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

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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 ε-opioid receptor (MOR) activation. We tested this by comparing complementary chemical sequela of long-term morphine treatment among cells in which short-term MOR activation induced 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 Gβγ phosphorylation, membrane protein kinase Cγ (PKCγ) translocation, and MOR Gβγ association. All converge, shifting the consequences of short-term MOR activation from Gαi/Gβγ inhibitory to AC stimulatory signaling. In contrast, overexpression of the Gβγ-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 Gβγ phosphorylation and PKCγ translocation while reversing the increase in AC phosphorylation and MOR Gβγ 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.
2003], increased membrane translocation of protein kinase Cγ (PKCγ) (Mao et al., 1995; Zeitz et al., 2001; Chakrabarti et al., 2005b), increased MOR Gaα 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-naïve 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” Gβγ-coupled receptors can be converted into stimulators of cAMP synthesis by overexpressing AC2 or AC7 (along with constitutively active Gaα) (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 biochemical 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 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 Genticin and plated at 3.8 × 10⁶ cells/150 mm² 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) for an additional 24 h at 37°C (both generously supplied by Dr. John Hildebrandt, Medical University of South Carolina, Charleston, SC) (Chakrabarti et al., 2005b). 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 or were 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 (14,000g 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, Düsseldorf, Germany) (Mollner and Pfueffer, 1988; Chakrabarti et al., 1998b). This antibody recognizes 23 amino acids of the second cytoplasmic domain (GKP/VAVG/VI/GARKPQYD/IVGNT; S. Mollner, personal communication). PKCy was immunoprecipitated using a mouse monoclonal antibody (6 μl/600 μg of protein; Sigma Chemical, St. Louis, MO), generated against rat PKCy 684 to 697 amino acid residues (Chakrabarti et al., 2005b). Gaα was immunoprecipitated from solubilized membrane using a rabbit anti-Gαs (bovine) polyclonal affinity-purified antibody generated against the C terminus of the Gaαs 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 ml of sample buffer (15 min at 85°C). Samples separated on 4 to 12% gradient Bis-Tris gels (Invitrogen) were electrophoresed 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). ³²P incorporation 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 (Chakrabarti et al., 2005b). Gaα and Gβγ proteins were visualized using a 1:10,000 and 1:15,000 dilution of anti-Gαs and anti-Gβγ polyclonal antibodies, respectively (both generously supplied by Dr. John Hildebrandt, Medical University of South Carolina, Charleston, SC) (Chakrabarti et al., 2005b). PKCy protein was visualized using a polyclonal antibody generated against the carboxyl terminus of PKCy (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, Gβγ, PKCγ, and Gαs 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-32P]cAMP from [α-32P]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 MgCl₂, 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 dolipram, 0.1% bovine serum albumin, and [α-32P]ATP; 1 μCi/sample) to cell membranes (50 or 5 μg) isolated from membranes obtained from opioid-naive and AC2-MOR-CHO, respectively. Membranes were preincubated with 1 μM GTP, 20 mM NaCl, 1 mM DTT, 5 mM MgSO₄, and bovine serum albumin (1.25 mg/ml) as described previously (Tang and Gilman, 1991). The activated Gαs-GTP•S was separated from free GTP•S using gel filtration (Sephadex G-25 spin columns). [α-32P]cAMP formation was terminated by the addition of 10 μl of 2.2 N HCl (4°C). Thereafter, [32P]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, [32P]cAMP formation was determined in membranes obtained from opioid-naive and long-term morphine-treated MOR-CHO, AC1-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 Gβγ in MOR-coupled modulation of AC was investigated by determining the effect of pretreating membranes with the Gβγ-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 Gβγ 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 Gβγ-stimulated AC isoform AC2 in MOR-CHO would reverse short-term MOR-coupled inhibition of AC activity to a Gβγ-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 produc-

lication 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 Gβγ-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 Gβγ-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 Gβγ 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 Gαs-Dependent Gβγ 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 rGαs (2.5, 5, 10, and 20 nM) is significantly augmented in membranes of opioid-naive versus opioid-naive MOR-CHO cells (Fig. 2A). ANOVA revealed significant effects for morphine, rGαs concentration, and their interaction (p < 0.001 for all comparisons). Regression analysis indicated that as the concentration of rGαs increased, so did the long-term morphine-induced increment in AC activity (p < 0.05; n = 4–9). To determine whether the increase in Gαs stimulation of AC after long-term morphine treatment is Gβγ-dependent, as has been reported for LMMP tissue (Chakrabarti et al., 1998a), we determined the effect of QEHA on rGαs 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 rGαs AC stimulation (p = 0.001). QEHA (50 μM) did not have any effect on the increment in AC activity produced by rGαs (5 nM) in membranes of opioid-naive cells. This indicated that the increased activity of AC resulted from its direct stimulation by rGαs (and not via Gβγ). In contrast, QEHA (50 μM) abolished the long-term morphine-induced increment in rGαs 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 Gαs-dependent Gβγ stimulation of AC.

Membranes obtained from opioid-naive and long-term morphine-treated AC2-MOR-CHO cells also manifested rGαs 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 Gαs stimulation of AC. ANOVA revealed a significant main effect for rGαs 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 rGαs stimulation of AC was observed for all rGαs concentrations (p > 0.17; n = 5). Thus, overexpression of AC2 in MOR-CHO cells resulted in the inability to manifest increased Gαs-dependent Gβγ 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 Gβγ-AC stimulation after long-term morphine treatment [i.e., increased phosphorylation of AC

![Graph](image)

**Fig. 1.** AC2 overexpression reverses MOR-coupled inhibitory modulation of AC activity to stimulatory. Formation of [α-32P]cAMP from [α-32P]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 mM, 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 Gβγ. 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.
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 32P-radio-labeled 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 LMMMP 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 LMMMP 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 Gβγ subunit differentially regulates AC1 and AC2 (Tang and Gilman, 1991). Gβγ 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 Gβγ stimulation of AC activity produced by long-term morphine treatment. The magnitude of AC stimulation produced by activated rGαs 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 < 0.001 for the magnitude of rGαs stimulation in long-term morphine-treated versus naive membranes (n = 4–9). Inset, effect of QEHA (50 μM) on the rGαs (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 rGαs 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 rGαs, which is abolished by QEHA. In contrast, long-term morphine treatment does not increase rGαs 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 $^{32}$P during the last 2 h of a 48-h treatment with either morphine or vehicle. Two radiolabeled bands of $\sim 155$ and $\sim 115$ 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 $\sim 155$ and $\sim 115$ to 120 kDa species present in AC IP obtained from long-term morphine-treated cells was significantly increased ($\sim 49\%$; $p = 0.002$), 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 ($\sim 21\%$, $p = 0.03$; Fig. 3C, bottom) was less than the observed increment in AC phosphorylation ($\sim 49\%$).

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 $\alpha\beta$. Long-term treatment with morphine augments PKC$\gamma$ phosphorylation of $\alpha\beta$ (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 $\alpha\beta$ subunit of $\alpha\beta\gamma$ is another important determinant of the balance between MON-coupled $\alpha\gamma$, inhibitory versus $\alpha\beta\gamma$-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 $\alpha\beta$ phosphorylation. Because a causal association has been established between PKC$\gamma$ and the augmented phosphorylation of $\alpha\beta$ 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 $\alpha\beta$ that was present in IP obtained using anti-PKC$\gamma$ antibodies (PKC$\gamma$ IP) from MOR-CHO, AC1-MOR-CHO, and AC2-MOR-CHO cells.

Autoradiographic analysis of the PKC$\gamma$ IP obtained from membranes of $^{32}$P 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 $\sim 37$ and $\sim 33$ kDa. These correspond to molecular masses of $\alpha\beta$ 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 $\sim 37$ and $\sim 33$ kDa $\alpha\beta$ bands by $\sim 73\%$ ($p = 0.026$) and $\sim 56\%$ ($p = 0.009$), 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 $\sim 37$ and $\sim 33$ kDa $\alpha\beta$ bands by $\sim 69\%$ ($p = 0.005$) and $\sim 76\%$ (0.018), respectively (lanes 6 versus 5).

In striking contrast to findings in MOR-CHO and AC1-MOR-CHO cells, the augmentation of $\alpha\beta$ 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 $\alpha\beta$ phosphorylation ($p > 0.3$ for $\sim 37$ and $\sim 33$ kDa $\alpha\beta$ species) (Fig. 5B, lane 4 versus 3).

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

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

PKC$\gamma$ Western analyses of membranes of MOR-CHO cells revealed a signal of $\sim 82$ 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 $\sim 120\%$ ($p = 0.002$) and $\sim 70\%$ ($p = 0.001$), respectively, in membrane PKC$\gamma$. However, in AC2-MOR-CHO cells, long-term morphine treatment failed to elicit a significant increase in membrane PKC$\gamma$ (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 Go,i that Occurs after Long-Term Morphine Exposure.** The increased association of MOR and Go,i 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-Go,i antibodies (Go,i 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
al., 2005a), was observed in the Gαs 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 Gαs 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-Gαs 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 Gαs (Fig. 7A, lane 4 versus 3). In fact, long-term morphine treatment of AC2-MOR-CHO cells resulted in a diminution of MOR Gαs coimmunoprecipitation. This diminution was modest (~15%) in magnitude but did reach statistical significance (p = 0.037; Fig. 7A shows a quantitative illustration). It is noteworthy that this occurred in the absence of any decrement in the Gαs content of the Gαs IP (Fig. 7A, compare lanes 3 and 4, bottom).

**Discussion**

Tolerance adaptations subserve a protective function by countering 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 Gβ/γ phosphorylation, PKCγ translocation, and MOR Gαs 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 Gβγ 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,
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 Gs 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 Gαi/Gs inhibitory to Gβγ stimulatory. At the same time, long-term morphine treatment also increases MOR signaling via Gs (Chakrabarti et al., 2005a; Chakrabarti and Gintzler, 2007). As a result, activation of MOR would stimulate AC via the generation of Gαs (from both Gi/Go and Gs). It is noteworthy that phosphorylation of AC increases its stimulatory

Fig. 7. Long-term morphine treatment augments MOR Gαs 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 Gαs 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, Gαs Western of the same sample shown above. Arrowhead indicates MOR (~85 kDa) (top) that coimmunoprecipitates with Gαs (bottom). The ability of long-term morphine to increase MOR-Gαs 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 Gαs 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 Gαs interaction but enhanced it (i.e., 121 versus 93% in AC1-MOR-CHO and MOR-CHO cells, respectively). The Gαs content of the Gαs 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 Gαs association. *, p < 0.001 for the increment in MOR-Gαs association produced by long-term morphine treatment of MOR-CHO and AC1-MOR-CHO cells. #, p < 0.001 for the decrement in MOR-Gαs 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 Gαs association in membranes of MOR-CHO/AC1-MOR-CHO and AC2-MOR-CHO cells, respectively.
responsiveness to $G_{i\alpha}$ and $G_{i\gamma}$ (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 PKC$\gamma$ translocation while reversing the increase in AC phosphorylation and MOR G$\gamma$ association. The reduced PKC$\gamma$ translocation in AC2-MOR-CHO after long-term morphine treatment could explain both the abolition of augmented G$\beta$ phosphorylation and the concomitant decrease in AC phosphorylation. This, together with the reduction in MOR G$\gamma$ 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 to 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.

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References


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