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Phosphorylation of $G\alpha_s$ Influences Its Association with the μ-Opioid Receptor and Is Modulated by Long-Term Morphine Exposure

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ABSTRACT

The recent biochemical demonstration of the association of the μ-opioid receptor (MOR) with $G\alpha_s$ that increases after long-term morphine treatment (*Mol Brain Res* **135:**217–224, 2005) provides a new imperative for studying MOR-$G\alpha_s$ interactions and the mechanisms that modulate it. A persisting challenge is to elucidate those neurochemical parameters modulated by long-term morphine treatment that facilitate MOR-$G\alpha_s$ association. This study demonstrates that 1) $G\alpha_s$ exists as a phosphoprotein, 2) the stoichiometry of $G\alpha_s$ phosphorylation decreases after long-term morphine treatment, and 3) in vitro dephosphorylation of $G\alpha_s$ increases its association with MOR. Furthermore, our data suggest that increased association of $G\alpha_s$ with protein phosphatase 2A is functionally linked to the long-term morphine treatment-induced reduction in $G\alpha_s$ phosphorylation. These findings are observed in MOR-Chinese hamster ovary and F11 cells as well as spinal cord, indicating that they are not idiosyncratic to the particular cell line used or a "culture " phenomenon and generalize to complex neural tissue. Taken together, these results indicate that the phosphorylation state of $G\alpha_s$ is a critical determinant of its interaction with MOR. Long-term morphine treatment decreases $G\alpha_s$ phosphorylation, which is a key mechanism underlying the previously demonstrated increased association of MOR and $G\alpha_s$ in opioid tolerant tissue.

The coupling of μ-opioid receptors (MOR) to G_s has long been controversial. The ability of MOR to signal via G_s, while persistently suggested by pharmacological experiments (Xu et al., 1989; Shen and Crain, 1990; Gintzler and Xu, 1991; Cruciani et al., 1993; Wang and Gintzler, 1997; Szucs et al., 2004), has met with considerable skepticism and has not been incorporated into commonly accepted models of short- and long-term opioid actions. This has largely resulted from the inability to demonstrate the physical association of MOR and $G\alpha_s$ in vivo. Our recent report that MOR is present in $G\alpha_s$ immunoprecipitate (IP), which increases after long-term morphine treatment (Chakrabarti et al., 2005a), provides a new imperative for studying MOR-$G\alpha_s$ interactions and mechanisms that modulate it. A remaining challenge is to identify the parameter(s) modulated by long-term morphine treatment and causally linked to the observed increased MOR-$G\alpha_s$ association during the tolerant condition.

Many biochemical parameters of receptors and G proteins could influence their functional interactions, of which phosphorylation has received much attention. Phosphorylation of G protein subunits has been shown to play a major role in adaptive changes in receptor signaling, altering their signaling patterns. Phosphorylation of $G\alpha_i$ suppresses the hormonal inhibition of adenylyl cyclase (AC) in human platelet membranes (Katada et al., 1985) and δ-opioid receptor mediated inhibition of AC activity in NG108-15 cells (Strassheim and Malbon, 1994). Recently, $G\alpha_{11}$ protein phosphorylation has been demonstrated to contribute to diminishing 5-HT2A receptor signaling (Shi et al., 2007). In addition, tyrosine phosphorylation of purified recombinant $G\alpha_s$ by immune-complexed pp60c-src enhances rates of β-adrenergic receptor-mediated binding of guanosine 5′-*O*-(2-[^{35}S]thio)triphosphate (GTPγS) as well as receptor-stimulated steady-state rate of GTP hydrolysis by G_s (Hausdorff et al., 1992).

Phosphorylation of G protein β and γ subunits has also been shown to be an important parameter of G protein signaling via $G\beta\gamma$. Protein kinase Cα phosphorylation of γ_{12} in the $\beta_1\gamma_{12}$ dimer regulates its activity in an effector-specific fashion (Yasuda et al., 1998). Threonine-phosphorylated $G\beta$

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ABBREVIATIONS: MOR, μ-opioid receptor; IP, immunoprecipitate; AC, adenylyl cyclase; CHO, Chinese hamster ovary; MOR-CHO, Chinese hamster ovary cells stably transfected with MOR; PP2A, protein phosphatase 2A; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; aa, amino acids; r$G\alpha_s$, recombinant purified $G\alpha_s$; PKC, protein kinase C; GTPγS, guanosine 5′-*O*-(2-[^{35}S]thio)triphosphate; P_i, inorganic phosphate.

has been demonstrated in spinal cord (Chakrabarti and Gintzler, 2003a), and histidine-phosphorylated Gβ has been demonstrated in membranes of bovine retinae (Wieland et al., 1991), liver, and brain and in human placental tissue (Nurnberg et al., 1996). It is noteworthy that long-term morphine treatment augments phosphorylation of Gβ in guinea pig longitudinal muscle myenteric plexus tissue (Chakrabarti et al., 2001), rat spinal cord (Chakrabarti and Gintzler, 2003a) and Chinese hamster ovary (CHO) cells stably transfected with MOR (MOR-CHO) (Chakrabarti et al., 2005b). Phosphorylation of Gβ has notable consequences on Gβγ signaling. It decreases the association of Gβγ with G protein receptor kinase (Chakrabarti et al., 2001) (which increases its availability for interaction with effectors, e.g., AC) and increases its potency to stimulate AC2 activity (Chakrabarti and Gintzler, 2003a).

The relevance of G protein subunit phosphorylation to G protein receptor-coupled signaling suggests that $G\alpha_s$ phosphorylation could be a regulatory parameter that is modulated by long-term morphine treatment and a determinant of the association of G_s with MOR. Accordingly, we investigated whether or not $G\alpha_s$ exists as a phosphoprotein, the modulation of its phosphorylation state by long-term morphine treatment, and the relevance of $G\alpha_s$ phosphorylation to its association with MOR. The results reveal that long-term morphine treatment decreases the phosphorylation of $G\alpha_s$ and that this is causally associated with the previously reported increased interaction of $G\alpha_s$ with MOR in opioid-tolerant tissue. Furthermore, our data suggest that increased association of $G\alpha_s$ (G_s) with protein phosphatase 2A (PP2A) is functionally linked to the long-term morphine treatment-induced reduction in $G\alpha_s$ phosphorylation.

Materials and Methods

Cell Culture and Transfection

MOR-CHO were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose with L-glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Nova-Tech Inc., Grand Island, NE), 100 units/ml penicillin/streptomycin, and 100 μg/ml G-418 (Geneticin; Mediatech, Herndon, VA). The neuroblastoma × dorsal root ganglia neuron hybrid F11 cell line was generously provided by Dr. Richard Ledeen (University of Medicine and Dentistry of New Jersey, Newark, NJ). These cells endogenously express μ-opioid receptors and manifest tolerance and dependence in response to long-term opioid treatment (Wu et al., 1995). Monolayer cultures of F11 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. To investigate whether dephosphorylated versus phosphorylated $G\alpha_s$ changes its stimulation of AC activity, MOR-CHO cells were transiently transfected with AC2 cDNA (AC2-pRC/CMV) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Morphine Treatment

Cell Culture. Cells were plated (4×10^6 cells/150 mm dishes) and grown at 37°C in a humidified atmosphere of 90% air/10% CO_2 for MOR-CHO and 95% air/5% CO_2 for F11 cells. Two days later, at 90 to 95% confluence, cells were treated with vehicle or morphine (1 μM) for 48 h. Media containing morphine or vehicle was replenished every 24 h.

Spinal Cord. Experiments employed male Sprague-Dawley rats (250–300 g; Charles River Laboratories, Kingston, NY) that were maintained in an approved controlled environment on a 12-h light/dark cycle. Food and water were available ad libitum. Studies were carried out in accordance with the *Guide for the Care and Use of laboratory Animals* as adopted and promulgated by the National Institutes of Health. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of SUNY Downstate Medical Center.

Morphine pellets were supplied by the National Institute on Drug Abuse, Bethesda, MD). Morphine tolerance was induced by subcutaneous implantation of one morphine pellets on day 1, two pellets on day 3, and three pellets on day 5 (each containing 75 mg of morphine base) (Bhargava and Villar, 1991) under sodium pentobarbital anesthesia (40 mg/kg, i.p., Anpro Pharmaceutical, Arcadia, CA). On the day 7 after pellet implantation, rats were decapitated, spinal cords were quickly expelled and washed extensively in Krebs buffer (4°C), and membranes were prepared.

$^{32}P_i$ Labeling of MOR-CHO Cells

On the day of harvest, cells were incubated for 2 h in phosphate- and serum-free DMEM at 37°C under normal culture conditions. Later, MOR-CHO cells were washed once with 10 ml of phosphate- and serum-free media and incubated with 10 ml of the same media containing [^{32}P]orthophosphate (100 μCi/ml; PerkinElmer Life and Analytical Sciences, Waltham, MA) for additional 2 h at 37°C under 90% air/10% CO_2.

Membrane Preparation and Immunoprecipitation

Cells were washed thoroughly (twice, 15 ml each) with ice-cold phosphate-buffered saline, pH 7.3, and harvested directly in 20 mM HEPES, pH 7.4, containing 10% sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT), 10 mM sodium pyrophosphate, 10 mM NaF; protease inhibitors 1 mM benzamidine, 0.2 mg/ml bacitracin, and 2 mg/l aprotinin; 3.2 mg/l each of trypsin inhibitor from soybean and leupeptin, 20 mg/l each of *N*-tosyl-L-phenyl-alanine chloromethyl ketone, Na-*p*-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, and complete cocktail inhibitor tablet/50 ml. Calyculin A, a protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) inhibitor was added after ^{32}P labeling before the onset of membrane preparation. Cells were homogenized and centrifuged at 1000*g*, 4°C for 10 min. Spinal tissue was homogenized in HEPES buffer of identical composition. Supernatants obtained from the low-speed spin were subjected to a high-speed spin at 30,000*g* for 40 min at 4°C.

Membrane fractions obtained were re-suspended in HEPES buffer, pH 7.4, containing 1 mM each EDTA, EGTA, and DTT, 10 mM sodium pyrophosphate, and the same protease and phosphatase inhibitors as mentioned above. Membranes were either stored at −80°C in aliquots or processed further. For immunoprecipitation, membranes were solubilized in the same buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol, agitated for 60 min at 4°C and centrifuged (14,000*g* for 20 min at 4°C). Clear supernatants were used for Protein Assay (Bradford) and immunoprecipitation. $G\alpha_s$ was immunoprecipitated from solubilized membrane using a rabbit anti-$G\alpha_s$ (bovine) polyclonal affinity purified antibody generated against the C terminus of the $G\alpha_s$ subunit (aa 385–394; US Biologicals, Swampscott, MA; 1 μl/100 μg protein). Prewashed Protein A-agarose (50 μl; Roche Molecular Biologicals, Indianapolis, IN) was used for immunoprecipitation overnight at 4°C. The beads were washed in 20 mM HEPES buffer, pH 7.4, containing 1 mM each DTT and EDTA, 150 mM NaCl, 0.05% Nonidet P-40, and the same protease inhibitors as mentioned above. Immunoprecipitates were eluted by heating samples in 30 μl of sample buffer (15 min at 85°C). Samples separated on 4–12% gradient Bis-Tris gels (Invitrogen) were electro-transferred onto nitrocellulose membranes and used for Western analyses or were exposed to Phosphorimager screens that were scanned in Phosphorimager Storm 860 (GE Healthcare, Chalfont St. Giles, Buckingham-

shire, UK). $^{32}P_i$ incorporated into phosphorylated samples was determined using densitometric analysis (Imagequant; GE Healthcare).

Western Analysis

MOR protein was visualized using a 1:5000 dilution of a rabbit polyclonal antibody (affinity-purified) generated against the C-terminal 50 aa of MOR (generously provided by Dr. Thomas Cote, Uniformed Services University of the Health Sciences, Bethesda, MD). $G\alpha_s$, PP2A, and phosphothreonine proteins were visualized by using a 1:10,000 dilution of a polyclonal anti-$G\alpha_s$ antibody (generously provided by Dr. J. Hildebrandt, Medical University of South Carolina, Charleston, SC), a 1:2000 dilution of an anti-PP2A monoclonal antibody (Upstate/Millipore, Charlottesville, VA), and a 1:1000 dilution of a polyclonal anti-phosphothreonine antibody (Invitrogen), respectively. The secondary antibody used was either a peroxidase-labeled donkey anti-rabbit or a peroxidase-labeled sheep anti-mouse antibody from GE Healthcare. Antibody-substrate complex was visualized using a Supersignal West Dura Chemiluminescence detection kit (Pierce, Rockford, IL). Specificity of Western signals was demonstrated via their diminution/elimination after incubation with antibodies while in the presence of a 3- to 5-fold excess of their respective blocking peptides. Specificity of $G\alpha_s$ immunoprecipitation was demonstrated via the diminution of MOR Western signal when $G\alpha_s$ immunoprecipitation was performed in the presence of 10 μg of the $G\alpha_s$ immunogen (aa 385–394 blocking peptide; Calbiochem, San Diego, CA). Sample pairs, obtained from opioid-naive and long-term morphine-treated MOR-CHO cultures were processed, electrophoresed, and blotted in parallel, after which they were exposed concomitantly to GeneGnome (CCD camera; Syngene, Frederick, MD) or to a Kodak X-Omat film (Denville Scientific, Metuchen, NJ). Intensity of signal was quantified using Syngene software in GeneGnome or NIH imaging software from films.

Stoichiometry of $G\alpha_s$ Phosphorylation

Stoichiometry of ^{32}P incorporation into $G\alpha_s$ by protein kinase C (PKC) was assessed after incubation of 160 ng of recombinant purified $G\alpha_s$ ($rG\alpha_s$; purified from *Escherichia coli*; generously provided by Dr. W-J Tang, University of Chicago, Chicago, IL) with catalytic subunits of PKC (PKCcat; purified from bovine brain; 20 mU/reaction; obtained from Calbiochem) and [γ-^{32}P]ATP (2.5 μCi/reaction; obtained from PerkinElmer Life and Analytical Sciences). The phosphorylation reaction was carried out in a 50-μl reaction mixture containing 20 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25% bovine serum albumin (BSA), 1 mM DTT, 40 mg/liter leupeptin, 40 mg/liter phenylmethylsulfonyl fluoride, and the phosphatase inhibitors 0.1 mM sodium vanadate, 0.5 μM okadaic acid, 25 nM calyculin A, and 100 μM ATP. The reaction was initiated by addition of PKCcat (as recommended by the manufacturer, Calbiochem, San Diego, CA), and incubated at 30°C for 2 h. The reaction was terminated by heating samples at 85°C for 8 min. Phosphorylated $G\alpha_s$ was resolved by gel electrophoresis (4–12% Bis-Tris gel), visualized by autoradiography using PhosphorImager analysis, and quantified by liquid scintillation spectroscopy. To assess whether $G\alpha_s$ exists as a phosphoprotein, the stoichiometry of $G\alpha_s$ phosphorylation was compared with that obtained using $rG\alpha_s$ that had been phosphatase-treated (see below).

In Vitro Dephosphorylation of Purified $G\alpha_s$ and Immunoprecipitation

Purified recombinant $G\alpha_s$ was dephosphorylated using commercially available PP1 and PP2A. Phosphatase reaction was initiated using 2 units each of purified PP1 and PP2A (from PP1/PP2A Toolbox kit; Upstate/Millipore) with 1 μg $rG\alpha_s$ in phosphatase reaction buffer (Upstate) at 30°C for 1h. Calyculin A (25 nM) was added to terminate the activity of the phosphatases. To assess the effect of dephosphorylating $rG\alpha_s$ on its association with MOR, phosphatase-treated and untreated $rG\alpha_s$ was incubated with solubilized membranes from opioid-naive MOR-CHO cells, and the content of MOR in $G\alpha_s$ IP obtained from each sample was determined and compared.

Preparation of AC2 Transfected MOR-CHO Cell Membranes and Determination of AC Activity

Forty-eight hours after transient transfection of AC2 in MOR-CHO cells, membranes were prepared as described above. Membrane pellets were resuspended in the homogenizing buffer without sucrose and stored in aliquots at −80°C for future use. $G\alpha_s$ stimulation of AC activity was determined in AC2-MOR-CHO membranes in the presence of $rG\alpha_s$ with or without dephosphorylation as described previously (Chakrabarti et al., 1998a). In brief, after the termination of dephosphorylation (or mock) reaction, $rG\alpha_s$ was activated by incubation (1 h at 30°C) with 100 μM GTPγS in 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT, 5 mM $MgSO_4$, and 1.25 mg/ml bovine serum albumin as described previously (Tang and Gilman, 1991). The activated $rG\alpha_s$-GTPγS was separated from free GTPγS using gel filtration (Sephadex G-25 spin columns). AC activity was determined by measuring the synthesis of [α-^{32}P]cAMP from [α-^{32}P]ATP (MP Biomedicals, Irvine, CA). Assays were initiated by the addition of the reaction mixture (50 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$, 20 mM creatine phosphate, 10 units/sample creatine phosphokinase, 0.1 μM ATP, 10 μM GTP, 20 mM NaCl, 1 mM DTT, 1 mM EGTA, 0.125 μM rolipram, 0.1% bovine serum albumin, and [α-^{32}P]ATP; 1 μCi/sample) to cell membranes (5 μg) with prior incubation (30°C, 15 min) with activated $rG\alpha_s$. Reactions (30°C, 15 min) were terminated by the addition of 10 μl of 2.2 N HCl (4°C). Thereafter, [^{32}P]cAMP generated was separated by neutral alumina column chromatography as described previously (Alvarez and Daniels, 1990) and quantified using liquid scintillation spectroscopy.

Protein Phosphatase 2A Assay

PP2A activity was measured by immunoprecipitation phosphatase Assay kit per the manufacturer's instructions (Upstate/Millipore,). PP2A was first immunoprecipitated from membranes as described above. Afterward the enzyme was used on phosphopeptide substrate to release phosphate molecules, which were measured colorimetrically and estimated from standard phosphate curves. It is important to note that all buffers and chemicals used for this procedure should be free of any contaminating phosphates.

Statistical Analysis

Significance of differences in the magnitude of autoradiographic and Western signals was assessed using paired two-tailed Student's *t* test. A repeated-measures ANOVA using a general linear mixed model was used to assess the effect of $G\alpha_s$ dephosphorylation on its ability to stimulate AC activity.

Results

$G\alpha_s$ Was Endogenously Expressed As a Phosphoprotein. $G\alpha_s$ was immunoprecipitated from $^{32}P_i$-metabolically labeled opioid-naive MOR-CHO cell membranes and subjected to sequential autoradiographic and Western analyses. Three radiolabeled bands of ≈45, 48, and 52 kDa were observed (Fig. 1A, lane 1). It is noteworthy that when the same sample was subjected to sequential autoradiographic and Western analyses, two of the three radiolabeled bands (≈45- and 48-kDa signals) coincided with signals observed in Westerns blotted with anti-$G\alpha_s$ antibodies (Fig. 1A, compare lanes 1 and 2 versus lanes 4/5 and 6/7). These observations in combination with the abolition/reduction of the Western signal when blotting was performed in the presence of a 3- to 5-fold excess of $G\alpha_s$ blocking peptide (Fig. 1A, lanes 8 and 9 indicates the ^{32}P-radiolabeled bands to be $G\alpha_s$ or its splice

variants. The ≈52-kDa radiolabeled band (comparable in molecular mass to $G\alpha_s$ "long"), which was much weaker than the ≈45- and 48-kDa signals, did not have a corresponding Western signal, suggesting that its protein content is extremely low, below the detection limits of the Western analysis employed.

The conclusion that $G\alpha_s$ exists as a phosphoprotein was validated by comparing the stoichiometry of ^{32}P incorporation into $rG\alpha_s$ before and after its treatment with PP1 and PP2A. The stoichiometry of $rG\alpha_s$ phosphorylation achieved by PKCcat after 2 h was 0.366 ± 0.03 mol of phosphate/mol of protein. It is noteworthy that stoichiometric phosphate increased by approximately 1-fold (to 0.71 ± 0.09) after $rG\alpha_s$ was in vitro dephosphorylated by the combined action of PP1 and PP2A. This indicates that $rG\alpha_s$ had been phosphorylated in vivo. It should be noted that when PP2A was used individually to dephosphorylate $rG\alpha_s$ before its phosphorylation by PKCcat, stoichiometric phosphate incorporation was ~80% of that observed after dephosphorylation by the combined action of PP2A and PP1. This suggests that PP2A might be the primary phosphatase dephosphorylating $G\alpha_s$.

$G\alpha_s$ Phosphorylation Decreased after Long-Term Morphine Treatment. $G\alpha_s$ IP was obtained in parallel from membranes of opioid-naive and long-term morphine-treated MOR-CHO cells that had been metabolically labeled with $^{32}P_i$. Sequential autoradiographic and Western analyses of $G\alpha_s$ IP obtained from membranes of long-term morphine-treated cells revealed the phosphorylation of ≈45-, 48-, and 52-kDa molecular mass forms of $G\alpha_s$, as was observed in $G\alpha_s$ IP obtained from membranes of opioid-naive MOR-CHO (Fig. 1A, lanes 1 and 2). However, densitometric analyses of radiolabeled bands revealed that long-term morphine treatment decreased ^{32}P incorporation into the predominant ≈45-kDa band by 47 ± 11% (Fig. 1A, lane 2 versus 1; $n = 3$; $p < 0.05$). ^{32}P incorporation into the 48- and 52-kDa minor molecular mass forms of $G\alpha_s$ was similarly decreased (40 and 59%, respectively; Fig. 1A, lane 2 versus 1; $n = 2$). It is noteworthy that sequential $G\alpha_s$ Western analyses revealed that the efficiency of $G\alpha_s$ immunoprecipitation was not altered by long-term morphine treatment (Fig. 1A, compare lanes 5 and 4). Thus, long-term morphine treatment results in the net decrease in $G\alpha_s$ phosphorylation. It is noteworthy that the decrement in $G\alpha_s$ phosphorylation after long-term morphine treatment is obliterated by the addition of calyculin A (25 nM) during the last 30 min of the ^{32}P radiolabeling period (Fig. 1A, lane 3 versus 2; $n = 3$) suggesting the preeminent importance of PP1/PP2A to the tolerant-associated decrement in $G\alpha_s$ phosphorylation.

The effect of chronic systemic morphine on the phosphorylation of $G\alpha_s$ that had been immunoprecipitated from spinal tissue was assessed via Western analyses using anti-phosphothreonine antibodies (Fig. 1B, lanes 1 and 2). One major band of ≈45 kDa was observed, the $G\alpha_s$ identity of which was confirmed by stripping and reprobing with anti-$G\alpha_s$ antibodies (Fig. 1B, lanes 3 and 4). It should be noted that although $G\alpha_s$ Western analysis revealed the expected ≈45- and 48-kDa $G\alpha_s$ species (Fig. 1B, lanes 3 and 4), only the ≈45-kDa molecular mass species appeared to be threonine-phosphorylated. Long-term systemic morphine administration in rats diminished spinal $G\alpha_s$ phosphorylation (27 ± 5.8%; Fig. 1B, lane 2 versus 1; $n = 3$; $p < 0.05$). This indicates that reduction of $G\alpha_s$ phosphorylation after long-term morphine treatment exposure generalizes to complex integrated neuronal systems and is not a cell culture phenomenon. The reduced magnitude of the long-term morphine treatment-induced decrement in $G\alpha_s$ phosphorylation in spinal cord versus MOR-CHO (27 versus 47%, respectively) could suggest a reduction in phosphorylation at sites in $G\alpha_s$ in addition to threonine.

Long-Term Morphine Treatment Enhanced Association of PP2A with $G\alpha_s$. A central role of PP2A in the decrement in $G\alpha_s$ phosphorylation after long-term morphine treatment is suggested by 1) the observation that PP2A pretreatment of $rG\alpha_s$ results in an increment in its in vitro phosphorylation that was 80% of that observed when using PP2A/PP1, and 2) the ability of calyculin A to abolish the decrement in $G\alpha_s$ phosphorylation after long-term morphine treatment. Thus, we explored the putative physiological relevance of PP2A to the in vivo phosphorylation state of $G\alpha_s$ and its modulation by long-term morphine treatment. This was investigated by assessing their association in vivo by quantifying the presence of PP2A in IP obtained with anti-

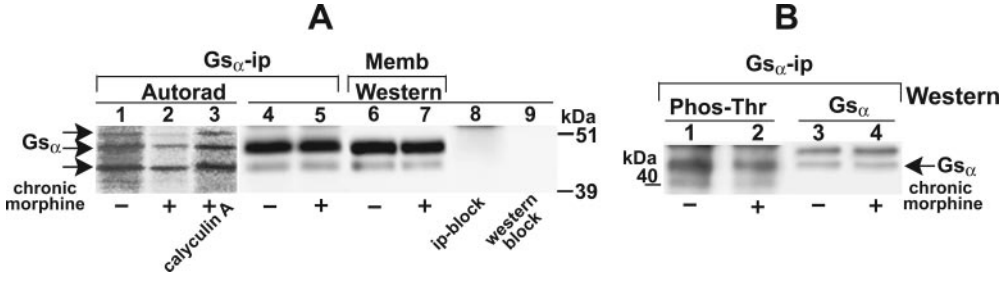

Fig. 1. $G\alpha_s$ was dephosphorylated after long-term morphine treatment. A, lanes 1, 2, and 3, autoradiographic analysis of $G\alpha_s$ IP obtained, in parallel, from opioid-naive and long-term morphine-treated MOR-CHO cells in the absence and presence of calyculin A, respectively. MOR-CHO cells were labeled with $^{32}P_i$, and membranes were prepared as described under *Materials and Methods*. $G\alpha_s$ immunoprecipitation was initiated using 400 μg of solubilized, radiolabeled MOR-CHO cell membranes and anti-$G\alpha_s$ antibodies (4 μl/400 μg). The involvement of PP1/PP2A in the long-term morphine treatment-induced decrement in $G\alpha_s$ phosphorylation is underscored by its abolition by calyculin A (lane 3). The $G\alpha_s$ identity of the ≈48- and 45-kDa radiolabeled bands was validated by demonstrating their coincidence with Western signals when the nitrocellulose membranes used for autoradiographic analyses was probed with anti-$G\alpha_s$ antibodies (lanes 4 and 5) or cell membranes obtained from MOR-CHO were subjected to Western blots using anti-$G\alpha_s$ antibodies (lanes 6 and 7). The absence of a $G\alpha_s$ Western signal that corresponds to the ≈52-kDa radiolabeled band most likely reflects its low protein content. Specificity of the $G\alpha_s$ IP and Western is indicated by the absence of signal when the immunoprecipitation (lane 8) or Western analysis (lane 9) is performed in the presence of 3- to 5-fold excess antigen. B, phosphothreonine Western analysis of $G\alpha_s$ IP from spinal cord membranes of opioid naive (lane 1) and long-term morphine treatment treated rats (lane 2). In lanes 3 and 4, the same immunoblot was reprobed with anti-$G\alpha_s$ antibodies after stripping off anti-phosphothreonine antibodies. $G\alpha_s$ phosphorylation decreased after long-term morphine treatment, which cannot be attributed to a decrement in the $G\alpha_s$ content of the $G\alpha_s$ IP. Results represent replicates of three observations.

$G\alpha_s$ antibodies. PP2A Western analysis of $G\alpha_s$ IP obtained from spinal cord, F11, and MOR-CHO cells, without or after long-term morphine treatment, revealed a single band of ≈36 kDa (Fig. 2). After long-term morphine treatment, there was a significant increase in the content of PP2A in $G\alpha_s$ IP obtained from all three sources (spinal cord, ≈109%; F11 cells, ≈57%; and MOR-CHO, ≈118%). It is noteworthy that the long-term morphine treatment-induced increased coimmunoprecipitation of PP2A with $G\alpha_s$ occurred in the absence of any detectable increase in the membrane content of PP2A (Fig. 2, top, lanes 7 and 8,) or $G\alpha_s$ (Fig. 2, bottom). Thus, long-term morphine treatment results in a net increment in the interaction between PP2A and $G\alpha_s$.

Long-Term Morphine Treatment Augmented PP2A Activity. PP2A activity was measured in membranes from naive and long-term morphine treatment treated MOR-CHO cells. Long-term morphine treatment increased PP2A activity (58 ± 10%; Fig. 3, lane 2 versus 1; $n = 4$; $p < 0.05$) compared with that in naive MOR-CHO cells. Similar increase in PP2A activity was also observed in spinal cord membrane samples from opioid-tolerant versus -naive rats (~70%, data not shown). The increase in PP2A activity occurred in the absence of any increment in its membrane concentration (Fig. 3B). This suggests that the observed increment in PP2A activity after long-term morphine treatment resulted from its allosteric activation.

In Vitro $G\alpha_s$ Dephosphorylation Enhanced Its Association with MOR. We previously reported that long-term morphine treatment enhances MOR-$G\alpha_s$ interaction (Chakrabarti et al., 2005a). Here, we explored the hypothesis that $G\alpha_s$ dephosphorylation is causally associated with this increased interaction. Purified $rG\alpha_s$ was mock-dephosphorylated (vehicle-treated) or dephosphorylated via incubation with PP2A and PP1 (2 U each), after which the reaction was terminated by placement on ice and the addition of calyculin A (25 nM). The dephosphorylated (or mock-dephosphorylated) $G\alpha_s$ was incubated with solubilized MOR-CHO membranes and subjected to immunoprecipitation using anti-$G\alpha_s$ antibodies. MOR Western analysis of $G\alpha_s$ IP revealed that its content of MOR was significantly augmented (83 ± 12% Fig. 4, lane 2 versus 1; $n = 3$; $p < 0.05$) after incubation of MOR-CHO membranes with dephosphorylated versus mock-dephosphorylated $G\alpha_s$. This strongly suggests that dephosphorylation of $G\alpha_s$ promotes its association with MOR.

Effect of $G\alpha_s$ Dephosphorylation on Its Ability to Stimulate AC2 Activity. Because AC phosphorylation state has been shown to be a critical determinant of the stimulatory responsiveness of some AC isoforms to $G\alpha_s$ (Jacobowitz and Iyengar, 1994; Watson et al., 1994), we determined whether the phosphorylation state of $G\alpha_s$ would similarly influence its ability to stimulate AC. $rG\alpha_s$ was either dephosphorylated with PP2A or mock-dephosphorylated, after which their ability to stimulate AC2 was determined and compared. As shown in Table 1, in vitro dephosphorylated $rG\alpha_s$ was more potent than mock-dephosphorylated $rG\alpha_s$ in stimulating cAMP production by AC2. The increment in cAMP production by 20, 40, and 60 nM dephosphorylated $rG\alpha_s$, although modest in magnitude, reached significance ($p < 0.05$).

Discussion

This study demonstrates that the $G\alpha_s$ subunit of G proteins exists as a phosphorylated protein. Data supporting this conclusion consists of 1) coincidence of radiolabeled and

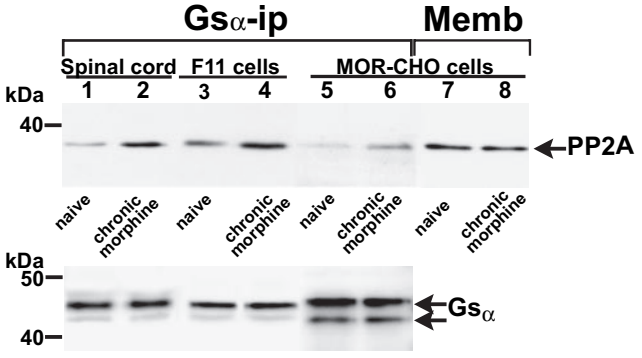

Fig. 2. PP2A coimmunoprecipitated with $G\alpha_s$ and their association increased after long-term morphine treatment. $G\alpha_s$ IP was performed as mentioned under *Materials and Methods*. Top, Western blot of PP2A in $G\alpha_s$ IP, obtained in parallel from membranes of opioid-naive and long-term morphine-treated rat spinal cord, F11, and MOR-CHO cells, respectively (lanes 1 through 6). Lanes 7 and 8 represent PP2A Western of membranes obtained from naive and long-term morphine-treated MOR-CHO cells (5 μg, respectively). Chronic morphine induced an increase in the PP2A that coimmunoprecipitated with $G\alpha_s$ (lanes 1–6). Bottom, $G\alpha_s$ Western analysis of the same blot after stripping and reprobing with anti-$G\alpha_s$ antibodies. The long-term morphine treatment-induced increment in coimmunoprecipitation of PP2A with $G\alpha_s$ occurred in the absence of increased content of $G\alpha_s$.

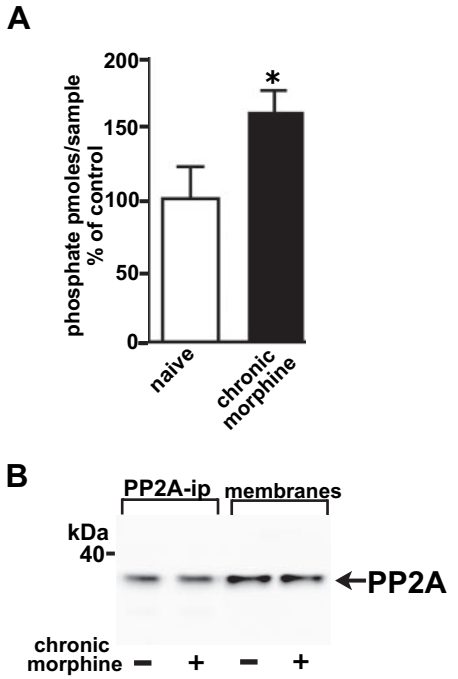

Fig. 3. PP2A activity increased in MOR-CHO cells after long-term morphine treatment. A, bar diagram denotes increased PP2A activity measured in PP2A IP from long-term morphine treated versus untreated MOR-CHO cell membranes (bar 2 versus 1). Details of PP2A activity assay are mentioned under *Materials and Methods*. In brief, anti-PP2A monoclonal antibody was used to IP PP2A, the activity of which was quantified using a commercially available phosphopeptide substrate. Phosphates liberated were measured colorimetrically (650 nm) using Malachite green. Results are expressed as percentage of control (picomoles of phosphate per sample). *, $p < 0.05$ for long-term morphine treatment versus naive; $n = 4$. B, Western analysis illustrating that the increased activity of PP2A after long-term morphine treatment is not accompanied by an increase in the MOR-CHO membrane content of PP2A or the efficiency of PP2A immunoprecipitation.

Western signals after sequential autoradiographic and $G\alpha_s$ Western analysis of the same sample that been metabolically labeled with $^{32}P_i$, 2) -fold increase in stoichiometry of $rG\alpha_s$ phosphorylation after phosphatase treatment, and 3) demonstration that (spinal cord) $G\alpha_s$ is immunoreactive with anti-phosphothreonine antibodies. The observed ^{32}P metabolic labeling of $G\alpha_s$ and its in vitro phosphorylation by PKCcat is consonant with the presence of multiple phosphorylation sites scattered throughout $G\alpha_s$ (e.g., seven serine, eight threonine, and three tyrosine; predicted by NetPhos 2.0; NetPhosk 1.0 predictions include 10 PKC/protein kinase A sites of >0.5 probability, of which three have a probability of >0.70).

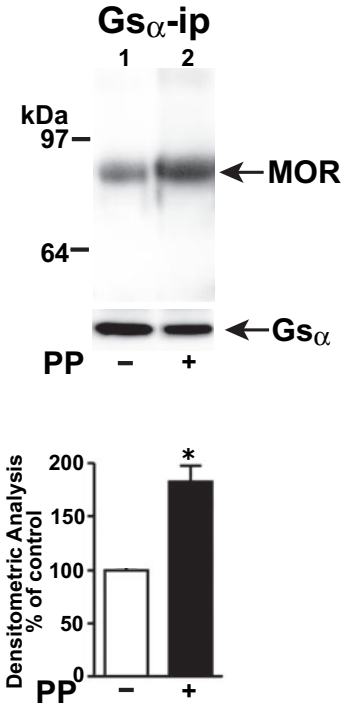

Fig. 4. In vitro dephosphorylation of $G\alpha_s$ enhanced its association with MOR. Western analysis of $G\alpha_s$ IP obtained from MOR-CHO membranes after incubation with purified recombinant $G\alpha_s$ that had been either dephosphorylated in vitro using protein PP1 and 2A or mock-dephosphorylated (PP+ and PP−, respectively). The content of MOR in the $G\alpha_s$ IP was quantified by Western analyses using anti-MOR antibodies. The $G\alpha_s$ Western blot of the $G\alpha_s$ IP, shown below the break in the gel, illustrates that the augmented coimmunoprecipitation of MOR with $G\alpha_s$ after its pretreatment with protein phosphatases did not result from differential loading. Bar graphs shown below illustrate that the content of MOR (~80 kDa) in $G\alpha_s$ IP was substantially increased after incubation of MOR-CHO membranes with in vitro dephosphorylated versus mock-dephosphorylated $rG\alpha_s$. * $p < 0.05$; $n = 3$.

TABLE 1

Effect of $G\alpha_s$ dephosphorylation on its ability to stimulate AC2 activity

ΔcAMP formation represents the differences in activity of AC2 when stimulated by mock (vehicle-treated) dephosphorylated versus dephosphorylated (via PP2A + PP1) $rG\alpha_s$. Analysis of variance and Tukey's post hoc test were used to assess significance of difference from zero for each indicated change in picomoles of cAMP formed.

$G\alpha_s$ (dose)	ΔcAMP Formation
	pmol/mg/min
10 nM	36.012
20 nM	45.91*
40 nM	40.98*
60 nM	76.46*

* $P < 0.05$

The second salient finding is that long-term morphine treatment decreases the stoichiometry of $G\alpha_s$ phosphorylation concomitant with its increased association with PP2A. This inference of their causal association is validated by the demonstration that the long-term morphine treatment-induced decrement in $G\alpha_s$ phosphorylation is abolished by short-term (30 min) pretreatment with calyculin A, an inhibitor of PP1/PP2A (data not shown). The third notable finding is that in vitro dephosphorylation of $G\alpha_s$ increases its association with MOR. It is noteworthy that augmented phosphorylation of $G\alpha_s$ (on tyrosine by pp60c-src) potentiates signaling via the β-adrenergic receptor (Hausdorff et al., 1992), which normally predominantly couples to G_s to stimulate AC, whereas a diminution in $G\alpha_s$ phosphorylation augments its association with MOR, which normally predominantly couples to G_i/G_o to inhibit AC activity. More extensive analysis will be required to assess if inverse consequences of $G\alpha_s$ phosphorylation generalizes to other "stimulatory" versus "inhibitory" receptors.

It is noteworthy that demonstration of findings in MOR-CHO and F11 cells as well as spinal cord indicate that they are not idiosyncratic to the particular cell line used or a culture phenomenon and generalize to complex neural tissue. Taken together, these results strongly suggest that the phosphorylation state of $G\alpha_s$ is a critical determinant of its interaction with MOR, which is regulated (decreased) by long-term morphine treatment. We previously demonstrated that long-term morphine treatment increases the association of MOR with $G\alpha_s$ (Chakrabarti et al., 2005a). The present results identify decreased $G\alpha_s$ phosphorylation as a mechanism central to this change in MOR-$G\alpha_s$ protein coupling.

Regulation of protein phosphorylation has long been recognized to be a key mechanism underlying opioid tolerance formation. Heretofore, most of the attention has been focused on augmented protein phosphorylation, predominantly via enhanced activity of protein kinase A/PKC pathways (Guitart and Nestler, 1989; Nestler, 1992; Zhang et al., 1996). More recently, we have demonstrated the causal association of tolerance formation and increased phosphorylation of multiple signaling proteins. These include the $G\beta$ subunit of G proteins, phospholipase Cβ3 and AC (Chakrabarti et al., 1998b, 2001; Chakrabarti and Gintzler, 2003a,b). A reduction in phosphorylation of phospholipase Cβ1 (Chakrabarti and Gintzler, 2003b) and mitogen-activated protein kinase (Schulz and Hollt, 1998) has been demonstrated after long-term morphine treatment and its withdrawal. However, so far, with a few notable exceptions, opioid tolerance has been associated mostly with enhancement in phosphorylation of multiple signaling protein(s). The current demonstration that long-term morphine treatment decreases phosphorylation of $G\alpha_s$ that in turn augments its association with MOR represents a novel dimension of adaptation to long-term morphine treatment.

Increased interaction of MOR with $G\alpha_s$ after long-term morphine treatment would act in parallel with and complement signaling consequences of adaptations to long-term morphine treatment we previously identified [i.e., increased availability of $G\beta\gamma$ (Chakrabarti et al., 2001), increased phosphorylation of $G\beta$ (Chakrabarti et al., 2001; Chakrabarti and Gintzler, 2003a), augmented AC isoform-specific synthesis and phosphorylation (Chakrabarti et al., 1998a,8b; Rivera and Gintzler, 1998)]. These converge to shift MOR-coupled

signaling from predominantly $G\alpha_i$-inhibitory to $G\beta\gamma$ AC stimulatory that would mitigate the persistent opioid inhibition of AC(s) via the opioid receptor-coupled generation of $G\alpha_i$ (for review, see Gintzler and Chakrabarti, 2006).

The tolerance-associated emergence of MOR-coupled $G\beta\gamma$ stimulatory AC signaling does not require a shift in G protein coupling. Nevertheless, it is well known that the presence of activated $G\alpha_s$ is also essential for a substantial component of $G\beta\gamma$ stimulation of AC (Tang and Gilman, 1991). In vivo, activated $G\alpha_s$ could be generated via the ongoing activation of a multitude of G_s-coupled receptors, independent of opioid receptor function. Nonetheless, the present report underscores that direct coupling of MOR to G_s represents an additional source of activated $G\alpha_s$; concomitant opioid receptor signaling via G_s as well as G_i/G_o would result in the coordinate generation of activated $G\alpha_s$ (from G_s) and $G\beta\gamma$ (from G_s as well as G_i/G_o). Thus, increased MOR-coupled generation of activated $G\alpha_s$ during morphine tolerance would be functionally coordinated with the previously described concomitant emergence of opioid receptor-coupled AC stimulatory $G\beta\gamma$ signaling. In addition, enhanced MOR-G_s coupling (the effects of which would be amplified by the modestly more AC stimulatory activity of dephosphorylated $G\alpha_s$; see Table 1) represent a parallel pathway for shifting opioid receptor signaling from predominantly inhibitory to stimulatory; direct stimulation of AC by MOR-coupled generation of activated $G\alpha_s$ would be additive with that resulting from the action of $G\beta\gamma$, and would thus further contribute to the neutralization of the predominant inhibitory G_i/G_o-coupled opioid receptor signaling (i.e., opioid tolerance formation).

Coimmunoprecipitation of proteins demonstrates their presence in stable high-affinity complexes. However, difficulties in distinguishing between a naturally occurring complex versus those that may form in vitro during lysate preparation and incubation can confound interpretation of results. In the present study, this concern is mitigated by the observation that the phosphorylation state of the $G\alpha_s$ that coprecipitates with increasing amounts of PP2A diminishes after long-term morphine treatment and can be blocked by calyculin A. This strongly suggests an increased functional association between $G\alpha_s$ and PP2A at the time of ^{32}P labeling, preceding lysate incubation. A priori, one would not expect a phosphatase to remain associated with its substrate after catalysis of its dephosphorylation. The coimmunoprecipitation of PP2A and dephosphorylated $G\alpha_s$ demonstrated in the present study can most easily be explained by postulating the presence of a third as-yet-unidentified protein/lipid that serves as an anchor for both PP2A and $G\alpha_s$.

The observed increased association of PP2A with $G\alpha_s$ after long-term morphine treatment most likely results from the translocation of one or both because their membrane content does not change after this treatment. We previously demonstrated that a macromolecular signaling complex containing PKCγ, Gβ, and AC underlies, in part, the previously reported shift from predominantly $G\alpha_i$-inhibitory to $G\beta\gamma$-stimulatory AC signaling (Chakrabarti et al., 2005b) and that long-term morphine treatment induces the concomitant phosphorylation of G protein-coupled receptor kinase 2/3, β-arrestin, and Gβ, signaling molecules that exist in a multimolecular complex, with attendant modulation of their association (Chakrabarti et al., 2001). The present study underscores the contribution of the concomitant modulation of multiple membrane microsignaling domains by long-term morphine treatment to opioid tolerance formation and that PP2A should be considered as a scaffolding molecule that facilitates the interaction between MOR and $G\alpha_s$, in addition to its enzymatic function.

It is important to note that tolerant-producing mechanisms demonstrated in this study are not mutually exclusive. Numerous adaptations to long-term morphine treatment have been observed and postulated to be causally associated with opioid tolerance. These include opioid receptor down-regulation/internalization (Chavkin and Goldstein, 1984; Chakrabarti et al., 1995; Cox and Crowder, 2004), MOR G protein uncoupling (Sim et al., 1996), and AC superactivation. Altered association/activity of regulators of G-protein signaling (Zachariou et al., 2003; Xu et al., 2004; Garzon et al., 2005; Xie and Palmer, 2005) has also been suggested to be a tolerance-producing adaptation. In general, studies designed to assess the relative contribution to opioid tolerance of the many proposed tolerant mechanisms are woefully lacking. Future studies will be required to parse the relative importance to opioid tolerance of the adaptations demonstrated in this study, the mechanisms suggested by the literature, and how they might intersect. This process should be facilitated by understanding tolerance within the context of physiological plasticity and the realization that opioid tolerance is the result of the combined effect of the loss of specific opioid receptor-coupled signaling sequelae as well as the emergence of novel signaling strategies.

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