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Phosphorylation of Gsα influences its association with the mu-opioid receptor and is modulated by chronic morphine

Sumita Chakrabarti and Alan R Gintzler

Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

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b) Corresponding Author: Dr. Alan Gintzler

Box 8, Department of Biochemistry

SUNY Downstate Medical Center

450 Clarkson Ave.

Brooklyn, NY 11203

Tel: 718 270 2129

Fax: 718 270 3316

E-mail: alan.gintzler@downstate.edu

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Abstract

The recent biochemical demonstration of the association of the μ-opioid receptor (MOR) with $G_{s\alpha}$ that increases following chronic morphine treatment (Chakrabarti, et al., Mol Brain Res, 135: 217-224, 2005) provides a new imperative for studying MOR $G_{s\alpha}$ interactions and mechanisms that modulate it. A persisting challenge is to elucidate those neurochemical parameters modulated by chronic morphine that facilitate MOR $G_{s\alpha}$ association. This study demonstrates that (1) $G_{s\alpha}$ exists as a phosphoprotein, (2) the stoichiometry of $G_{s\alpha}$ phosphorylation decreases following chronic morphine and (3) *in vitro* de-phosphorylation of $G_{s\alpha}$ increases its association with MOR. Furthermore, our data suggest that increased association of $G_{s\alpha}$ with protein phosphatase 2A is functionally linked to the chronic morphine-induced reduction in $G_{s\alpha}$ phosphorylation. These findings are observed in MOR-CHO 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. In the aggregate, these results indicate that the phosphorylation state of $G_{s\alpha}$ is a critical determinant of its interaction with MOR. Chronic morphine treatment decreases $G_{s\alpha}$ phosphorylation, which is a key mechanism underlying the previously demonstrated increased association of MOR and $G_{s\alpha}$ in opioid tolerant tissue.

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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 (Cruciani et al., 1993; Gintzler and Xu, 1991; Shen and Crain, 1990; Szucs et al., 2004; Wang and Gintzler, 1997; Xu et al., 1989), has met with considerable skepticism and has not been incorporated into commonly accepted models of acute and chronic opioid actions. This has to a large extent resulted from the inability to demonstrate the physical association of MOR and $G_{s\alpha}$ *in vivo*. Our recent report that MOR is present in $G_{s\alpha}$ immunoprecipitate (IP), which increases following chronic morphine treatment (Chakrabarti et al., 2005a), provides a new imperative for studying MOR $G_{s\alpha}$ interactions and mechanisms that modulate it. A remaining challenge is to identify the parameter(s) that is (are) modulated by chronic morphine and are causally linked to the observed increased MOR $G_{s\alpha}$ 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 $G_{i\alpha}$ 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). Additionally, tyrosine phosphorylation of purified recombinant $G_{S\alpha}$ by immune-complexed pp6Oc-src enhances rates of β-adrenergic receptor-mediated binding of guanosine 5'-[γ-thio-^{35}S] triphosphate as well as receptor-stimulated steady-state rate of GTP hydrolysis by G_s (Hausdorff et al., 1992).

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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 β_1 γ_{12} dimer regulates its activity in an effector-specific fashion (Yasuda et al., 1998). Threonine-phosphorylated G_β has been demonstrated in spinal cord (Chakrabarti and Gintzler, 2003a) and histidine-phosphorylated G_β has been demonstrated in membranes of bovine retinae (Wieland et al., 1991), liver, and brain, and human placental tissue (Nurnberg et al., 1996). Importantly, chronic morphine augments phosphorylation of G_β 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 G_β has notable consequences on $G_{\beta\gamma}$ signaling. It decreases the association of $G_{\beta\gamma}$ 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 $G_{s\alpha}$ phosphorylation could be a regulatory parameter that is modulated by chronic morphine and a determinant of the association of G_s with MOR. Accordingly, we investigated whether or not $G_{s\alpha}$ exists as a phosphoprotein, the modulation of its phosphorylation state by chronic morphine and the relevance of $G_{s\alpha}$ phosphorylation to its association with MOR. The results reveal that chronic morphine decreases the phosphorylation of $G_{s\alpha}$ and that this is causally associated with the previously reported increased interaction of $G_{s\alpha}$ with MOR in opioid tolerant tissue. Furthermore, our data suggest that increased association of $G_{s\alpha}$ (G_s) with protein phosphatase 2A (PP2A) is functionally linked to the chronic morphine-induced reduction in $G_{s\alpha}$ 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 Geneticin (Mediatech). The neuroblastoma x dorsal root ganglia neuron hybrid F11 cell line was generously provided by Dr. Richard Ledeen (University of Medicine and Dentistry of New Jersey, NJ). These cells endogenously express μ-opioid receptors and manifest tolerance and dependence in response to chronic 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. In order to investigate whether de-phosphorylated vs. phosphorylated $G_{s\alpha}$ changes its stimulation of AC activity, MOR-CHO cells were transiently transfected with AC2 cDNA (AC2-pRC/CMV) using Lipofeactamine 2000 reagent (Invitrogen) as per manufacturer's instruction.

Morphine treatment.

(a) Cell culture. Cells were plated (4.0×10^6 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 - 95% confluency, cells were treated with vehicle or morphine (1 μM) for 48 h. Media containing morphine or vehicle was replenished every 24 h.

(b) Spinal cord. Experiments employed male Sprague-Dawley rats (Charles River, Kingston, NY; 250-300 g) that were maintained in an approved controlled environment on a 12 hr 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 U.S. 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. 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 morphine base) (Bhargava and Villar, 1991) under sodium pentobarbital anesthesia (40 mg/kg, i.p., Anpro Pharmaceutical). On the 7th day following pellet implantation, rats were decapitated, spinal cords were quickly expelled, washed extensively in Krebs buffer (4^{o}C) and membranes prepared.

^{32}Pi 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; Perkin Elmer Life Sciences, Boston, MA) for additional 2h 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 Na-pyrophosphate, 10 mM NaF; protease inhibitors 1 mM Benzamidine, 0.2 mg/ml bacitracin, 2 mg/l Aprotinin, 3.2 mg/l each of trypsin inhibitor from soybean and Leupeptin, 20 mg/l each of N-tosyl-l-phenylalanine chloromethyl ketone, Na -p-tosyl-l-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride (PMSF), and complete cocktail inhibitor tablet/50 ml. Calyculin A, a protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) inhibitor was added following ^{32}P labeling prior to the onset of membrane preparation.

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Cells were homogenized and centrifuged at 1000 x 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 of EDTA, EGTA and DTT, 10 mM Na-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 (NP-40), 0.5% Na-deoxycholate, 0.1% Na-dodecyl sulfate and 10% glycerol, agitated 60 min at 4°C and centrifuged (14,000 x g for 20 min at 4°C). Clear supernatants were used for Protein Assay (Bradford) and immunoprecipitation. $G_{s\alpha}$ was immunoprecipitated from solubilized membrane using a rabbit anti-$G_{s\alpha}$ (bovine) polyclonal affinity purified antibody generated against the C-terminus of the $G_{s\alpha}$ subunit (aa 385-394; US Biologicals, Swampscott, MA; 1 μl /100 μg protein). Pre-washed 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 of DTT and EDTA, 150 mM NaCl, 0.05% NP-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 Phosphor-imager screens that were scanned in Phosphor-imager Storm 860 (Molecular Dynamics, Sunnyvale, CA. ^{32}Pi incorporated into phosphorylated samples was determined using densitometric analysis (Imagequant, Molecular Dynamics).

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Western analysis.

MOR protein was visualized using a 1:5,000 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, Maryland). $G_{s\alpha}$, PP2A and phospho-threonine proteins were visualized by using a 1:10,000 dilution of a polyclonal anti-$G_{s\alpha}$ antibody (generously provided by Dr. J. Hildebrandt, Medical University of South Carolina), a 1: 2,000 dilution of an anti-PP2A monoclonal antibody (Upstate/Millipore, Charottesville, VA), and a 1:1000 dilution of a polyclonal anti-phoshothreonine antibody (Zymed/Invitrogen), respectively. The secondary antibody utilized was either a peroxidase-labeled donkey anti-rabbit or a peroxidase-labeled sheep anti-mouse antibody from Amersham/GE Healthcare (Piscataway, NJ). 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 following incubation with antibodies while in the presence of a 3-5-fold excess of their respective blocking peptides. Specificity of $G_{s\alpha}$ immunoprecipitation was demonstrated via the diminution of MOR Western signal when $G_{s\alpha}$ immunoprecipitation was performed in the presence of 10 μg of the $G_{s\alpha}$ immunogen (aa 385-394 blocking peptide, Calbiochem, San Diego, CA). Sample pairs, obtained from opioid naïve and chronic 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 $G_{s\alpha}$ phosphorylation.

Stoichiometry of ^{32}P incorporation into $G_{s\alpha}$ by protein kinase C (PKC) was assessed following incubation of 160 ng of recombinant purified $G_{s\alpha}$ ($rG_{s\alpha}$; purified from E. Coli; generously provided

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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 Perkin Elmer). 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, 1 mM DTT, 40 mg/L leupeptin, 40 mg/L PMSF, 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 $G_{s\alpha}$ was resolved by gel electrophoresis (4-12% Bis-Tris gel), visualized by autoradiography using PhosphorImager analysis and quantified by liquid scintillation spectroscopy. In order to assess if $G_{s\alpha}$ exists as a phosphoprotein, the stoichiometry of $G_{s\alpha}$ phosphorylation was compared with that obtained using $rG_{s\alpha}$ that had been phosphatase-treated (see below).

***In vitro* de-phosphorylation of purified $G_{s\alpha}$ and immunoprecipitation.**

Purified recombinant $G_{s\alpha}$ was de-phosphorylated 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 $rG_{s\alpha}$ in phosphatase reaction buffer (Upstate) at 30°C for 1h. Calyculin A (25 nM) was added to terminate the activity of the phosphatases. In order to assess the effect of de-phosphorylating $rG_{s\alpha}$ on its association with MOR, phosphatase treated and un-treated $rG_{s\alpha}$ was incubated with solubilized membranes from opioid naïve MOR-CHO cells and the content of MOR in $G_{s\alpha}$ 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 earlier. Membrane pellets were re-suspended in the homogenizing buffer without

sucrose and stored in aliquots at -80 °C for future use. $G_{s\alpha}$ stimulation of AC activity was determined in AC2-MORCHO membranes in the presence of $rG_{s\alpha}$ with or without de-phosphorylation as described previously (Chakrabarti et al., 1998a). Briefly, after the termination of de-phosphorylation (or mock) reaction, $rG_{s\alpha}$ was activated by incubation (1h at 30°C) with 100 μM GTPγS in 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, 1mM DTT, 5 mM MgSO4, and BSA (1.25 mg/ml) as described previously (Tang and Gilman, 1991). The activated $rG_{s\alpha}$-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 $MgCl_2$, 20mM 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% BSA, and [α-^{32}P]ATP; 1 μCi/sample) to cell membranes (5 μg) with prior incubation (30°C, 15 min) with activated $rG_{s\alpha}$. 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 as per manufacturer's instruction (Upstate/Millipore, Charlottesville, VA). PP2A was first immunoprecipitated from membranes as described above. Afterwards 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.

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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 $G_{s\alpha}$ de-phosphorylation on its ability to stimulate AC activity.

Results

$G_{s\alpha}$ is endogenously expressed as a phosphoprotein. $G_{s\alpha}$ was immunoprecipitated from ^{32}Pi-metabolically labeled opioid naïve MORCHO 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). Notably, 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-$G_{s\alpha}$ antibodies (compare lanes 1 and 2 vs. lanes 4 / 5 and 6 / 7 of Fig. 1A). These observations in combination with the abolition/reduction of the Western signal when blotting was performed in the presence of a 3-5 fold excess of $G_{s\alpha}$ blocking peptide (Fig. 1A, lanes 8 and 9 indicates the ^{32}P-radiolabeled bands to be $G_{s\alpha}$ or its splice variants. The ≈52 kDa radiolabeled band, (comparable in molecular mass to $G_{s\alpha}$ 'long') that 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 detection limits of the Western analysis employed.

The conclusion that $G_{s\alpha}$ exists as a phosphoprotein was validated by comparing the stoichiometry of ^{32}P incorporation into purified recombinant $G_{s\alpha}$ ($rG_{s\alpha}$) before and following its treatment with PP1 and PP2A. The stoichiometry of $rG_{s\alpha}$ phosphorylation achieved by PKCcat after 2 h was 0.366 ± 0.03 mol phosphate/mol protein. Notably, stoichiometric phosphate increased by approximately

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one-fold (to 0.71 ± 0.09) after $rG_{s\alpha}$ was *in vitro* de-phosphorylated by the combined action of PP1 and PP2A. This indicates that $rG_{s\alpha}$ had been phosphorylated *in vivo*. It should be noted that when PP2A was used individually to de-phosphorylate $rG_{s\alpha}$ prior to its phosphorylation by PKCcat, stoichiometric phosphate incorporation was ~80% of that observed after de-phosphorylation by the combined action of PP2A and PP1. This suggested that PP2A might be the primary phosphatase dephosphorylating $G_{s\alpha}$.

$G_{s\alpha}$ phosphorylation decreases following chronic morphine. $G_{s\alpha}$ IP was obtained in parallel from membranes of opioid naïve and chronic morphine-treated MOR-CHO cells that had been metabolically labeled with ^{32}Pi. Sequential autoradiographic and Western analyses of $G_{s\alpha}$ IP obtained from membranes of chronic morphine-treated cells revealed the phosphorylation of ≈45, 48 and 52 kDa molecular mass forms of $G_{s\alpha}$, as was observed in $G_{s\alpha}$ IP obtained from membranes of opioid naïve MOR-CHO (Fig. 1A, lanes 1 and 2). However, densitometric analyses of radiolabeled bands revealed that chronic morphine treatment decreased ^{32}P incorporation into the predominant ≈45 kDa band by 47 ± 11% (Fig. 1A, lane 2 vs. 1; n=3; $p<0.05$). ^{32}P incorporation into the 48 and 52 kDa, minor molecular mass forms of $G_{s\alpha}$ was similarly decreased (40 and 59%, respectively; Fig. 1A, lane 2 vs. 1; n=2). Importantly, sequential $G_{s\alpha}$ Western analyses revealed that the efficiency of $G_{s\alpha}$ immunoprecipitation was not altered by chronic morphine treatment (compare lane 5 vs. 4 of Fig. 1A). Thus, chronic morphine results in the net decrease in $G_{s\alpha}$ phosphorylation. Notably, the decrement in $G_{s\alpha}$ phosphorylation following chronic 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 vs. 2; n=3) suggesting the preeminent importance of PP1/PP2A to the tolerant-associated decrement in $G_{s\alpha}$ phosphorylation.

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

Chronic morphine enhances association of PP2A with $G_{s\alpha}$. A central role of PP2A in the decrement in $G_{s\alpha}$ phosphorylation after chronic morphine is suggested by (1) the observation that PP2A pretreatment of $rG_{s\alpha}$ 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 $G_{s\alpha}$ phosphorylation following chronic morphine. Thus, we explored the putative physiological relevance of PP2A to the *in vivo* phosphorylation state of $G_{s\alpha}$ and its modulation by chronic morphine. This was investigated by assessing their association *in vivo* by quantifying the presence of PP2A in IP obtained with anti-$G_{s\alpha}$ antibodies. PP2A Western analysis of $G_{s\alpha}$ IP obtained from spinal cord, F11 and MOR-CHO cells, without or following chronic morphine treatment, revealed a single band of ≈36 kDa (Fig. 2). Strikingly, after chronic morphine, there was a significant increase in the content of PP2A in $G_{s\alpha}$ IP obtained from all three sources (Spinal cord: ≈109%; F11 cells:

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≈57%; MOR-CHO: ≈118%). Importantly, the chronic morphine-induced increased co-immunoprecipitation of PP2A with $G_{s\alpha}$ occurred in the absence of any detectable increase in the membrane content of PP2A (see lanes 7 and 8, Fig. 2 upper panel) or $G_{s\alpha}$ (Fig. 2 bottom panel). Thus, chronic morphine results in a net increment in the interaction between PP2A and $G_{s\alpha}$.

Chronic Morphine augments PP2A activity. PP2A activity was measured in membranes from naïve and chronic morphine treated MOR-CHO cells. Chronic morphine treatment increased PP2A activity (58 ± 10%; Fig. 3, lane 2 vs. 1; n=4; $p<0.05$) compared to that in naïve MOR-CHO cells. Similar increase in PP2A activity was also observed in spinal cord membrane samples from opioid tolerant vs. naïve 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 following chronic morphine resulted from its allosteric activation.

***In vitro* $G_{s\alpha}$ de-phosphorylation enhances its association with MOR.** We previously reported that chronic morphine enhances MOR-$G_{s\alpha}$ interaction (Chakrabarti et al., 2005a). Here, we explored the hypothesis that $G_{s\alpha}$ de-phosphorylation is causally associated with this increased interaction. Purified $rG_{s\alpha}$ was 'mock' de-phosphorylated (vehicle-treated) or de-phosphorylated 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 de-phosphorylated (or mock de-phosphorylated) $G_{s\alpha}$ was incubated with solubilized MOR-CHO membranes and subjected to immunoprecipitation using anti-$G_{s\alpha}$ antibodies. MOR Western analysis of $G_{s\alpha}$ IP revealed that its content of MOR was significantly augmented (83 ± 12% Fig. 4, lane 2 vs. 1; n=3; $p<0.05$) following incubation of MOR-CHO membranes with de-phosphorylated vs. mock de-phosphorylated $G_{s\alpha}$. This strongly suggests that de-phosphorylation of $G_{s\alpha}$ promotes its association with MOR.

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Effect of $G_{s\alpha}$ de-phosphorylation on its ability to stimulate AC2 activity. Since AC phosphorylation state has been shown to be a critical determinant of the stimulatory responsiveness of some AC isoforms to $G_{s\alpha}$ (Jacobowitz and Iyengar, 1994; Watson et al., 1994), we determined if the phosphorylation state of $G_{s\alpha}$ would similarly influence its ability to stimulate AC. $rG_{s\alpha}$ was either de-phosphorylated with PP2A or 'mock' de-phosphorylated, after which their ability to stimulate AC2 was determined and compared. As shown in table 1, *in vitro* de-phosphorylated $rG_{s\alpha}$ was more potent than 'mock' de-phosphorylated $rG_{s\alpha}$ in stimulating cAMP production by AC2. The increment in cAMP production by 20, 40, 60 nM de-phosphorylated $rG_{s\alpha}$, although modest in magnitude, reached significance ($p<0.05$).

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Discussion

This study demonstrates that the $G_{s\alpha}$ subunit of G proteins exists as a phosphorylated protein. Data supporting this conclusion consists of (1) coincidence radiolabeled and Western signals following sequential autoradiographic and $G_{s\alpha}$ Western analysis of the same sample that been metabolically labeled with ^{32}Pi, (2) fold increase in stoichiometry of $rG_{s\alpha}$ phosphorylation following phosphatase treatment and (3) demonstration that (spinal cord) $G_{s\alpha}$ is immunoreactive with anti-phosphothreonine antibodies. The observed ^{32}P metabolic labeling of $G_{s\alpha}$ and its *in vitro* phosphorylation by PKCcat is consonant with the presence of multiple phosphorylation sites scattered throughout $G_{s\alpha}$ (e.g., 7 serine, 8 threonine and 3 tyrosine; predicted by NetPhos 2.0; NetPhosk 1.0 predictions include 10 PKC/PKA sites of >0.5 probability, of which 3 have a probability of >0.70).

The second salient finding is that chronic morphine decreases the stoichiometry of $G_{s\alpha}$ phosphorylation concomitant with its increased association with PP2A. This inference of their causal association is validated by the demonstration that the chronic morphine-induced decrement in $G_{s\alpha}$ phosphorylation is abolished by acute (30 min) pretreatment with Calyculin A, an inhibitor of PP1/PP2A (data not shown). The third notable finding is that *in vitro* de-phosphorylation of $G_{s\alpha}$ increases its association with MOR. Interestingly, augmented phosphorylation of $G_{s\alpha}$ (on tyrosine by pp6Oc-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 $G_{s\alpha}$ 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 $G_{s\alpha}$ phosphorylation generalizes to other 'stimulatory' vs. 'inhibitory' receptors.

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Importantly, 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. In the aggregate, these results strongly suggest that the phosphorylation state of $G_{s\alpha}$ is a critical determinant of its interaction with MOR, which is regulated (decreased) by chronic morphine. We previously demonstrated that chronic morphine increases the association of MOR with $G_{s\alpha}$ (Chakrabarti et al., 2005a). The present results identify decreased $G_{s\alpha}$ phosphorylation as a mechanism central to this change in MOR $G_{s\alpha}$ 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 PKA/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_{β} subunit of G proteins, phospholipase Cβ3 and AC (Chakrabarti and Gintzler, 2003a; Chakrabarti and Gintzler, 2003b; Chakrabarti et al., 2001; Chakrabarti et al., 1998b). A reduction in phosphorylation of phospholipase Cβ1 (Chakrabarti and Gintzler, 2003b) and MAP kinase (Schulz and Hollt, 1998) has been demonstrated after chronic morphine and its withdrawal. However, to date, with a few notable exceptions, opioid tolerance has been associated mostly with enhancement in phosphorylation of multiple signaling protein(s). The current demonstration that chronic morphine decreases phosphorylation of $G_{s\alpha}$ that in turn augments its association with MOR represents a novel dimension of adaptation to chronic morphine.

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Increased interaction of MOR with $G_{s\alpha}$ following chronic morphine would act in parallel with and compliment signaling consequences of adaptations to chronic morphine we previously identified, i.e., increased availability of $G_{\beta\gamma}$ (Chakrabarti et al., 2001), increased phosphorylation of G_{β} (Chakrabarti and Gintzler, 2003a; Chakrabarti et al., 2001), augmented AC isoform-specific synthesis and phosphorylation (Chakrabarti et al., 1998a; Chakrabarti et al., 1998b; Rivera and Gintzler, 1998). These converge to shift MOR-coupled signaling from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ AC stimulatory that would mitigate the persistent opioid inhibition of AC(s) via the opioid receptor-coupled generation of $G_{i\alpha}$ (see Gintzler and Chakrabarti, 2006 for review).

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 $G_{s\alpha}$ is also essential for a substantial component of $G_{\beta\gamma}$ stimulation of AC (Tang and Gilman, 1991). *In vivo,* activated $G_{s\alpha}$ could be generated via the ongoing activation of a multitude of G_s-coupled receptors, independent of opioid receptor function. This notwithstanding, the present report underscores that direct coupling of MOR to G_s represents an additional source of activated $G_{s\alpha}$; concomitant opioid receptor signaling via G_s as well as G_i/G_o would result in the coordinate generation of activated $G_{s\alpha}$ (from G_s) and $G_{\beta\gamma}$ (from G_s as well as G_i/G_o). Thus, increased MOR-coupled generation of activated $G_{s\alpha}$ during morphine tolerance would be functionally coordinated with the previously described concomitant emergence of opioid receptor-coupled AC stimulatory $G_{\beta\gamma}$ signaling. Additionally, enhanced MOR-G_s coupling (the effects of which would be amplified by the modestly more AC stimulatory activity of de-phosphorylated $G_{s\alpha}$; 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 $G_{s\alpha}$ 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.

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Co-immunoprecipitation of proteins demonstrates their presence in stable high-affinity complexes. However, difficulties in distinguishing between a naturally occurring complex vs. 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 $G_{s\alpha}$ that co-precipitates with increasing amounts of PP2A diminishes following chronic morphine and can be blocked by Calyculin A. This strongly suggests an increased functional association between $G_{s\alpha}$ 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 following catalysis of its de-phosphorylation. The co-immunoprecipitation of PP2A and de-phosphorylated $G_{s\alpha}$ demonstrated in the present study can most easily be explained by postulating the presence of a third, as of yet unidentified, protein/lipid that serves as an anchor for both PP2A and $G_{s\alpha}$.

The observed increased association of PP2A with $G_{s\alpha}$ following chronic morphine most likely results from the translocation of one or both since their membrane content does not change after this treatment. We previously demonstrated that a macromolecular signaling complex containing PKCγ, G_{β} and AC underlies, in part, the previously reported shift from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory AC signaling (Chakrabarti et al., 2005b) and that chronic morphine induces the concomitant phosphorylation of G protein-coupled receptor kinase 2/3, β-arrestin and G_{β}, 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 micro-signaling domains by chronic morphine to opioid tolerance formation and that PP2A should be considered as a scaffolding molecule that facilitates the interaction between MOR and $G_{s\alpha}$, in addition to its enzymatic function.

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It is important to note that tolerant-producing mechanisms demonstrated in this study are not mutually exclusive. Numerous adaptations to chronic morphine have been observed and postulated to be causally associated with opioid tolerance. These include opioid receptor downregulation/internalization (Chakrabarti et al., 1995; Chavkin and Goldstein, 1984; Cox and Crowder, 2004), MOR G protein uncoupling (Sim et al., 1996) and AC superactivation. Altered association/activity of RGS (regulators of G-protein signaling) proteins (Garzon et al., 2005; Xie and Palmer, 2005; Xu et al., 2004; Zachariou et al., 2003) 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 in the field. Clearly, future studies will be required to parse the relative importance to opioid tolerance of the adaptations demonstrated in this study and mechanisms suggested by the literature and how they may 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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Legends for Figures

Fig. 1. $G_{s\alpha}$ is dephosphorylated following chronic morphine treatment. A (lanes 1, 2, and 3), autoradiographic analysis of $G_{s\alpha}$ IP obtained, in parallel, from opioid naïve and chronic morphine-treated MOR-CHO cells in the absence and presence of calyculin A, respectively. MOR-CHO cells were labeled with ^{32}Pi and membranes were prepared as described in Materials and Methods. $G_{s\alpha}$ immunoprecipitation was initiated using 400 μg of solubilized, radiolabeled MOR-CHO cell membranes and anti-$G_{s\alpha}$ antibodies (4 μl/400 μg). The involvement of PP1/PP2A in the chronic morphine-induced decrement in $G_{s\alpha}$ phosphorylation is underscored by its abolition by calyculin A (lane 3). The $G_{s\alpha}$ 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-$G_{s\alpha}$ antibodies (lanes 4 and 5) or cell membranes obtained from MOR CHO were subjected to Westerns using anti-$G_{s\alpha}$ antibodies (lanes 6, 7). The absence of a $G_{s\alpha}$ Western signal that corresponds to the ≈52 kDa radiolabeled band most likely reflects its low protein content. Specificity of the $G_{s\alpha}$ 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 3-5 fold excess antigen. B, Phospho-threonine Western analysis of $G_{s\alpha}$ IP from spinal cord membranes of opioid naïve (lane 1) and chronic morphine treated rats (lane 2). In lanes 3 and 4, the same immunoblot was re-probed with anti-$G_{s\alpha}$ antibodies after stripping off anti-phospho-threonine antibodies. $G_{s\alpha}$ phosphorylation decreases following chronic morphine treatment, which cannot be attributed to a decrement in the $G_{s\alpha}$ content of the $G_{s\alpha}$ IP. Results represent replicates of three observations.

Fig. 2. PP2A co-immunoprecipitates with $G_{s\alpha}$ and their association increases following chronic morphine treatment. $G_{s\alpha}$ IP was performed as mentioned in Materials and Methods. Top: Western

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blot of PP2A in $G_{s\alpha}$ IP, obtained in parallel from membranes of opioid naïve and chronic 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 naïve and chronic morphine-treated MOR-CHO cells (5 μg, respectively). Chronic morphine induces an increase in the PP2A that co-immunoprecipitates with $G_{s\alpha}$ (lanes 1-6). Lower panel represents $G_{s\alpha}$ Western analysis of the same blot after striping and re-probing with anti-$G_{s\alpha}$ antibodies. The chronic morphine-induced increment in co-immunoprecipitation of PP2A with $G_{s\alpha}$ occurs in the absence of increased content of $G_{s\alpha}$.

Fig. 3. PP2A activity increases in MOR-CHO cells after chronic morphine. A, bar diagram denotes increased PP2A activity measured in PP2A IP from chronic morphine treated vs. untreated MOR-CHO cell membranes (bar 2 vs. 1). Details of PP2A activity assay are mentioned in Methods and Materials. Briefly, 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 % control (pmoles phosphate/sample). *$p<0.05$ for chronic morphine vs. naïve; n=4. B, Western analysis illustrating that the increased activity of PP2A after chronic morphine treatment is not accompanied by an increase in the MOR-CHO membrane content of PP2A or the efficiency of PP2A immunoprecipitation.

Fig. 4. *In vitro* de-phosphorylation of $G_{s\alpha}$ enhances its association with MOR. Western analysis of $G_{s\alpha}$ IP obtained from MOR-CHO membranes following incubation with purified recombinant $G_{s\alpha}$ that had been either de-phosphorylated *in vitro* using protein phosphatases and 2A or mock de-phosphorylated, PP+ and PP-, respectively. The content of MOR in the $G_{s\alpha}$ IP was quantified by Western analyses using anti-MOR antibodies. The $G_{s\alpha}$ Western blot of the $G_{s\alpha}$ IP, shown below the

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break in the gel, illustrates that the augmented co-immunoprecipitation of MOR with $G_{s\alpha}$ following its pretreatment with protein phosphatases did not result from differential loading. Bar graphs shown below illustrate that the content of MOR (~80 kDa) in $G_{s\alpha}$ IP was substantially increased following incubation of MOR-CHO membranes with *in vitro* de-phosphorylated vs. mock de-phosphorylated $rG_{s\alpha}$. * $p<0.05$; n=3.

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Table 1. Effect of Gsα dephosphorylation on its ability to stimulate AC2 activity

Gsα (dose)	Δ cAMP formation (pmoles/mg/min)
10 nM	36.012
20 nM	45.91*
40 nM	40.98*
60 nM	76.46*

Δ cAMP formation represents the differences in activity of AC2 when stimulated by 'mock' (vehicle-treated) dephosphorylated vs. de-phosphorylated (via PP2A + PP1) rG_{sa}. ANOVA and Tukey's post hoc test were used to assess significance of difference from zero for each indicated change in pmoles cAMP formed. *=$p<0.05$

Fig. 1

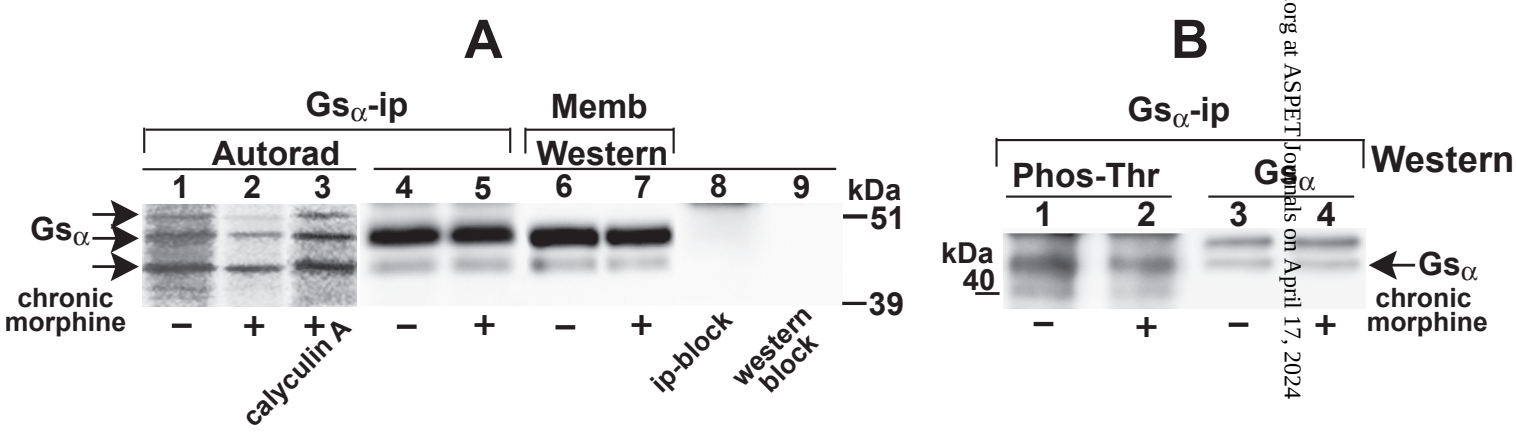

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Fig. 2

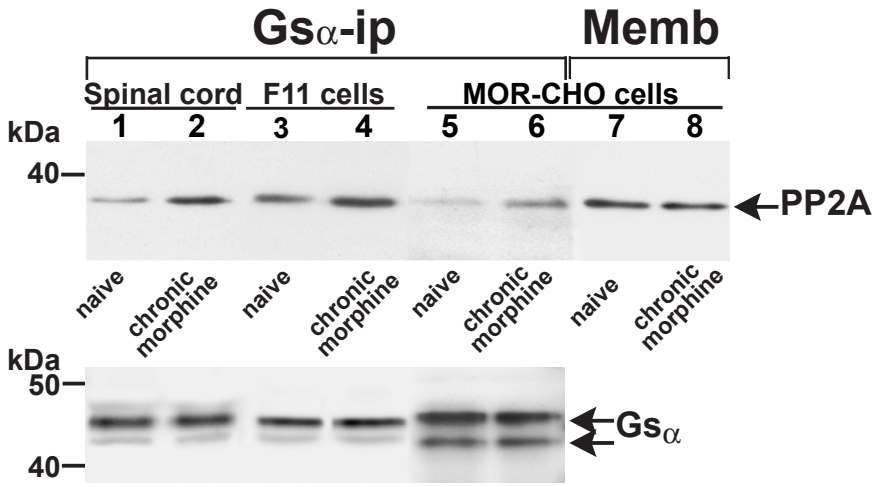

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Fig. 3

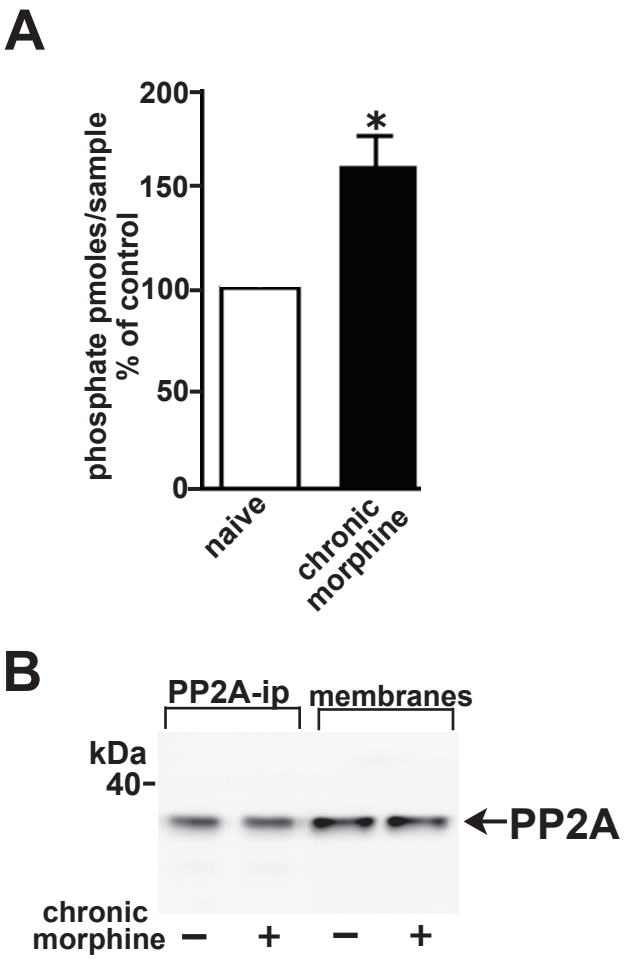

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Fig. 4