0026-895X/08/7303-868–879$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2008 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 73:868–879, 2008

Vol. 73, No. 3
42184/3304038
Printed in U.S.A.

Plasticity of Adenylyl Cyclase-Related Signaling Sequelae after Long-Term Morphine Treatment

Michael Shy, Sumita Chakrabarti, and Alan R. Gintzler

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

Received September 26, 2007; accepted November 27, 2007

ABSTRACT

Adaptations to long-term morphine treatment resulting in tolerance are protective by counteracting the consequences of sustained opioid receptor activation. Consequently, the manifestation of specific adenylyl cyclase (AC)-related neurochemical sequelae of long-term morphine treatment should depend on the consequences of short-term μ-opioid receptor (MOR) activation. We tested this by comparing complementary chemical sequelae of long-term morphine treatment among cells in which short-term MOR activation inhibited instead of stimulated AC activity. Short-term activation of MOR in Chinese hamster ovary (CHO) cells stably transfected with MOR (MOR-CHO) inhibits AC activity. Long-term morphine treatment of these cells increased AC and Gβ phosphorylation, membrane protein kinase Cγ (PKCγ) translocation, and MOR G_s association. All converge, shifting the consequences of short-term MOR activation from $G\alpha_i/G\alpha_o$ inhibitory to AC stimulatory signaling. In contrast, overexpression of the G$\beta\gamma$-stimulated AC isoform AC2 (which converted MOR-coupled inhibition to stimulation of AC) eliminated or reversed these adaptations to long-term morphine treatment; it negated the increase in Gβ phosphorylation and PKCγ translocation while reversing the increase in AC phosphorylation and MOR G_s association. These adaptations greatly attenuated MOR-coupled stimulation of AC activity. Altered overexpression of AC protein per se was not a confounding factor because MOR-CHO overexpressing AC1, which is inhibited by short-term MOR activation, manifested adaptations to long-term morphine treatment qualitatively identical with those of MOR-CHO. These results reveal that adaptations elicited by long-term morphine treatment depend on the effects of short-term MOR activation. This dynamic and pliable nature of tolerance mechanisms could represent a new paradigm for pharmacotherapeutics.

All formulations of opioid tolerance are based on the tenet that it is adaptive. All identified neurochemical adaptations to long-term morphine enable cell survival by reinstating initial steady-state conditions. For example, up-regulation of the adenylyl cyclase (AC) system, believed to underlie the AC "superactivation" that is manifest upon short-term precipitated opioid withdrawal of long-term opioid-treated tissue (Chavkin and Goldstein, 1984; Chakrabarti et al., 1995; Cox and Crowder, 2004), would neutralize the persistent ongoing opioid receptor-coupled inhibition of AC. Opioid receptor uncoupling and its arrestin-mediated internalization that has been demonstrated in some preparations after persistent opioid exposure (Appleyard et al., 1999; Bohn et al., 2000) has been postulated to compensate for the sustained inhibitory action of drugs like morphine that are resistant to proteolytic degradation and thus persist in the extracellular milieu. Augmented AC stimulatory signaling via G$\beta\gamma$ (Chakrabarti et al., 1998a,b, 2005b; Chakrabarti and Gintzler, 2003) and increased interaction of the μ-opioid receptor (MOR) with G_s (Chakrabarti et al., 2005a) that occurs after persistent exposure to morphine would neutralize the functional consequences of ongoing opioid receptor-coupled inhibitory signaling and thereby reinstate naive steady-state cAMP conditions.

A protective function of diminished responsiveness to opioids requires that adaptations to long-term morphine treatment should not be unidirectional and invariant. Instead, mechanisms cells use to cope with the persistent presence of morphine should be pliable and depend on the initial steady-state conditions. We hypothesized that the strategies that cells use to rescue themselves from the sustained activation of opioid receptors would depend on the consequences of their short-term activation in the opioid-naive state. This formulation predicts that previously demonstrated sequelae of long-term morphine treatment [e.g., augmented phosphorylation of AC (Chakrabarti et al., 1998b) and the Gβ subunit of G proteins (Chakrabarti et al., 2001, 2005b; Chakrabarti and Gintzler,

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.042184.

ABBREVIATIONS: AC, adenylyl cyclase; MOR, μ-opioid receptor; MOR-CHO, Chinese hamster ovary cells stably expressing μ-opioid receptor; AC1-MOR-CHO, Chinese hamster ovary cells stably expressing μ-opioid receptor overexpressing AC1; AC2-MOR-CHO, Chinese hamster ovary cells stably expressing μ-opioid receptor overexpressing AC2; IP, immunoprecipitate; LMMP, longitudinal muscle myenteric plexus; PKCγ, protein kinase Cγ; GPCR, G protein-coupled receptor; DTT, dithiothreitol; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; ANOVA, analysis of variance.

2003], increased membrane translocation of protein kinase Cγ (PKCγ) (Mao et al., 1995; Zeitz et al., 2001; Chakrabarti et al., 2005b), increased MOR $G\alpha_s$ association (Chakrabarti et al., 2005a), all of which shift short-term MOR-coupled signaling from AC inhibitory to stimulatory (Gintzler and Chakrabarti, 2006), are not hard-wired and in fact will be negated, or reversed, in opioid-naive cells manifesting short-term stimulatory responsiveness to MOR activation.

One determinant of the direction of G protein-coupled receptor (GPCR) signaling is the relative abundance of AC isoform protein. For example, agonists that act through "inhibitory" G_i-coupled receptors can be converted into stimulators of cAMP synthesis by overexpressing AC2 or AC7 (along with constitutively active $G\alpha_s$) (Federman et al., 1992; Yoshimura et al., 1996). Likewise, the cloned δ-opioid receptor has been shown to inhibit or stimulate AC activity depending on the AC isoforms present (Tsu et al., 1995).

The current study uses the dependence of the consequence of GPCR (i.e., opioid receptor) activation on AC isoform expression to test our postulate that the direction of short-term opioid responsiveness (inhibitory versus stimulatory) is a critical determinant of the mechanism(s) cells use to adapt to the persistent presence of morphine. We determined the effect of AC isoform state on targeted neurochemical adaptations to long-term morphine that impinge on the cAMP system identified previously; these adaptations were compared among Chinese hamster ovary (CHO) cells stably expressing MOR (MOR-CHO) and MOR-CHO overexpressing either AC1 or AC2 (AC1-MOR-CHO and AC2-MOR-CHO, respectively). AC1 and AC2 isoforms were selected because they are differentially regulated by $G\beta\gamma$, which would result in their qualitatively opposite MOR-coupled short-term regulation. Results reveal that the initial cellular starting conditions (i.e., MOR stimulatory versus inhibitory AC responsiveness) are a major determinant of postreceptor cAMP-associated adaptations to long-term morphine treatment underscoring their plasticity and their dependence on cell physiology.

Materials and Methods

Cell Culture, Transfection, and Morphine Treatment. MOR-CHO cells were maintained in Dulbecco's modified Eagle's medium high glucose with L-glutamine supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 100 μg/ml Geneticin and plated at 3.8×10^6 cells/150 mm^2 dishes. At 65 to 70% confluence, MOR-CHO cells were transiently transfected with AC2 cDNA (AC2-pRC/CMV), AC1 cDNA (pcDNA 3.1), or empty vector (untransfected) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The next day, at 90 to 95% confluence, cells were further divided into groups and treated with vehicle or morphine (1 μM) for 48 h. Media containing morphine or vehicle were replenished every 24 h.

$^{32}P_i$-Labeling of MOR-CHO . On the day of harvest, cells were incubated for 2 h in phosphate- and serum-free Dulbecco's modified Eagle's medium 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 an 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, and 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, sodium-*p*-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, and complete cocktail inhibitor tablet per 50 ml. Calyculin A, a protein phosphatase 1 and protein phosphatase 2A inhibitor, was added after ^{32}P-labeling before the onset of membrane preparation. Cells were homogenized and centrifuged at 1000*g* at 4°C for 10 min. Supernatants obtained from the low-speed spin were subjected to a high-speed spin at 30,000*g* for 40 min at 4°C. Morphine (0.1 μM) was maintained in all solutions for preparation of membranes from long-term morphine-treated cells.

Membrane fractions obtained were resuspended in HEPES buffer, pH 7.4, containing 5 mM EDTA, 1 mM concentration each of 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 method) and immunoprecipitation.

AC was immunoprecipitated using BBC-4 monoclonal antibodies, which is generated against AC purified from bovine brain (6 μl/600 μg of membrane protein; generously provided by Dr. S. Mollner, Heinrich Heine University, Dusseldorf, Germany) (Mollner and Pfeuffer, 1988; Chakrabarti et al., 1998b). This antibody recognizes 23 amino acids of the second cytoplasmic domain (KGPVVAGVIGARKPQYDIVVGNT; S. Mollner, personal communication). PKCγ was immunoprecipitated using a mouse monoclonal antibody (6 μl/600 μg of protein; Sigma Chemical, St. Louis, MO), generated against rat PKCγ 684 to 697 amino acid residues (Chakrabarti et al., 2005b). $G\alpha_s$ 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 (385–394 amino acids; U.S. Biologicals, Swampscott, MA; 1 μl/100 μg of protein) (Chakrabarti et al., 2005b; Chakrabarti and Gintzler, 2007). 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 to 12% gradient Bis-Tris gels (Invitrogen) were electrotransferred 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, Buckinghamshire, UK). ^{32}Pi incorporated into phosphorylated samples was determined using densitometric analysis (ImageQuant; GE Healthcare).

Western Analysis. Standard procedures were used for Western analyses as used previously by this laboratory (Chakrabarti et al., 1998a). When autoradiographic analyses were performed, nitrocellulose membranes initially processed for densitometric analyses in the PhosphorImager was sequentially subjected to Western analyses. Coincidence of autoradiographic and Western signals was used to validate the chemical identity of radiolabeled bands (Chakrabarti and Gintzler, 2003, 2007; Chakrabarti et al., 2005b). MOR protein was visualized using a 1:10,000 dilution of a rabbit polyclonal antibody (affinity-purified) generated against the C-terminal 50 amino acids of MOR (generously provided by Dr. Thomas Cote, Uniformed Services University of the Health Sciences, Bethesda, MD). $G\alpha_s$ and Gβ proteins were visualized using a 1:10,000 and 1:15,000 dilution of C-terminal anti-$G\alpha_s$ and anti-Gβ polyclonal antibodies, respectively (both generously supplied by Dr. John Hildebrandt, Medical University of South Carolina, Charleston, SC) (Chakrabarti et al., 2005b). PKCγ protein was visualized using a polyclonal antibody generated against the carboxyl terminus of PKCγ (Santa Cruz Biotechnology,

Santa Cruz, CA) (Chakrabarti et al., 2005b). AC protein was visualized using monoclonal antibodies BBC-4 (generously provided by Dr. S. Mollner, Heinrich Heine University, Dusseldorf, Germany) (Chakrabarti et al., 2005b). The secondary antibody used was either a peroxidase-labeled donkey anti-rabbit or a peroxidase-labeled sheep anti-mouse antibody from GE Healthcare. In all instances, sample pairs, obtained from opioid-naive and long-term morphine-treated MOR-CHO cultures were processed, electrophoresed, and blotted in parallel. Antibody-substrate complex was visualized using a Supersignal West Dura Chemiluminescence detection kit (Pierce, Rockford, IL) and a charge-coupled device camera (GeneGnome, Syngene, Frederick, MD). Intensity of signal was quantified using Syngene software. Because specificity of AC, Gβ, PKCγ, and Gα_s Western signals obtained with these antibodies in MOR-CHO membranes had been demonstrated previously (Chakrabarti et al., 1998b, 2005a,b), these controls were not repeated in the current study.

Determination of AC Activity. AC activity was determined (30°C, 15 min) 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 U/sample creatine phosphokinase, 0.1 mM 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 (50 or 5 μg for membranes from MOR-CHO or AC2-MOR-CHO, respectively). Membranes were preincubated with rGα_s (30°C, 15 min), activated previously by prior 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 bovine serum albumin (1.25 mg/ml) as described previously (Tang and Gilman, 1991). The activated Gα_s-GTPγS was separated from free GTPγS using gel filtration (Sephadex G-25 spin columns). [α-^{32}P]cAMP formation was 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. To determine the influence of AC overexpression state on MOR-coupled regulation of AC activity and modulation thereof, [^{32}P]cAMP formation was determined in membranes obtained from opioid-naive and long-term morphine-treated MOR-CHO, ACl-MOR-CHO, and AC2-MOR-CHO in the absence or presence (5-min pretreatment) of the MOR agonist sufentanil (3, 10, 100, and 1000 nM). The role of G$\beta\gamma$ in MOR-coupled modulation of AC was investigated by determining the effect of pretreating membranes with the G$\beta\gamma$-blocking peptide QEHA (50 μM; 20 min on ice) (Chen et al., 1995; Chakrabarti et al., 1998a).

Statistical Analyses. Either one-way or factorial mixed model analysis of variance (ANOVA) was used to locate statistically significant interactions between the main effects of opioid treatment, AC overexpression, and the G$\beta\gamma$ blocking peptide QEHA. Analysis of covariance was used to assess AC phosphorylation while adjusting for AC protein changes. Thereafter, post hoc analyses using least significant difference tests were performed to detect the sources of the significant interactions. To assess the effect of opioid treatments within an AC expression group, t test was used.

Results

Overexpression of AC2 in MOR-CHO Shifted the Consequences of Short-Term MOR Activation from Inhibition to Stimulation of AC. Experiments were conducted to validate that overexpression of the G$\beta\gamma$-stimulated AC isoform AC2 in MOR-CHO would reverse short-term MOR-coupled inhibition of AC activity to a G$\beta\gamma$-mediated stimulation. The potent MOR-selective agonist sufentanil was used to maximize differences in effects of MOR activation in MOR-CHO versus AC2-MOR-CHO. As expected, short-term MOR activation in MOR-CHO inhibited AC activity; a decrement in cAMP production was observed at all sufentanil concentrations tested (3, 10, 100, and 1000 nM). In contrast, in AC2-MOR-CHO, short-term MOR activation did not inhibit AC activity. Instead, the opposite response was observed; ANOVA analysis indicated that sufentanil dose-dependently stimulated AC activity ($n = 8$, $p < 0.001$; Fig. 1).

The G$\beta\gamma$-dependence of MOR-coupled AC stimulation in AC2-MOR-CHO was revealed by assessing the effect of short-term MOR activation on AC activity in the presence versus the absence of the G$\beta\gamma$-blocking peptide QEHA (Fig. 1, inset). ANOVA analysis revealed that QEHA (50 μM) abolished the increment in AC activity produced by short-term activation of MOR ($p < 0.001$). This indicates that the observed stimulation of AC by sufentanil in AC2-MOR-CHO is dependent on G$\beta\gamma$ regulation.

We used ANOVA analysis to assess cross-tolerance between the AC stimulatory effect of MOR activation in AC2-MOR-CHO and morphine. This revealed that sufentanil (3, 10, 100, and 1000 nM) was not able to stimulate AC activity in AC2 MOR-CHO after treatment with morphine (1 μM for 48 h; $n = 4$, $p > 0.05$; Fig. 1). The magnitudes of the sufentanil stimulation of AC activity were substantially attenuated at all concentrations tested ($n = 4$–8; $p < 0.01$), suggesting cross-tolerance with morphine.

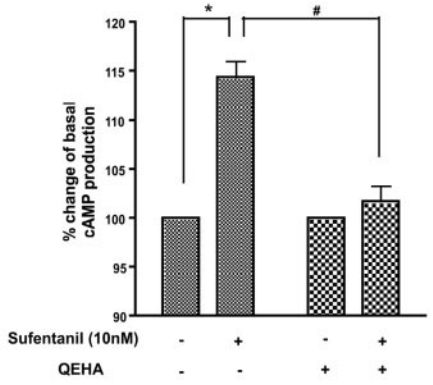
Membranes from long-term morphine-treated AC2-MOR-CHO were maintained in the presence of low levels of morphine (0.1 μM) to prevent the onset of withdrawal. This could have confounded conclusions of cross-tolerance between AC excitatory effects of sufentanil and morphine because the low morphine levels could have produced a maximal stimulation of AC, which would mask stimulation of AC produced by sufentanil. This possibility was eliminated by demonstrating equivalent basal cyclase activity in membranes of opioid-naive versus long-term morphine-treated AC2-MOR-CHO cells maintained in the presence of 0.1 μM morphine (134.8 ± 15.1 and 132.8 ± 6.3, cAMP pmol/mg/min, respectively). Dose-dependent stimulation of AC by sufentanil and its cross-tolerance with morphine validated the use of AC2-MOR-CHO cells to test whether or not initial starting conditions are relevant to adaptations elicited by long-term morphine.

Ability of Long-Term Morphine Treatment to Augment Gα_s-Dependent G$\beta\gamma$ Stimulation of AC Is Significantly Attenuated in AC2-MOR-CHO Cells. As was first observed in membranes obtained from longitudinal muscle myenteric plexus (LMMP) tissue (Chakrabarti et al., 1998a), the magnitude of stimulation of AC activity produced by rGα_s (2.5, 5, 10, and 20 nM) is significantly augmented in membranes obtained from long-term morphine-treated versus opioid-naive MOR-CHO cells (Fig. 2A). ANOVA revealed significant effects for morphine, rGα_s concentration, and their interaction ($p < 0.001$ for all comparisons). Regression analysis indicated that as the concentration of rGα_s increased, so did the long-term morphine-induced increment in AC activity ($p < 0.05$; $n = 4$–9).

To determine whether the increase in Gα_s stimulation of AC after long-term morphine treatment is G$\beta\gamma$-dependent, as has been reported for LMMP tissue (Chakrabarti et al., 1998a), we determined the effect of QEHA on rGα_s stimulation of AC activity in membranes obtained from opioid-naive and long-term morphine-treated MOR-CHO cells. The ANOVA analysis revealed a significant interaction between the morphine treatment condition (absence or presence of morphine)

and the ability of QEHA to alter rGα_s AC stimulation ($p = 0.001$). QEHA (50 μM) did not have any effect on the increment in AC activity produced by rGα_s (5 nM) in membranes of opioid-naive cells. This indicated that the increased activity of AC resulted from its direct stimulation by rGα_s (and not via G$\beta\gamma$). In contrast, QEHA (50 μM) abolished the long-term morphine-induced increment in rGα_s AC stimulation in membranes obtained from long-term morphine-treated MOR-CHO cells; in the presence of QEHA, the magnitude of the increment in AC activity produced in membranes of long-term morphine-treated and opioid-naive MOR-CHO cells was indistinguishable ($p = 0.421$) (Fig. 2A, inset). Thus, in MOR-CHO cells as in LMMP tissue, long-term morphine treatment augments Gα_s-dependent G$\beta\gamma$ stimulation of AC.

Membranes obtained from opioid-naive and long-term morphine-treated AC2-MOR-CHO cells also manifested rGα_s dose-dependent stimulation of AC activity (Fig. 2B). However, in AC2-MOR-CHO cells, unlike MOR-CHO cells, long-term morphine treatment failed to augment the magnitude of Gα_s stimulation of AC. ANOVA revealed a significant main effect for rGα_s concentration ($p < 0.001$) but not for the presence or absence of long-term morphine treatment or for the interaction between these two variables. The inability of long-term morphine to alter rGα_s stimulation of AC was observed for all rGα_s concentrations ($p > 0.17$; $n = 5$). Thus, overexpression of AC2 in MOR-CHO cells resulted in the inability to manifest increased Gα_s-dependent G$\beta\gamma$ stimulation of AC activity in response to long-term morphine. This indicated the utility of investigating the influence of AC2 overexpression in MOR-CHO cells on those adaptations that underlie augmented G$\beta\gamma$-AC stimulation after long-term morphine treatment [i.e., increased phosphorylation of AC



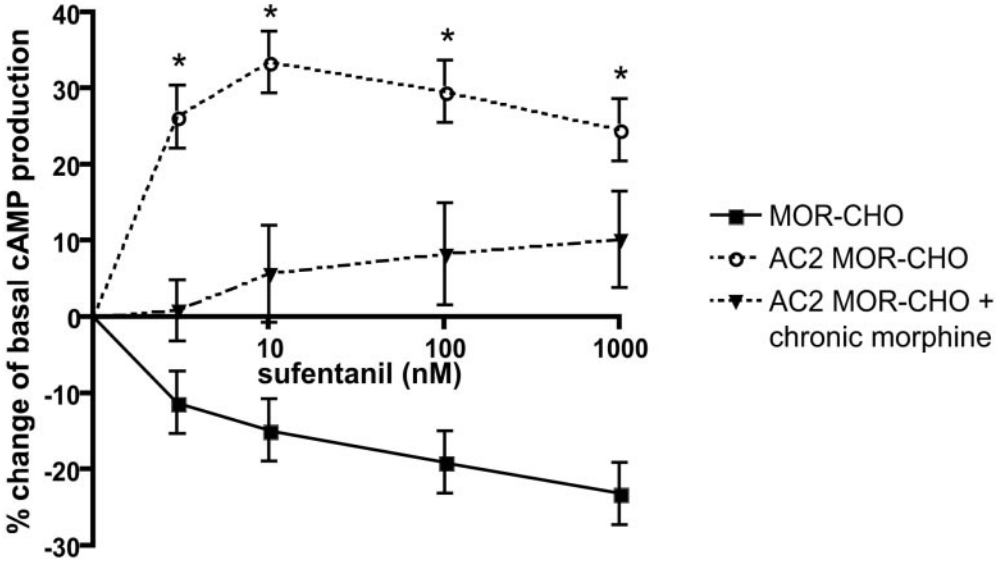


Fig. 1. AC2 overexpression reverses MOR-coupled inhibitory modulation of AC activity to stimulatory. Formation of [α-^{32}P]cAMP from [α-^{32}P]ATP was used to reflect AC activity as described under *Materials and Methods*. Synthesis of radiolabeled cAMP was determined in membranes obtained from MOR-CHO (■), AC2-MOR-CHO (○), and long-term morphine-treated (1 μM, 48 h) AC2-MOR-CHO (▼), each without and with the indicated concentrations of sufentanil. *, $p < 0.01$ for effect of MOR activation on AC activity in membranes of naive AC2-MOR-CHO versus long-term morphine-treated AC2-MOR-CHO ($n = 4$) and MOR-CHO ($n = 8$). ANOVA revealed a significant three-way interaction between effects of MOR activation, AC overexpression, and long-term morphine exposure ($p < 0.001$). Basal AC activity (cAMP pmol/mg/min) in AC2-MOR-CHO membranes in the absence and presence of long-term morphine exposure was 134.8 ± 15.1 and 132.8 ± 6.3 ($n = 4$), respectively. AC activity (cAMP pmol/mg/min) in MOR-CHO membranes with 1 μM forskolin was 18.7 ± 1.6 ($n = 8$). Inset, effect of QEHA (50 μM) on the stimulatory responsiveness of AC2-MOR-CHO membranes to sufentanil (10 nM). #, $p < 0.001$ for the effect of sufentanil (10 nM) on AC activity in the presence versus absence of QEHA in membranes of AC2-MOR-CHO ($n = 6$). MOR activation inhibits AC activity in MOR-CHO cells yet enhances AC activity in AC2-MOR-CHO cells, which is attenuated after long-term morphine. Abolishment of the MOR-coupled increment in AC activity by QEHA indicates its mediation by G$\beta\gamma$. Loss of sufentanil inhibition of AC activity in MOR-CHO after long-term morphine treatment was not shown because this effect has been extensively demonstrated.

(Chakrabarti et al., 1998b), and Gβ subunit of G$\beta\gamma$ (Chakrabarti et al., 2001; Chakrabarti and Gintzler, 2003; Chakrabarti et al., 2005b) and membrane translocation of PKCγ (Chakrabarti et al., 2005b)].

AC2 Overexpression Abolished the Ability of Long-Term Morphine Treatment to Augment AC Phosphorylation. We quantified metabolic phosphorylation of AC in opioid-naive versus long-term morphine-treated (1 μM, 48h) AC2-MOR-CHO and MOR-CHO cells. Autoradiograms of immunoprecipitate (IP) obtained using anti-AC antibodies (AC IP) from membranes of MOR-CHO contained two radiolabeled bands of ≈155 kDa and ≈130 kDa (Fig. 3A, lanes 1 and 2). Analogous bands were observed in AC IP from membranes of AC2-MOR-CHO cells. As seen in previous studies (Böl et al., 1997), in contrast to MOR-CHO cells, the molecular mass of the predominant band in AC2-MOR-CHO encompassed molecular mass that ranged from ≈110 to ≈130 kDa (Fig. 3B, lanes 3 to 4), reflecting phosphorylation of the more abundant AC2 (≈ 120 kDa) that had been overexpressed.

Densitometric analyses were performed on the ^{32}P-radiolabeled AC bands present in AC IP obtained from membranes of MOR-CHO and AC2-MOR-CHO cells that had been maintained with and without long-term morphine. In MOR-CHO cells, long-term morphine treatment increased AC phosphorylation (≈72%; $p = 0.002$, Fig. 3A, lane 2 versus 1), as was initially reported for LMMP tissue (Chakrabarti et al., 1998b). In contrast, in AC2-MOR-CHO cells, the long-term morphine-induced increment in AC phosphorylation was no longer observed; on the contrary, long-term morphine treatment decreased AC phosphorylation (≈34%; $p < 0.001$, Fig. 3B, lanes 4 versus 3).

There was an increment in AC protein immunoprecipitated from long-term morphine-treated MOR-CHO cells ($p = 0.05$; Fig. 3A, bottom), consistent with previous observations (Chakrabarti et al., 2005b). It is noteworthy, however, that its magnitude (≈25%) was less than that of the observed increase in AC phosphorylation (≈72%). This indicates that long-term morphine treatment resulted in a net increase in AC phosphorylation in these cells, as was reported for LMMP tissue (Chakrabarti et al., 1998b). Long-term morphine treatment of AC2-MOR-CHO also resulted in an increment in the magnitude of immunoprecipitated AC (≈41%, $p = 0.023$; Fig. 3B, bottom). It is interesting that in contrast to MOR-CHO cells, this occurred concomitant with a diminution in AC phosphorylation. Thus, the observed decrement in AC phosphorylation produced by long-term morphine treatment in these cells (≈36%) is an underestimate of its true magnitude. These results suggested that the effect of long-term morphine treatment on AC phosphorylation is dependent on the consequences of short-term activation of MOR (inhibitory versus stimulatory).

Overexpression of AC1 Failed to Suppress Long-Term Morphine Treatment-Induced Augmentation of AC Phosphorylation. The G$\beta\gamma$ subunit differentially regulates AC1 and AC2 (Tang and Gilman, 1991). G$\beta\gamma$ stimulates AC2 whereas it inhibits AC1. As a consequence, a shift from MOR-coupled inhibition to stimulation of AC should not occur as a result of AC1 overexpression. As a result, compari-

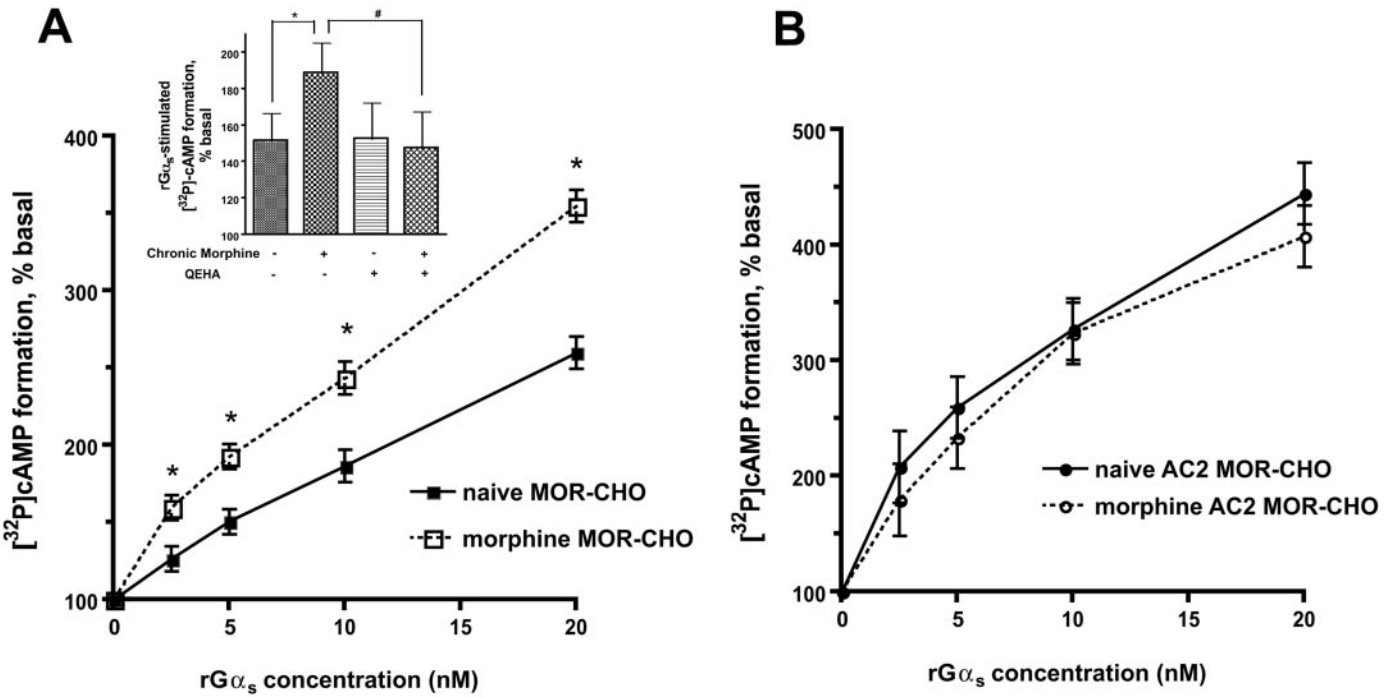


Fig. 2. Overexpression of AC2 in MOR-CHO abolished the increment in G$\beta\gamma$ stimulation of AC activity produced by long-term morphine treatment. The magnitude of AC stimulation produced by activated rGα_s was determined in membranes of opioid-naive and long-term morphine-treated MOR-CHO (A) and AC2-MOR-CHO (B). Basal AC activity (cAMP pmol/mg/min) in AC2-MOR-CHO membranes in the absence and presence of long-term morphine treatment was 107.4 ± 20.3 and 108.1 ± 18.4 ($n = 5$), respectively. Basal AC activity (cAMP pmol/mg/min) in MOR-CHO membranes without and with long-term morphine treatment was 2.3 ± 0.3 and 2.1 ± 0.4 ($n = 9$), respectively. *, $p < 0.002$ for the magnitude of rGα_s stimulation in long-term morphine-treated versus naive membranes ($n = 4$–9). Inset, effect of QEHA (50 μM) on the rGα_s (5 nM) stimulatory responsiveness of membranes obtained from opioid-naive versus long-term morphine-treated MOR-CHO. #, $p = 0.001$ for effect of QEHA on rGα_s stimulatory responsiveness in long-term morphine-treated MOR-CHO membranes ($n = 4$). In membranes obtained from MOR-CHO, long-term morphine treatment augments AC stimulatory responsiveness to rGα_s, which is abolished by QEHA. In contrast, long-term morphine treatment does not increase rGα_s stimulatory responsiveness of AC in membranes from AC2-MOR-CHO.

son of the effect of overexpressing AC1 versus AC2 on the ability of long-term morphine to augment AC phosphorylation can be used to differentiate between consequences of AC overexpression per se versus those that result from qualitatively altering default responsiveness to MOR. Therefore, autoradiograms of AC IP were obtained from AC1-MOR-CHO cells metabolically labeled with ^{32}Pi during the last 2 h of a 48-h treatment with either morphine or vehicle. Two radiolabeled bands of ≈155 and ≈115 to 120 kDa were prominent (Fig. 3C, lanes 5 and 6) in autoradiograms of AC IP obtained from membranes obtained from treated and untreated AC1-MOR-CHO cells. However, the intensity of radiolabeling of both the ≈155 and ≈115 to 120 kDa species present in AC IP obtained from long-term morphine-treated cells was significantly increased (≈49%; $p = 0.002$), analogous to observations made in MOR-CHO cells. It is noteworthy that the magnitude of the increment in AC protein immunoprecipitated from long-term morphine-treated AC1-MOR-CHO cells (≈21%, $p = 0.03$; Fig. 3C, bottom) was less than the observed increment in AC phosphorylation (≈49%).

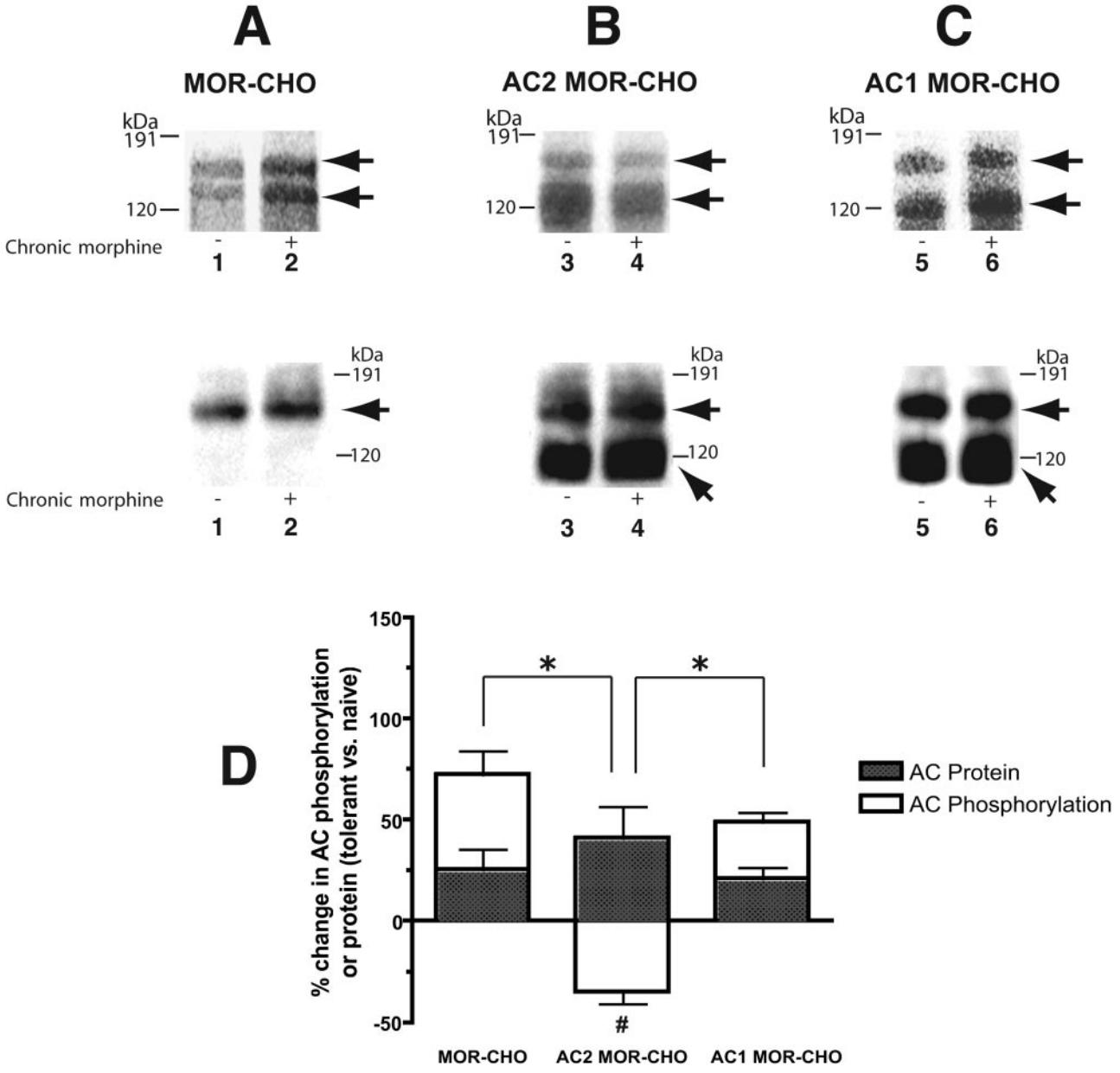


Fig. 3. Long-term morphine augments AC phosphorylation in MOR-CHO and AC1-MOR-CHO but not in AC2-MOR-CHO cells. A–C, top, autoradiograms of ^{32}P incorporation into AC IP obtained from membranes of opioid-naive (−) and long-term morphine-treated (+) MOR-CHO (A), AC2-MOR-CHO (B), and AC1-MOR-CHO cells (C). Arrowheads represent phosphorylated AC protein in MOR-CHO (~155 kDa, ~130 kDa), AC2-MOR-CHO (~155 kDa, ~120 kDa), and AC1-MOR-CHO (~155 kDa, ~115 kDa). A–C, bottom, sequential AC Western analyses of corresponding autoradiograms shown immediately above. D, quantification of the influence of AC overexpression state on the ability of long-term morphine to modulate AC phosphorylation (□) and AC protein (■) present in the AC IP. Changes in AC phosphorylation produced by long-term morphine among the AC-expressing groups was found to be significant using ANOVA ($p < 0.001$). Specifically, a significant difference was found between the effects of long-term morphine treatment on AC phosphorylation in MOR-CHO versus AC2-MOR-CHO) and in AC1-MOR-CHO versus AC2-MOR-CHO ($p < 0.001$ for all comparisons). *, $p < 0.001$ for the effect of long-term morphine treatment on AC phosphorylation in AC2-MOR-CHO versus MOR-CHO or AC1-MOR-CHO ($n = 4–6$ for MOR-CHO and AC1-MOR-CHO; $n = 11$ for AC2-MOR-CHO). #, $p < 0.001$, for the effect of long-term morphine treatment on AC phosphorylation in AC2-MOR-CHO.

Thus, long-term morphine treatment produces a net increase in AC phosphorylation in AC1-MOR-CHO cells.

AC2 Overexpression Levels Influenced the Magnitude of the Decrement in AC Phosphorylation Produced by Long-Term Morphine Treatment. In AC2-MOR-CHO, long-term morphine elicited decrements in AC phosphorylation of varying magnitudes. To identify the variable responsible for this variance, we performed an ANOVA. This revealed a significant interaction between levels of AC2 overexpression and the magnitude of change in AC phosphorylation ($p = 0.031$). A scatter plot of levels of AC2 overexpression versus the magnitude of the long-term morphine-induced decrement in AC phosphorylation depicts the relationship between these two variables (Fig. 4); a greater magnitude of AC2 overexpression is associated with a greater decrement in AC phosphorylation after long-term morphine treatment ($r = -0.648$, $p = 0.031$).

Overexpression of AC2, but Not AC1, Abolished the Ability of Long-Term Morphine Treatment to Augment the Phosphorylation of Gβ. Long-term treatment with morphine augments PKCγ phosphorylation of Gβ (Chakrabarti et al., 2005b), increasing both its availability and potency to stimulate AC (Chakrabarti et al., 2001; Chakrabarti and Gintzler, 2003). Thus, the phosphorylation state of the Gβ subunit of Gβγ is another important determinant of the balance between MOR-coupled $G\alpha_i$-inhibitory versus Gβγ-stimulatory AC signaling (Chakrabarti and Gintzler, 2003). Therefore, we investigated the relevance of AC overexpression state on the ability of long-term morphine treatment to augment Gβ phosphorylation. Because a causal association has been established between PKCγ and the augmented phosphorylation of Gβ that occurs after long-term morphine treatment (Chakrabarti and Gintzler, 2003; Chakrabarti et al., 2005b), we compared the influence of this treatment on phosphorylation of Gβ that was present in IP obtained using anti-PKCγ antibodies (PKCγ IP) from MOR-CHO, AC1-MOR-CHO, and AC2-MOR-CHO cells.

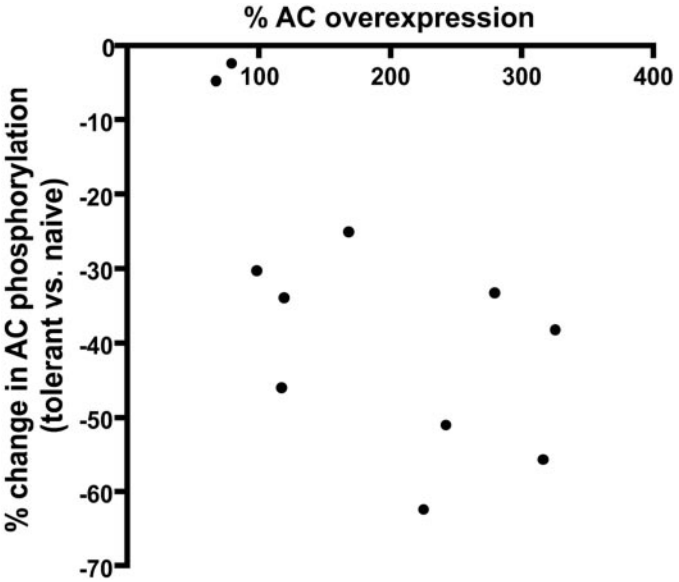


Fig. 4. Relationship between AC2 overexpression levels and the magnitude of the long-term morphine-induced decrement in AC phosphorylation. The ability of long-term morphine to attenuate ^{32}P incorporation into AC was plotted as a function of AC2 overexpression levels, which resulted from random variation of AC2 overexpression observed in repeated experiments using the same dose of cDNA. Regression analysis revealed a linear relationship between AC2 overexpression level and change in AC phosphorylation (slope = −0.129, $p = 0.031$). These two factors were negatively correlated ($r = -0.648$).

Autoradiographic analysis of the PKCγ IP obtained from membranes of ^{32}Pi metabolically radiolabeled MOR-CHO, AC2-MOR-CHO, and AC1-MOR-CHO cells, each maintained with and without long-term morphine, revealed two radiolabeled bands of ≈37 and ≈33 kDa. These correspond to molecular masses of Gβ observed previously (Chakrabarti et al., 2001, 2005b) (Figs. 5, A–C, lanes 1–6). Long-term morphine treatment of MOR-CHO cells augmented phosphorylation of the ≈37 and ≈33 kDa Gβ bands by ≈73% ($p = 0.026$) and ≈56% ($p = 0.009$), respectively (Fig. 5A, lane 2 versus 1), confirming observations reported previously in MOR-CHO cells and rat spinal cord (Chakrabarti et al., 2005b). Analogous results were obtained in AC1-MOR-CHO cells (Fig. 5C) in which long-term morphine treatment increased phosphorylation of the ≈37 and ≈33 kDa Gβ bands by ≈69% ($p = 0.005$) and ≈76% (0.018), respectively (lanes 6 versus 5).

In striking contrast to findings in MOR-CHO and AC1-MOR-CHO cells, the augmentation of Gβ phosphorylation after long-term morphine treatment of metabolically radiolabeled AC2-MOR-CHO cells was not discernible (Fig. 5B); autoradiographic analyses revealed no significant effect of long-term morphine treatment on Gβ phosphorylation ($p > 0.3$ for ≈37 and ≈33 kDa Gβ species) (Fig. 5B, lane 4 versus 3).

Gβ Western analysis of the membranes used to quantify autoradiographic bands was performed to validate their chemical identity and to assess whether altered coimmunoprecipitation of Gβ with PKCγ could confound the interpretation of the effect of AC2 overexpression on the increment in Gβ phosphorylation that occurs after long-term morphine. Long-term morphine did not produce any discernible change (<10%) in the protein content of either the ≈37 or ≈33 kDa Gβ species that coimmunoprecipitates with PKCγ from either MOR-CHO (Fig. 5A, bottom), AC2-MOR-CHO (Fig. 5B, bottom), or AC1-MOR-CHO cells (Fig. 5C, bottom). Therefore, changes in Gβ protein did not confound interpretation of the Gβ phosphorylation data.

AC2 Overexpression Obliterated the Ability of Long-Term Morphine Treatment to Augment Membrane PKCγ Translocation. Augmentation of membrane translocation of PKCγ is a well-established sequela of long-term morphine treatment (Mayer et al., 1995) that underlies the expansion of the pool of PKCγ functionally linked with augmented phosphorylated Gβ in MOR-CHO cells after long-term morphine exposure (Chakrabarti et al., 2005b). The inability of long-term morphine treatment to increase Gβ (and AC) phosphorylation in AC2-MOR-CHO cells could result from the absence of increased PKCγ translocation in these cells. This possibility was investigated by performing semiquantitative PKCγ Western analyses of membranes of MOR-CHO, AC1-MOR-CHO, and AC2-MOR-CHO cells.

PKCγ Western analyses of membranes of MOR-CHO cells revealed a signal of ≈82 to 85 kDa (Fig. 6A), consistent with previous reports (Yasuda et al., 1998; Chakrabarti et al., 2005b). In MOR-CHO (Fig. 6A, lanes 1 and 2) and AC1-MOR-CHO cells (Fig. 6A, lanes 5 and 6), long-term morphine treatment produced an increment of ≈120% ($p = 0.002$) and ≈70% ($p = 0.001$), respectively, in membrane PKCγ. However, in AC2-MOR-CHO cells, long-term morphine treatment failed to elicit a significant increase in membrane PKCγ (Fig. 6A, lanes 3 and 4; $p = 0.180$; see Fig. 6B for quantitative

illustration). In fact, long-term morphine exposure did produce a numeric decrement in membrane PKCγ translocation, but this did not achieve statistical significance.

AC2 Overexpression Reverses Increased Association of MOR with $G\alpha_s$ that Occurs after Long-Term Morphine Exposure. The increased association of MOR and $G\alpha_s$ after long-term morphine treatment (Chakrabarti et al., 2005a; Chakrabarti and Gintzler, 2007) represents a parallel mechanism for shifting MOR-coupled signaling from inhibition to stimulation of AC (Gintzler and Chakrabarti, 2006). Thus, it should not be observed in AC2-MOR-CHO cells after long-term morphine exposure. To test this prediction, we quantified the content of MOR in IP obtained using anti-$G\alpha_s$ antibodies ($G\alpha_s$ IP) from membranes of opioid-naive and long-term morphine-treated MOR-CHO, AC1-MOR-CHO, and AC2-MOR-CHO cells.

MOR Western signal of ≈82 to 85 kDa, shown previously to correspond to the presence of a MOR species (Chakrabarti et

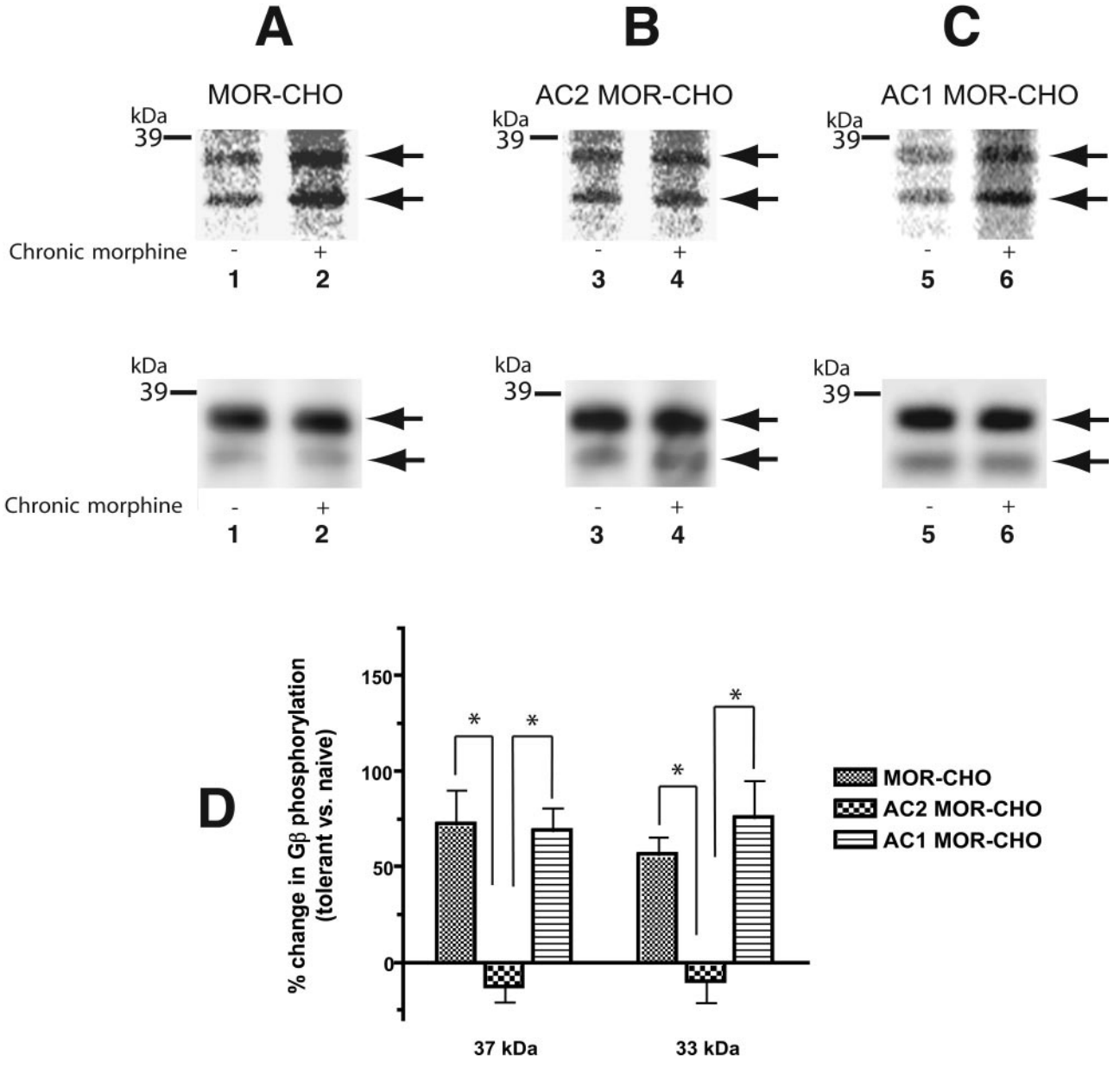


Fig. 5. AC2 overexpression abolishes the ability of long-term morphine to augment Gβ phosphorylation. A–C, top, autoradiograms of ^{32}P incorporation into Gβ that coimmunoprecipitates with PKCγ IP from membranes of opioid-naive (−) and long-term morphine-treated (+) MOR-CHO (A), AC2-MOR-CHO (B), and AC1-MOR-CHO cells (C). Arrowheads represent ^{32}P incorporated into Gβ protein obtained from MOR-CHO (~37 kDa, ~33 kDa), AC2-MOR-CHO (~37 kDa, ~33 kDa), and AC1-MOR-CHO cells (~37 kDa, ~33 kDa). A–C, bottom, sequential Gβ Western analyses of corresponding autoradiograms shown above. Gβ chemical identity of autoradiographic signals (indicated by arrowheads) is validated by the coincidence of their molecular mass with Western signals. D, quantification of the influence of AC1 versus AC2 overexpression on the ability of long-term morphine to modulate phosphorylation of Gβ in MOR-CHO (stippled bars), AC1-MOR-CHO (lined bars), and AC2-MOR-CHO (checkered bars). Changes in Gβ phosphorylation after long-term morphine among the AC-expressing groups was found to be significant for both the ≈37 and ≈33 kDa bands using ANOVA ($p < 0.001$ for both). Specifically, long-term morphine treatment significantly enhanced Gβ phosphorylation in MOR-CHO versus AC2-MOR-CHO cells and in AC1 versus AC2-MOR-CHO cells ($p < 0.01$ for all comparisons). *, $p < 0.01$ for the effect of long-term morphine treatment on Gβ phosphorylation in AC2-MOR-CHO versus MOR-CHO or AC1-MOR-CHO cells ($n = 4–5$ for MOR-CHO and AC1-MOR-CHO cells; $n = 9$ for AC2-MOR-CHO cells).

al., 2005a), was observed in the $G\alpha_s$ IP obtained from the membranes of all cell groups (Fig. 7A, top). Long-term morphine treatment significantly increased the quantity of MOR that coimmunoprecipitated with $G\alpha_s$ from membranes of MOR-CHO and AC1-MOR-CHO cells (≈93 and ≈121%, respectively; Fig. 7A, lanes 2 versus 1 and 6 versus 5, respectively) ($p = 0.013$ for MOR-CHO; $p < 0.001$ for AC1-MOR-CHO). These results validate previous findings that long-term morphine treatment produced a net increase in MOR-$G\alpha_s$ association (Chakrabarti et al., 2005a).

In striking contrast to MOR-CHO and AC1-MOR-CHO cells, long-term morphine treatment of AC2-MOR-CHO cells did not result in increased coimmunoprecipitation of MOR with $G\alpha_s$ (Fig. 7A, lane 4 versus 3). In fact, long-term morphine treatment of AC2-MOR-CHO cells resulted in a diminution of MOR $G\alpha_s$ coimmunoprecipitation. This diminution was modest (≈15%) in magnitude but did reach statistical significance ($p = 0.037$; Fig. 7B shows a quantitative illustration). It is noteworthy that this occurred in the absence of any decrement in the $G\alpha_s$ content of the $G\alpha_s$ IP (Fig. 7A, compare lanes 3 and 4, bottom).

Discussion

Tolerance adaptations subserve a protective function by counteracting the effects of sustained opioid receptor activation. Based on this, we hypothesized that adaptations to long-term morphine exposure are not hard-wired and invariant but instead depend on dynamic interactions among signaling molecules. To test this hypothesis, we studied AC/cAMP-related cellular adaptations to long-term morphine treatment in cells in which short-term MOR activation either inhibited or stimulated AC activity. Observations of multiple complementary signaling parameters underscored that adaptations elicited by long-term morphine treatment, which involve the AC/cAMP signaling pathway, are not hard-wired but instead are conditional on the directionality of the short-term MOR-mediated modulation.

Changes in components of the AC/cAMP signaling pathway that occur upon prolonged morphine exposure are just a part of the spectrum of adaptations to long-term morphine that occur in vivo. These would include but are not limited to 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 altered association/activity of regulators of G-protein signaling proteins (Zachariou et al., 2003; Xu et al., 2004; Garzón et al., 2005; Xie and Palmer, 2005). In addition, altered functionality of nonopioid receptor-bearing neurons that are interconnected with those bearing opioid receptors and changes in the overall balance of brain inhibitory and stimulatory circuits will contribute to the loss of opioid responsiveness.

In the current study, we focused on changes in AC and $G\beta$ phosphorylation, PKCγ translocation, and MOR G_s association because these represent an interrelated, convergent set of complementary adaptations. Thus, demonstration of their synchronized plasticity in MOR-CHO and AC1-MOR-CHO versus AC2-MOR-CHO cells would reflect the validity of our hypothesis within a definable subgroup of adaptations and provide a rationale for exploring whether or not current observations of the pliability of tolerant mechanisms generalize to other sets of adaptations.

Because $G\beta\gamma$ inhibits AC1 activity, its overexpression in MOR-CHO should not alter the default responsiveness to short-term MOR activation. Therefore, the demonstration that AC/cAMP related adaptations in MOR-CHO and AC1-MOR-CHO cells did not qualitatively differ indicates that altered AC overexpression per se was not a confounding factor. In contrast, transfection of MOR-CHO cells with AC2,

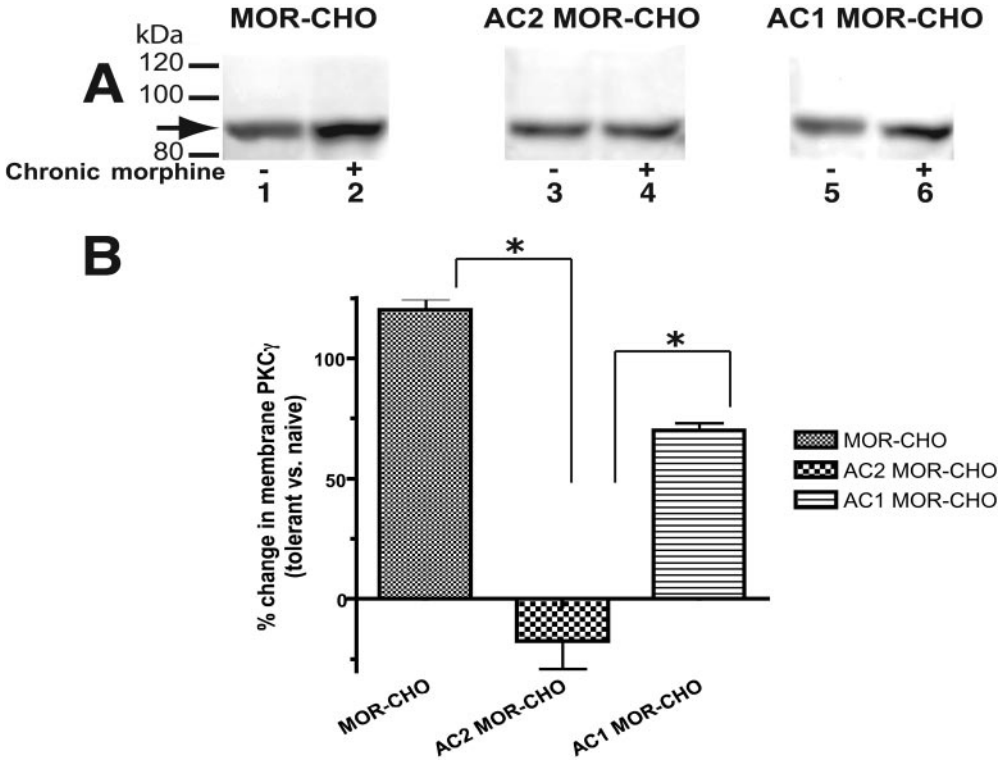


Fig. 6. AC2 overexpression abolishes long-term morphine augmentation of membrane translocation of PKCγ. A, PKCγ Western analyses of membrane obtained from opioid-naive (−) and long-term morphine-treated (+) MOR-CHO, AC2-MOR-CHO, and opioid-naive and AC1-MOR-CHO cells. Arrowhead indicates membrane PKCγ protein (~85 kDa). B. quantification of the influence of AC overexpression state on the ability of long-term morphine treatment to modulate membrane content of PKCγ. Changes in the ability of long-term morphine to increase PKCγ membrane content among AC-expressing groups was found to be significant using ANOVA ($p < 0.001$). Specifically, post hoc analysis revealed a significant difference in the effect of long-term morphine exposure on membrane PKCγ content in MOR-CHO versus AC2-MOR-CHO cells ($p < 0.001$) and in AC1 versus AC2-MOR-CHO cells ($p = 0.001$). *, $p < 0.001$, for the effect of long-term morphine treatment on membrane PKCγ in AC2-MOR-CHO versus MOR-CHO or AC1-MOR-CHO ($n = 3$ for MOR-CHO and AC1-MOR-CHO cells; $n = 7$ for AC2-MOR-CHO cells).

which is stimulated by Gβγ, should shift MOR-coupled modulation of AC activity from inhibition to stimulation. Thus, if we were correct that adaptations to long-term morphine treatment depend on the directionality of short-term opioid responsiveness, overexpression of AC2 should eliminate or reverse those AC/cAMP-related adaptations to long-term morphine manifested in MOR-CHO and AC1-MOR-CHO cells.

The adaptations to long-term morphine exposure examined in this study converge to shift the consequences of MOR activation from predominantly inhibitory to stimulatory AC signaling (Gintzler and Chakrabarti, 2006). This results from augmenting the ability of Gβγ to stimulate AC and increasing MOR G_s coupling. Membrane translocation of PKCγ increases after long-term morphine treatment (Mao et al., 1995; Narita et al., 2004). This underlies the augmented phosphorylation of both AC and Gβ after long-term morphine treatment (Chakrabarti et al., 1998b, 2005b). Phosphorylation of AC increases its stimulatory responsiveness to Gβγ (Zimmermann and Taussig, 1996), whereas increased phosphorylation of Gβ increases the availability and potency of Gβγ to stimulate AC (Chakrabarti et al., 2001, 2005b; Chakrabarti and Gintzler, 2003). These changes shift MOR-coupled signaling from $G_i\alpha/G_o\alpha$ inhibitory to Gβγ stimulatory. At the same time, long-term morphine treatment also increases MOR signaling via G_s (Chakrabarti et al., 2005a; Chakrabarti and Gintzler, 2007). As a result, activation of MOR would stimulate AC via the generation of $G_s\alpha$ and via the generation of Gβγ (from both G_i/G_o and G_s). It is noteworthy that phosphorylation of AC increases its stimulatory

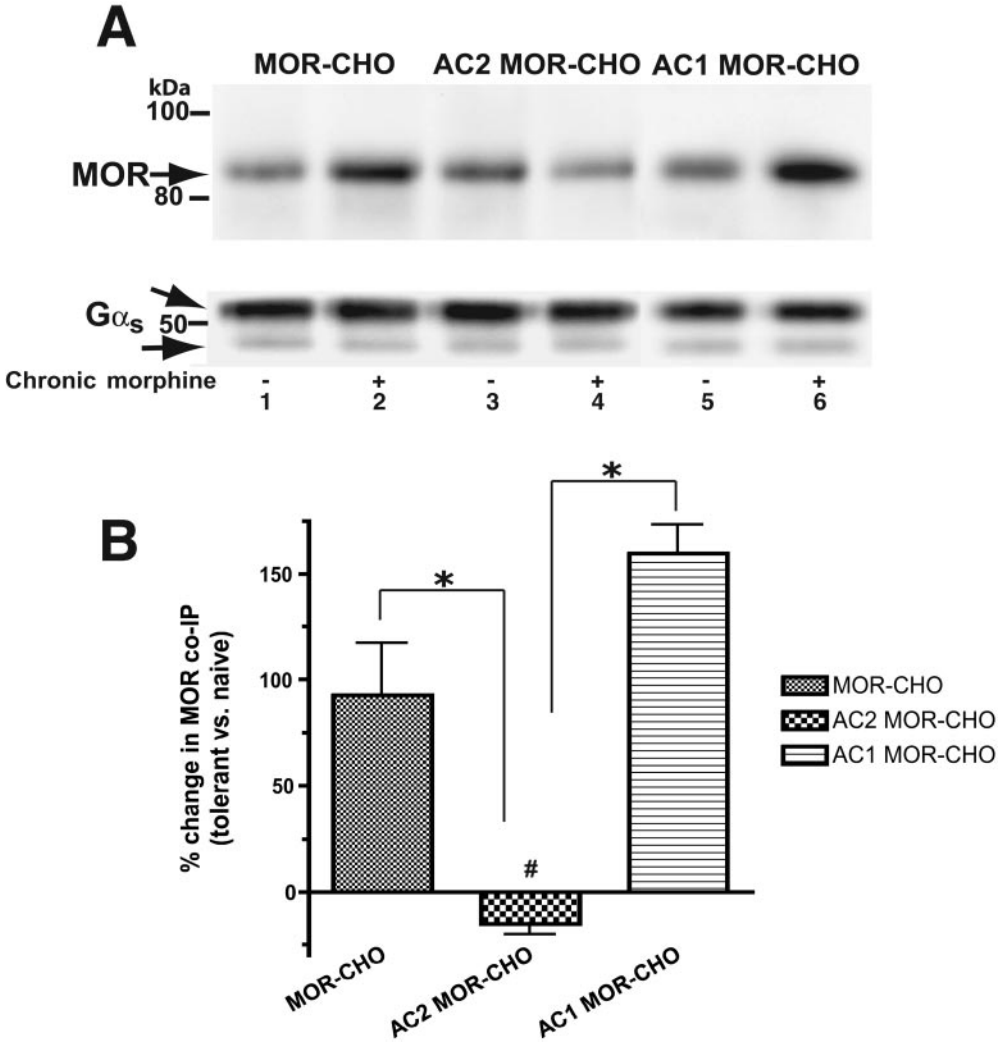


Fig. 7. Long-term morphine treatment augments MOR $G\alpha_s$ association in membranes of MOR-CHO and AC1-MOR-CHO cells but decreases that association in membranes of AC2-MOR-CHO cells. A, top, MOR Western analysis of $G\alpha_s$ IP obtained from membranes of opioid-naive (−) and long-term morphine-treated (+) MOR-CHO (lanes 1 and 2), AC2-MOR-CHO (lanes 3 and 4), and AC1-MOR-CHO cells (lanes 5 and 6), respectively. A, bottom, $G\alpha_s$ Western of the same sample shown above. Arrowhead indicates MOR (~85 kDa) (top) that coimmunoprecipitates with $G\alpha_s$ (bottom). The ability of long-term morphine to increase MOR-$G\alpha_s$ coimmunoprecipitation among the AC-expressing groups was found to be significant using ANOVA ($p < 0.001$). Post hoc analysis revealed a significant difference in the ability of long-term morphine treatment to alter MOR $G\alpha_s$ coimmunoprecipitation in membranes of MOR-CHO versus AC2-MOR-CHO, AC1 versus AC2-MOR-CHO, and MOR-CHO versus AC1-MOR-CHO cells ($p < 0.04$ for all comparisons). AC1 overexpression not only fails to negate the ability of long-term morphine to augment MOR G_s interaction but enhanced it (i.e., 121 versus 93% in AC1-MOR-CHO and MOR-CHO cells, respectively). The $G\alpha_s$ content of the $G\alpha_s$ IP was not influenced by long-term morphine. B, quantification of the effect of AC overexpression state on the influence of long-term morphine treatment on MOR G_s association. *, $p < 0.001$ for the increment in MOR-$G\alpha_s$ association produced by long-term morphine treatment of MOR-CHO and AC1-MOR-CHO cells. #, $p < 0.001$ for the decrement in MOR $G\alpha_s$ association after long-term morphine treatment of AC2-MOR-CHO ($n = 5–6$). Long-term morphine treatment produces a net increment and decrement in MOR $G\alpha_s$ association in membranes of MOR-CHO/AC1-MOR-CHO and AC2-MOR-CHO cells, respectively.

responsiveness to $G_s\alpha$ and $G\beta\gamma$ (Jacobowitz et al., 1993; Jacobowitz and Iyengar, 1994; Watson et al., 1994; Zimmermann and Taussig, 1996).

The present findings add a new dimension to the above tolerance adaptations by demonstrating that their manifestation depends on the effects of short-term MOR activation in opioid-naive cells. It was striking that overexpression of AC2, which converts MOR inhibition to stimulation of AC, obliterated the above adaptations to long-term morphine that were manifested in MOR-CHO cells (and AC1-MOR-CHO cells); it negated the increase in $G\beta$ phosphorylation and PKCγ translocation while reversing the increase in AC phosphorylation and MOR G_s association. The reduced PKCγ translocation in AC2-MOR-CHO after long-term morphine treatment could explain both the abolishment of augmented $G\beta$ phosphorylation and the concomitant decrease in AC phosphorylation. This, together with the reduction in MOR G_s coupling, would explain the loss of AC stimulatory effects after long-term morphine treatment.

It is physiologically significant that the directionality of short-term MOR-coupled effects can influence opioid tolerance mechanisms. When MOR activation results in a predominantly inhibitory modulation of AC activity (in MOR-CHO and AC1-MOR-CHO cells), adaptations are elicited by long-term opioid treatment that augment the stimulatory effects of MOR-G protein coupling, offsetting the persistent inhibition of AC activity by the sustained activation of MOR. This gives the appearance of the loss of MOR-coupled signaling, often considered to be a predominant, if not exclusive, underpinning of opioid tolerance. On the contrary, in AC2-MOR-CHO cells, which manifest short-term AC stimulatory responsiveness to MOR activation, adaptations that augment that response, as occurs in MOR-CHO and AC1-MOR-CHO cells, would only exacerbate the initial perturbing influence of opioids. This necessitates that in such cells, a qualitatively different set of adaptive mechanisms be harnessed in response to long-term morphine treatment. Thus, coordination of the tolerant mechanisms used with opioid responsiveness has adaptational advantages.

The manifestation of cellular adaptations that have opposing functional consequences in MOR-CHO and AC1-MOR-CHO versus AC2-MOR-CHO cells demonstrate that tolerance mechanisms elicited by long-term morphine, which involve the AC/cAMP pathway, use the plasticity inherent in G protein-coupled receptor signaling to oppose the consequences of the short-term activation of MOR. This indicates that tolerance mechanisms can be dynamic, pliable, and interconnected with cell physiology.

Current results do not shed any light on the long-held debate regarding the relationship between opioid tolerance-producing mechanisms and physical dependence. It seems likely, however, that the directionality of short-term MOR-coupled effects will also influence the nature of the mechanism(s) underlying physical dependence. Postreceptor adaptations to long-term morphine are unlikely be restricted to opioid receptor-coupled systems. Phosphorylation and subsequent altered activity of AC and other signaling proteins could alter responsiveness to a myriad of neurotransmitters. Short-term opioid withdrawal after long-term morphine-treatment would reveal disrupted signal transduction via other GPCRs that modulate AC activity. Thus, the default responsiveness to short-term MOR activation would be expected to influence neurochemical underpinnings of opioid withdrawal and tolerance mechanisms.

It is interesting that there was a significant interaction between AC2 overexpression levels and the magnitude of long-term morphine-induced decrease in AC phosphorylation. This reveals that the dependence on the cellular milieu of AC/cAMP-related cellular adaptations to long-term morphine treatment is not all-or-none. As a result, in most biochemical studies, a spectrum of adaptations to long-term morphine treatment would be averaged across regions of the brain and spinal cord. This would also explain, at least in part, observations that many electrophysiological manifestations of the loss of opioid responsiveness are observed in only a percentage of cells examined.

The engineered cell populations overexpressing AC2 and AC1 used in these studies represent an extreme example of AC isoform distribution. Most, if not all, in vivo circumstances represent a gradient of relative preponderance of one AC isoform versus another. Extrapolation from our cell culture models suggests that AC/cAMP-related adaptations in the central nervous system to long-term morphine treatment would also be multifactorial and that the relative contributions of each will vary depending on the functional state of each cell. The task of future studies will be to establish whether or not the multitude of adaptive mechanisms elicited by long-term morphine manifest plasticity comparable with those that involve the AC/cAMP pathway.

Acknowledgments

We thank Hans von Gizycki, Scientific Computing, State University of New York, Downstate Medical Center, for statistical analyses and helpful discussions regarding data presentation.

References

Alvarez R and Daniels DV (1990) A single column method for the assay of adenylate cyclase. *Anal Biochem* **187:**98–103.
Appleyard SM, Celver J, Pineda V, Kovoor A, Wayman GA, and Chavkin C (1999) Agonist-dependent desensitization of the κ opioid receptor by G protein receptor kinase and β-arrestin. *J Biol Chem* **274:**23802–23807.
Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408:**720–723.
Böl GF, Hulster A, and Pfeuffer T (1997) Adenylyl cyclase type II is stimulated by PKC via C-terminal phosphorylation. *Biochim Biophys Acta* **1358:**307–313.
Chakrabarti S and Gintzler AR (2003) Phosphorylation of $G\beta$ is augmented by chronic morphine and enhances $G\beta\gamma$ stimulation of adenylyl cyclase activity. *Brain Res Mol Brain Res* **119:**144–151.
Chakrabarti S and Gintzler AR (2007) Phosphorylation of Gsα influences its association with the μ-opioid receptor and is modulated by chronic morphine. *Mol Pharmacol* **72:**753–760.
Chakrabarti S, Law PY, and Loh HH (1995) Neuroblastoma neuro2A cells stably expressing a cloned μ-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. *Mol Brain Res* **30:**269–278.
Chakrabarti S, Oppermann M, and Gintzler AR (2001) Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: a novel mechanism for modulating cell signaling. *Proc Natl Acad Sci U S A* **98:**4209–4214.
Chakrabarti S, Regec A, and Gintzler AR (2005a) Biochemical demonstration of mu-opioid receptor association with Gsalpha: enhancement following morphine exposure. *Brain Res Mol Brain Res* **135:**217–224.
Chakrabarti S, Regec A, and Gintzler AR (2005b) Chronic morphine acts via a protein kinase Cγ-Gβ-adenylyl cyclase complex to augment phosphorylation of $G\beta$ and $G\beta\gamma$ stimulatory adenylyl cyclase signaling. *Brain Res Mol Brain Res* **138:**94–103.
Chakrabarti S, Rivera M, Yan SZ, Tang WJ, and Gintzler AR (1998a) Chronic morphine augments $G\beta\gamma$/Gsα stimulation of adenylyl cyclase: relevance to opioid tolerance. *Mol Pharmacol* **54:**655–662.
Chakrabarti S, Wang L, Tang WJ, and Gintzler AR (1998b) Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signaling during tolerance/dependence. *Mol Pharmacol* **54:**949–953.
Chavkin C and Goldstein A (1984) Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proc Natl Acad Sci U S A* **81:**7253–7257.
Chen J, DeVivo M, Dingus J, Harry A, Li J, Sui J, Carty DJ, Blank JL, Exton JH,

Stoffel RH, et al. (1995) A region of adenylyl cyclase 2 critical for regulation by G protein $\beta\gamma$ subunits. *Science* **268:**1166–1169.
Cox BM and Crowder AT (2004) Receptor domains regulating μ opioid receptor uncoupling and internalization: relevance to opioid tolerance. *Mol Pharmacol* **65:**492–495.
Federman AD, Conklin BR, Schrader KA, Reed RR, and Bourne HR (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein $\beta\gamma$ subunits. *Nature* **356:**159–161.
Garzón J, Rodriguez-Munoz M, and Sanchez-Blazquez P (2005) Morphine alters the selective association between mu-opioid receptors and specific RGS proteins in mouse periaqueductal gray matter. *Neuropharmacology* **48:**853–868.
Gintzler AR and Chakrabarti S (2006) Post-opioid receptor adaptations to chronic morphine; altered functionality and associations of signaling molecules. *Life Sci* **79:**717–722.
Jacobowitz O, Chen J, Premont RT, and Iyengar R (1993) Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. *J Biol Chem* **268:**3829–3832.
Jacobowitz O and Iyengar R (1994) Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. *Proc Natl Acad Sci U S A* **91:**10630–10634.
Mao J, Price DD, Phillips LL, Lu J, and Mayer DJ (1995) Increases in protein kinase Cγ immunoreactivity in the spinal cord of rats associated with tolerance to the analgesic effects of morphine. *Brain Res* **677:**257–267.
Mayer DJ, Mao J, and Price DD (1995) The development of tolerance and dependence is associated with translocation of protein kinase C. *Pain* **61:**365–374.
Mollner S and Pfeuffer T (1988) Two different adenylyl cyclases in brain distinguished by monoclonal antibodies. *Eur J Biochem* **171:**265–271.
Narita M, Suzuki M, Yajima Y, Suzuki R, Shioda S, and Suzuki T (2004) Neuronal protein kinase Cγ-dependent proliferation and hypertrophy of spinal cord astrocytes following repeated in vivo administration of morphine. *Eur J Neurosci* **19:**479–484.
Sim LJ, Selley DE, Dworkin SI, and Childers SR (1996) Effects of chronic morphine administration on mu opioid receptor-stimulated [^{35}S]GTPgammaS autoradiography in rat brain. *J Neurosci* **16:**2684–2692.
Tang WJ and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science* **254:**1500–1503.
Tsu RC, Chan JSC, and Wong YH (1995) Regulation of multiple effectors by the cloned delta-opioid receptor: stimulation of phospholipase C and type II adenylyl cyclase. *J Neurochem* **64:**2700–2707.
Watson PA, Krupinski J, Kempinski AM, and Frankenfiedl CD (1994) Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. *J Biol Chem* **269:**28893–28898.
Xie GX and Palmer PP (2005) RGS proteins: new players in the field of opioid signaling and tolerance mechanisms. *Anesth Analg* **100:**1034–1042.
Xu H, Wang X, Wang J, and Rothman RB (2004) Opioid peptide receptor studies. 17. Attenuation of chronic morphine effects after antisense oligodeoxynucleotide knock-down of RGS9 protein in cells expressing the cloned Mu opioid receptor. *Synapse* **52:**209–217.
Yasuda H, Lindorfer MA, Myung CS, and Garrison JC (1998) Phosphorylation of the G protein γ12 subunit regulates effector specificity. *J Biol Chem* **273:**21958–21965.
Yoshimura M, Ikeda H, and Tabakoff B (1996) μ-Opioid receptors inhibit dopamine-stimulated activity of type V adenylyl cyclase but enhance dopamine-stimulated activity of type VII adenylyl cyclase. *Mol Pharmacol* **50:**43–51.
Zachariou V, Georgescu D, Sanchez N, Rahman Z, DiLeone R, Berton O, Neve RL, Sim-Selley LJ, Selley DE, Gold SJ, et al. (2003) Essential role for RGS9 in opiate action. *Proc Natl Acad Sci U S A* **100:**13656–13661.
Zeitz KP, Malmberg AB, Gilbert H, and Basbaum AI (2001) Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC g mutant mice. *Pain* **94:**245–253.
Zimmermann G and Taussig R (1996) Protein kinase C alters the responsiveness of adenylyl cyclase to G protein α and $\beta\gamma$ subunits. *J Biol Chem* **271:**27161–27166.

Address correspondence to: Dr. Alan Gintzler, Box 8, Department of Biochemistry, SUNY Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203. E-mail: alan.gintzler@downstate.edu