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Enhanced Stimulatory Adenylyl Cyclase Signaling during Opioid Dependence Is Associated with a Reduction in Palmitoylated $G_{s\alpha}$

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SUMMARY

Chronic opioid treatment of stably μ-opioid receptor transfected human mammary epidermoid A431 carcinoma cells (clone A431/μ13) results in sensitization of adenylyl cyclase (AC), a cellular adaptation associated with drug dependence. Up-regulation of AC is characterized by significantly increased levels of both basal and post-receptor-stimulated effector activities, which develop without any apparent change in the quantity of stimulatory G proteins and the maximum catalytic activity of AC. Here, we report that detergent extracts from membranes of chronically morphine-treated (10 μM; 2 days) A431/μ13 cells display higher stimulatory AC activities as assessed in the S49*cyc*$^-$ reconstitution assay. This finding is most likely due to an increased functional activity of $G_{s\alpha}$ because the addition of exogenous $G_{\beta\gamma}$ subunits, which *per se* stimulate AC in S49*cyc*$^-$ membranes, failed to affect the difference in reconstitutive AC activity. Moreover, both chemical depalmitoylation by hydroxylamine and inhibition of palmitoyl-CoA transferase *in vivo* by tunicamycin treatment increased the reconstitutive activity of detergent extracts and eliminated the differences between native and opioid-dependent cells, indicating that the increase in stimulatory activity is due to depalmitoylation of $G_{s\alpha}$. Indeed, metabolic labeling studies with [^{3}H]palmitic acid revealed that chronic opioid treatment reduces considerably the fraction of palmitoylated $G_{s\alpha}$ in the plasma membrane. Furthermore, high affinity [^{3}H]forskolin binding experiments demonstrated that depalmitoylated $G_{s\alpha}$ is able to associate directly with AC during the state of opioid dependence even without preceding receptor activation. These results suggest that post-translational palmitoylation of $G_{s\alpha}$ provides a potential regulator of transmembrane signaling. Moreover, accumulation of the depalmitoylated form of $G_{s\alpha}$ in the plasma membrane as reported herein may contribute to the increase in stimulatory AC signaling, as is characteristic for the state of opioid dependence.

Opioid dependence is characterized by an enhanced neuronal excitability toward stimulatory input (1). The underlying cellular mechanisms involve up-regulation of the cAMP second messenger system (2), which results from sensitization of AC activity (1, 3, 4). Although the role of cAMP in drug addiction is well recognized, the regulatory mechanism leading to an increase in AC activity is largely unknown.

Opioid receptors belong to the family of seven-transmembrane domain receptors that regulate their appropriate intracellular effector systems via inhibitory G proteins (4, 5). Acute activation of an opioid receptor leads to the inhibition of AC and subsequently to a reduction of intracellular cAMP levels (6). During the course of chronic opioid treatment, however, initially attenuated cAMP levels begin to recover and, in some cell systems (7–9) and brain areas (10), even exceed those originally observed in control cells. The increase in AC activity is generally referred to as "sensitization" of AC (4) and is mediated by an active counter-regulation of stimulatory receptor systems (7, 8, 11). The individual regulatory changes found comprise alterations in the quantity of stimulatory receptors (7, 8, 11) and G proteins (7) as well as an enhanced functional coupling efficiency between both entities (7, 11). However, there also were some cell systems (8) and brain areas (10) in which sensitization of AC develops without any apparent quantitative changes in stimulatory signal transduction components, suggesting the existence of additional functional mechanisms.

Stimulation of AC is mediated by the activated, GTP-bound form of $G_{s\alpha}$ (12). As a variety of other signal transduction proteins (13–15), the $G_{s\alpha}$ subunit undergoes post-translational palmitoylation near the amino terminus (16–18). Palmitoylation of $G_{s\alpha}$ is reversible and turns over rapidly after receptor activation (18–20). Thus, palmitoylation possesses the potential to regulate $G_{s\alpha}$ signaling. Indeed, palmitoylation of $G_{s\alpha}$ is required for intact receptor signaling (17) and has been implicated in the regulation of subcellular localization of the protein (21). However, despite these infor-

ABBREVIATIONS: AC, adenylyl cyclase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate.

mations, the role of $G_{s\alpha}$ palmitoylation for intracellular signaling remains unclear (13).

To investigate whether changes in $G_{s\alpha}$ palmitoylation may contribute to the enhancement of stimulatory signal transduction during the state of opioid dependence, we used human mammary epidermoid A431 carcinoma cells (22) stably transfected with the rat μ-opioid receptor cDNA (23). Chronic opioid treatment of clonal A431/μ13 cells largely enhances the capacity of stimulatory AC signaling, which develops without any apparent quantitative changes at the level of stimulatory G proteins and AC (8). Thus, A431/μ13 cells represent a useful model system for studying functional changes in stimulatory AC signaling. Here, we report that chronic opioid treatment of A431/μ13 cells enhances stimulatory AC signaling by reducing the palmitoylation state of $G_{s\alpha}$. Deacylation of $G_{s\alpha}$ was found (i) to increase intrinsic $G_{s\alpha}$ activity and (ii) to promote $G_{s\alpha}$/AC interaction. These results support the concept that changes in stimulatory transmembrane signaling contribute to the state of opioid dependence.

Experimental Procedures

Materials. [^{3}H]Forskolin (31 Ci/mmol) and [9,10-^{3}H]palmitic acid (30 Ci/mmol) were from NEN DuPont (Dreieich, Germany). ^{125}I-cAMP tracer (2000 Ci/mmol) from Amersham International (Braunschweig, Germany). Rabbit anti-cAMP antibody was purchased from BioMakor (Rehovot, Israel). Geneticin (G418) and tissue culture reagents were from GIBCO BRL (Eggenstein, Germany). CNBr-activated Sepharose 4B was from Pharmacia (Freiburg, Germany). (*R*)-(−)-Isoproterenol bitartrate and Ro 20–174 (4-[(butoxy-4-methoxyphenyl)-methyl]2-imidazolidinone) were from Research Biochemicals International (Köln, Germany). PGE_1, cAMP, ATP, GTP, hydroxylamine, and Tunicamycin (mixture of isomers A, B, C, and D; catalogue No. T-7765), as well as all standard laboratory reagents, were obtained from Sigma Chemical (Deisenhofen, Germany).

Cell culture, chronic opioid treatment, and membrane preparation. Parental human mammary epidermoid carcinoma (A431) cells were stably transfected with plasmid pRC/CMV (InVitrogen, San Diego, CA) containing the rat μ-opioid receptor cDNA (18). Clones resistant to G418 were isolated and screened for μ-opioid receptor expression by [^{3}H]diprenorphine binding (8). All experiments reported here were performed with clone A431/μ13 (B_{max} = 302.9 ± 46 fmol/mg of membrane protein; K_d = 1.3 ± 0.6 nM; six experiments). A431/μ13 cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml G418 in a humidified atmosphere of 95% air/5% CO_2 at 37°. At 50% confluency, morphine (10 μM) was added to the medium for 2 days to induce opioid dependence (8). Parallel flasks of the same passage, which were kept in the absence of morphine, served as controls. Cells were harvested after trypsination and membranes were prepared as described previously (24). Murine S49*cyc*$^-$ lymphoma cells were grown in DMEM containing 10% heat-inactivated horse serum. Membranes were prepared as described previously (25) and stored in aliquots (10 mg/ml in 5 mM Tris·HCl buffer, pH 7.4, containing 1 mM dithiothreitol and 1 mM EGTA) at −70° until use.

Determination of AC activity. Membrane-bound AC activity was determined in a reaction mixture (100 μl volume) containing 40 mM Tris·HCl, pH 7.4, 0.2 mM EGTA, 0.2 mM dithiothreitol, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATP, 5 μg/ml phosphocreatine, 5 IU/ml creatine phosphokinase, 10 μM GTP, and 30 μM Ro 20–1724. Reactions were started by the addition of 10 μg of membrane protein, incubated for 10 min at 28°, and stopped with 500 μl of 0.01 M HCl. In some cases, $[AlF_4]^-$ (30 μM) or the stable guanine nucleotide Gpp(NH)p (100 μM) was included to determine receptor-independent stimulation of AC activity. Membranes from opioid-dependent cells were measured in the presence of morphine (10 μM) to avoid spontaneous withdrawal. The amount of cAMP generated was determined by radioimmunoassay (26).

S49*cyc*$^-$ reconstitution assay. Membranes of A431/μ13 cells were extracted for 1 hr at 4° with sodium cholate (1% w/v) in NMT buffer (50 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$ and 100 mM NaCl). Insoluble material was removed by centrifugation (10,000 × *g*; 15 min). $G_{s\alpha}$-deficient S49*cyc*$^-$ membranes (10 μg/tube) were reconstituted on ice for 20 min with 10 μg of detergent-extracted proteins from A431/μ13 cell membranes. In some experiments, 50 ng of purified bovine brain $G_{\beta\gamma}$ was added to the tubes. Subsequently, $[AlF_4]^-$ (30 μM)-stimulated AC activity was determined as described previously (27). All assays were done in triplicate.

Depalmitoylation of $G_{s\alpha}$. $G_{s\alpha}$ from control or opioid-dependent A431/μ13 cells was chemically depalmitoylated in a cell-free system (28). Sodium cholate extracts (10 μg of protein/μl) were incubated for 30 min on ice in the presence of neutral hydroxylamine (1 M; pH 8.0). Controls received Tris·HCl, pH 8.0. The samples were diluted 10-fold in NMT buffer before AC activity was determined in the S49*cyc*$^-$ reconstitution assay. In a second approach, palmitoyl-$G_{s\alpha}$ was depalmitoylated *in vivo* by blocking a palmitoyl-CoA transferase activity (29). Cells were washed serum free and cultured for an additional 3 hr with DMEM containing tunicamycin (25 μg/ml) and 1% defatted bovine serum albumin. Tunicamycin treatment did not significantly affect cell viability as determined by trypan blue exclusion. Opioid-dependent cells were incubated in the presence of morphine (10 μM). Subsequently, the cells were harvested, sodium cholate (1% w/v) extracts were prepared, and reconstitutive AC activity was determined as above.

Metabolic labeling with [^{3}H]palmitic acid. Steady state levels of $G_{s\alpha}$ palmitoylation were determined by metabolic labeling with [^{3}H]palmitic acid under saturating conditions (30). A431/μ13 cells were plated onto 24-well culture dishes and grown for 2 days in the absence (control) or presence of morphine (10 μM) to induce dependence. Some wells received morphine together with the opioid antagonist naloxone (10 μM). On the day of experimentation, the cells were washed three times with prewarmed DMEH (DMEM plus 25 mM HEPES, pH 7.4) and incubated for 1 hr at 37° with DMEH containing 5% dialyzed fetal calf serum and 5 mM sodium pyruvate in the absence or presence of the drugs given chronically. Metabolic labeling was initiated by the addition of 0.5 mCi/ml [^{3}H]palmitic acid for an additional 3 hr. Incubations were stopped with 2 ml/well of ice-cold phosphate buffered saline. All subsequent steps were performed at 4°. The cells were washed three times with phosphate-buffered saline and lysed in 100 μl buffer A (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) plus 0.5% sodium dodecyl sulfate. After 1 hr, the solubilate was diluted 5-fold with buffer A and centrifuged for 10,000 × *g* for 15 min. Immunoprecipitation for 4 hr was performed with Protein A-purified anti-$G_{s\alpha}$ antibodies (11) coupled to CNBr-activated Sepharose 4B beads (10 mg/ml; 20 μl/tube). The pellets were washed three times with buffer A, boiled for 3 min in Laemmli sample buffer without a reducing agent (17) and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were soaked in ENHANCE (Amersham), dried, and fluorographed for 3–6 weeks. Incorporation of the radiolabel into $G_{s\alpha}$ was determined by videodensitometry of the films using the Herolab E.A.S.Y. system (Wiesloch, Germany).

[^{3}H]Forskolin binding studies. High affinity [^{3}H]forskolin binding to intact cells was performed essentially as described previously (31). Naive, chronically morphine-treated (10 μM; 2 days) or tunicamycin-treated (25 μg/ml; 3 hr) A431/μ13 cells were collected by trypsination, washed three times with ice-cold DMEH, pH 7.4, and equilibrated for 30 min at 4°. Binding reactions (500 μl) were performed for 60 min at 4° in the presence of 40 nM [^{3}H]forskolin and 5 × 10^6 cells/tube. Receptor-mediated stimulation of $G_{s\alpha}$/AC interaction was achieved with 10 μM isoproterenol, whereas basal binding

of $G_{s\alpha}$ to AC was measured in the absence of a stimulatory ligand. Specific binding was obtained with 10 μM forskolin. In case of opioid-dependent cells, all steps were performed in the presence of morphine (10 μM). Binding reactions were stopped by rapid filtration over Whatman GF/C filters followed by four washes with 5 ml of ice-cold 50 mM Tris·HCl buffer, pH 7.4. Incorporated radioactivity was determined by scintillation counting at 60% efficiency (LS 1801; Beckman Instruments, Columbia, MD). All reactions were done in triplicate.

Results and Discussion

Opioid dependence in A431/μ13 cells. Stimulatory AC-coupled receptor systems play an important role in the cellular mechanisms underlying opioid dependence (7, 11). Using human mammary epidermoid carcinoma A431 cells stably expressing the rat μ-opioid receptor (clone A431/μ13) as a model system, we demonstrated previously that chronic opioid-induced sensitization of AC is associated with an increased signaling activity of the endogenous β_2-adrenoceptor system (8). Up-regulation of stimulatory AC signaling in A431/μ13 cells is characterized by significantly elevated levels of both basal (3.5 ± 0.4 versus 4.9 ± 1.1 fmol of cAMP/mg of membrane protein/min) and isoproterenol (10 μM)-stimulated AC activity (41.9 ± 6 versus 55.6 ± 4 fmol of cAMP/mg of membrane protein/min; mean ± standard deviation; four or more experiments). These changes are prevented by pertussis toxin pretreatment (16 ng/ml; 2 days) and by coincubation of the cells with the opioid antagonist naloxone (10 μM; 2 days), indicating a specific opioid receptor-mediated effect. We originally attributed the increase in AC activity to the presence of an increased number of β_2-adrenoceptors because no additional changes were found for both the quantity of $G_{s\alpha}$ and the maximum catalytic activity of AC (8). This conclusion was substantiated by the finding that ICI-118,551 [(±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methyleth-yl)amino]-2-butanol hydrochloride], an inverse agonist at the β_2-adrenoceptor, largely reversed the increase in basal cAMP accumulation. However, as reported here, further experiments revealed that chronic opioid treatment also produced an increase in AC activity after direct activation of $G_{s\alpha}$ by either 30 μM $[AlF_4]^-$ or 100 μM Gpp(NH)p (Fig. 1), and these effects were not sensitive to ICI-118,551. These observations indicate the existence of an additional postreceptor mechanism involved in sensitization of AC.

To investigate the site of adaptation within the $G_{s\alpha}$/AC unit responsible for the enhancement of AC activity, we determined the dose-response relationship for Gpp(NH)p-stimulated AC. Although chronic opioid treatment results in a ~40% increase in the maximum capacity of the stable guanine-nucleotide analogue Gpp(NH)p to stimulate AC (8.2 ± 1.8 versus 13.9 ± 1.3 fmol of cAMP/mg of membrane protein/min; mean ± standard deviation; four experiments, no change in its potency is observed (ED_{50} = 5.7 versus 5.8 μM). Thus, chronic opioid-induced sensitization of AC in A431/μ13 cells seems to be mediated by an increased stimulatory activity of $G_{s\alpha}$ rather than an enhanced coupling efficiency between $G_{s\alpha}$ and AC. This finding indicates that multiple functional mechanisms may underlie the phenomenon of sensitization of AC; that is, chronic treatment of both intact animals (32) and C6–2B glioma cells (33) with tricyclic antidepressants has been shown to enhance stimulatory AC signaling by a more productive $G_{s\alpha}$/AC interaction.

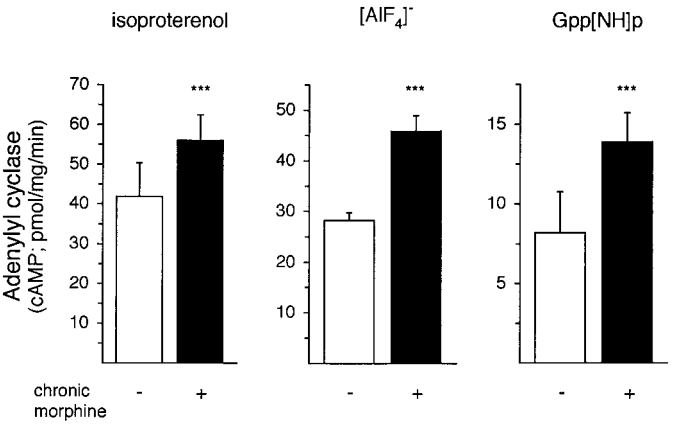

Fig. 1. Chronic opioid-induced sensitization of AC-A431/μ13 cells were chronically treated with (*filled bars*) or without (*open bars*) 10 μM morphine for 2 days. Membranes were prepared, and AC activity was determined in the presence of isoproterenol (10 μM), $[AlF_4]^-$ (30 μM), or Gpp(NH)p (100 μM). Opioid-dependent cells were measured in the presence of morphine (10 μM) to prevent spontaneous withdrawal. AC activity is expressed in pmol of cAMP formed/min/mg of membrane protein. Values are mean ± standard deviation from at least four individual experiments. ***, Significantly different from untreated controls ($p < 0.001$; Student's *t* test).

Chronic opioid treatment alters the stimulatory activity of $G_{s\alpha}$. To confirm whether the increase in post-receptor-stimulated AC activity is indeed due to an altered functional activity of $G_{s\alpha}$, the reconstitutive activity of sodium cholate (1% w/v) extracts prepared from membranes of A431/μ13 cells was determined in the S49*cyc*$^-$ assay. Measurements were done in the presence of 30 μM $[AlF_4]^-$, which constitutively activates $G_{s\alpha}$. Under these conditions, complementation of $G_{s\alpha}$-deficient S49*cyc*$^-$ membrane AC (25) with detergent extracts from opioid-dependent cells results in ~2-fold higher effector activities compared with control cell extracts (Fig. 2). Western blotting of the deter-

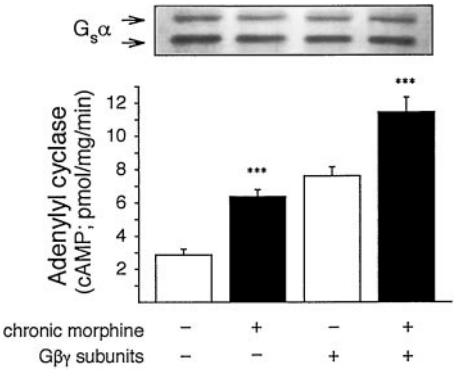

Fig. 2. Chronic opioid treatment of A431/μ13 cells increases intrinsic $G_{s\alpha}$ activity. Sodium cholate (1% w/v) extracts were prepared from control or chronically morphine-treated (10 μM; 2 days) A431/μ13 cells. Reconstitution of S49*cyc*$^-$ membranes with detergent extracts was performed in the absence (−) or presence (+) of purified bovine brain $G_{\beta\gamma}$ subunits (50 ng/tube). Reconstitutive AC activity was determined after activation of $G_{s\alpha}$ with 30 μM $[AlF_4]^-$. AC activity is expressed in pmol of cAMP generated/min/mg of detergent extract. Values are mean ± standard error from six ($-G_{\beta\gamma}$) or three ($+G\beta\gamma$) experiments. ***, Significantly different compared with control cell extract ($p < 0.001$, Student's *t* test). *Inset*, representative immunoblot of detergent extracts used for reconstitution. Staining was performed with a carboxyl-terminal anti-$G_{s\alpha}$ antibody as described previously (9).

gent extracts was used to verify that identical amounts of $G_{s\alpha}$ were present. Because S49*cyc*$^-$ cells contain an AC isoform that is sensitive to stimulation by $G_{\beta\gamma}$ subunits (34), we had to exclude the possibility that an altered $G_{\beta\gamma}$ content of the sodium cholate extracts could mediate the increase in reconstitutive AC activity. For this, we added a maximal effective amount of purified bovine brain $G_{\beta\gamma}$ (50 ng) to detergent extracts from both control and opioid-dependent A431/μ13 cells and determined the effect on reconstitutive AC activity. As expected, the addition of $G\beta\gamma$ resulted in a ~2-fold increase in reconstitutive AC activity regardless of whether extracts from control or chronically morphine-treated cells were measured (Fig. 2), indicating that the difference in reconstitutive AC activities observed for sodium cholate extracts from naive and opioid-dependent A431/μ13 cells is not due to an altered $G_{\beta\gamma}$ content. Although we cannot rule out entirely any other additional factors present in the detergent extracts, such as inhibitory G protein α subunits, these results suggest that the increased reconstitutive activity of detergent extracts from opioid-dependent cells is due to an increased functional activity of $G_{s\alpha}$. Thus, besides an increase in β_2-adrenoceptor levels, sensitization of AC in A431/μ13 cells is likely to involve an additional regulatory mechanism at the level of $G_{s\alpha}$.

Palmitoylation attenuates the stimulatory activity of $G_{s\alpha}$. The $G_{s\alpha}$ subunit is subject to post-translational palmitoylation (16–18), a covalent lipid modification that has been shown to regulate the function of a series of membrane proteins participating in signal transduction, such as G protein-coupled receptors (15), G protein α subunits (13, 17, 18), effector molecules (14), and tyrosine protein kinases (35). Palmitoylation is reversible due to the lability of the thioester bond (13) and thus provides a potential mechanism that could regulate the activity of $G_{s\alpha}$. In a first step to investigate whether alterations in $G_{s\alpha}$ palmitoylation may account for the increase in the stimulatory activity observed for $G_{s\alpha}$ from opioid-dependent cells, two functional approaches were used to modulate the palmitoylation state of $G_{s\alpha}$: (i) chemical depalmitoylation *in vitro* by hydroxylamine treatment (28), and (ii) inhibition of palmitoyl-CoA transferase activity *in vivo* by tunicamycin (29). Because neither approach involves specific modulation of $G_{s\alpha}$ palmitoylation (tunicamycin also inhibits *N*-linked glycosylation; hydroxylamine cleaves every thioester bond) and would also affect the functional properties of other signal transduction components, such as receptors, inhibitory G protein α subunits, and AC (14, 15, 18), specific effects of these treatments on the activity of $G_{s\alpha}$ were determined in the S49*cyc*$^-$ reconstitution assay after solubilization and persistent activation of $G_{s\alpha}$ by $[AlF_4]^-$. Neutral hydroxylamine has been used frequently to remove the palmitate residue from $G_{s\alpha}$ by cleaving the thioester bond (14, 16–18). Depalmitoylation of detergent-solubilized $G_{s\alpha}$ from control cells with hydroxylamine (1 M; 30 min; 4°) was found to considerably enhance its reconstitutive AC activity by ~3.5-fold. Although native $G_{s\alpha}$ from opioid-dependent cells *per se* exhibits a ~2-fold higher reconstitutive activity compared with control cell $G_{s\alpha}$, depalmitoylation by hydroxylamine treatment further increased its stimulatory activity, reaching values almost identical to those obtained for depalmitoylated control cell $G_{s\alpha}$ (Fig. 3). Similar results were obtained after depalmitoylation *in vivo* by tunicamycin treatment (25 μg/ml; 3 hr). Again, depalmitoylation was found to largely increase the stimulatory activity of $G_{s\alpha}$. In addition, the difference in the stimulatory activity of $G_{s\alpha}$ between control and opioid-dependent cells disappears after depalmitoylation (Fig. 3). These data not only confirm that depalmitoylated $G_{s\alpha}$ is active *in vitro* (13) but also demonstrate that palmitoylation of $G_{s\alpha}$ attenuates its ability to activate AC. This finding is somewhat unexpected because a previous study showed that removal of the palmitoylation site by mutagenesis reduced the stimulatory activity of a constitutively activated form of $G_{s\alpha}$ (17). However, the same laboratory also reported that a depalmitoylated form of $G_{z\alpha}$, the G protein α subunit mediating pertussis toxin-insensitive inhibition of AC, possesses an increased functional capacity to inhibit AC (30). Although it is not possible currently to determine the actual intrinsic activities of palmitoylated and deacylated $G_{s\alpha}$ because of the inability to provide stably palmitoylated $G_{s\alpha}$, our results indicate that post-translational palmitoylation seems to affect the stimulatory activity of $G_{s\alpha}$.

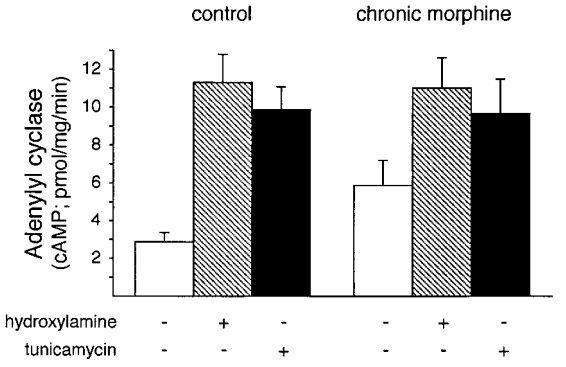

Fig. 3. Depalmitoylation increases the intrinsic activity of $G_{s\alpha}$. Depalmitoylation of $G_{s\alpha}$ was performed either *in vitro* by treatment of detergent extracts with neutral hydroxylamine (*hatched bars*) or *in vivo* by exposure of the cells to tunicamycin before membrane preparation and solubilization with 1% (w/v) sodium cholate (*filled bars*). Intrinsic activity of depalmitoylated $G_{s\alpha}$ was determined by the S49 *cyc*$^-$ reconstitution assay in the presence of 30 μM $[AlF_4]^-$. Native detergent extracts from control and opioid-dependent cells served as controls (*open bars*). Values are mean ± standard error from three independent experiments.

Chronic opioid treatment reduces the palmitoylation state of $G_{s\alpha}$. Palmitoylated $G_{s\alpha}$ is located exclusively in the plasma membrane, whereas depalmitoylated $G_{s\alpha}$ is found in both the membrane and cytosol (18–20). Based on the observation that chronic opioid treatment does not affect the abundance of membrane-bound $G_{s\alpha}$ (8), the finding of an increased stimulatory activity of $G_{s\alpha}$ may suggest that opioid dependence is associated with a reduced fraction of palmitoylated $G_{s\alpha}$ in the plasma membrane. To test this prediction, we performed metabolic labeling studies with [^{3}H]palmitic acid. Because palmitoylation of $G_{s\alpha}$ is dynamic (18, 20), we first investigated the time course of incorporation of [^{3}H]palmitate over 15 min to 4 hr to determine the time required to reach equilibrium labeling conditions. As shown in Fig. 4A, the greatest incorporation of the radiolabel was achieved within 2 hr of exposure to [^{3}H]palmitic acid. Thus, all subsequent experiments were performed for 3 hr to ensure reliable examination of the steady state levels of $G_{s\alpha}$ palmitoylation.

Radiolabeling of control cells resulted in strong incorpora-

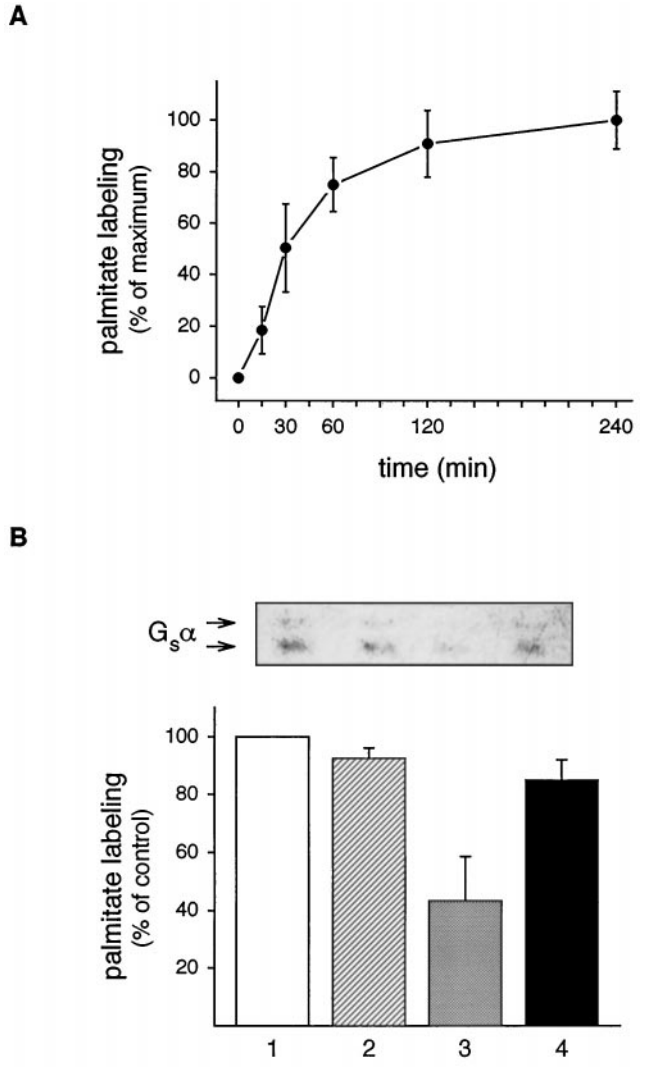

Fig. 4. Regulation of steady state palmitoylation of $G_{s\alpha}$ by chronic opioid treatment. A, Time course of [^{3}H]palmitate incorporation into $G_{s\alpha}$ from naive A431/μ13 cells. Cells were incubated with 0.5 mCi/ml [^{3}H]palmitate for 15 min to 4 hr before cells were lysed, and $G_{s\alpha}$ was immunoprecipitated using a carboxyl-terminal anti-$G_{s\alpha}$ antibody. Immunocomplexes were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was fluorographed for 3 weeks. The relative intensity of the respective $G_{s\alpha}$ bands was determined by videodensitometry using the Herolab E.A.S.Y. system and is expressed in percent of maximum labeling, which was set at 100%. Values are mean ± variation from one experiment performed in duplicate. B, Effect of acute and chronic opioid treatment on steady state $G_{s\alpha}$ palmitoylation. Naive or chronically morphine pretreated cells were metabolically labeled with 0.5 mCi/ml [^{3}H]palmitic acid for 3 hr. After radiolabeling, $G_{s\alpha}$ was immunoprecipitated and fluorographed as above. *Lane 1*, naive cells. *Lane 2*, acute morphine (10 μM; 30 min). *Lane 3*, chronic morphine pretreatment (10 μM; 2 days). *Lane 4*, chronic morphine and naloxone pretreatment (10 μM each; 2 days). Values are mean ± standard deviation from one representative experiment performed in triplicate. Similar results were obtained in three separate experiments.

tion of [^{3}H]palmitate into both the 45- and 48-kDa isoforms of $G_{s\alpha}$ present in A431/μ13 cells. After chronic opioid treatment, however, the steady state levels of $G_{s\alpha}$ palmitoylation were found to be largely reduced (Fig. 4B). Coincubation of the cells with naloxone (10 μM; 2 days), which blocks the development of dependence (8), prevented the decrease in $G_{s\alpha}$ palmitoylation. Treatment of the cells with naloxone alone (10 μM) had no effect (not shown). These findings indicate that the reduction in $G_{s\alpha}$ palmitoylation represents a specific μ-opioid receptor-mediated effect. Because depalmitoylation of $G_{s\alpha}$ after activation of a stimulatory receptor occurs within minutes (20), we further investigated whether acute activation of an inhibitory opioid receptor would also produce this effect. However, the addition of an acute dose of morphine (10 μM) during the last 30 min of the metabolic labeling period had no effect on the palmitoylation statues of $G_{s\alpha}$ (Fig. 4B). In addition, short term activation of the μ-opioid receptor failed to affect the reconstitutive activity of sodium cholate-extracted $G_{s\alpha}$ in the S49*cyc*$^-$ assay (2.9 ± 0.6 versus 3.4 ± 0.5 pmol of cAMP/min/mg of sodium cholate extract, mean ± standard deviation; three experiments). These results indicate that long term activation of μ-opioid receptors in A431/μ13 cells is required to reduce the overall palmitoylation state of $G_{s\alpha}$. Moreover, the decrease in $G_{s\alpha}$ palmitoylation during the state of opioid dependence and the finding that depalmitoylated $G_{s\alpha}$ displays enhanced stimulatory AC activity may suggest that sensitization of AC is mediated by an increased fraction of depalmitoylated $G_{s\alpha}$ in the plasma membrane of opioid-dependent cells.

The effects of tunicamycin and hydroxylamine treatment on the palmitoylation status of $G_{s\alpha}$ were also investigated in metabolic labeling studies with [^{3}H]palmitic acid. Inhibition of palmitoyl-CoA transferase activity by tunicamycin treatment (25 μg/ml; 3 hr) during metabolic labeling completely prevented incorporation of the radiolabel into $G_{s\alpha}$. The ability of hydroxylamine treatment to remove palmitate from $G_{s\alpha}$ was tested in membranes from prelabeled cells. Exposure of [^{3}H]palmitoylated $G_{s\alpha}$ for 30 min to 1 M neutral hydroxylamine completely removed the radiolabel from $G_{s\alpha}$ (Fig. 5). These results show that both tunicamycin and hydroxylamine treatments are useful tools with which to regulate the palmitoylation status of $G_{s\alpha}$.

The finding that chronic activation of μ-opioid receptors reduces the level of $G_{s\alpha}$ palmitoylation raises the question whether this effect is specific for $G_{s\alpha}$ or reflects a more general effect of chronic opioid treatment on the overall palmitoylation of membrane proteins. In an attempt to clarify this issue, we investigated the effects of acute and chronic opioid treatment on the palmitoylation state of inhibitory G protein α subunits, which couple directly to the μ-opioid receptor. This

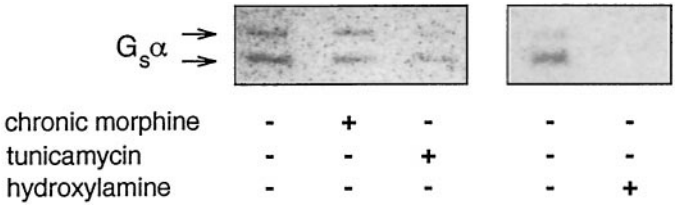

Fig. 5. Effect of tunicamycin and hydroxylamine treatment on $G_{s\alpha}$ palmitoylation. Inhibition of $G_{s\alpha}$ palmitoylation by tunicamycin was investigated by coincubation of A431/μ13 cells for 3 hr with 25 μg/ml tunicamycin and 0.5 mCi/ml [^{3}H]palmitic acid. Cells were lysed and incorporation of the radiolabel into $G_{s\alpha}$ was analyzed as in Fig. 4. Sensitivity of [^{3}H]palmitoyl-$G_{s\alpha}$ to hydroxylamine treatment was analyzed in membranes from A431/μ13 cells that were prelabeled with [^{3}H]palmitic acid. Membrane aliquots were incubated for 30 min at 4° either in the presence of 1 M NH_2OH, pH 8.0, or 1 M Tris·HCl, pH 8.0 (control). Membranes were washed and solubilized, $G_{s\alpha}$ was immunoprecipitated, and fluorography was performed as described. Films were exposed for 3 weeks at −70°.

was of particular interest because palmitoylation of inhibitory G protein α subunits is also reversible (18). However, most probably due to the relative low abundance of G_i proteins in this cell system, we were not able to obtain reliable information about this issue so far. One possible explanation for the reduction in overall $G_{s\alpha}$ palmitoylation during the state of opioid dependence would be the fact that chronic opioid treatment increases the functional activity of stimulatory receptor systems (7, 11). Because the turnover of $G_{s\alpha}$ palmitoylation is accelerated after activation of a stimulatory receptor (18, 19), an enhanced stimulatory receptor activity could result in the reduction of $G_{s\alpha}$ palmitoylation, as observed during the state of opioid dependence.

Depalmitoylation promotes $G_{s\alpha}$/AC interaction. The regulatory cycle of acylation and deacylation of $G_{s\alpha}$ is well established and closely linked to the activation state of the G protein. On activation, the GTP-bound form of $G_{s\alpha}$ dissociates from $G_{\beta\gamma}$ and becomes rapidly depalmitoylated (20). After hydrolysis of GTP, the depalmitoylated and GDP-bound form of $G_{s\alpha}$ may either reassociate with $G_{\beta\gamma}$ in the plasma membrane and become rapidly repalmitoylated (36) or redistribute into the cytosol (17, 21). On this basis, it could be anticipated that the reduction in $G_{s\alpha}$ palmitoylation observed after chronic morphine treatment would result in a loss of $G_{s\alpha}$ from the plasma membrane. However, in a previous study (8), we failed to detect any change in the abundance of membrane-bound $G_{s\alpha}$, indicating that depalmitoylated $G_{s\alpha}$ in opioid-dependent A431/μ13 cells does not redistribute into the cytosol but instead redistributes laterally in the plasma membrane. Because depalmitoylated $G_{s\alpha}$ displays higher stimulatory activity than palmitoyl-$G_{s\alpha}$ and chronic morphine treatment increases basal cAMP accumulation in A431/μ13 cells, we investigated whether depalmitoylated $G_{s\alpha}$ might bind directly to AC. For this, we performed high affinity [^{3}H]forskolin binding studies, which provide a measure for the number of complexes formed between $G_{s\alpha}$ and AC (31). In untreated A431/μ13 cells, specific binding of [^{3}H]forskolin is detectable only after activation of β_2-adrenoceptors by isoproterenol. In contrast, in chronically opioid treated cells, there is substantial [^{3}H]forskolin binding in the absence of any stimulatory ligand, whereas the maximum number of β_2-adrenoceptor-stimulated $G_{s\alpha}$/AC complexes remains unchanged. Depalmitoylation of intracellular $G_{s\alpha}$ in control cells by tunicamycin treatment (25 μg/ml; 3 hr) mimics the increase in basal [^{3}H]forskolin binding (Fig. 6). The lack of β_2-adrenoceptor-stimulated high affinity [^{3}H]forskolin binding after tunicamycin treatment may reflect receptor depalmitoylation, which has been reported recently to attenuate receptor signaling (15). However, these results also demonstrate that depalmitoylated $G_{s\alpha}$, which accumulates in the plasma membrane during the state of opioid dependence or after tunicamycin treatment, is able to associate directly with AC, even in the absence of preceding receptor activation. This observation is the first example of the regulation of G protein activity by modulation of its palmitoylation state. The most plausible explanation for this altered protein/protein interaction of depalmitoylated and presumably GDP-bound $G_{s\alpha}$ would be a change in its affinity for $G_{\beta\gamma}$ subunits and/or AC. Indeed, depalmitoylated $G_{s\alpha}$ has been shown recently to possess ~5-fold lower affinity for $G_{\beta\gamma}$ subunits than palmitoyl-$G_{s\alpha}$ (37). However, inactivated and depalmitoylated $G_{s\alpha}$ is still able to associate with $G_{\beta\gamma}$ subunits in the plasma membrane, a critical step in the forward reaction of the palmitoylation cycle that enhances susceptibility of $G_{s\alpha}$ for repalmitoylation by membrane-bound palmitoyl-CoA transferases (36, 37). Binding of depalmitoylated $G_{s\alpha}$ to other membrane proteins, such as AC, could be affected by limiting the availability of free $G_{\beta\gamma}$ subunits. Such a mechanism seems likely because $G_{\beta\gamma}$ subunits have been shown to contribute to sensitization of AC by an unidentified indirect mechanism (38). Alternatively, because signal transduction molecules are organized in functional compartments within the plasma membrane (39), depalmitoylation of $G_{s\alpha}$ could simply increase its mobility and allow access to additional AC molecules (13).

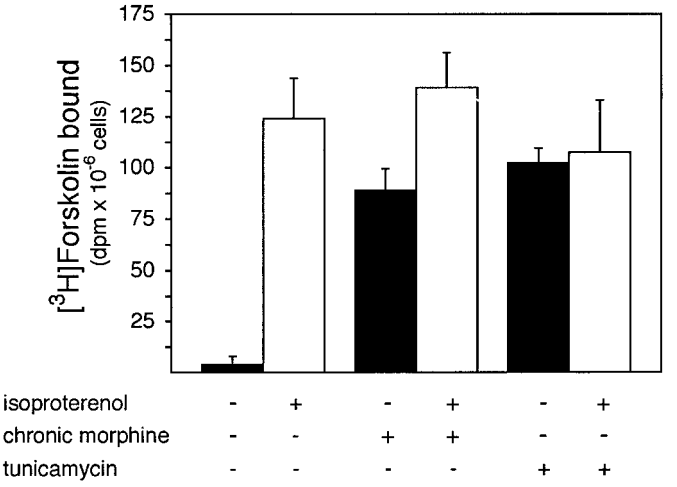

Fig. 6. Chronic opioid and tunicamycin treatment promotes association of $G_{s\alpha}$ with AC. Formation of $G_{s\alpha}$/AC complexes was determined by high affinity [^{3}H]forskolin binding. Naive, chronically morphine-treated (10 μM; 2 days) or tunicamycin-treated (25 μg/ml; 3 hr) A431/μ13 cells were analyzed in the absence (*filled bars*) or presence of the β-adrenoceptor agonist isoproterenol (10 μM; *open bars*). Data are given in dpm of specific [^{3}H]forskolin binding/10^6 cells. Values are mean ± standard error from four experiments.

By analyzing an altered stimulatory signal transduction during the state of opioid dependence, we revealed some functional consequences of palmitoylation on the signaling activity of $G_{s\alpha}$. Chronic opioid-induced depalmitoylation of $G_{s\alpha}$ has been shown (i) to increase its stimulatory activity and (ii) to promote direct binding to AC without preceding receptor activation. Both regulatory changes are suggested to contribute to the phenomenon of sensitization of AC. Although the increase in basal as well as post-receptor-stimulated AC activity may be mediated by the increased stimulatory activity of depalmitoylated $G_{s\alpha}$, preformation of $G_{s\alpha}$/AC complexes could be responsible for the enhanced neuronal sensitivity toward stimulatory input observed during the state of opioid dependence.

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