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Chronic Morphine Augments Adenylyl Cyclase Phosphorylation: Relevance to Altered Signaling during Tolerance/Dependence

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ABSTRACT

Despite the demonstration that chronic morphine increases phosphorylation of multiple substrate proteins, their identity has, for the most part, remained elusive. Thus far, chronic morphine has not been shown to increase the phosphorylation of any identified effector protein. This is the first demonstration that persistent activation of opioid receptors has profound effects on phosphorylation of adenylyl cyclase (AC). A dramatic increase in phosphorylation of AC (type II family) was observed in ileum longitudinal muscle myenteric plexus preparations obtained from chronic morphine-treated guinea pigs. Analogous results were obtained when AC was immunoprecipitated using two differentially directed AC antibodies. The magnitude of the augmented AC phosphorylation was substantially attenuated by chelerythrine, a protein kinase C-selective inhibitor. These results suggest the potential relevance of increased phosphorylation (protein kinase C-mediated) of AC to opioid tolerant/dependent mechanisms. Because phosphorylation of AC isoforms (type II family) can significantly increase their stimulatory responsiveness to $G_{s\alpha}$ and $G_{\beta\gamma}$, this mechanism could underlie, in part, the predominance of opioid AC stimulatory signaling observed in opioid tolerant/dependent tissue. Moreover, in light of the fact that many G protein-coupled receptors signal through common effector proteins, this effect provides a mechanism for divergent consequences of chronic morphine treatment and could explain the well documented complexity of changes that accompany the opioid tolerant/dependent state.

Altered protein phosphorylation has long been considered to underlie, at least in part, many of the physiological sequelae of persistent activation of opioid receptors. Studies in rat locus ceruleus have demonstrated that after chronic systemic morphine administration, the phosphorylation state of multiple proteins (substrate for PKA) is augmented (Guitart and Nestler, 1989; Nestler, 1992). Recently, in mammalian cells, acute (Zhang *et al.*, 1998) and chronic (Zhang *et al.*, 1996) opioid treatment has been shown to increase phosphorylation of overexpressed μ-opioid receptors. However, to date, no effector protein has been identified whose phosphorylation state is increased by chronic morphine.

The relevance of augmented protein phosphorylation to the sequelae of chronic morphine exposure is also indicated by pharmacological analyses. In the guinea pig ileum LMMP preparation, persistent *in vivo* activation of opioid receptors has been shown to shift the consequences of opioid receptor signaling from predominantly inhibitory to excitatory modulation of transmitter release (Gintzler *et al.*, 1987) and adenylyl cyclase (AC) activity (Wang and Gintzler, 1995; Wang and Gintzler, 1997). This consequence of chronic *in vivo* morphine is accompanied by up-regulation of PKC and can be mimicked by acute treatment of opioid naive LMMP tissue with a phorbol ester. Phorbol 12-myristate 13-acetate not only abolishes high dose sufentanil inhibition of AC activity, but also reverses it to an enhancement (Wang *et al.*, 1996). Chelerythrine, a PKC-selective inhibitor (Herbert *et al.*, 1990), blocks the above-mentioned qualitative changes in opioid responsiveness after either chronic morphine or acute phorbol ester treatment (Wang *et al.*, 1996). Collectively, these data indicate that the phosphorylation state of one or

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ABBREVIATIONS: PKA, cyclic AMP-dependent protein kinase; LMMP, longitudinal muscle myenteric plexus; PKC, protein kinase C; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

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more proteins can be a critical determinant of the consequences of opioid receptor signal transduction.

Opioid agonist-induced receptor phosphorylation is associated with its uncoupling, desensitization (Zhang *et al.*, 1996), and internalization (Zhang *et al.*, 1998). Thus, it seems unlikely that phosphorylation of this protein would also be causally related to augmented opioid receptor AC stimulatory signaling after chronic morphine treatment. This suggests the requirement for another protein kinase substrate(s) that would subserve this function.

This and other laboratories have demonstrated the potential importance of AC isoform-specific signaling to opioid tolerant/dependent mechanisms. Previous studies have demonstrated its relevance to the manifestation of one aspect of the opioid withdrawal phenomenon, AC 'superactivation.' In COS-7 cells co-transfected with the μ-opioid receptor and various AC isoforms, the cAMP 'overshoot' characteristic of opioid withdrawal is dependent on the particular isoform present (Avidor-Reiss *et al.*, 1996; Avidor-Reiss *et al.*, 1997). Moreover, opioid tolerance has been shown to involve AC isoform-specific adaptations. In LMMP tissue, chronic morphine up-regulates specific AC isoforms that are stimulated by $G_{\beta\gamma}$, but not AC I, which is inhibited by this G protein subunit (Chakrabarti *et al.*, 1998; Rivera and Gintzler, 1998). Such changes could underlie, in part, the recently demonstrated augmented $G_{\beta\gamma}/G_{s\alpha}$ AC stimulatory interactivity that ensues in chronic morphine-treated LMMP tissue (Chakrabarti *et al.*, 1998). As a consequence, a shift from predominantly opioid receptor-G_i inhibitory signaling to opioid receptor-$G_{\beta\gamma}$ (G_i-derived) stimulatory signaling could ensue.

Augmented stimulatory opioid receptor AC signaling could also result from increased phosphorylation of AC isoforms. Phorbol ester-activated (PKC-mediated) phosphorylation of AC II and AC VII augments the stimulatory responsiveness of these isoforms to $G_{s\alpha}$ (Jacobowitz and Iyengar, 1994; Watson *et al.*, 1994). PKC-mediated phosphorylation of AC II also results in its augmented stimulation by $G_{\beta\gamma}$ [$G_{s\alpha}$-independent (Tsu and Wong, 1996; Zimmermann and Taussig, 1996)]. Both would contribute to the observed shift from predominantly inhibitory to stimulatory opioid receptor AC signaling in tolerant/dependent tissue (Gintzler *et al.*, 1987; Wang and Gintzler, 1995; Wang and Gintzler, 1997).

Accordingly, this study was undertaken to assess the effect of chronic morphine treatment on the phosphorylation state of AC. The results provide the first demonstration in a complex mammalian neuronal tissue that AC is phosphorylated. Moreover, the level of phosphorylation is greatly augmented by chronic *in vivo* morphine exposure. The consequences of this covalent modification to opioid receptor AC signaling and the relevance of these changes to mechanisms underlying opioid tolerance/dependence are discussed.

Materials and Methods

LMMP preparation and phosphorylation method. Morphine tolerance/dependence was induced by subcutaneous implantation of five morphine pellets (generously supplied by National Institute on Drug Abuse, each containing a 75-mg morphine base) into each guinea pig under light anesthesia. On the sixth day after pellet implantation, animals were killed and ilea were removed and washed with phosphate-free Krebs buffer (pH 7.5). Myenteric plexus and attached longitudinal muscle were removed and equilibrated in the same buffer with continuous gassing (95% O_2/5% CO_2) at 35° for 30 min. For tolerant/dependent LMMP tissues, morphine (100 nM) was included in the buffer. LMMP strips were then incubated with 1 mCi/ml of ^{32}Pi (New England Nuclear, Boston, MA) in phosphate-free Krebs (2 hr at 35°) with continuous gassing. Subsequently, LMMP tissues were washed extensively with phosphate-free Krebs buffer and homogenized in Tris buffer (10 mM, pH 7.6) containing 10% sucrose, 2 mM DTT, 5 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, protease inhibitors (Bacitracin, 100 mg/liter; 20 mg/liter each of *N*-tosyl-L-phenylalanine chloromethyl ketone, sodium-*p*-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride; 3.2 mg/liter each of leupeptin and soybean trypsin inhibitor; 0.5 mg/liter of Aprotinin and 1 mM Benzamidine), protein phosphatase inhibitors, 25 nM calyculin A, 1 μM okadaic acid and 100 μM of sodium orthovanadate. Supernatant obtained from a low-speed centrifugation (1,000 $\times$ *g*, 4°, 10 min) was subjected to a high-speed centrifugation (30,000 $\times$ *g*, 4°, 30 min). Membranes obtained were re-suspended in 50 mM Tris buffer containing 1 mM DTT, 10 mM sodium pyrophosphate, and the above-mentioned protease and phosphatase inhibitors.

Immunoprecipitation of AC protein. Membranes were solubilized in the above mentioned buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 150 mM NaCl (60 min on ice), vortexed three times, and centrifuged (14,000 $\times$ *g* for 30 min). Immunoprecipitation of AC from supernatants was accomplished using the monoclonal AC antibody 6C6 [generated against the AC IIC_2 domain (Chakrabarti *et al.*, 1998)] and AC antibodies (BBC-4) generated against the carboxyl terminus, common to most AC isoforms (generously provided by Dr. T. Pfeuffer, Heinrich Heine University, Düsseldorf, Germany; Mollner and Pfeuffer, 1988; Bol *et al.*, 1997). Protein amounts were determined by the method of Bradford using bovine serum albumin as a standard. Solubilized membrane protein (1 mg) from each sample was incubated with 20 μg of either 6C6 or BBC-4 (1:200) antibody (60 min at 4°). Afterwards, 30 μl of a protein A-Agarose slurry (50%, prewashed; Life Technologies, Gaithersburg, MD) was added. The mixture was agitated and incubated overnight at 4°. An analogous procedure was performed using 6C6 hybridoma extract preadsorbed on a Ni-NTA column (Yan *et al.*, 1996; Chakrabarti *et al.*, 1998). On the next day, the beads were washed extensively with wash buffer (50 mM Tris and 150 mM NaCl, pH 7.6) containing 1 mM DTT, 5 mM EDTA, 1 mM EGTA, 0.05% Nonidet P-40 and the same protease inhibitors used in the lysis buffer. The immunoprecipitate was eluted using 30 μl of Laemmli sample buffer (62.5 mM Tris buffer, pH 6.8, 2% SDS in 10% glycerol) and heating samples at 85° for 6 min. Samples were treated with *N*-ethylmaleimide (50 mM for 15 min at room temperature) to avoid smearing of proteins. After addition of 1% β-mercaptoethanol and traces of bromphenol blue (0.001%), samples were electrophoresed in 7.5% SDS polyacrylamide gels (10 mA/gel). Gels were dried and exposed to PhosphorImager screens that were scanned in a PhosphorImager Storm 860 (Molecular Dynamics, Sunnyvale, CA). The amount of ^{32}P incorporated into the phosphorylated protein bands was determined using densitometric analysis (Imagequant software, Molecular Dynamics).

Results

Phosphorylation of AC protein. In the absence of chronic morphine treatment, ^{32}P incorporation into 6C6 (generated against AC IIC_2) immunoprecipitable material was minimal (Fig. 1A, *lane 1*). However, 6C6 immunoprecipitates from LMMP tissue obtained from chronic morphine-treated animals revealed a dramatic increase in phosphorylation of a ~155-kDa protein (Fig. 1A, *lane 2*). Because ^{32}P incorporation into this protein obtained from opioid naive LMMP tissue was at the margin of detectability, accurate assessment of the -fold increase in its phosphorylation after chronic mor-

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phine treatment was not possible. The intensity of the ~155 kDa band in 6C6 immunoprecipitate obtained from opioid tolerant/dependent LMMP extract was markedly reduced (>90%) when immunoprecipitation was performed using preadsorbed 6C6 hybridoma extract (Fig. 1A, *lane 2* versus *lane 3*). Chronic morphine-induced augmented phosphorylation of a ~155-kDa protein was also observed when immunoprecipitation was performed using another AC antibody, BBC-4, generated against the carboxyl terminus common to most AC isoforms (Mollner and Pfeuffer, 1988; Bol *et al.*, 1997). The magnitude of the increment in phosphorylation after chronic morphine was analogous to that observed using 6C6 (Fig. 1B).

To investigate the potential role of PKC in chronic morphine-induced AC phosphorylation, the effect of a PKC-selective inhibitor on the increment in AC phosphorylation was determined. Chelerythrine (5 μM), included during the last 30 min of the ^{32}P labeling period, essentially abolished the increased ^{32}P incorporation into the ~155-kDa band obtained from BBC-4 immunoprecipitate (Fig. 2). Because BBC-4 recognizes all AC isoforms, these results indicate that the chronic morphine-induced augmented phosphorylation of all potential AC substrates is mediated via a chelerythrine-sensitive (presumably PKC) kinase.

Discussion

The present study demonstrates a dramatic increase in phosphorylation of a ~155-kDa protein in the LMMP tissue after chronic systemic morphine treatment. This protein is identified as AC based on multiple criteria: (1) the molecular mass of the phosphorylated protein is strikingly similar to that of AC isoforms that have been identified in brain (Krupinski *et al.*, 1989; Orlando *et al.*, 1992; Sunahara *et al.*, 1995; Lane-Ladd *et al.*, 1997) and LMMP tissue (Chakrabarti *et al.*, 1998); (2) the intensity of the ~155-kDa band was markedly reduced after immunoprecipitation with preadsorbed 6C6 hybridoma cell extract; (3) the molecular mass of a ^{32}P-labeled protein immunoprecipitated with a differentially directed AC antibody, BBC-4, as well as the magnitude of its increased phosphorylation after chronic morphine treatment, was analogous to that obtained using 6C6.

This is the first demonstration that chronic morphine augments the phosphorylation of an identified effector protein. A recent study (Schulz and Hollt, 1998) demonstrated that the phosphorylation of another effector protein, mitogen-activated protein kinase, is diminished by chronic morphine. Both studies underscore the importance of altered phosphorylation state of effector proteins to tolerant/dependent mechanisms.

Of the nine AC isoforms identified in brain, six have, thus far, been either directly (Jacobowitz and Iyengar, 1994; Iwami *et al.*, 1995; Zimmermann and Taussig, 1996; Wei *et al.*, 1996; Chen *et al.*, 1997) or indirectly (Yoshimura and Cooper, 1993; Watson *et al.*, 1994; Tsu and Wong, 1996; Bol *et al.*, 1997) shown to be substrates for protein kinases. Thus, this modification could be a critical endogenous control mechanism for the regulation of AC signaling pathways. The ability of opioids to regulate AC-cAMP-PKA signaling cascades via G protein-coupled mechanisms has long held a pivotal position in formulations of models of acute and chronic opioid actions. The present study adds a new dimension to this mechanism by revealing that continual activation of opioid receptors results in a covalent modification of AC which in turn alters its G protein subunit responsiveness (see below). Given the myriad of cell functions that can be influenced by cAMP-dependent protein phosphorylation, chronic morphine-induced phosphorylation of AC could underlie, at least in part, a host of physiological sequelae of opioid tolerance/dependence.

In an earlier study (Chakrabarti *et al.*, 1998), we demonstrated that chronic morphine increases AC protein (56%) in LMMP tissues. The relationship of this increase in AC pro-

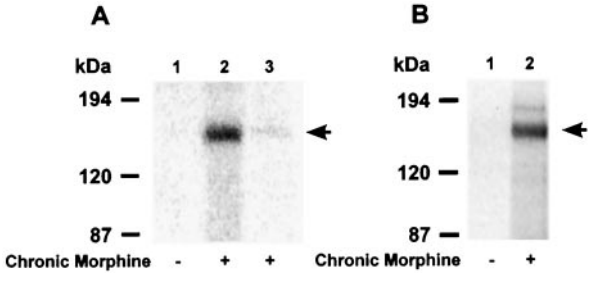

Fig. 1. Chronic morphine augments phosphorylation of AC protein. A, Autoradiogram of ^{32}P incorporation into 6C6 (AC IIC_2-directed) immunoprecipitate from LMMP tissue obtained from opioid naive (*lane 1*) and chronic morphine-treated (*lane 2*) guinea pigs. *Lane 3*, LMMP tissue extract used in *lane 2* that was immunoprecipitated using preadsorbed 6C6 hybridoma extract. B, Autoradiogram of ^{32}P incorporation into immunoprecipitate obtained from LMMP tissue extracts used in *lanes 1* and *2* of A using BBC-4 (directed against the carboxyl terminus common to AC isoforms). All *lanes* contain immunoprecipitate from 1 mg of solubilized LMMP membrane protein. The autoradiograms shown are representative of one of three experiments, each using separate pairs of naive and morphine-treated animals. Immunoprecipitate from one pair of control and morphine-treated LMMP tissue were obtained in parallel. Radioactive signal was visualized by their concomitant autoradiography (18-hr exposure) using storage phosphor imaging techniques. *Arrowhead*, molecular mass of phosphorylated AC protein (~155 kDa). A prestained protein ladder (10–200 kDa; Life Technologies) was included in each run.

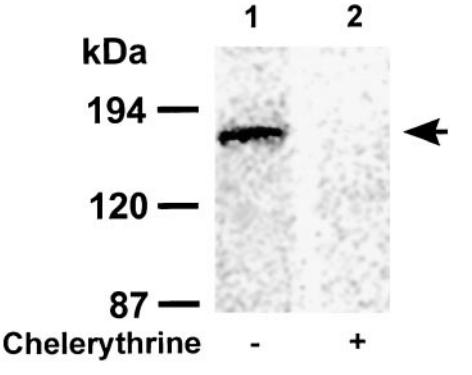

Fig. 2. Chelerythrine abolishes the chronic morphine-induced phosphorylation of AC. Autoradiogram of ^{32}P incorporation into BBC-4 immunoprecipitate from LMMP tissue obtained from a chronic morphine-treated guinea pig. LMMP tissue from the same ileum was randomly divided into two samples, one of which contained chelerythrine (5 μM) during the last 30 min of the 2-hr ^{32}P labeling period. Thereafter, membranes were prepared, solubilized, and immunoprecipitated using BBC-4 as described in Materials and Methods. All *lanes* contain immunoprecipitate from 1 mg of solubilized LMMP membrane protein obtained from sample incubated in the absence (*lane 1*) or presence (*lane 2*) of chelerythrine. The autoradiogram shown is representative of one of three experiments, each using a separate morphine-treated animal. Radioactive signal was visualized by their concomitant autoradiography (18-hr exposure) using storage phosphor imaging techniques. *Arrowhead*, absence of signal at the molecular mass of phosphorylated AC protein (~155 kDa). A prestained protein ladder (10–200 kDa; Life Technologies) was included in each run.

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tein to its augmented phosphorylation remains unclear. The relative susceptibility of newly synthesized versus pre-existing AC protein to phosphorylation remains to be determined.

The 6C6 AC antibody recognizes the type II AC enzyme family (AC II, IV and VII) and AC I but not AC types III, V, VI, VIII, IX and rutabaga (Chakrabarti *et al.*, 1998). Consequently, of the plethora of AC isoforms that have been identified to date, only four are potential substrates for the chronic morphine-induced augmented phosphorylation demonstrated in the current study. Of these four, only those belonging to the AC type II family have thus far been shown to be substrates for a protein kinase(s) (Yoshimura and Cooper, 1993; Jacobowitz and Iyengar, 1994; Watson *et al.*, 1994; Zimmermann and Taussig, 1996). Furthermore, the activity of AC I is not altered after phorbol ester treatment (Yoshimura and Cooper, 1993). Thus, ACs of the type II family are the most likely substrates for the present demonstration of augmented AC phosphorylation. However, other AC isoforms, such as AC III, V, and VI, have also been shown to be kinase substrates (Iwami *et al.*, 1995; Wei *et al.*, 1996; Chen *et al.*, 1997). The present results do not preclude the possibility that chronic morphine also results in their augmented phosphorylation, the regulatory consequences of which could contribute to opioid tolerant/dependent sequelae.

Several lines of evidence are consistent with the importance of PKC in both the chronic morphine-induced augmented phosphorylation of AC and the shift from predominantly inhibitory to stimulatory opioid receptor AC signaling observed in tolerant/dependent tissue. First, PKC can phosphorylate ACs of the type II family. Consequently, G protein subunit responsiveness is altered in a manner consistent with the predominance of opioid stimulatory signaling in tolerant/dependent LMMP tissue. Phosphorylated AC II and VII manifest increased stimulatory responsiveness to $G_{s\alpha}$ (Jacobowitz and Iyengar, 1994; Zimmermann and Taussig, 1996; Watson *et al.*, 1994). Moreover, $G_{s\alpha}$-independent $G_{\beta\gamma}$ stimulation of AC II is augmented after its phosphorylation (Tsu and Wong, 1996; Zimmermann and Taussig, 1996). It should be noted that PKC-mediated phosphorylation of AC IV diminishes stimulatory responsiveness to $G_{s\alpha}/G_{\beta\gamma}$ (Zimmermann and Taussig, 1996). However, the recent demonstration that chronic morphine-treated LMMP tissue manifests augmented AC $G_{s\alpha}/G_{\beta\gamma}$ stimulatory interactivity (Chakrabarti *et al.*, 1998) would suggest that AC IV does not represent that predominant AC isoform that undergoes augmented phosphorylation in the current study. Second, PKC α and PKC β are up-regulated in LMMP tissue obtained from chronic morphine-treated animals (Wang *et al.*, 1996). Third, chronic morphine-induced augmented AC phosphorylation is substantially reduced by chelerythrine, which also obliterates the predominance of opioid AC stimulatory signaling observed in these preparations and reinstates inhibitory responses (Wang *et al.*, 1996). It should be noted that the concentration of chelerythrine used in the current study (5 μM) is 7.5-fold higher than its K_i value for PKC (0.66 μM) but 34-fold less than its K_i for PKA. Recently, chelerythrine was shown to activate/inhibit certain phosphodiesterases (Eckly-Michel *et al.*, 1997). However, the minimum concentration of chelerythrine required to observe these effects (10 and 300 μM for activation and inhibition, respectively) was above that used in the current experiments. Moreover, kinase inhibitors selective for PKA versus PKC are devoid of any effect on the chronic morphine-induced, chelerythrine-sensitive reversal of AC inhibition to enhancement (Wang *et al.*, 1996). Thus, it seems unlikely that any potential effect of chelerythrine on phosphodiesterase activity (and consequently cAMP levels) would confound interpretation of the current results. Fourth, autoradiographic analyses of two dimensional gel electrophoresis of LMMP tissue extract revealed a chronic morphine-induced augmented phosphorylation of a ~150-kDa protein that was also chelerythrine-sensitive (Wang L and Gintzler AR, unpublished observations). Collectively, these data suggest a causal relationship between AC phosphorylation and the predominance of opioid excitatory effects and underscore the potential relevance of (PKC-mediated) increased phosphorylation of AC to opioid tolerant/dependent mechanisms.

The chronic morphine-induced AC phosphorylation mechanism would operate in parallel with increased AC isoform-specific synthesis (Chakrabarti *et al.*, 1998; Rivera and Gintzler, 1998), the combination of which would shift opioid receptor signaling from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory. The latter would explain the recently reported chronic morphine-induced augmented $G_{s\alpha}/G_{\beta\gamma}$ stimulatory interactivity (Chakrabarti *et al.*, 1998). Chronic morphine-induced (PKC-mediated) phosphorylation of AC II and/or AC VII could underlie the previously reported effect of this treatment on opioid responses mediated via receptors differentially coupled to G_s and G_i (Gintzler and Xu, 1991; Wang and Gintzler, 1997). The increased stimulatory responsiveness of phosphorylated AC II and/or VII to $G_{s\alpha}$ would explain enhanced AC stimulatory responses to low-dose sufentanil (choleratoxin-sensitive, $G_{s\alpha}$-mediated) in tolerant/dependent LMMP tissue (Wang and Gintzler, 1997). Similarly, augmented $G_{s\alpha}$- independent stimulation of these phosphorylated isoforms by G_i-derived $G_{\beta\gamma}$ would explain the choleratoxin-insensitivity of the high dose sufentanil facilitation of AC activity (presumably mediated via $G_{s\alpha}$-independent $G_{\beta\gamma}$ stimulation) also reported in these preparations (Wang and Gintzler, 1997).

Opioid receptor phosphorylation resulting in desensitization via internalization and/or altered coupling to G proteins has been a predominant focus of attention regarding opioid tolerant/dependent mechanisms. The present study demonstrates that chronic morphine also has profound effects on events downstream from receptor-G protein interactions. Biochemical modification of effector enzymes that are central to cell signaling pathways could alter responsiveness to a myriad of neurotransmitters that signal via G protein-coupled receptors. Thus, phosphorylation of AC provides a mechanism for the well documented heterologous consequences of chronic morphine exposure.

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