immunoprecipitation of MOR, endogenous RGS4 remained intact and associated with MOR (i.e., it was not degraded), and therefore GTP γ S could only modestly increase the binding of endogenous RGS4 to MOR. We speculate that receptor activation (and subsequent G protein activation) causes RGS4 to bind to the MOR/G protein complex and that, after a period of time, RGS4 undergoes ubiquitination and proteolysis by the proteasome (Wang and Traynor 2011). The fact that His₆RGS4 could be detected in material immunoprecipitated with MOR suggests that His₆RGS4 is less susceptible to proteolysis than

endogenous RGS4. In preliminary experiments performed with His₆RGS4, we had observed a significant amount of ‘smearing’ in the location at and below ~26 kDa on western blots. We found that preparing our own cocktail of protease inhibitors that also included phosphatase inhibitors resulted in far less breakdown of His₆RGS4 as is indicated by the presence of discrete bands of His₆RGS4 around 26 kDa (as seen in Figs. 1 and 2). Clearly, even higher levels of inhibitors (2 \times) plus MG132 were needed to protect endogenous RGS4 from degradation (Fig. 3). Future studies will focus on the interactions of endogenous RGS proteins with MOR in distinct regions of the brain.

Association of $G\alpha$ with RGS4 – influence of MOR

The interaction of RGS4 with Go was also studied in a preparation of solubilized rat brain membranes that had been passed through a SephadexG50 column pre-equilibrated with 4 mM CHAPS in buffer A. Passage of the solubilized material through the SephadexG50 column removes all small molecules (including free peptides and nucleotides), reduces the CHAPS concentration to 4 mM, and maintains the MOR in a high affinity binding state that is sensitive to guanine nucleotides (Weems *et al.* 1996). In this soluble preparation, neither GTP nor GDP had an effect on the binding of $G\alpha$ to His₆RGS4-Ni²⁺-NTA agarose, but GTP γ S (0.3 μ M) caused a three-fold increase in the binding of $G\alpha$ to His₆RGS4 (Fig. 4a). GTP γ S caused a concentration-dependent increase in the binding of $G\alpha$ to RGS4 with an EC₅₀ of 0.1 μ M and a near maximal effect occurring at 0.3 μ M (Fig. 4b). Interestingly, an excess of GDP (100 μ M) blocked the activation of $G\alpha$ GTP γ S (0.3 μ M) and diminished the subsequent binding of $G\alpha$ to His₆RGS4. However, activation of MOR with DAMGO during the incubation with GDP and GTP γ S increased the binding of $G\alpha$ to His₆RGS4 (Fig. 4c and d). Presumably, 0.3 μ M GTP γ S activated the majority of solubilized Go-type G proteins, just as 0.3 μ M GTP γ S caused a near maximal inhibition of adenylyl cyclase activity (Figure S6b). GDP (100 μ M) blocks access of GTP γ S to $G\alpha$ and reduces the binding of $G\alpha$ to RGS4, whereas activation of MOR by DAMGO decreases the affinity of 40–50% of the $G\alpha$ molecules for GDP and resulted in the subsequent activation of these $G\alpha$ molecules by GTP γ S causing them ($G\alpha$ -GTP γ S) to bind to His₆RGS4-Ni²⁺-NTA agarose (Fig. 4d, right bar graph). Previously, we demonstrated that activation of MOR decreased the affinity of Gi/o-type G proteins for GDP (and thus caused GDP to dissociate from G proteins) because GDP could prevent GTP γ S from inhibiting adenylyl cyclase activity in the absence, but not in the presence of morphine (Gosse *et al.* 1989). Of course it has been accepted for decades that all GPCRs work by inducing the dissociation of GDP from their G proteins and allowing intracellular GTP to bind to, and to activate, their cognate G proteins (Gilman 1987). Indeed, in the experiment shown in Figure S6c, DAMGO stimulated the

binding of [³⁵S]GTPγS to SHSY5Y cell membranes in the presence of 10 μM GDP. Importantly, this experiment demonstrates that MOR activation of Goα caused GDP to dissociate from Goα, allowing GTPγS to bind to Goα and for Goα-GTPγS to bind to RGS4. These findings clearly demonstrate that RGS4 is capable of binding to G proteins that have been activated by MOR.

In agreement with our findings, Leontiadis *et al.* (2009) demonstrated that when MOR and RGS4 were co-transfected into HEK 293 cells, RGS4 could be co-immunoprecipitated with MOR, and more interestingly, when aluminum, fluoride, and magnesium ions were included during the MOR immunoprecipitation, the amount of RGS4 in the immunoprecipitate tripled.

Influence of RGS4 on MOR Signaling

RGS4 caused a concentration-dependent attenuation of MOR-mediated inhibition of adenylyl cyclase (Figure S5). We assume that the ability of RGS4 to attenuate DAMGO-mediated inhibition of adenylyl cyclase activity stems from its ability to accelerate the GTPase of G_{i/o}-type G proteins since RGS4 has a similar EC50 in both of these systems (Berman *et al.* 1996b; Watson *et al.* 1996). The inhibitory effect of RGS4 was found to be primarily noncompetitive because RGS4 diminished the efficacy, but not the potency, of DAMGO in inhibiting adenylyl cyclase activity (Fig. 5, left panel). In contrast, naltrexone, an opioid receptor antagonist, diminished the potency, but not the efficacy of DAMGO (Fig. 5, right panel). The noncompetitive nature of the RGS4 inhibition is consistent with the hypothesis that RGS4 stabilizes the transition state of the intrinsic GTPase activity of G_{i/o}-type G proteins as opposed to its having a direct effect on the ligand binding site on the receptor (Berman *et al.* 1996a,b; Watson *et al.* 1996; Tesmer *et al.* 1997). In contrast to its attenuating effect on MOR signaling, RGS4 had no effect on GTPγS-induced inhibition adenylyl cyclase, a finding consistent with the resistance of GTPγS to hydrolysis by GTPase. Also, RGS4 did not affect DAMGO-stimulated [³⁵S]GTPγS binding to SHSY5Y membranes (Figure S6c). The rate-limiting step in stimulating the binding of GTP to G proteins is the dissociation of GDP from the G protein (Gilman 1987). As DAMGO activation of MOR stimulates the release of GDP from Gi and Go in enhancing the binding of [³⁵S]GTPγS to Gi and Go, we conclude that RGS4 has no major effect on the coupling of MOR to its G proteins or on the activation of these G proteins by the receptor and guanosine triphosphates. Also, RGS4 had no significant effect on either the affinity of MOR for [³H]DAMGO or on the maximal number of [³H]DAMGO binding sites (Figure 6d). Because high affinity agonist binding requires the association of the receptor with its G proteins, these results provide further evidence that RGS4 does not interfere with the coupling of MOR to its G proteins.

Proposed mechanism of RGS4 attenuation of MOR signaling

In the absence of an agonist, MOR (blue serpentine line) is associated weakly with its cognate G proteins, Gi1, Gi3, and Go (represented by αβγ) and perhaps is also temporarily associated with RGS4 (until RGS4 is proteolyzed) (Fig. 6). When DAMGO, a mu opioid agonist, binds to and activates MOR, Go (αβγ) binds more tightly to MOR, and MOR causes a decrease in the affinity of Goα for GDP. The dissociation of GDP from Goα allows GTP to bind to, and to activate, Goα. A Goα-GTP (circle) dissociates slightly from Gβγ and slightly from MOR, and Goα-GTP triggers downstream signaling (e.g., inhibition of adenylyl cyclase activity, opening of K⁺ channels, etc.). RGS4 binds to activated Goα-GTP and perhaps simultaneously to the C-terminal region of MOR (Leontiadis *et al.* 2009); the binding of RGS4 to Goα-GTP increases the intrinsic GTPase of Goα [RGS4 stabilizes the transition state of Goα (Kimple *et al.* 2011)]. Once GTP is hydrolyzed to GDP, Goα-GDP becomes weakly associated with MOR, and we speculate that RGS4 remains associated with the C-terminal region of MOR where it is in position to rapidly inactivate the next round of Goα-GTP produced by the activation of MOR. After a period of time, RGS4 is removed from the MOR/G protein complex by ubiquitination and proteolysis by the proteasome.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Anti-MOR349–398 identifies MOR immunohistochemically in sections of rat brain.

Figure S2. Immunoprecipitation of the MOR/G protein complex Panel A.

Figure S3. Specificity of anti-RGS4170–205 in western blots and ability of anti-RGS4170–205 to recognize endogenous RGS4 A.

Figure 4. GTPγS stimulated the binding of Goα and Gi3α to RGS4.

Figure S5. RGS4 caused a concentration-dependent attenuation of DAMGO-mediated inhibition of adenylyl cyclase activity.

Figure S6. Influence of RGS4 on the signaling of MOR in SHSY5Y cell membranes.

Table S1. Antibodies used in the current study.

Table S2. Pre-treatment of SHSY5Y cells with DAMGO enhanced the ability of RGS4 to attenuate DAMGO-mediated inhibition of adenylyl cyclase activity in cell homogenates.

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