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Chronic Morphine Augments $G_{\beta\gamma}/G_{s\alpha}$ Stimulation of Adenylyl Cyclase: Relevance to Opioid Tolerance

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ABSTRACT

In the current study, we investigated the neurochemical basis for the previously reported predominance of stimulatory μ-opioid signaling in guinea pig longitudinal muscle/myenteric plexus (LMMP) preparations after chronic *in vivo* morphine exposure. As expected, recombinant $G_{s\alpha}$ ($rG_{s\alpha}$) dose-dependently stimulated adenylyl cyclase (AC) activity in LMMP membranes obtained from opioid naive as well as tolerant LMMP tissue. However, the magnitude of the increase was significantly greater in the latter than in the former. The $G_{\beta\gamma}$ blocking peptide QEHA (50 μM) essentially abolished stimulation by $rG_{s\alpha}$ in LMMP membranes obtained from both opioid naive and tolerant animals. Interestingly, after partial blockade by lower QEHA concentrations, the incremental AC stimulation by $rG_{s\alpha}$ in tolerant LMMP membranes was no longer observed, indicating augmented $G_{\beta\gamma}$ stimulatory responsiveness. Concomitant changes in the content of AC isoform protein are consistent with these biochemical observations. After chronic systemic morphine, AC protein is augmented significantly (56%). This increment is most likely to be composed of AC isoforms that are stimulated by $G_{\beta\gamma}$. This is the first demonstration in a complex mammalian tissue that persistent activation of opioid receptors results in augmented $G_{\beta\gamma}/G_{s\alpha}$ AC stimulatory interactiveness. The relevance of such changes to the manifestation of opioid tolerance is discussed.

Despite the delineation of many of the physiological, biochemical, and molecular biological sequelae of persistent exposure to opioids, elucidation of the biochemical underpinnings of tolerance formation remains incomplete. AC superactivation (Sharma *et al*., 1975), diminution of spare opioid receptors (Chavkin and Goldstein, 1984), altered opioid receptor density (Chakrabarti *et al*., 1995), and altered G protein content (Ammer and Schulz, 1993) have long been explored as possible biochemical bases for the physiological sequelae of chronic morphine exposure. Additionally, we have focused attention on the contribution to opioid tolerant mechanisms of the recently discovered ability of opioids to regulate transmitter release and second messenger formation bimodally. These studies made use of the guinea pig ileum LMMP preparation. This tissue contains both enkephalin and dynorphin neurons, as well as a considerable density of the three predominant types of opioid receptor (μ, δ, and κ). It has long been used as a model system for acute and chronic effects of opioids.

LMMP opioid bimodality is concentration dependent (Gintzler and Xu, 1991; Wang and Gintzler, 1994). Low concentrations of sufentanil (nanomolar) facilitate evoked transmitter release (Gintzler and Xu, 1991) and cAMP accumulation (Wang and Gintzler, 1994), whereas higher concentrations (micromolar) are inhibitory. After chronic *in vivo* exposure to morphine, there is a qualitative in shift opioid responsiveness such that previously inhibitory concentrations of sufentanil facilitate neuronal function (Gintzler *et al*., 1987; Wang and Gintzler, 1995).

The shift from inhibitory to excitatory opioid modulation after chronic *in vivo* treatment with morphine could result from the loss of inhibitory responsiveness. This possibility, however, was eliminated by the observation that in these preparations, there seems to be a paradoxical augmentation of μ-opioid receptor coupling to the inhibitory (presumably G_i mediated) opioid pathway (Wang and Gintzler, 1997). Alternatively, the qualitative shift in opioid responsiveness could result from an augmentation of the opioid-facilitatory, G_s pathway. However, the inability of CTX to mitigate high dose (micromolar) sufentanil facilitation of AC activity in tolerant/

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ABBREVIATIONS: AC, adenylyl cyclase; LMMP, longitudinal muscle/myenteric plexus; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DTT, dithiothreitol; BSA, bovine serum albumin; SSC, standard saline citrate; TES, *N*-Tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid; $rG_{s\alpha}$, recombinant $G_{s\alpha}$.

dependent LMMP tissue (Wang and Gintzler, 1997) argues against the relevance of this mechanism. Such considerations fuel the hypothesis that chronic *in vivo* exposure to morphine results in the amplification or induction of an opioid receptor-coupled signal transduction pathway or pathways that are either poorly expressed in or absent from opioid naive LMMP tissue.

Consistent with this formulation, we recently demonstrated that chronic morphine augments LMMP levels of mRNA encoding AC in an isoform selective-fashion. Levels of mRNA encoding AC I do not change, but that encoding AC IV is increased significantly (Rivera and Gintzler, 1998). This has substantial qualitative implications regarding the consequences on AC activity of opioid receptor/G_i signal transduction because the former is inhibited by both $G_{i\alpha}$ and $G_{\beta\gamma}$ (Tang and Gilman, 1991a; Taussig *et al*., 1993a, 1993b), whereas the latter is activated conditionally by $G_{\beta\gamma}$ derived from G_i proteins (Gao and Gilman, 1991). Indeed, the relevance of AC isoform-specific $G_{\beta\gamma}$ stimulatory signaling to the directionality (excitatory or inhibitory) of receptor-coupled regulation of AC activity has been demonstrated amply in several cell lines (Federman *et al*., 1992; Matsuoka *et al*., 1994; Avidor-Reiss *et al*., 1997).

Collectively, these observations support the conceptual rubric that chronic morphine exposure induces a shift in the relative preponderance of opioid receptor/$G_{i\alpha}$ inhibition to $G_{\beta\gamma}$ (G_i-derived) stimulation of AC activity ($G_{s\alpha}$ dependent). This formulation presupposes enhanced AC $G_{\beta\gamma}$ stimulatory responsiveness in chronic morphine-treated tissue. Accordingly, the current study investigates whether such augmentation could be detected in LMMP tissue after chronic *in vivo* morphine exposure. Additionally, the occurrence of concomitant alterations in AC isoform protein as well as in G protein subunits, consistent with such changes in AC signaling was investigated.

Materials and Methods

Chronic *in vivo* morphine exposure and tissue preparation. Male albino guinea pigs weighing 375 to 450 g were used. Chronic *in vivo* morphine exposure (6 days) was accomplished via the subcutaneous implantation of five morphine pellets (75 mg of morphine base each; generously supplied by National Institutes of Drug Abuse) into animals under light anesthesia [ketamine/xylazine (7:1)]. LMMP tissue were prepared, in parallel, from control and morphine-treated ilea as described previously (Wang and Gintzler, 1997).

Preparation of LMMP membranes for assessment of AC activity. LMMP tissue was homogenized in Na-HEPES buffer (25 mM, pH 8.0) containing 10% sucrose, 1 mM EDTA, and the protease inhibitors bacitracin (100 mg/liter), leupeptin and soybean trypsin inhibitors (3.2 mg/liter each), aprotinin (0.5 mg/liter), and benzamidine (1 mM). Supernatant obtained from low speed centrifugation (1000 × *g*, 10 min, 4°) was subjected to higher speed centrifugation (30,000 × *g*, 30 min, 4°). The resulting membrane pellet was resuspended in the same buffer containing 8% sucrose, aliquoted, and stored at −70° for future use.

$G_{s\alpha}$ stimulation of AC activity. Recombinant $G_{s\alpha}$ ($rG_{s\alpha}$) was expressed in and purified from *Escherichia coli* (Lee *et al*., 1994). $rG_{s\alpha}$ (5 μg) was activated by incubation (60 min at 30°) with 100 μM GTPγS in 50 mM Na-HEPES buffer, pH 8.0, containing 1 mM EDTA, 1 mM DTT, 5 mM $MgSO_4$, and BSA (1.25 mg/ml) as described previously (Tang *et al*., 1991). Separation from free GTPγS was achieved through gel filtration (Sephadex G-25). Recovery of activated $rG_{s\alpha}$ (~50%) was determined using [^{35}S]GTPγS (New England Nuclear, Boston, MA). To determine whether any altered stimulatory responsiveness to $G_{s\alpha}$ requires the participation of $G_{\beta\gamma}$ subunits, the effects of the $G_{\beta\gamma}$ blocking peptide QEHA (Chen *et al*., 1995) on activated $rG_{s\alpha}$ stimulation of AC also were investigated. Stimulatory responsiveness to $rG_{s\alpha}$ was determined after treatment of the membranes with QEHA (50 μM; 20 min on ice) or its inactive control peptide, SKEE (50 μM).

Determination of AC activity in membrane preparations. AC activity was determined by measuring the synthesis of [^{32}P]cAMP from [α-^{32}P]ATP (ICN, Costa Mesa, CA). Assays were initiated by the addition of the reaction mixture (50 mM HEPES buffer, pH 8.0, containing 10 mM $MgCl_2$, 20 mM creatine phosphate, 10 units/sample creatine phosphokinase, 0.1 mM ATP, 10 μM GTP, 20 mM NaCl, 1 mM DTT, 50 mM EGTA, 0.125 mM rolipram, 0.1% BSA, and [α-^{32}P]ATP; 1 μCi/sample) to cell membranes (10 μg) with or without prior incubation (30°, 10 min) with activated $rG_{\alpha s}$. Reactions (30°, 15 min) were terminated by the addition of 10 μl of 2.2 N HCl (4°). Thereafter, [^{32}P]cAMP generated was separated by neutral alumina column chromatography as described previously (Alvarez and Daniels, 1990). [^{3}H]cAMP (0.005 μCi), added to each sample before fractionation, was used as an internal standard to correct for column recovery of the [^{32}P]cAMP. Radioisotopes were quantified using liquid scintillation spectroscopy (LKB 1209).

Production and characterization of AC IIC2 (6C6) monoclonal antibody. The C2 domain of rat AC type II (IIC2 protein) was expressed in *E. coli* BL21(DE3) and purified by Ni-NTA affinity chromatography followed by Q-Sepharose column chromatography as described previously (Yan *et al*., 1996). The purified IIC2 protein (100 μg) was emulsified in complete Freund's adjuvant and injected subcutaneously into 8-week-old Balb/c mice. Four additional immunization were administered subcutaneously in incomplete Freund's adjuvant at 2-week intervals. Lymphocytes isolated from immunized mice were fused with murine myeloma cell line SP2/0 using PEG1500. Hybridoma supernatant were screened in enzyme-linked immunosorbent assay plates coated with 100 ng of purified IIC2. Hybridomas were cloned by limiting dilution into 96-well microculture plates containing Dulbecco's modified Eagle's medium, 15% fetal bovine serum, and hypoxanthine-thymidine. Of the five positive clones isolated, hybridoma 6C6 was the best antibody producer. Hybridoma supernatant was collected and purified using Q-Sepharose column chromatography. The isotype of heavy and light chain for monoclonal antibody 6C6 was determined to be IgG_1 and κ, respectively (Id-sp Kit; Zymed Lab, San Francisco, CA).

To obtain cell membranes with which to determine the specificity of the 6C6 antibody, Sf9 cells were infected with recombinant baculoviruses encoding one of AC types II–VI, 48 hr after which cell membranes were harvested and 20 μg was reduced and alkylated as described previously (Tang *et al*., 1991). Additionally, constructs for the expression of the C2 domain of AC I, II, VII, VIII, and IX were transformed into *E. coli* BL21(DE3) and used to extend the 6C6 specificity profile. The resulting clones were used for the induction and preparation of the corresponding C2 domain protein (obtained from the 200,000 × *g* supernatant of *E. coli* lysate; Yan *et al*., 1996). Plasmid constructs for the expression of IC2 (bovine AC I: amino acids 806-1134) and IIC2 (rat AC II: amino acids 855-1090) were described previously (Yan *et al*., 1996). The coding sequence for VIIC2 (human AC VII: amino acids 864-1080) was amplified using polymerase chain reaction and cloned to pProExH6 vector, whereas that for VIIIC2 (mouse AC VIII: amino acids 946-1248) and IXC2 (mouse AC IX: amino acids 1011–1353) was amplified and cloned to pProEx-HAH6 vector.

AC isoform selectivity of 6C6 was determined using Western blot analysis. Sf9 cell membranes and *E. coli* lysates were loaded onto 11% and SDS-7.5% polyacrylamide gels, respectively, followed by electrophoreses and electrotransfer onto a nitrocellulose membrane. Recognition by 6C6 of AC isoforms or C2 domains thereof was visualized using ECL detection (Amersham, Arlington Heights, IL). To validate expression of constructs containing C2 domains, these ni-

trocellulose membranes also were blotted with antibody 12CA5 (Yan *et al*., 1996), which recognizes the hemagglutinin epitope.

Preparation of preadsorbed hybridoma supernatant. Preadsorbed AC monoclonal antibody was prepared through incubation (60 min, 27°) of 5 μg of 6C6 antibody with excess peptide (AC IIC_2; 50 μg) in 200 μl of buffer A (20 mM Tris·HCl, pH 8.0, 5 mM β-mercaptoethanol, 100 mM NaCl) followed by Ni-NTA affinity chromatography (Yan *et al*., 1996). The column was sequentially eluted with 900 μl of buffer A (FT1A), twice with the same volume of buffer containing 20 mM imidazole (FT2 and FT2A), and once with buffer containing 150 mM imidazole (FT3). An aliquot (50 μl) of each column eluate was electrophoresed (SDS-7.5% polyacrylamide gel) and electrotransferred onto a nitrocellulose membrane. AC antibody protein was visualized using a secondary peroxidase-labeled anti-mouse antibody and ECL detection. The remainder of fractions FT1 and FT1A (from which 6C6 had been preadsorbed) was concentrated to a volume of ~200 μl, pooled, and used as a negative control in Western blot analysis.

LMMP membrane preparation for Western blot analysis. Membranes from LMMP tissue obtained from control and morphine-treated animals were prepared at 0–4° as described previously (Sternweis and Robishaw, 1984). Briefly, the LMMP tissue was homogenized in buffer A containing a previously described cocktail of protease inhibitors (Tang *et al*., 1991). Supernatants from a low speed centrifugation (8,000 × *g* for 30 min) were recentrifuged (20,000 × *g*; 60 min). Membranes were resuspended in 300–500 μl of the same buffer, and the protein concentration was determined by the Bradford Assay (BioRad, Hercules, CA).

Gel electrophoresis and Western blot analysis. Membrane samples were heated (80° for 5 min) in Laemmli's buffer (50 mM Tris·HCl, pH 6.8, containing 2% SDS and 0.2 mM DTT), after which they were treated with 50 mM *N*-ethylmaleimide (10 min at room temperature) and subjected to SDS-polyacrylamide gel electrophoresis. Gels were electroeluted onto nitrocellulose membranes, which were incubated with blocking solution (5% skim milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 hr). Thereafter, selected lanes were incubated (room temperature for 2 hr) with either the AC monoclonal antibody 6C6 (0.87 μg/4 ml) or the polyclonal G protein subunit antibodies (Makhlouf *et al*., 1996) BC-1 (anti-G_β, carboxyl terminus, 1:2000) or ASC-A (anti-$G_{s\alpha}$, carboxyl terminus, 1:1500), each dissolved in blocking solution. The secondary antibody used was either peroxidase-labeled anti-mouse (for 6C6) or anti- rabbit (for G protein subunits). Antibody/substrate complex was visualized using ECL detection. Sample pairs, obtained from opioid naive and chronic morphine-treated LMMP tissues, were processed, electrophoresed, and blotted in parallel, after which they were exposed concomitantly to the same X-ray film (X-Omat film, Kodak). Signal intensity was quantified using NIH imaging software.

Preparation of total RNA extract. LMMP tissue was homogenized in 5 volumes of guanidinium thiocyanate (5.7 M) containing sodium citrate (5 mM, pH 7.0) and β-mercaptoethanol (1 mM). Extraction of total RNA via CsCl centrifugation and ethanol precipitation was performed as described previously (Rivera and Gintzler, 1998). LMMP tissues from control and morphine-treated animals were processed in parallel. All RNA preparations yielded ratio of absorption (260:280 nm) of ~2.0.

Preparation of RNA probes. Full-length cDNA for rat AC type II (4008 bp; Feinstein *et al*., 1991) was provided as a plasmid in pBluescript SK^- (courtesy of Dr. Randall Reed). The excised cDNA/*Hin*dIII/*Sac*I fragment was subcloned into the pSP64 and pSP65 vectors. Restriction enzyme mapping indicated that the AC II/pSP64 and AC II/pSP65 plasmids that were obtained were of the sense and antisense orientation, respectively. Both plasmids were linearized with *Sac*I and *Hin*dIII to generate the respective templates. An SP6 transcription system (Promega, Madison, WI) and [α-^{32}P]GTP (New England Nuclear, Boston, MA) were used to form radiolabeled AC II (~8.2×10^8 cpm/μg) riboprobe antisense. CF11 column chromatography was used to purify the RNA transcripts (Franklin, 1966).

Solution hybridization assay for mRNA encoding AC II. The solution hybridization/ribonuclease treatment/TCA precipitation protocol used to quantify RNA levels was a modification of that described previously (Franklin *et al*., 1991) as reported earlier (Rivera and Gintzler, 1998). Briefly, duplicate aliquots of total RNA extract (40 μg) were incubated (75° overnight) with AC II ^{32}P-labeled riboprobe (200,000 cpm) contained in 2× TESS (20 mM TES, pH 7.4, 20 mM EDTA, 0.6 M NaCl, 1% SDS). Thereafter, samples were incubated (30° for 1 hr) with a high salt buffer (10 mM Tris·HCl, pH 7.5, 0.3 M NaCl, 5 mM EDTA) containing 40 μg/ml RNase A (Worthington Biochemicals, Freehold, NJ) and 2 μg/ml RNase T1 (Calbiochem, San Diego, CA). TCA precipitates, obtained via the addition (1 ml) of 5% TCA containing 0.75% sodium pyrophosphate and one drop of 0.5% BSA, were collected onto glass microfiber filters. Radioactivity was quantified by liquid scintillation spectroscopy. Extract from a chronic morphine-treated LMMP tissue always was assayed in parallel with its paired opioid naive tissue. AC II mRNA levels in LMMP tissue extract were quantified from an AC II mRNA standard curve that was generated using the "sense transcript" (5, 10, 20, 40, 60, 80, 160, 320 pg) containing 10 μg of *E. coli* tRNA as a carrier as described previously (Rivera and Gintzler, 1998). Standard AC II RNA was synthesized using unlabeled ribonucleotides and the SP6 RNA polymerase. Regression analysis of the standards was carried out to determine the least-squares best fit, from which values for assigning concentrations to the tissue samples were obtained by interpolation.

Northern blot analysis. Total RNA from LMMP tissue as well as AC I and AC IV "sense transcript" was subjected to 1% agarose/2.2 M formaldehyde gel electrophoresis and transferred onto nitrocellulose membranes as described previously (Rivera and Gintzler, 1998). Membranes were hybridized with an α-^{32}P-GTP-labeled cRNA probe specific for mRNA encoding AC II (10^6 cpm/ml; 60° overnight in sodium phosphate buffer, 0.4 M, pH 7.0, containing 3% SDS, 1 mM EDTA, 43% formamide, 0.025% acetylated BSA). Subsequently, membranes were washed once for 5 min followed by a 20-min wash (60°) and a 30-min wash (75°) with 0.5× standard saline citrate buffer (15 mM sodium citrate, 150 mM sodium chloride, pH 7.2) containing 1% SDS and 1 mM EDTA. Hybridized signal was visualized by autoradiography using storage PhosphorImaging techniques (Molecular Dynamics, Sunnyvale, CA).

Results

Effect of chronic morphine on $G_{s\alpha}$ stimulation of AC activity. As expected, $rG_{s\alpha}$ stimulated AC activity in LMMP membranes obtained from both opioid naive and tolerant LMMP tissue in a dose-dependent manner ($p < 0.05$ for all concentrations of $rG_{s\alpha}$; Fig. 1A). Of particular relevance to the current study, the magnitude of the increase in the latter group was significantly greater than that observed in the former compared with all $rG_{s\alpha}$ concentrations tested (1, 2.5, 5, 10 nM). However, the increment in $G_{s\alpha}$ stimulation between groups did not vary across concentrations tested. A two-way analysis of variance revealed a group and dose effect ($F_{1,6} = 10.25$ and $F_{3,18} = 119.28$; $p < 0.02$ for both) but no group × dose interaction ($F_{3,18} = 2.77$; $p > 0.05$).

Effect of the $G_{\beta\gamma}$ blocking peptide QEHA on $G_{s\alpha}$ stimulation of AC. To investigate the role of $G_{\beta\gamma}$ in the altered $G_{s\alpha}$ stimulation of AC after chronic morphine, the effect of the $G_{\beta\gamma}$ blocking peptide QEHA (a peptide corresponding to residues 956–982 of AC II; Chen *et al*., 1995) on $G_{s\alpha}$-stimulated AC activity was determined in opioid naive and tolerant membranes (Fig. 1B). A two-way analysis of variance revealed a dose effect of QEHA ($F_{1,10} = 186.39$, $p < 0.001$).

QEHA (50 μM) essentially abolished stimulation by $G_{s\alpha}$ in membranes obtained from opioid naive and morphine-treated LMMP membranes. Lower QEHA concentrations (10 and 20 μM) incompletely attenuated $G_{s\alpha}$ stimulation of AC activity. Interestingly, after partial blockade by QEHA, the difference in the magnitude of AC stimulation by $G_{s\alpha}$ between naive and tolerant LMMP membranes was no longer observed ($F_{1,4} = 1.83$, $p > 0.2$; Fig. 1A, *inset*). This effect did not vary across concentrations of QEHA ($F_{1\ 10} = 1.64$; $p > 0.2$). Moreover, in LMMP membranes obtained from four animals (two opioid naive and two chronic morphine treated), coincubation with QEHA and purified $G_{\beta\gamma}$ (50 nM) reversed the QEHA block by 69–99%. In these experiments, the concentration of Lubrol PX present in the buffer containing $G_{\beta\gamma}$ was maintained at 0.01% in all assays.

Interestingly, QEHA failed to lower the basal AC activity in either control or morphine-treated preparations despite the presence of 10 μM GTP, a concentration sufficient to generate activated $G_{s\alpha}$ and $G_{\beta\gamma}$. However, it should be noted that higher concentrations of GTP (up to 100 μM) failed to produce any further increase in basal AC activity in both preparations (three experiments), which remained significantly lower than that observed after the addition of 5 nM activated $G_{s\alpha}$. This suggests that although GTP (10 μM) results in the generation of activated $G_{s\alpha}$, the concentration achieved is <5 nM and below that required for AC stimulation by $G_{\beta\gamma}$. In the absence of $G_{\beta\gamma}$, stimulation of AC, QEHA should not be expected to alter AC activity.

Analogous to $G_{s\alpha}$ stimulatory responsiveness, forskolin (10 μM) stimulation of AC activity also was augmented after chronic morphine (~17%, $p < 0.05$, Fig. 1B). However, in contrast to $G_{s\alpha}$ stimulation of AC activity, QEHA (50 μM) did not alter the forskolin activation of this enzyme (Fig. 1B). To explore further the specificity of the effect of QEHA, the effect of a related peptide, SKEE, was determined. This peptide corresponds to the region of AC III (not stimulated by $G_{\beta\gamma}$) that is analogous to the $G_{\beta\gamma}$ binding region on AC II (stimulated by $G_{\beta\gamma}$), the amino acid sequence of which composes QEHA (Chen *et al.*, 1995). A concentration of SKEE equal to that used for QEHA (50 μM) was devoid of any effect on $rG_{s\alpha}$ AC stimulation (Fig. 1B).

Specificity of monoclonal antibody 6C6 raised against AC IIC2 domain. Antibody 6C6 was able to detect recombinant types II and IV ACs but not types III, V, VI, or rutabaga (Fig. 2A) in membranes obtained from Sf9 cells transfected with these isoforms. Western blot analysis of the C2 protein domain of various adenylyl cyclases expressed in *E. coli* indicated that 6C6 recognized IC2, IIC2, and VIIC2 but not VIIIC2 and IXC2 (Fig. 2B). Expression of VIIIC2 and IXC2 was confirmed by demonstrating that lysate obtained from *E. coli* infected with either gave a positive signal, at the expected molecular mass, when blotted with antibody 12CA5 (Fig. 2C; Yan *et al.*, 1996). Thus, antibody 6C6 recognizes the type II AC enzyme family (type II, IV, and VII) and AC I but not AC types III, V, VI, VIII, IX, and rutabaga.

Specificity of AC Western blot analysis. Specificity of the signal visualized using 6C6 antibody in Western blot analysis was determined using preadsorbed hybridoma cell extract. Fig. 3A illustrates the elution profile of the adsorbed AC monoclonal antibody from a Ni-NTA column that had been incubated with AC antibody/IIC_2 peptide complex. The absence of any signal after incubation with goat anti-mouse

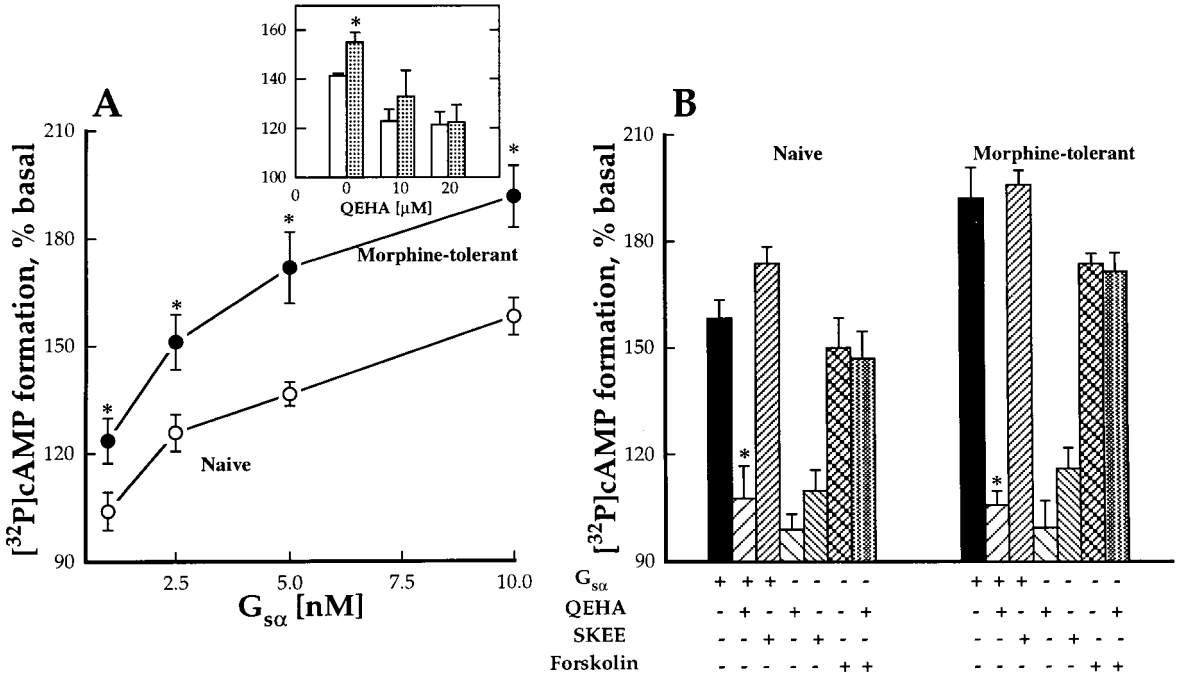

Fig. 1. A, $rG_{s\alpha}$ stimulatory responsiveness of AC in membranes obtained from opioid naive (○) and chronic morphine-treated LMMP tissue (●). B, Effect of the $G_{\beta\gamma}$ blocking peptide QEHA and its control peptide SKEE (50 μM each) on AC stimulation by $rG_{s\alpha}$ (5 nM) and forskolin (10 μM) in membranes obtained from LMMP used in A. Basal AC activity (cAMP formed expressed as pmol × mg of protein^{-1} × min^{-1}) in LMMP membranes obtained from opioid naive and chronic morphine-treated tissue was 4.62 ± 0.38 and 4.98 ± 0.49, respectively ($p > 0.05$). A, *Inset*, effect of submaximal concentrations of QEHA on the differential $rG_{s\alpha}$ stimulatory responsiveness of membranes obtained from opioid naive (*open bars*) versus chronic morphine-treated LMMP tissue (*stippled bars*). Morphine treatment, $rG_{s\alpha}$ activation, stimulation of AC activity, and QEHA pretreatment were accomplished as described in the text. *, $p < 0.05$ for $rG_{s\alpha}$ stimulation in tolerant versus naive membranes (A) and for $rG_{s\alpha}$ stimulation of AC in the absence versus the presence of QEHA (B).

immunoglobulin indicates that column eluate obtained with imidazole-free buffer (FT1 and FT1A) was devoid of all immunoglobulin. In contrast, AC antibody was eluted with buffer containing imidazole (fractions FT2 and FT3); therefore, fractions FT1 and FT1A were pooled and used for adsorption control immunoblotting.

Fig. 3B illustrates the Western blots obtained using either preadsorbed hybridoma cell extracts (FT1 and FT1A) or unabsorbed 6C6 monoclonal antibody that was equivalently diluted. The predominant signal visualized by Western blot analysis using unadsorbed 6C6 had a molecular mass of ~150 kDa. In addition, a faster moving band was detected at ~140 kDa (*lane 1*). Neither of these bands was detected when LMMP extract was blotted with preadsorbed hybridoma extract (Fig. 3B, *lane 2*). The molecular weight difference between LMMP ACs recognized by 6C6 versus that expressed in Sf9 cells (Fig. 2A) most likely is due to variations in the degree of glycosylation, sites for which are present in ACs. Indeed, it has been shown that the molecular masses of ACs vary, depending on the source (Mollner and Pfeuffer, 1988)

Effect of chronic morphine treatment on AC protein. The signal intensity of the 150-kDa band was augmented significantly in LMMP extract obtained from guinea pigs chronically treated with morphine (56%; $p < 0.05$, four experiments; Fig. 3C). The content of the 140-kDa species was quite variable among experiments. In fact, it was detectable in only two of four experiments. Therefore, a comparison of signal intensity between samples obtained from opioid naive and chronic morphine-treated tissues was restricted to the larger molecular mass species.

Effect of chronic morphine treatment on G protein subunits. Western blot analyses of $G_{s\alpha}$ and G_{β} (Fig. 4A) revealed two bands for the former (45 and 48 kDa) and one for the latter (36 kDa). This is consistent with results from previous studies using these as well as other antibodies (Makhlouf *et al*., 1996; Ammer and Schulz, 1997). Neither the intensity of the two immunoreactive bands corresponding to $G_{s\alpha}$ nor that corresponding to G_{β} differed among cholate extracts of membranes obtained from opioid naive versus chronic morphine-treated LMMP tissue (*lane 1* versus *lane 2* for each blot).

Specificity of the AC II riboprobe. Northern blot analysis was used to determine the size of AC II mRNA in guinea pig LMMP, as well as the specificity of the radiolabeled riboprobe used in these experiments (Fig. 4B). For AC II, Northern blots of total RNA obtained from guinea pig LMMP tissue revealed an abundant hybridized transcript of ~4.0 kb (Fig. 4B, *lane* 4). This is consistent with the 4.1-kb size reported previously for the type II isoform isolated from rat brain and olfactory bulb (Feinstein *et al*., 1991), as well as the size of the sense AC II RNA transcribed in this study (Fig. 4B, *lane 3*). Additionally, a hybridized transcript estimated to be 1.9 kb was observed. Because it survived high stringency washes (1% SDS, 0.5× standard saline citrate buffer, 1 mM EDTA at 70° for 60 min), it most likely does not result from nonspecific hybridization. This band could represent splice variants of the original transcript or alternative transcription initiation sites. An analogous pattern of the size distribution for AC II mRNA has been reported for rat tissues (Feinstein *et al*., 1991).

Specificity of the riboprobe for AC II was evaluated further by assessing the extent to which AC II antisense cross-hybridizes with AC I or AC IV sense. Northern blot analyses (Fig. 4B, *lanes 1 and 2*) and solution hybridization (data not shown) revealed that the radiolabeled riboprobe for AC II does not cross-hybridize with sense RNA encoding AC I or IV.

Quantification of AC II mRNA in opioid naive and chronic morphine-treated tissue. Solution hybridization assays revealed that levels of mRNA encoding AC II in opioid naive LMMP tissue are 0.93 ± 0.16 pg/μg total RNA. After

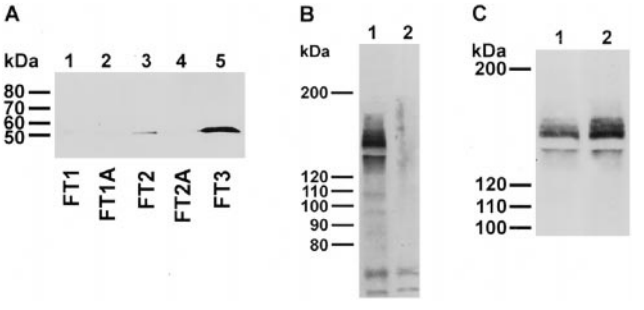

Fig. 3. Western blot analysis of AC protein in opioid and chronic morphine-treated LMMP tissue. A, AC monoclonal antibody was incubated with IIC2 peptide, after which it was applied to a Ni-NTA column as described in the text. The presence or absence of antibody protein in column fractions was determined by ECL/Western blot analysis using a peroxidase-labeled anti-mouse antibody. *Lane 1*, fraction FT1, column flow-through. *Lane 2*, fraction FT1A, eluting buffer was imidazole free. *Lanes 3 and 4*, eluting buffer contained 20 mM imidazole, fractions FT2 and FT2A, respectively. *Lane 5*, eluting buffer contained 150 mM imidazole, FT3. Molecular mass markers (20–200 kDa) were included in the run. Fractions FT1 and FT1A do not contain 6C6 antibody protein. B, Western blot of LMMP protein (120 μg) using either unabsorbed 6C6 monoclonal antibody (*lane 1*) or equivalently diluted 6C6-free column eluate (pooled FT1 and FT1A fractions; *lane 2*). C, Effect of chronic morphine on LMMP tissue content of AC protein. Samples (240 μg) were obtained from opiate naive (*lane 1*) and chronic *in vivo* morphine-treated animals (*lane 2*). LMMP tissues were blotted concomitantly with 6C6 and exposed to the same X-ray film. The X-ray film was scanned, and signal intensity was quantified using NIH imaging software. Molecular mass markers (20–200 kDa) were included in each run. The Western blot shown is representative of one of four experiments. Signal intensity of 6C6-recognizable protein was found to be linear between 60 and 240 μg of membrane protein. Comparable increases in AC protein also were observed at lower concentrations of membrane protein.

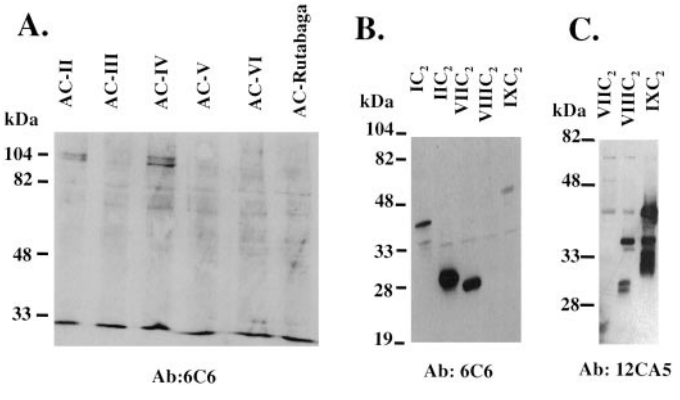

Fig. 2. Specificity of monoclonal antibody 6C6. A, Immunoblot using antibody 6C6 with Sf9 cell membranes (30 μg) containing either AC type II –VI or rutabaga. B, Immunoblot using antibody 6C6 with *E. coli* lysates (20 μg) containing the C2 domain of AC types I, II, VII, VIII, and IX. For AC I, II, and VII, a single predominant band was visualized. C, Immunoblot using antibody 12CA5 with *E. coli* lysates containing VIIC2, VIIIC2, and IXC2. Specificity of 12CA5 is indicated by the lack of a positive signal when lysate obtained from VIIC2 infected *E. coli* (which does not contain the hemagglutinin epitope) is blotted with this antibody.

persistent *in vivo* activation of opioid receptors, levels of AC II mRNA were increased slightly to 1.0 ± 0.10 pg/μg total RNA, but this numerical difference was not statistically significant ($p = 0.6$, five experiments; see Table 1 for a comparison with mRNA encoding AC I and IV). In these assays, the standard curves for AC II sense RNA had a correlation coefficient of ≥0.98. It should be noted that the riboprobe for AC II contains complementary sequences encoding bovine AC II (Feinstein *et al.*, 1991). Because these sequences are, undoubtedly, not perfectly complementary to guinea pig AC II mRNA, their quantification in the current study probably is an underestimation. It is not possible to determine the precise correction factor to be used, so assay values are expressed as "pg equivalents" (Franklin *et al.*, 1991).

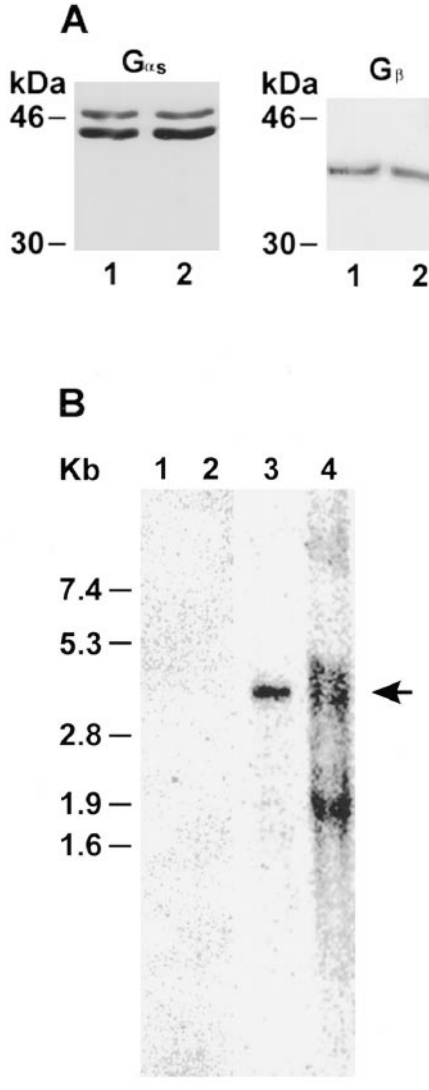

Fig. 4. Quantification of G protein subunits and AC II mRNA in opioid naive and chronic morphine-treated LMMP tissue. A, Western blot analysis of $G_{s\alpha}$ and G_β subunit protein in membranes obtained from opioid naive (*lane 1*) and chronic morphine-treated LMMP tissue (*lane 2*). Cholate extract from membrane samples (30 and 8 μg for $G_{s\alpha}$ and G_β, respectively) were electrophoresed in SDS-10% polyacrylamide gels. Immunoblots shown in A and B were generated using ASC-A (anti-$G_{s\alpha}$) and BC-1 (anti-G_β), respectively, in combination with a peroxidase-labeled goat anti-rabbit secondary antibody. Antibody/substrate complex was visualized using Western blotting and ECL detection. Blot is representative of one of four experiments. Neither the intensity of the two immunoreactive bands corresponding to $G_{s\alpha}$ nor that corresponding to G_β differed among cholate extracts of membranes obtained from opioid naive versus chronic morphine-treated LMMP tissue. B, Northern blot analysis using an α-^{32}P-GTP-labeled cRNA probe specific for mRNA encoding AC II. *Lanes 1–3*, AC "sense" cRNA for AC I, IV, and II, respectively (35 pg for each). *Lane 4*, total LMMP RNA (50 μg). Hybridized signal was visualized by autoradiography (24-hr exposure) using storage phosphorimaging techniques. A 1.6–7.4-kb ladder was used as molecular weight standards. The α-^{32}P-GTP-labeled cRNA probe specific for AC II mRNA does not cross-hybridize with AC I or IV mRNA. Blot is representative of one of three experiments.

Discussion

It has been demonstrated that ACs from murine S49 cells and platelets, insensitive to $G_{\beta\gamma}$, are stimulated by $G_{s\alpha}$ (Tang and Gilman, 1992). These observations are consistent with the more recent finding that $rG_{s\alpha}$ stimulated purified AC in the absence of $G_{\beta\gamma}$ (Yan *et al.*, 1997). Therefore, the abolishment by QEHA of $rG_{s\alpha}$ and stimulation of AC in membranes prepared from opioid naive and tolerant tissue indicate that in both preparations, it is mediated via $G_{\beta\gamma}$ ($G_{s\alpha}$ dependent); that is, stimulation of LMMP AC by exogenous $rG_{s\alpha}$ (1–10 nM) results from its ability to augment stimulation by endogenous $G_{\beta\gamma}$ (Tang and Gilman, 1991b). Thus, chronic morphine-induced augmented $G_{\beta\gamma}$ stimulation ($G_{s\alpha}$ dependent) of AC is most likely reflected by the increased $rG_{s\alpha}$ stimulatory responsiveness of tolerant preparations. This inference is supported by the ability of submaximal concentrations of QEHA to obliterate the incremental $rG_{s\alpha}$ stimulatory response that occurs after chronic morphine because a similar degree of $G_{\beta\gamma}$ neutralization by QEHA in both preparations would have a greater effect on $rG_{s\alpha}$ stimulation in the latter.

The current study was unable to directly demonstrate augmented $G_{\beta\gamma}$ stimulation of AC activity after chronic morphine treatment. In fact, stimulation of AC activity by exogenous $G_{\beta\gamma}$ in either opioid naive or tolerant LMMP membranes was not detectable. This most likely results from the fact that the LMMP tissue contains multiple AC isoforms, some of which are inhibited by $G_{\beta\gamma}$. For example, in addition to AC II and IV (which are stimulated by $G_{\beta\gamma}$), the LMMP tissue also contains AC I (Rivera and Gintzler, 1998). All would be stimulated by activated exogenous $G_{s\alpha}$. However, $G_{\beta\gamma}$ inhibition of AC I (Tang and Gilman, 1991b) could mask any augmented $G_{\beta\gamma}$ stimulation of ACs of the type II family. In this regard, it is relevant to note that to date, virtually all studies demonstrating direct $G_{\beta\gamma}$ stimulation of AC activity have been conducted with cultured cells overexpressing a single AC isoform.

The effect of chronic morphine on LMMP levels of AC isoform-specific mRNA and AC isoform protein is consistent

TABLE 1
Levels of mRNA encoding adenylyl cyclase isoforms in LMMP tissue obtained from opiate naive and chronic morphine-treated guinea pigs

AC isoform	Isoform mRNA/total RNA	
	Opiate naive	Chronic morphine
	pg/μg	
AC II	0.93 ± 0.16 (4)	1.0 ± 0.1 (4)
AC I[a]	0.64 ± 0.14 (4)	0.71 ± 0.13 (4)
AC IV[a]	0.49 ± 0.04 (4)	0.67 ± 0.04[b] (4)

Data are represented as mean ± standard error pg equivalents/μg of total RNA. The number of animals in each group is indicated in parentheses.

[a] Reprinted from Molecular Brain Research (1998) **54:** 165–169 by Rivera and Gintzler, with permission from Elsevier Science.

[b] $p < 0.05$ for AC IV mRNA in chronic morphine-treated tissue versus control.

with the augmented $G_{\beta\gamma}$ stimulatory AC signaling demonstrated in the current study. Furthermore, these data suggest a mechanism by which such changes could occur. The antibody used to quantify AC protein recognizes ACs of the type II family (AC II, IV, and VII), all of which are stimulated by $G_{\beta\gamma}$, and AC I. It is unlikely that cross-reactivity with AC I confounds interpretation of the Western blot analyses because levels of mRNA encoding the latter do not change after chronic morphine (Rivera and Gintzler, 1998). This supposition is supported by a previous report (Hellevuo *et al*., 1996) that in several brain areas, the mRNA levels of six AC isoforms were predictive of the regulatory characteristics of AC activity in corresponding membrane preparations. Similarly, it also is unlikely that AC II contributes to the chronic morphine-induced up-regulation of AC protein because its corresponding mRNA fails to increase after this treatment.

In contrast, the LMMP content of AC IV mRNA is increased (~37%) after chronic systemic morphine treatment (Rivera and Gintzler, 1998). Interestingly, the magnitude of the increase in AC protein observed in the current study (56%) is of comparable magnitude. Thus, it seems plausible to infer that the morphine-dependent increase in AC protein observed in the current study reflects elevated levels of AC IV. The effect of chronic morphine on levels of mRNA encoding AC VII has not been determined. Consequently, its contribution to the morphine-induced up-regulation of AC protein remains unknown. This notwithstanding, distinction between these isoforms is not relevant to the current study because an increase in either would have the same $G_{\beta\gamma}$ regulatory implications (Gao and Gilman, 1991; Yoshimura *et al*., 1996). Thus, the increased $G_{\beta\gamma}$ stimulatory signaling observed in LMMP tissue obtained from chronic morphine-treated animals most likely results from the elevated content of the $G_{\beta\gamma}$-stimulated AC isoforms AC IV, VII, or both.

Paradoxically, basal AC activity did not differ between LMMP tissue obtained from opioid naive versus morphine-treated animals, despite the increase in AC protein that occurred in the latter group. However, it should be noted that after agonist removal from chronic morphine-treated COS-7 cells, activity of transfected AC II, IV, or VII not only failed to manifest superactivation but actually decreased (Avidor-Reiss *et al*., 1997). This presumably also would occur as a result of the removal of morphine from chronic morphine-treated LMMP tissue during the preparation of LMMP membranes. Thus, any expected increase in basal AC activity resulting from chronic morphine-induced elevated levels of AC IV and/or VII protein could have been masked by a concomitant decrement in AC basal specific activity.

As a consequence of increased levels of AC isoforms of the type II family, a shift from predominantly opioid receptor/G_i inhibitory coupling to opioid receptor $G_{\beta\gamma}$ (G_i derived) stimulatory signaling could ensue. Such a shift would be consistent with and could underlie both the CTX-insensitive excitatory effects of previously inhibitory concentrations of sufentanil and the paradoxical increase in μ-opioid receptor coupling to G_i that has been reported in these preparations (Wang and Gintzler, 1997). This mechanism also would explain enhanced $G_{\beta\gamma}$ stimulatory AC responsiveness in the absence of any increase in the LMMP content of this G protein subunit (Fig. 4A).

In a previous study (Lane-Ladd *et al*., 1997), the effects of chronic morphine on AC isoform content was investigated in one brain region, the locus ceruleus. Chronic morphine was found to up-regulate AC I and AC VIII protein, the magnitude of which was comparable to that observed in the current study. In contrast, levels of AC III, IV, or V were not altered. These findings in combination with the current report suggest that up-regulation of AC protein by chronic morphine may be a generalizable consequence of persistent opioid receptor activation. However, the nature of the specific isoform or isoforms so affected and, consequently, the physiological significance of such changes vary across tissues and, presumably, brain regions.

A qualitative shift in opioid receptor signaling from inhibitory (mediated via $G_{i\alpha}$) to stimulatory (mediated via $G_{\beta\gamma}$) would compensate for the blunted stimulated formation of cAMP in the absence of opioid agonist that occurs in chronic morphine-treated LMMP tissue (Wang and Gintzler, 1995), as well as in COS-7 cells transfected with AC isoforms II, IV, and VII (Avidor-Reiss *et al*., 1997). This would result in the restoration of cAMP levels that is characteristic of opioid naive tissue, despite the continued presence of inhibitory concentrations of opioid (i.e., tolerance) (Wang and Gintzler, 1995).

The importance of the relative abundance of $G_{\beta\gamma}$-stimulated AC isoform or isoforms to the directionality of opioid responsiveness has been demonstrated in several cell lines. For example, dopamine activation of AC types V and VII can be inhibited and stimulated, respectively, by morphine in a dose-dependent manner (Yoshimura *et al*., 1996). The stimulatory effects of morphine on AC VII are blocked by pertussis toxin, as well as the α subunit of transducin, suggesting the importance of $G_{\beta\gamma}$ derived from G_i. Similarly, in COS-7 cells, acute opioid treatment inhibits or stimulates transfected AC activity in an isoform-specific fashion (Avidor-Reiss *et al*., 1997), the pattern of which correlates with that of $\beta\gamma$ stimulatory responsiveness.

The current demonstration of enhanced $rG_{s\alpha}$ stimulation of AC activity after chronic morphine is consonant with results obtained using A431 carcinoma cells stably transfected with μ-opioid receptors (Ammer and Schulz, 1997). This study reported increased $G_{s\alpha}$ stimulation of AC activity after chronic morphine treatment, albeit via a different mechanism than that reported here. In this case, augmented $G_{s\alpha}$ stimulation of AC activity was attributed to increased $G_{s\alpha}$ depalmitoylation. The current results do not preclude the occurrence of this mechanism in the LMMP tissue because the consequences of any chemical modification of endogenous $G_{s\alpha}$ would not be reflected by assessing stimulatory responses to exogenous $rG_{s\alpha}$, as was done in the current study.

Chronic morphine treatment does not alter the content of $G_{s\alpha}$ in LMMP tissue (Fig. 4A), as has been observed in A431 carcinoma cells (Ammer and Schulz, 1997). Thus, the previously reported (Wang and Gintzler, 1997) increased low dose ($G_{s\alpha}$ mediated) sufentanil stimulation of AC activity in the LMMP tissue after chronic morphine most likely results from increased AC stimulatory responsiveness to $G_{s\alpha}$. This could result from augmented $G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulation of AC, increased $G_{s\alpha}$ depalmitoylation, or a combination.

Heretofore, attempts to elucidate neurochemical mechanisms that underlie narcotic tolerance have focused predominantly on altered G proteins and opioid receptor content, as well as changes in their coupling. The current report points to the relevance of augmented AC $G_{\beta\gamma}$ stimulatory signaling

to mechanisms that underlie opioid tolerance. Such changes reflect that chronic morphine qualitatively as well as quantitatively alters opioid receptor-coupled signaling.

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