Activation of \(\mu\)-Opioid Receptors Transfers Control of Ga Subunits to the Regulator of G-protein Signaling RGS9-2

ROLE IN RECEPTOR DESSENSITIZATION*

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In mouse periaqueductal gray matter (PAG) membranes, the \(\mu\)-opioid receptor (MOR) coprecipitated the \(\alpha\)-subunits of the \(G_{i/o/q}^{\text{GAP}}\) proteins, the \(G_{\beta}\) subunits, and the regulator of G-protein signaling RGS9-2 and its partner protein \(G_{\gamma}\). RGS7 and RGS11 present in this neural structure showed no association with MOR. In vivo intracerebroventricular injection of morphine did not alter MOR immunoreactivity, but 30 min and 3 h after administration, the coprecipitation of Ga subunits with MORs was reduced by up to 50%. Furthermore, the association between Ga subunits and RGS9-2 proteins was increased. Twenty-four hours after receiving intracerebroventricular morphine, the Ga subunits left the RGS9-2 proteins and re-associated with the MORs. However, doses of the opioid able to induce tolerance promoted the stable transfer of Ga subunits to the RGS9-2 control. This was accompanied by Ser phosphorylation of RGS9-2 proteins, which increased their coprecipitation with 14-3-3 proteins. In the PAG membranes of morphine-desensitized mice, the capacity of the opioid to stimulate G-protein-related guanosine 5'-O-(3-[\(^{35}\)S]thiotriphosphate) binding as well as low \(K_m\) GTPase activity was attenuated. The in vivo knockdown of RGS9-2 expression prevented morphine from altering the association between MORs and G-proteins, and tolerance did not develop. In PAG membranes from RGS9-2 knockdown mice, morphine showed full capacity to activate G-proteins. Thus, the tolerance that develops after administration, the coprecipitation of Ga subunits with MORs was reduced by up to 50%. Furthermore, the association between Ga subunits and RGS9-2 proteins was increased. Twenty-four hours after receiving intracerebroventricular morphine, the Ga subunits left the RGS9-2 proteins and re-associated with the MORs. However, doses of the opioid able to induce tolerance promoted the stable transfer of Ga subunits to the RGS9-2 control. This was accompanied by Ser phosphorylation of RGS9-2 proteins, which increased their coprecipitation with 14-3-3 proteins. In the PAG membranes of morphine-desensitized mice, the capacity of the opioid to stimulate G-protein-related guanosine 5'-O-(3-[\(^{35}\)S]thiotriphosphate) binding as well as low \(K_m\) GTPase activity was attenuated. The in vivo knockdown of RGS9-2 expression prevented morphine from altering the association between MORs and G-proteins, and tolerance did not develop. In PAG membranes from RGS9-2 knockdown mice, morphine showed full capacity to activate G-proteins. Thus, the tolerance that develops following an adequate dose of morphine is caused by the stabilization and retention of MOR-activated Ga subunits by RGS9-2 proteins. This multistep process is initiated by the morphine-induced transfer of MOR-associated Ga subunits to the RGS9-2 proteins, followed by Ser phosphorylation of the latter and their binding to 14-3-3 proteins. This regulatory mechanism probably precedes the loss of MORs from the cell membrane, which has been observed with other opioid agonists.

The \(\mu\)-opioid receptor (MOR), which belongs to the family of seven-transmembrane G-protein-coupled receptors (GPCRs), is a heavily N-glycosylated protein (1, 2) that regulates \(G_{i/o/q}^{\text{GAP}}\) proteins (Ref. 3 and references therein) through a direct interaction (4, 5). In the central nervous system, MORs play an important role in the antinociceptive action of opioids, but they become desensitized after repeated administration. Such tolerance is observed even after a single dose of an opioid agonist and can persist for 3 days (6, 7). This common characteristic of opioids that act via MORs is a serious drawback with respect to their long-term use as analgesics for the treatment of chronic pain. The phosphorylation of intracellular residues, followed by internalization, is the most widely accepted mechanism involved in GPCR desensitization. Thus, upon agonist challenge and the release of activated Ga-GTP subunits, GPCRs undergo phosphorylation by \(G_{\beta}\)-bound G-protein-coupled receptor kinases (GRKs), followed by arrestin binding (8). This has been amply documented for the \(\mu\)-, \(\delta\)-, and \(\kappa\)-opioid receptors in in vitro cell expression systems (for example, see Ref. 9).

The desensitization and internalization of MOR are agonist-dependent processes. In contrast to what has been observed for opioids such as etorphine and \(\delta\)-Ala\(^\delta\), NMe-Phe\(^\delta\), Gly\(^\delta\)-ol enkephalin, a number of reports describe the ability of morphine to activate the MAPK (mitogen-activated protein kinase) pathway and to desensitize the MORs without causing receptor phosphorylation and arrestin/dynamin-dependent internalization (10–15). It is therefore possible that receptor down-regulation is promoted only by agonists with high binding affinity for these receptors (16). Indeed, tightly bound agonists increase the probability that GRKs will inactivate the GPCRs since these kinases act only on agonist-occupied receptors (for example, see Ref. 17). The in vivo attained desensitization of MORs can be influenced by agents targeted to the specific RGS (regulator of G-protein signaling) proteins belonging to the R7 subfamily. The mammalian RGS proteins that act as GTPase-activating proteins (GAP) for Ga-GTP subunits are grouped into five subfamilies according to structural and genetic similarities: Rz, R4, R7, R12, and RA (18). The members of the RGS-R7 subfamily in the central nervous system (RGS6, RGS7, RGS9-2, and RGS11) associate mostly with cell membranes. Their sequences contain the GGL (G-protein \(\gamma\)-subunit-like) domain that binds to the \(G_{\gamma}\) protein, but not to the other \(G_{\beta}\) subunits (19, 20). In nervous tissues, the RGS-R7 and \(G_{\gamma}\) proteins are always found as dimers, indicating that this association is required for their GAP function on the corresponding Ga-GTP subunits. The RGS-R7 proteins show negative regulatory activity on the intensity of signals originating at MORs; thus, they participate in the development of tolerance to ago-
nist effects (21, 22). RGS9-2 is particularly important with respect to this function because its impairment provokes an increase in the potency of μ-opioid agonists and prevents (or at least delays) the appearance of MOR desensitization (7, 21, 23).

The RGS-R7 proteins display stronger affinity for the G_{oq} subunits in their transition state, when the Ga subunit initiates the spontaneous metabolism of GTP into the effector-inactive GDP form, than for the Ga-GTP form (24). Because RGS-R7 proteins can efficiently activate only the GTPase in sonicated (two cycles of 5 s each) PAG membranes in a volume of 400 μl, RGS9-2 proteins serve to control the intensity of agonist signaling by reducing the pool of receptor-regulated G-proteins (22). In this scenario, a fraction of the morphine-activated G_{oq} subunits is retained by the RGS9-2 proteins, leading to MOR desensitization. To determine whether this is the case, we analyzed the influence of RGS9-2 proteins on the changes that morphine induces in MOR regulation of G-proteins. The study was performed using membranes isolated from periaqueductal gray matter (PAG), a neural structure that plays a major role in mediating the effects of opioids when administered by the intracerebroventricular route (26). Morphine was found to induce a dose-dependent transfer of Ga subunits from the MORs to the RGS9-2 proteins that correlates with the attenuation of the morphine-induced activation of Ga subunits and its antinociceptive effects.

**EXPERIMENTAL PROCEDURES**

**Preparation of Membranes from Mouse PAG—**Male albino CD-1 mice (Charles River Laboratories España, S. A., Barcelona, Spain) weighing 22–25 g were killed by cervical dislocation, and the PAG was removed. After the PAG was removed around the aqueduct was taken from 2-mm-thick coronal sections (MP-600 micropunch, Activational Systems Inc.). The structures from 20 mice (for direct analysis) or from six mice (for immunoprecipitation studies) were washed and pooled in ice-cold 25 mM Tris-HCl (pH 7.7), 1 mM EDTA, and 0.32 M sucrose supplemented with a protease inhibitor mixture (catalog no. P8340, Sigma), a phosphatase inhibitor mixture (catalog no. P2850, Sigma), and H-89 (catalog no. B1427, Sigma). The PAG membranes were then obtained. The tissue was homogenized in a Polytron homogenizer (Model PT 10/35) for 15 s at setting 3. The homogenate was centrifuged (Sorvall RC5C centrifuge, SS-34 rotor) at 1000 × g for 10 min to remove the nuclear fraction. After the pellet was discarded, the supernatant was centrifuged at 20,000 × g for 20 min to obtain the crude synaptosomal pellet (P2). After two cycles of washing, followed by resuspension in buffer and centrifugation, the final pellet was diluted in Tris buffer and analyzed as described below.

**Co-immunoprecipitation of Signaling Proteins—**Affinity-purified IgGs raised against MORs (2, 3) and RGS9-2 proteins (catalog nos. sc-8142 and sc-8143, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were labeled with biotin (catalog no. B1022, Sigma) following the manufacturer’s instructions. MORs and RGS9-2 proteins were immunoprecipitated from the solubilized P2 fraction of mouse PAG as described (22) with minor modifications. Negative controls were performed with IgGs heated for 10 min at 100 °C or pre-absorbed with 0.1 mg of antigenic peptide for 1 h at room temperature. Pilot assays were performed to adjust the amount of IgGs and sample protein and to determine the incubation period required to precipitate the desired protein in a single run. Thus, in any second precipitation, only a remnant of the immunoprecipitation was expected to remain. The precipitated protein (MOR or RGS9-2) was performed in 400 μl of ice-cold buffer containing 50 μM Tris-HCl (pH 7.7), 50 mM NaCl, 1% Nonidet P-40, 50 μM of protease and phosphatase inhibitor mixtures, and H-89. Membranes were solubilized overnight at 4 °C and centrifuged at 10,000 × g for 10 min. The supernatant was then cleared with 20 μl of streptavidin-agarose and then centrifuged at 4 °C, followed by centrifugation at 3000 × g for 5 min. The solubilized material was incubated overnight at 4 °C with 10 μl (3 μg) of affinity-purified biotinylated IgGs raised against either MORs or RGS9-2 proteins. Fifty microliters of streptavidin-agarose were added, and incubation was continued for an additional 90 min at 4 °C. The samples were then centrifuged at 3000 × g for 5 min, and the supernatant was removed. The agarose pellets were subjected to five cycles of washing, followed by centrifugation and resuspension in 1 ml of Nonidet P-40 buffer. At the end of this process, the agarose pellets were heated in 300 μl of 40 mM Tris-HCl and 1% SDS for 10 min at 100 °C to denature the proteins contained in the immunocomplexes. The mixture was then cooled to room temperature, and the streptavidin-agarose was separated in centrifugal filter devices with a 0.45-μm pore (Ultracel-MC, Millipore Iberica S. A.). To prevent interference in the visualization of Western blots, the biotinylated IgGs detached during the initial heating were selectively removed by the addition of octyl thioglycoside at a final percentage of 0.65% in 400 μl plus 30 μl of fresh streptavidin-agarose. After 2 h at 4 °C, the samples were centrifuged for 5 min at 10,000 × g, and the streptavidin-agarose with the attached biotinylated IgGs was discarded. The proteins in the soluble fraction were concentrated in centrifugal filter devices (10,000-Da nominal molecular mass limit; Amicon Microcon YM-10, catalog no. 42407, Millipore Iberica S. A.). The proteins were solubilized in 2× Laemmli buffer with mercaptoethanol by heating at 100 °C for 3 min and then left to cool to room temperature before resolving by SDS-PAGE (10–16% total acrylamide concentration, 2.6% bisacrylamide cross-linker concentration). The above procedure supplied enough protein to load four to six gel lanes. The proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) for Western blot analysis and incubated with the antibodies in Hoefer TM Deco-Probe chambers (catalog no. PR150, Amersham Biosciences, Barcelona).

**Detection of Signaling Proteins in Mouse PAG Membranes by Electrophoresis and Immunoblotting—**The proteins from P2 membranes were resolved by SDS-PAGE on 8×11×1.5-cm gel slabs (10–20%). For immunodetection, 40–60 μg of PAG protein/lane were typically used. The separated proteins were transferred to 0.2-μm polyvinylidene difluoride membranes (Bio-Rad) for Western blot analysis and incubated with the antibodies in Hoefer TM Deco-Probe chambers (catalog no. PR150, Amersham Biosciences, Barcelona).

The antibodies were diluted in Tris-buffered saline and 0.5% Tween 20 and incubated with the transfected membranes at 6 °C for 2 h. Primary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L; catalog no. 170-6515, Bio-Rad), horseradish peroxidase-conjugated donkey anti-goat IgG (catalog no. sc-2012, Santa Cruz Biotechnology, Inc.) (7, 21) diluted 1:2000; and antibodies mapping a 14-3-3 epitope at the N terminus (recognizing isoforms β, γ, θ, ε, ζ, η, and σ; sc-629G, Santa Cruz Biotechnology, Inc.) (22) diluted 1:3000. The phosphoserin detection kit (catalog no. 525282, Calbiochem) recognizes phosphoserine in different amino acid environments. The mouse monoclonal antibodies (anti-IC5, 4A5, 4A9, and 4A8) were used at 0.1 μg/ml to study Ser phosphorylation of RGS9-2 proteins.

The antibodies were diluted in Tris-buffered saline and 0.05% Tween 20 and incubated with the transfected membranes at 6 °C for 2 h. Primary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L; catalog no. 170-6515, Bio-Rad), horseradish peroxidase-conjugated donkey anti-goat IgG (catalog no. sc-2012, Santa Cruz Biotechnology, Inc.) (7, 21) diluted 1:2000; and antibodies mapping a 14-3-3 epitope at the N terminus (recognizing isoforms β, γ, θ, ε, ζ, η, and σ; sc-629G, Santa Cruz Biotechnology, Inc.) (22) diluted 1:3000. The phosphoserin detection kit (catalog no. 525282, Calbiochem) recognizes phosphoserine in different amino acid environments. The mouse monoclonal antibodies (anti-IC5, 4A5, 4A9, and 4A8) were used at 0.1 μg/ml to study Ser phosphorylation of RGS9-2 proteins.
MOR-selective Linkage of RGS Proteins—In synaptosomal preparations of mouse PAG, the antibodies against MORs precipitated glycoylated proteins of 55–65, 70–80, and 100–110 kDa. These proteins were recognized by antibodies directed against different epitopes of the receptor protein. N-Glycosidase F increased the electrophoretic mobility of all these bands to the size of the protein predicted from the MOR amino acid sequence (2). Thus, the different sized moieties corresponded to degrees of glycosylation of MOR. No immunoprecipitation was seen when the anti-MOR IgGs were pre-absorbed with the antigenic peptides or when heat-inactivated IgGs were used (Fig. 1A).

The MORs coprecipitated various signaling elements: Gα subunits, Gβγ subunits, and RGS9-2 and its associated Gβ protein (Fig. 1B). Notably, other members of the RGS-R family present in PAG synaptosomal fractions (RGS7 and RGS11) showed no association with this receptor (Fig. 1C).

Although, in the mouse, the 77-kDa RGS9-2 protein is found in large amounts in striatal membranes, it is also present in other neural structures such as the cerebral cortex and PAG (7, 23, 30). The antibody directed against the RGS9-2 N-terminal sequence gave more intense signals than that directed against the C-terminal domain (Fig. 1C). The 675-residue RGS9-2 protein bears Ser467, Thr457, and Thr458 in its C terminus, groups associated with high phosphorylation potential (NetPhos Version 2.0 Prediction Server, Center for Biological Sequence Analysis). Thus, specific phospho-specific GTPase assay and immunoblot analysis of endogenous regulation of this protein could account for the differences in RGS9-2 immunosignals obtained with the anti-C-terminal domain antibody in the cerebral cortex and PAG (Fig. 1C).

Effect of in Vivo Intracerebroventricular Injection of Morphine on the Association of MOR with Go Subunits in PAG—The effect of the acute administration of morphine on the association of G signaling proteins with MORs was analyzed. Two different doses of morphine (3 and 10 nmol) were studied in the production of acute tolerance. These morphine priming doses were given to distinct groups of mice by the intracerebroventricular route; and at the time the effect peaked (after 30 min), they produced analgesic effects of −50 and 80% of the maximum possible effect for this test (cutoff time of 10 s). At the 120-min interval, only the 10-nmol morphine dose produced a remnant 20% of the maximum possible effect (Fig. 2, left panel) at 24 h, no analgesic activity was detected (data not shown). To determine whether the morphine priming doses produced tolerance, the effect of the same doses given 24 h later (test doses) was examined. Only the 10-nmol morphine dose greatly reduced the response of the test dose (Fig. 2, right panel). The 3-nmol morphine priming dose induced no tolerance to a test dose of 10 nmol (data not shown). This phenomenon, known as acute tolerance, appears within hours of agonist administration and lasts for 2–3 days. The threshold dose needed to produce this long-lasting tolerance to morphine is about three to four times greater than that required to produce detectable analgesia in this test (6, 21, 31).

The administration of the desensitizing dose of 10 nmol of morphine promoted no noticeable changes in the pattern of precipitated MORs when studied in PAG membranes from mice killed 24 h later (Fig. 3A), and the opioid did not alter the levels of the Go subunits present in the PAG membranes (data not shown). However, the number of Go subunits that coprecipitated with the MORs was reduced at the 30-min, 3-h, and
24-h post-opioid intervals. These reductions ranged from 40 to 60% for G/i/o/z subunits and somewhat less for G/q/11 subunits ([20%]. Fig. 3B). 3 nmol of morphine also reduced the association of MORs with the G/i/o/z subunits, but to a lesser extent. This effect was observed 30 min and 3 h after injecting the opioid, but not at 24 h (Fig. 3C). This dose of the opioid was devoid of desensitizing capacity 24 h after its intracerebroventricular injection (Fig. 2).

**FIG. 1.** Coprecipitation of RGS9-2 proteins with MORs in PAG membranes. PAG membranes were solubilized with 1% Nonidet P-40 and incubated overnight at 4 °C with affinity-purified biotinylated IgGs raised against the second external loop (amino acids 208–216) or the N terminus (amino acids 2–16) of MOR. Immunocomplexes were precipitated with streptavidin-agarose, resolved by SDS-PAGE, and visualized by Western blotting. A, upper panel, lanes 1–3, immunoprecipitation was performed with affinity-purified IgGs directed against the N-terminal epitope. Blots were probed with IgGs directed against the second external (2nd-Ext) loop sequence on MOR. Lanes 4–6, the second external loop epitope was used to precipitate the immunocomplexes. The probe was directed against the N-terminal sequence. Specificity of precipitation was determined by boiling MOR-directed IgGs at 100 °C for 10 min (lanes 1 and 6) or preincubating the IgGs with 0.1 mg of the corresponding antigenic peptide for 1 h at room temperature (lanes 2 and 5) before starting the immunoprecipitation procedure. Lower panel, the proteins coprecipitated with the MORs were assayed with antibodies to G/α/β/γ subunits. B, immunoprecipitates were also probed with anti-RGS7, anti-RGS9-2, anti-RGS11, and anti-G/α/β/γ antibodies. No G/α/β/γ or RGS proteins were coprecipitated with pre-absorbed or heat-inactivated anti-MOR IgGs. C, control PAG membranes were SDS-solubilized, PAGE-resolved, Western-blotted, and probed with antibodies to the RGS and G/α/β/γ proteins. Inset, synaptosomal membranes from the cerebral cortex, PAG, and striatum were probed with antibodies directed against the N-terminal (N) and C-terminal (C) sequences of RGS9-2 proteins.

**FIG. 2.** Absence of desensitizing capacity of morphine in mice with reduced levels of RGS9-2. The capacity of morphine to produce acute tolerance was studied in mice that had received the active ODN against RGS9-2 mRNA, a mismatched ODN (control), or saline (control) for 5 consecutive days (upper panels). On day 6, the time course for the analgesic effects of priming doses of 3 and 10 nmol of morphine was studied by the warm water (52 °C) tail-flick test. After 24 h, identical doses of the opioid (test dose) served to evaluate the development of tolerance in these animals. Since no differences were observed in the responses of saline- and mismatched ODN-injected mice to morphine, only the data from the mismatched ODN tests are shown as a control. Data are expressed as a percentage of the maximum possible analgesic effect (cutoff time of 10 s) and are the means ± S.E. from groups of 10–15 mice. *, significantly different from the corresponding control group injected with the mismatched ODN (ANOVA/Student-Newman-Keuls test, p < 0.05). The diminishing effects of the active ODN directed against RGS9-2 mRNA are shown (lower panels). The data are representative of three experiments performed in PAG membranes obtained from different groups of mice. At the end of the ODN treatment, the mice were killed on day 6, and the PAG membranes were obtained. **ODN:** M, mismatched ODN; **ODN:** RGS9-2, active ODN. The Western blots were probed with antibodies directed against members of the RGS-R7 subfamily (RGS9-2 N- and C-terminal sequences, RGS7, and RGS11) and the G/α/β/γ subunits.
and in the flanking regions establish critical contacts with the S (33). Residues in this motif consist of a consensus sequence, (K/R)DpSY(P/A), which is present in RGS-R7 subfamily members and has a protein kinase A phosphorylation site. This putative 14-3-3-binding motif is conserved in RGS domains (32). Phosphorylation in RGS9-2 function was therefore analyzed. In mouse PAG, RGS9-2 coprecipitates a series of signaling molecules, including the Ga12, Ga13, and Ga16 subunits; the Gβ2 subunit that associates with RGS9-2 proteins; and the phosphoprotein-binding 14-3-3 proteins (22). Although the electrophoretic mobilities of these proteins are clearly different from that of RGS9-2, the possibility exists that unidentified phosphoproteins might coprecipitate with RGS9-2 and interfere with the phosphoserine analysis. We therefore sought to detach any accompanying proteins from the RGS9-2 proteins prior to performing immunoprecipitation. The PAG synaptosomal membranes were thus heated in 40 mM Tris-HCl and 1% SDS for 10 min at 100 °C under reducing conditions. This mixture was then cooled to room temperature; the SDS concentration was reduced by adding octyl thioglucoside to a final percentage of 0.65%. Under these conditions, there is only a low probability that the denatured proteins will re-associate. The RGS9-2 immunoprecipitation was then performed with an antisera directed against the N terminus. Of the four anti-Ser(P) antibodies studied, only clone 1C8 gave immunosignals on RGS9-2 proteins. This clone recognizes Ser(P) in sequences acted upon by protein kinases A and C. In PAG from control mice, this protein showed a certain level of Ser phosphorylation; after receiving the dose of morphine that produced acute tolerance (10 nmol), the phosphorylation was notably increased at 30 min (200%), 3 h (100%), and 24 h (50%) (Fig. 4B).

After the solubilization of PAG membranes under nonreducing conditions, the Ga12, Ga13, and 14-3-3 proteins coprecipitated with RGS9-2 proteins (Fig. 4C). The experimental conditions we used were aimed at maintaining all existing associations between RGS9-2 proteins and Go subunits. Therefore, solubilization of PAG membranes and immunoprecipitation were conducted in the absence of guanine nucleotides or agents that could force the Go subunits to copy the transition state. This situation is not directly comparable with that used to detect the binding of RGS domains or complete sequences to different classes of Go subunits in protein mixtures or when coupled to a gel matrix. Under these circumstances, the addition of GDP and AlF4- is critical for the Go subunits to switch to the hydrolytic transition state and hence to promote their binding to the RGS domains (for example, see Refs. 35 and 36). In our assays, we pulled down physiological protein complexes formed before the animals were killed. In these complexes, it is probable that the endogenous guanine nucleotides determined whether the Go subunits associated with either MORs or RGS9-2 proteins. However, if RGS domains can associate only with the GTP- or GDP-bound forms of Go, and were Go-GTP to be metabolized into GDP during the immunoprecipitation procedure at 4 °C, no association of RGS domains with Go would be observed. Since, at the end of the immunoprecipitation, we obtained RGS9-2-Go complexes, it is probable that a durable association was favored by the post-translational modification of these proteins or an interaction with third partner proteins.

Twenty-four hours after intracerebroventricular injection of 10 nmol of morphine, a tendency to increase the association between Ga12, Ga13, and Ga16 subunits; the Gβ2 subunit that associates with RGS9-2 proteins; and the phosphoprotein-binding 14-3-3 proteins was observed. The 14-3-3 proteins and Ga12 subunits showed statistically significant time-dependent increases in their coprecipitation with RGS9-2 proteins. At the 30-min post-opioid interval, this was augmented by 140 and 100%, respectively; at 3 and 24 h, it diminished to ~60% in both cases (Fig. 4C). This pattern was paralleled by RGS9-2 Ser(P) signals. These were also attenuated at the later post-morphine intervals (Fig. 4B). The lower dose of 3 nmol of morphine produced a slight RGS9-2 phosphorylation plus a moderate association of Go and 14-3-3 proteins with these RGS9-2 proteins. This was observed at the 30-min and 3-h post-opioid intervals; but 24 h later, no changes were detectable (data not shown).
Notably, at the 24-h post-opioid interval, the 10-nmol (but not the 3-nmol) dose promoted significant reductions in the number of Gα subunits that remained associated with the MORs (Fig. 3B). Therefore, the loss of G-proteins that coprecipitated with MORs brought about by high doses of morphine correlated with increases in their association with RGS9-2 proteins and also with MOR desensitization.

The influence of RGS9-2 proteins on the regulation of MOR signaling was then studied by reducing the expression of these RGS proteins in PAG. The efficacy and selectivity of the ODN treatment used have been previously assessed using antibodies directed against RGS9-2 (7). In the present work, after mouse PAG proteins were resolved by SDS-PAGE, the same antibodies detected reductions of some 50% of the 77-kDa RGS9-2 protein caused by the ODN treatment (Fig. 2). In mice with reduced PAG levels of RGS9-2 proteins, the antinociception promoted by 10 nmol of morphine increases, and notably, tolerance to the acute dose of morphine does not develop (7, 21–23). In the present work, we observed that RGS9-2 knockdown prevented MORs from becoming desensitized by 10 nmol of intracerebroventricular morphine (Fig. 2). This correlated with the MORs maintaining their association with the G-proteins in the PAG membranes from these morphine-treated RGS9-2 knockdown mice (Fig. 4D).

**Influence of RGS9-2 on MOR-stimulated GTPγS Binding and GTPase Activity in Mouse PAG Membranes**—The above results suggest that RGS9-2 is involved in the processes that, upon agonist challenge, bring about MOR desensitization. The efficacy and selectivity of the ODN treatment used have been previously assessed using antibodies directed against RGS9-2 (7). In the present work, after mouse PAG proteins were resolved by SDS-PAGE, the same antibodies detected reductions of some 50% of the 77-kDa RGS9-2 protein caused by the ODN treatment (Fig. 2). In mice with reduced PAG levels of RGS9-2 proteins, the antinociception promoted by 10 nmol of morphine increases, and notably, tolerance to the acute dose of morphine does not develop (7, 21–23). In the present work, we observed that RGS9-2 knockdown prevented MORs from becoming desensitized by 10 nmol of intracerebroventricular morphine (Fig. 2). This correlated with the MORs maintaining their association with the G-proteins in the PAG membranes from these morphine-treated RGS9-2 knockdown mice (Fig. 4D).
FIG. 5. Basal and stimulated [35S]GTPγS binding by morphine in PAG membranes from mice not exposed to the opioid. PAG synaptosomal membranes from mice treated with saline (Control), the mismatched ODN, and active ODN-RGS9-2 were incubated with (A) or without (B) increasing concentrations of morphine and 50 μM [35S]GTPγS for 2 h at 25 °C. Non-specific binding is defined as that obtained in the presence of 40 μM unlabeled GTPγS. The process was terminated by rapid filtration and washing. Data are expressed as picomoles of [35S]GTPγS specifically bound per mg of protein. The values shown are the means ± S.E. from three experiments, each carried out in triplicate. *, significantly different from the PAG control group (mice treated with the mismatched ODN; ANOVA/Student-Newman-Keuls test, p < 0.05).

FIG. 6. Morphine stimulation of [35S]GTPγS binding to PAG membranes. Shown is the influence of in vivo injection of desensitizing doses of morphine and RGS9-2 down-regulation. A, mice were intracerebroventricularly injected with saline (Control) or 10 nmol of morphine, and their PAG membranes were obtained 24 h later. The in vitro stimulating capacity of morphine was then evaluated as described under “Experimental Procedures” and in the legend to Fig. 5. B, shown is the base line-specific binding of [35S]GTPγS (in the absence of morphine in the incubation medium) to PAG membranes from mice treated with saline (Control), the mismatched ODN, and active ODN-RGS9-2. On day 6, the animals were intracerebroventricularly injected with a dose of 10 nmol of morphine and killed 24 h later to obtain the PAG membranes. AT, PAG from mice gently treated with morphine. C, shown is the morphine-stimulated binding of [35S]GTPγS. Details were as described for B, *, significantly different from the controls (A). PAG from mice treated with saline (control); B and C, PAG from mice treated with the mismatched ODN and 10 nmol of morphine (ANOVA/Student-Newman-Keuls test, p < 0.05).

**FIG. 7.** The effect of morphine on the association of G-proteins with MORs in PAG membranes. A, was carried out in triplicate. *, significantly different from the PAG control group (mice treated with the mismatched ODN; ANOVA/Student-Newman-Keuls test, p < 0.05).

**DISCUSSION**

MORs are transmembrane proteins of ~40 kDa that, after N-glycosylation, generate apparent sizes of 55–65, 75–85, and 100–110 kDa. Since the immunoprecipitated MORs were processed under reducing conditions, the diversity of molecular species observed for the chromatographically resolved MORs probably reflects different degrees of glycosylation of a single protein. In fact, the deglycosylated MOR moiety is of a size consistent with that predicted from its amino acid sequence (2).

In the PAG membranes, MOR coprecipitated a series of signaling elements, including Gα and Gβ subunits and RGS9-2 proteins. RGS7 and RGS11 present in PAG membranes showed no association with this receptor. Alternative splicing of RGS9 yields the RGS9-1 and RGS9-2 isoforms. RGS9-1 is found in the retina, whereas RGS9-2 is present in the central nervous system, with the highest levels in the striatum (7, 23, 37). RGS9-2 forms heterodimers with the short form of Gβγ proteins and binds activated Gαi/o subunits, although it has only a moderate GAP activity on Gαi/o subunits (Refs. 21 and 22 and references therein).

Although there are reports describing agonist-selective MOR down-regulation in neurons, this has usually been achieved with doses of agonists greater than those that induce opioid desensitization. In the central nervous system, strong tolerance to morphine and related opioids such as heroin develops as a consequence of a reduced affinity of MORs for opioid agonists, but no loss of MORs is observed (this work and Refs. 38–43). In fact, agonist-induced desensitization of κ-opioid receptors is achieved by phosphorylation, but without their internalization, and resensitization requires replacement by newly synthesized receptors rather than dephosphorylation (44). MOR substitution has also been proposed after morphine induces MOR desensitization in mouse brain (45). In recent years, elements of the MOR transduction system involved in

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the molecular mechanisms responsible for desensitization independent of receptor down-regulation have come to light. These include G-proteins (45), regulators of Gβγ dimers such as the phoshducin-like proteins (31), and the RGS proteins (this work and Refs. 7 and 21–23).

The in vivo administration of a single dose of 3 nmol of morphine caused reductions in MOR-associated Gαβγ proteins that reverted to control levels upon the extinction of the effects of the agonist. The recovery of the G-protein pool associated with MORs was not observed when a dose of 10 nmol of morphine (which promotes long-term MOR desensitization) was used instead. This phenomenon probably increases the population of uncoupled MORs and hence of those displaying low affinity for agonist binding. Because morphine mostly activates G2 and G4 proteins to produce MOR-mediated supraspinal antinociception (for example, see Refs. 3 and 46), RGS9-2 could favor the onset of desensitization at MORs by binding and sequestering agonist-activated Gα subunits (7, 21, 22). The possibility of this transference of Go subunits upon morphine challenge has now been substantiated. The reductions in MOR-regulated Gα subunits corresponded with increases in those bound to RGS9-2 proteins, in particular those of the Go class. Accordingly, the acutely tolerant MORs also showed a reduced response to morphine, including poor stimulation of GTPγS binding and/or low Km GTPase activity, both of which are related to the capacity of the agonist to activate Gα subunits.

Knockdown or knockout of the RGS9-2 proteins provokes an increase in the potency of μ-opioid agonists and strongly reduces the extent of MOR desensitization (this work and Refs. 7, 21, and 23). This suggests that the knockdown of RGS9-2 proteins makes a larger number of G-proteins available to MOR regulation. Thus, in PAG membranes from these RGS9-2 knockdown control mice, morphine-stimulated GTPγS binding increased. In the presence of 0.3 μM GTP, a tendency to increase the morphine-activated GTPase activity was observed as well. Morphine doses that produced MOR tolerance in naïve mice failed to promote this phenomenon in RGS9-2 knockdown mice. Twenty-four hours after morphine injection, the in vitro assays performed in PAG membranes from these mice (Ga coprecipitation with MORs, morphine-induced GTPγS binding, and stimulation of GTPase activity) indicated that the MORs maintained a control-like association with the G-proteins.

Together, these observations indicate a role for RGS9-2 proteins in morphine-induced desensitization of MOR. The transfer of MOR-regulated Ga subunits to the RGS9-2 control is insufficient for long-term desensitization; stabilization of the RGS9-2-Gα complexes is required. Studies performed on the physiology and structure of the retinal short C-tailed RGS9-1 protein may help in understanding the processes that lead RGS9-2 to sequester receptor-activated Ga subunits. In the retina, cGMP phosphodiesterase activity is inhibited by its γ-subunit (Pγ) (47). This negative regulation is removed by the binding of the activated GTP-bound α-subunit of transducin to Pγ (48, 49). The N-terminal sequence of Pγ establishes contacts with determined residues in Go, although this binding is not affected by the nucleotide bound to Go. This is followed by interaction with a Pγ C-terminal region that recognizes the activated state of Goα-GTP (50, 51). Notably, RGS9-1 recognizes only Goα5L subunits when they are bound to Pβγ subunits. The Gβγ long splice variant that binds the GGL domain on RGS9-1 determines this selectivity (36). The RGS9-1-Gβ5L complex establishes an initial contact with both the Pγ and Goα subunits through residues in the RGS9-1 RGS domain that are not directly involved in its GAP activity (51, 52). Pγ and RGS9-1 bind to distinct non-overlapping Goα residues located on the GTPase pocket (53) and stabilize the Goα-GDP hydrolytic intermediate or transition state before it is bound by the GAP region of the RGS domain (51). Thus, to perform its GAP activity on activated Goα subunits, RGS9-1 first requires the interaction of Goα-GTP with Pγ. It next requires cooperative binding with the Goα-GTP-Pγ complex to induce and stabilize the Goα-GDP-Pγ transition state. Finally, the binding of the RGS GAP section to produce Goα-GDP must take place. This leads to the dissociation of the signaling elements Goα-GDP, RGS9-1-Gβ5L, and Pγ, re-establishing negative control over phosphodiesterase function. This sequence of events warrants that Ga remains active until it reaches and regulates the effector target. Later, since certain residues in the RGS domain can bind to the effector (52), the RGS domain binds to the Go-effector complex, inactivating the Go subunit.

The 18 C-terminal residues of RGS9-1 are replaced in the central nervous system RGS9-2 by a 209-amino acid proline-rich domain (37). The Pγ subunit that increases the affinity between RGS9-1 and activated Goα (36) is structurally similar...
The RGS proteins that delay or inhibit Gq protein activation lead to receptor desensitization (62). The GAP function of RGS proteins is reduced and even blocked by mechanisms that involve phosphorylation of specific residues inside or near the RGS box and binding to 14-3-3 proteins. RGS9-1 and RGS7 are acted upon by protein kinase C blocked by mechanisms that involve phosphorylation of specific residues should produce the effector-inactive Gq subunits. RGS9-1 and RGS7 are acted upon by protein kinase C blocked by mechanisms that involve phosphorylation of specific residues in Gq subunits, but exhibit no GAP activity on Gq subunits, blocking the regulation of phospholipase Cβ (63). Similarly, RGS-Rz proteins bind receptor-activated Gα subunits, but exhibit no GAP activity on them, and this interaction reduces Gαq-mediated calcium mobilization (64). Thus, the sequestering of activated Gα subunits by RGS9-2 proteins may better explain MOR desensitization promoted by single doses of morphine (this study and Ref. 22).

In summary, the number of MOR-associated Gαq proteins was reduced during the time course of morphine effects. However, it was not recovered when morphine produced MOR desensitization independent of down-regulation. By retaining mostly MOR-activated Gαq subunits, the RGS9-2 proteins play a key role in the onset of this phenomenon. Thus, effects of certain magnitude induce the transfer of MOR-associated Gαq subunits to RGS9-2 proteins and also bring about the phosphoserine-dependent binding of 14-3-3 that stabilizes their interaction. These processes could exclude or precede the loss of the agonist-activated MORs.

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Activation of $\mu$-Opioid Receptors Transfers Control of $G\alpha$ Subunits to the Regulator of $G$-protein Signaling RGS9-2: ROLE IN RECEPTOR DESENSITIZATION
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