Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: A novel mechanism for modulating cell signaling

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Traditional mechanisms thought to underlie opioid tolerance include receptor phosphorylation/down-regulation, G-protein uncoupling, and adenylyl cyclase superactivation. A parallel line of investigation also indicates that opioid tolerance development results from a switch from predominantly opioid receptor $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory signaling. As described previously, this results, in part, from the increased relative abundance of $G_{\beta\gamma}$-stimulated adenylyl cyclase isoforms as well as from a profound increase in their phosphorylation [Chakrabarti, S., Rivera, M., Yan, S.-Z., Tang, W.-J. & Gintzler, A. R. (1998) *Mol. Pharmacol.* 54, 655–662; Chakrabarti, S., Wang, L., Tang, W.-J. & Gintzler, A. R. (1998) *Mol. Pharmacol.* 54, 949–953]. The present study demonstrates that chronic morphine administration results in the concomitant phosphorylation of three key signaling proteins, G protein receptor kinase (GRK) 2/3, β-arrestin, and G_{β}, in the guinea pig longitudinal muscle myenteric plexus tissue. Augmented phosphorylation of all three proteins is evident in immunoprecipitate obtained by using either anti-GRK2/3 or G_{β} antibodies, but the phosphorylation increment is greater in immunoprecipitate obtained with G_{β} antibodies. Analyses of coimmunoprecipitated proteins indicate that phosphorylation of GRK2/3, β-arrestin, and G_{β} has varying consequences on their ability to associate. As a result, increased availability of and signaling via $G_{\beta\gamma}$ could occur without compromising the membrane content (and presumably activity) of GRK2/3. Induction of the concomitant phosphorylation of multiple proteins in a multimolecular complex with attendant modulation of their association represents a novel mechanism for increasing $G_{\beta\gamma}$ signaling and opioid tolerance formation.

tolerance | dependence | opioid | phosphorylation | protein kinases A and C

Responsiveness to opioids includes initial perturbations that are followed by the reestablishment of "normal" physiological equilibrium despite its continued presence. Despite the delineation of many of the physiological, biochemical, and molecular biological sequela of persistent exposure to opioids, elucidation of the cellular events that are causally associated with the reestablishment of homeostasis following chronic morphine remains incomplete. There is now growing evidence that although traditional explanations for opioid tolerance (e.g., receptor down-regulation, desensitization, G-protein uncoupling) do contribute, these mechanisms are not the exclusive underpinnings of opioid tolerance (see ref. 1 for review).

Opioid receptors are predominantly coupled to adenylyl cyclase (AC) via the $G_{i\alpha}$ subunit of G proteins. Previous reports, however, have underscored the relevance of enhanced opioid receptor signaling via $G_{\beta\gamma}$ to the biochemical underpinnings of opioid tolerance. In the ileum longitudinal muscle myenteric plexus preparation (LMMP), chronic *in vivo* morphine treatment results in augmented levels of mRNA encoding AC IV (2) and VII (S.C., L. Wang, and A.R.G., unpublished observations), both of which are stimulated by $G_{\beta\gamma}$ (3). In the LMMP tissue, persistent *in vivo* activation of opioid receptors also induces a profound increase in AC (type II family) phosphorylation (4) that can significantly increase their stimulatory responsiveness to $G_{\beta\gamma}$ (as well as $G_{s\alpha}$) (5–7). These changes contribute to enhanced $G_{\beta\gamma}$ stimulatory AC signaling that occurs following chronic morphine treatment (8) and thus underlie the altered opioid regulation of transmitter release (9) and cAMP formation (10, 11) in these preparations.

In addition to AC, $G_{\beta\gamma}$ also interacts with other cell-signaling proteins, one group of which is G protein-coupled receptor kinase (GRK) 2/3 (12). These are cytosolic serine/threonine kinases that participate in homologous G protein-coupled receptor (GPCR) desensitization via their phosphorylation upon agonist binding. Translocation, membrane attachment, and subsequent activation of GRK2/3 require the $G_{\beta\gamma}$ subunit of heterotrimeric G protein (13). Thus, the formation of GRK/$G_{\beta\gamma}$ complexes is critical to desensitization of cellular signaling mediated via GPCRs (14).

The formation of GRK/$G_{\beta\gamma}$ complexes also has significant functional implications regarding the increased role of $G_{\beta\gamma}$ signaling in opioid tolerance. The carboxyl third of GRK2/3, the region that contains the $G_{\beta\gamma}$ binding sequences, can inhibit $G_{\beta\gamma}$ stimulation of AC II (15), and presumably the other ACs of the type II family (AC IV and VII) as well as other effectors known to be regulated by $G_{\beta\gamma}$ (16). Thus, changes in the dynamics of GRK/$G_{\beta\gamma}$ interactions could be critical to increased chronic morphine-induced opioid receptor-coupled $G_{\beta\gamma}$ stimulatory AC signaling (4, 8) as well as changes in other $G_{\beta\gamma}$-sensitive signaling cascades (e.g., phospholipid metabolism, mitogen-activated protein kinase) that have been associated with opioid tolerance/dependence (17).

We find that chronic morphine augments the concomitant phosphorylation of GRK2/3, β-arrestin, and G_{β}. These proteins are coimmunoprecipitated by using two distinct, highly selective antisera. Phosphorylation has opposing consequences on their association; as a result, availability of $G_{\beta\gamma}$ is increased without compromising the membrane content of GRK2/3. Opioid-induced concomitant phosphorylation of several proteins in a multimolecular complex with attendant modulation of their association represents a novel mechanism for increasing $G_{\beta\gamma}$ signaling and opioid tolerance formation.

Abbreviations: AC, adenylyl cyclase; GRK, G protein receptor kinase; LMMP, longitudinal muscle myenteric plexus; GPCR, G protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C.

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Materials and Methods

Materials. Morphine pellets were supplied by the National Institute on Drug Abuse. ^{32}P-labeled inorganic phosphorus (^{32}Pi) was obtained from New England Nuclear. All protein kinase and phosphatase inhibitors were purchased from Alexis (San Diego, CA). All protease inhibitors were purchased from Sigma, except for complete protease inhibitor mixture, which was purchased from Roche Molecular Biochemicals. Anti-β-arrestin2 antibodies, recombinant GRK2 protein, and transformed *Escherichia coli* containing the expression plasmid pGEX/β-arrestin-CT were generously supplied by R. J. Lefkowitz (Duke University, Durham, NC). Anti-G_β antibodies and purified $G_{\beta\gamma}$ dimer were generously supplied by J. Hildebrandt (Medical University of South Carolina, Charleston, SC). Anti-μ-opioid receptor antibody was generously provided by Robert Elde (University of Minnesota, Minneapolis, MN).

LMMP Preparation and Incubation with ^{32}Pi. Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Morphine tolerance/dependence was induced by s.c. implantation of five morphine pellets (each containing 75 mg morphine base) into male albino guinea pigs (375–450 g) under light anesthesia. On the 6th day following 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 (LMMP) were removed and equilibrated in the same buffer with continuous gassing (95% O_2, 5% CO_2) at 35°C for 30 min (11). For tolerant/dependent LMMP tissue, morphine (100 nM) was included in the buffer. LMMP strips were then incubated with 1 mCi/ml (1 Ci = 37 GBq) ^{32}Pi in phosphate-free Krebs (2 h at 35°C) with continuous gassing. To determine the effect of acute opioid treatment on protein phosphorylation, opioid naïve LMMP strips were incubated with morphine (100 nM) during the last 5 min of ^{32}Pi incubation. The kinase(s) required for chronic morphine-induced augmented phosphorylation was inferred from the effect of inhibitors on the incorporation of ^{32}P into protein in LMMP tissue obtained from morphine-treated animals. LMMP tissue from the same ileum was randomly divided into two samples, one of which contained a kinase inhibitor that was able to discriminate between protein kinase C (PKC) or protein kinase A (PKA). Inhibitors were added during the last 30 min of the 2-h ^{32}P labeling period. Chelerythrine and calphostin C were used as PKC-selective inhibitors; HA-1004 and H-89 were used as PKA-selective inhibitors. Inhibitor concentrations were selected to maximize discrimination between PKC and PKA as well as inhibition of the targeted kinase.

Following incubation with ^{32}Pi, 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 (100 μg/liter bacitracin; 20 μg/liter each of chloromethyl ketone, N^α-*p*-tosyl-L-lysine chloromethyl ketone, and PMSF; 3.2 μg/liter each of leupeptin and soybean trypsin inhibitor; 0.5 μg/liter aprotinin, 1 mM benzamidine and complete inhibitor mixture, one tablet/50 ml)/protein phosphatase inhibitors (25 nM calyculin A, 1 μM okadaic acid, 100 μM sodium orthovanadate). Supernatant obtained from a low-speed centrifugation (1,000 $\times$ *g*, 4°C, 10 min) was subjected to a high-speed centrifugation (30,000 $\times$ *g*, 4°C, 30 min). Membranes obtained were resuspended in 50 mM Tris buffer containing 1 mM DTT, 10 mM sodium pyrophosphate and the above-mentioned protease and phosphatase inhibitors.

Immunoprecipitation of GRK2/3, β-Arrestin, and G_β Protein. Immunoprecipitation of specific proteins used a mouse monoclonal antibody generated against rat GRK2/3 (last 221 C-terminal residues) (18) or polyclonal antibodies generated against a glutathione *S*-transferase fusion protein encoding the last 75 residues (336–411) of the carboxyl terminus of rat β-arrestin2 (19) or the C-terminal last 11 aa residues of G_β (20). Membranes were solubilized in buffer containing phosphatase and protease inhibitors, as described above, containing 1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS/5% glycerol/150 mM NaCl (60 min on ice), vortexed three times, and centrifuged (14,000 $\times$ *g* for 30 min). Protein amounts were determined by the Bradford method (21) by using BSA as a standard. Solubilized membrane protein (1.4 mg) from each sample was incubated with either 24 μg of anti-GRK2/3 antibodies or a 1:200 dilution of anti-β-arrestin or G_β antibodies (each for 60 min at 4°C). Immunoprecipitation and SDS/PAGE (10%) were performed as previously described (4). A prestained protein ladder (10–200 kDa, Life Technologies, Grand Island, NY) was included in each electrophoresis to control for variation in running time and/or subtle differences in gel composition, running temperature, etc. among experiments. Molecular mass of the proteins being monitored, calculated by extrapolation from the mobility of molecular mass standards that were included with each run, were very comparable among all experiments. Gels were dried and exposed to PhosphorImager screens that were scanned in a PhosphorImager Storm 860 (Molecular Dynamics). Amounts of ^{32}P incorporated into the phosphorylated protein bands were determined by using densitometric analysis (Imagequant, Molecular Dynamics).

Gel Electrophoresis and Western Blot Analysis. Standard procedures were used for Western analyses as used previously by this laboratory (8). Selected lanes from polyacrylamide gels were incubated (room temperature for 2 h) with either a mouse monoclonal antibody generated against GRK2/3 (C5/1, 4 μg/ml) or polyclonal antibodies generated against G_β or β-arrestin/preadsorbed anti-β-arrestin antisera (each dissolved in blocking solution, 1:1,000). The secondary antibody used was either a peroxidase-labeled anti-mouse (for GRK2/3) or anti-rabbit (for β-arrestin) antibody. Antibody–substrate complex was visualized by using an enhanced chemiluminescence detection kit (Amersham Pharmacia). Sample pairs, obtained from opioid naïve and chronic morphine-treated LMMP tissues, were processed, electrophoresed, and blotted in parallel, after which they were exposed concomitantly to the same Kodak X-Omat film (New England Nuclear). Signal intensity was quantified by using National Institutes of Health imaging software.

Preparation of Preadsorbed Anti-β-Arrestin Antibodies. Transformed *E. coli* containing the expression plasmid pGEX/β-arrestin-CT was grown in a large scale in LB/ampicillin media. Following induction of the cultures with 1 mM isopropyl-1-thio-β-D-galactopyranoside (37°C, 3 h), cells were harvested by centrifugation (5,000 $\times$ *g* for 10 min). Cells were lysed in PBS containing protease inhibitors (0.1 mM PMSF/0.31 mg/ml benzamidine/4 μg/ml leupeptin/5 μg/ml aprotinin/1 μg/ml pepstatin A) by repeated freeze-thawing, which was followed by sonication and centrifugation (10,000 $\times$ *g* for 10 min; 4°C). glutathione *S*-transferase–β arrestin2–CT fusion protein from the supernatant was immobilized on glutathione-Sepharose (Amersham Pharmacia), on which diluted (1:1) anti-β-arrestin antibodies were adsorbed. The void volume was collected and concentrated such that the dilution of preadsorbed and nonadsorbed antiserum was the same.

Purification of $G_{\beta\gamma}$ Subunits and *in Vitro* Phosphorylation of Purified $G_{\beta\gamma}$. $G_{\beta\gamma}$ subunits were purified from bovine brain by a modification of procedures originally described for the isolation and purification of G protein after heterotrimer activation (22). Briefly, G protein was extracted from bovine brain membranes with 1% sodium cholate and separated from other proteins by DEAE chromatography, followed by AcA34/Octyl-agarose chromatography and Mono-Q FPLC.

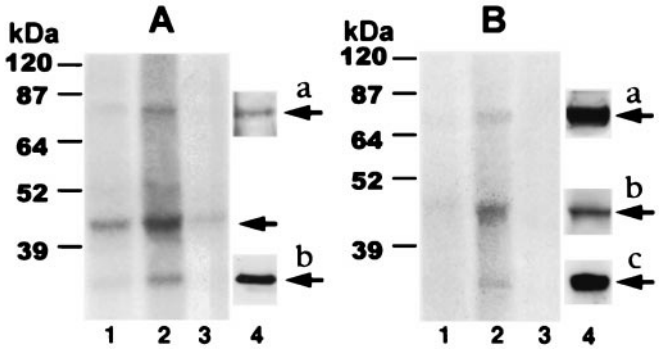

Fig. 1. Effect of chronic morphine on phosphorylation of proteins immunoprecipitated using anti-G_β (*A*) or anti-GRK2/3 (*B*) antibodies. LMMP tissue obtained from opioid naïve (lane 1) and chronic morphine-treated (lane 2) guinea pigs was incubated with ^{32}P for 2 h. Membranes were prepared, solubilized, and immunoprecipitated using antibodies selective for either G_β (*A*) or GRK2/3 (*B*). In lane 3 of both panels, preadsorbed antiserum was used for immunoprecipitation of LMMP tissue obtained from chronic morphine-treated animals. Immunoprecipitates were subjected to SDS/PAGE, and radiolabeled proteins were visualized by their concomitant autoradiography (18-h exposure) using storage phosphorimaging techniques. Quantitative densitometric analysis was used to assess magnitude of ^{32}P incorporation. Lane 4 in *A* shows Western analysis of GRK protein in G_β immunoprecipitate (a) or purified G_β protein (b). Lane 4a in *B* illustrates Western analysis of purified recombinant GRK2 protein. The presence of β-arrestin and G_β proteins in GRK immunoprecipitate is illustrated in lanes 4b and 4c, respectively. Immunoblots were obtained as described in *Materials and Methods*. Figure is representative of four experiments. Molecular mass was assessed using a prestained protein ladder (10–200 kDa; Life Technologies) that was included in each run. Chemical identity of radiolabeled proteins was based on calculated molecular mass of autoradiographic signal, loss of signal following antibody preadsorption, and comparison of calculated molecular mass of signal obtained with autoradiographic vs. Western analysis. The apparent variability in the relative mobility of monitored protein in *A* vs. *B* results from variability in running time and other subtle aspects of SDS/PAGE among experiments. Protein molecular masses, calculated by extrapolation from the mobility of molecular mass standards, were very comparable among all experiments.

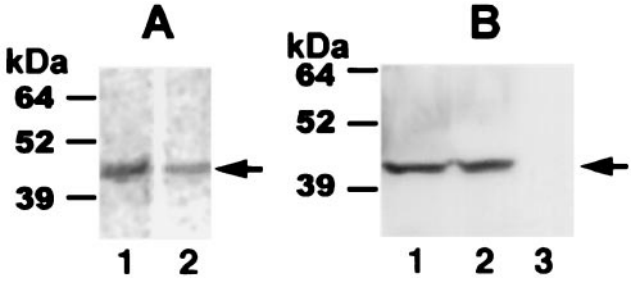

Fig. 2. Effect of chronic morphine on the phosphorylation of noncomplexed β-arrestin. (*A*) Autoradiographic analysis of ^{32}P incorporation into LMMP tissue obtained from opioid naïve (lane 1) and chronic morphine-treated (lane 2) guinea pigs was processed and analyzed as described for Fig. 1 except that anti-β-arrestin antibodies were used for immunoprecipitation. Panel is representative of four experiments. (*B*) Western blot analysis of LMMP membranes obtained from opioid naïve (lane 1) and chronic morphine-treated guinea pigs (lane 2) using anti-β-arrestin antibodies. Lane 3 represents Western analysis of LMMP membranes obtained from chronic morphine-treated tissue using preadsorbed anti-β-arrestin antibodies. Panel is representative of four experiments. All procedures were performed as described in *Materials and Methods*. The β-arrestin antibody used was generated against the 75-aa C-terminal domain of β-arrestin2 ($\approx$80% homologous to the corresponding region of β-arrestin1). Furthermore, because this antiserum was polyclonal, it would undoubtedly contain antibody generated against multiple epitopes. It is thus highly unlikely that it differentiates between β-arrestin1 and -2 when used for immunoprecipitation vs. Western analyses.

Phosphorylation of purified $G_{\beta\gamma}$ (10 μg) was carried out (30 min at 30°C) in a final volume of a 0.1-ml reaction mixture containing 20 mM Tris, pH 7.6/10 mM $MgCl_2$/1.0 mM $CaCl_2$/0.25% BSA/1.0 mM DTT/40 μg/ml leupeptin/40 μM PMSF/phosphatase inhibitors (0.1 mM sodium vanadate, 1 μM okadaic acid, 25 nM calyculin A, 100 μM ATP). The reaction was initiated by addition of PKA (150 U) and/or PKC (18 mU) catalytic subunits (as recommended by the manufacturer, Calbiochem) or an equivalent volume (20 μl) of water. Following phosphorylation, the reaction mixture was incubated with purified recombinant GRK2 protein (18 μg per reaction; 1 h at 5°C), after which immunoprecipitation by using anti-G_β antibodies and Western analysis by using anti-GRK2/3 antibodies commenced as described above. In separate, parallel experiments, the stoichiometry of ^{32}P incorporation into G_β subunit was determined under identical conditions by incubating the purified protein with [γ-^{32}P]ATP (5 μCi per reaction). Phosphorylated G_β protein was resolved by SDS/PAGE, visualized by PhosphorImager analysis, and quantified by liquid scintillation spectroscopy.

Statistical Analysis. Significance of autoradiographic and Western analyses was assessed by using 2-tailed Student's *t* test. Percent inhibition of phosphorylation by kinase inhibitors was evaluated by using a one-tailed *t* test.

Results

Coimmunoprecipitation of Phosphorylated GRK2/3, β-Arrestin, and G_β. Immunoprecipitate of ^{32}P-radiolabeled LMMP tissue obtained from opioid naïve and chronic morphine-treated tissue by using either anti-G_β or anti-GRK2/3 antibodies (G_β immunoprecipitate, GRK2/3 immunoprecipitate, respectively) contained predominantly three phosphorylated proteins of $\approx$80, 45, and 33 kDa (Fig. 1). Preincubation of anti-G_β antibodies with a 10-fold excess of purified bovine $G_{\beta\gamma}$ protein before immunoprecipitation markedly reduced (>90%) the radiolabel intensity of the $\approx$33-kDa band consistent with its G_β identity (Fig. 1*A*, lane 3). Similarly, preincubation of anti-GRK2/3 antibodies with a 10-fold excess of synthetic peptide corresponding to (Asn-478–Glu-491 of bovine GRK2/3), which was recognized by anti-GRK2/3 antibodies in ELISA assays (Martin Oppermann, personal communication), markedly reduced (>90%) the radiolabel intensity of the $\approx$80-kDa band consistent with its GRK2/3 identity (Fig. 1*B*, lane 3). Consistency of calculated molecular mass of autoradiographic signals and those obtained in Western analysis of purified protein and GRK/G_β immunoprecipitate further confirms that the phosphorylated $\approx$80-, $\approx$45-, and $\approx$33-kDa phosphorylated bands are GRK2/3, β-arrestin, and G_β, respectively (data shown in lane 4 of Fig 1 panels). The presence of all three proteins in immunoprecipitate obtained by using anti-GRK2/3 or G_β antibodies combined with their absence in immunoprecipitate obtained with preadsorbed antiserum indicates that they are present in a multimolecular complex.

In contrast to G_β and GRK2/3, immunoprecipitate that was obtained with anti-β-arrestin antibodies did not contain phosphorylated bands of $\approx$80 or $\approx$33 kDa (Fig. 2*A*). β-arrestin immunoprecipitate only contained one phosphorylated band, the molecular mass of which ($\approx$45 kDa; Fig. 2*A*) is identical to that of the intermediate size band observed in the autoradiogram of both G_β and GRK2/3 immunoprecipitate (Fig. 1). It is also identical to the signal observed in Western analysis of GRK2/3 immunoprecipitate (Fig. 1*B*, lane 4b) and LMMP membranes (Fig. 2*B*) using anti-β-arrestin antibodies. The $\approx$45-kDa signal was no longer detected in Western analysis by using preadsorbed β-arrestin antiserum (Fig. 2*B*, lane 3).

Effect of Chronic Morphine on ^{32}P Incorporation into GRK2/3, β-Arrestin, and G_β Proteins. Chronic morphine altered the phosphorylation state of GRK2/3, β-arrestin, and G_β, but the nature and

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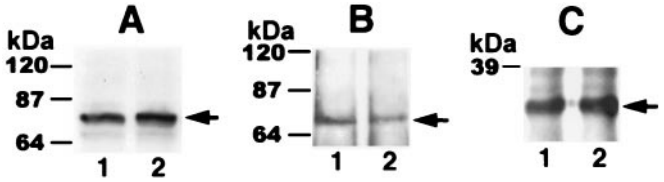

Fig. 3. Effect of chronic morphine on the content of GRK2/3 protein in LMMP membranes (*A*) and G_β immunoprecipitate (*B*). (*C*) The effect of chronic morphine on the content of G_β in immunoprecipitate obtained using anti-G_β antibody. Lanes 1 and 2 in all panels represent samples obtained from opioid naïve vs. chronic morphine-treated animals, respectively. Western analysis and immunoprecipitation were performed as described in *Materials and Methods*. Figure is representative of four experiments.

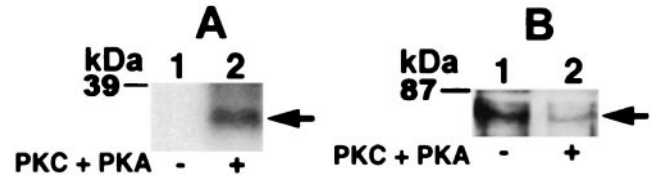

Fig. 4. Phosphorylation of purified $G_{\beta\gamma}$ reduces its association with purified GRK2. *In vitro* phosphorylation of purified $G_{\beta\gamma}$ protein was accomplished using ATP and the catalytic subunits of PKC and PKA. (*A*) Autoradiography of purified $G_{\beta\gamma}$ following incubation with [γ-^{32}P]ATP in the absence (lane 1) or presence (lane 2) of kinase catalytic subunits. Both catalytic subunits were used to mimic the chronic morphine-induced phosphorylation of G_β. (*B*) Western analysis of recombinant GRK2 protein in G_β immunoprecipitate. Immunoprecipitation was performed following incubation of recombinant GRK2 protein with nonphosphorylated (lane 1) or phosphorylated (lane 2) purified $G_{\beta\gamma}$ protein. All procedures were performed as described in *Materials and Methods*. Figure is representative of four experiments.

magnitude of the change that was observed depended on the antibody used for immunoprecipitation. Chronic morphine augmented phosphorylation of the GRK2/3, β-arrestin, and G_β that was present in immunoprecipitate obtained using either anti-G_β or GRK2/3 antibodies, but this phosphorylation increment was greater in the anti-G_β immunoprecipitate. In G_β immunoprecipitate, chronic morphine increased incorporation of ^{32}P into GRK2/3, β-arrestin, and G_β by 194 ± 16.4%, 115 ± 28.6%, and 199.5 ± 27%, respectively (Fig. 1*A*, compare lanes 1 and 2; $n = 4$, $P < 0.05$). However, the phosphorylation increment of GRK2/3, β-arrestin, and G_β observed in immunoprecipitate obtained using anti-GRK2/3 antibodies was only 44 ± 7.6%, 74.3 ± 19%, and 76.5 ± 25%, respectively (Fig. 1*B*, compare lanes 1 and 2; $n = 5$, $P < 0.05$). Although chronic morphine increased phosphorylation of β-arrestin that was coimmunoprecipitated with anti-GRK2/3 or G_β antibodies, this treatment decreased (43 ± 7.1%; $n = 3$, $P < 0.05$) phosphorylation of (free) β-arrestin that was immunoprecipitated with anti-β-arrestin antibody (Fig. 2*A*, compare lanes 1 and 2).

In contrast to observations made in tolerant/dependent tissue, increased ^{32}P incorporation into proteins of ≈80, ≈45, and ≈33 kDa was not observed following acute *in vitro* morphine treatment of opioid naïve tissue (100 nM during the last 5 min of ^{32}Pi incubation; $n = 2$; data not shown). These parameters of morphine exposure are sufficient to produce a >50% reduction of the electrically induced LMMP contracture (23). The augmented phosphorylation of these proteins following chronic but not acute exposure to morphine suggests its relevance to mechanisms underlying tolerance.

Chronic Morphine Does Not Alter the LMMP Membrane Content of β-Arrestin or GRK2/3 but Reduces the Content of GRK2/3 in G_β Immunoprecipitates. Western analyses of LMMP membranes reveal that chronic morphine does not alter their content of β-arrestin (Fig. 2*B*, lanes 1 and 2) or GRK2/3 (Fig. 3*A*). This laboratory previously reported analogous findings for the LMMP membrane content of G_β (8). Thus, their altered phosphorylation following chronic morphine results from the ability of this treatment to change the equilibrium between phosphorylated and nonphosphorylated forms.

Western analysis of G_β immunoprecipitates using anti-GRK2/3 antibodies indicates that chronic morphine reduces the total amount of GRK2/3 (phosphorylated and nonphosphorylated) that coimmunoprecipitates with G_β (≈40%, $n = 4$, $P < 0.05$; Fig. 3*B*). This notwithstanding, the phosphorylation of the GRK that is present in G_β immunoprecipitate obtained from chronic morphine-treated LMMP tissue is increased ≈2-fold. Thus, this augmented phosphorylation is manifested by a reduced pool of protein indicating its enhanced interaction with G_β. The preferential binding of phosphorylated GRK2/3 to G_β is consistent with reports of its increased membrane translocation following phosphorylation by PKC (24). It should be noted that, consistent with quantitative Western analysis of LMMP membrane G_β, equivalent amounts of G_β protein were present in G_β immunoprecipitate obtained from opioid naïve vs. chronic morphine-treated LMMP tissue (Fig. 3*C*, lanes 1 and 2). Consequently, G_β phosphorylation, augmented in LMMP tissue following chronic morphine, does not alter antibody recognition of this protein, immobilized or in solution. Thus, it does not confound interpretation of the reduced presence of GRK2/3 in G_β immunoprecipitate following chronic morphine.

The reduced content of GRK2/3 in G_β immunoprecipitate obtained from chronic morphine-treated tissue in combination with the augmented phosphorylation of G_β in these preparations suggests that phosphorylation of G_β reduces its ability to associate with GRK2/3. To validate this inference, we performed *in vitro* phosphorylation experiments that assessed the effect of purified bovine $G_{\beta\gamma}$ phosphorylation on its association with purified recombinant GRK2. Studies with inhibitors of PKA and PKC indicate that the activity of both contributes to the observed chronic morphine-induced increased phosphorylation of monitored proteins (see below). To simulate this *in vitro*, both kinases and purified $G_{\beta\gamma}$ were used. The stoichiometry of phosphorylation was 0.45 ± 0.01 mol of phosphate incorporated/mol of G_β subunit ($n = 3$). *In vitro* phosphorylation of G_β (Fig. 4*A*) attenuated its association with recombinant GRK2. This was indicated by quantitative analysis of Western blots in which G_β immunoprecipitate contained ≈50% less GRK2 when this protein was incubated with phosphorylated vs. nonphosphorylated $G_{\beta\gamma}$ protein, respectively ($n = 2$; Fig. 4*B*). When purified G_β protein was phosphorylated with the catalytic subunit of either PKA or PKC, individually, G_β immunoprecipitate contained ≈33% less GRK2 ($n = 2$), indicating the summation of their effects.

Kinase Inhibitors Selective for PKC or PKA Attenuate Chronic Morphine-Induced Phosphorylation of GRK2/3, β-Arrestin, and G_β. The kinase inhibitors used in this study were selected on the basis of their relative selectivity for PKC vs. PKA. Concentrations were selected to maximize selectivity and inhibition of the targeted kinase. Attenuation (≈50–80%), but not abolishment, of chronic morphine-induced phosphorylation of the ≈80-, ≈45-, and ≈33-kDa bands was observed following a 30-min treatment with all inhibitors used (Fig. 5). In contrast, concomitant incubation with chelerythrine and H-89 abolished chronic morphine-induced ^{32}P incorporation into immunoprecipitated proteins (data not shown).

Discussion

This laboratory previously demonstrated that chronic morphine augments opioid receptor-coupled $G_{\beta\gamma}$ stimulatory AC signaling. This was shown to result, at least in part, from the increased synthesis of ACs of the type two family (2, 8) as well as a profound

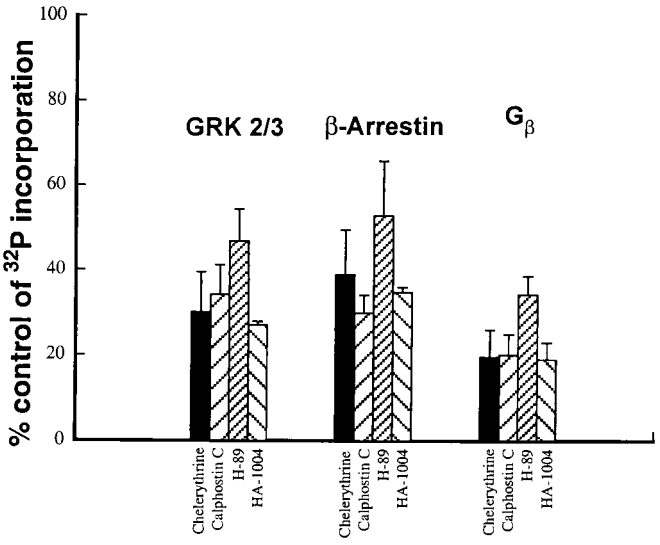

Fig. 5. Kinase inhibitors selective for PKC vs. PKA inhibit the chronic morphine-induced phosphorylation of GRK2/3, β-arrestin, and G_β. LMMP tissue from the same chronic morphine-treated guinea pig was randomly divided such that ^{32}P incorporation into G_β-immunoprecipitated protein could be concomitantly determined in the absence as well as presence of kinase inhibitors (added during the last 30 min of the 2-h ^{32}P labeling period). Membranes were prepared and solubilized, and proteins were immunoprecipitated using anti-G_β antibodies. Immunoprecipitates were subjected to electrophoresis, and radiolabeled proteins were visualized by their concomitant autoradiography (18-h exposure) using storage phosphorimaging techniques. Quantitative densitometric analysis was used to assess magnitude of ^{32}P incorporation into ≈80-, 45-, and 33-kDa signals. Chemical identity of each was assessed as described in Fig. 1. Data are expressed as a percent inhibition of the ^{32}P incorporation observed in the absence of kinase inhibitors. $n = 4$, 3, 3, and 2 for chelerythrine, calphostin C, H-89, and HA1004, respectively. All reductions in phosphorylation are significant ($P < 0.05$).

increase in their phosphorylation state (4). The present study demonstrates that chronic treatment with morphine also results in the concomitant phosphorylation of GRK2/3, β-arrestin, and G_β, the identities of which were unambiguously identified by using targeted immunoprecipitation in combination with Western analysis of (*i*) purified protein, (*ii*) immunoprecipitates, and (*iii*) LMMP membranes. All three proteins are coimmunoprecipitated using anti-GRK2/3 or G_β. Phosphorylation alters the association of these signaling molecules, one consequence of which would be to maximize the availability of $G_{\beta\gamma}$ for interaction with AC and other effectors.

The magnitude of the augmented phosphorylation that is observed as a function of chronic morphine treatment was found to vary with the antibody used to immunoprecipitate proteins. However, chronic morphine-induced phosphorylation increments of monitored protein was always determined by using the same antibody to process, in parallel, opioid naïve vs. chronic morphine-treated LMMP tissue. Therefore, interpretation of antibody-dependent differences (see below) should not be influenced by variations in immunoprecipitation efficiency among the antibodies.

Chronic morphine-induced phosphorylation of GRK2/3 seems to alter its association with G_β. This conclusion is based on the fact that the GRK2/3 immunoprecipitate should contain predominantly (albeit not exclusively) free GRK2/3 in contrast to the exclusive presence of complexed GRK2/3 in immunoprecipitate obtained using anti-G_β antibody. The smaller chronic morphine-induced increment in phosphorylation of GRK2/3 that is present in GRK2/3 vs. G_β immunoprecipitate (≈44% vs. ≈194%, respectively) implies that phosphorylation of GRK2/3 increases its association with $G_{\beta\gamma}$, consistent with the increased translocation of phosphorylated GRK to the membrane (24).

Chronic morphine-induced phosphorylation of β-arrestin produces an analogous effect. Autoradiography of β-arrestin immunoprecipitate reveals only a single phosphorylated band (≈45 kDa); there is no detectable presence of either phosphorylated GRK2/3 or G_β. This would be expected because the C-terminal antibody recognition site (19) is the region known to bind to phosphorylated GPCRs (25). The decrease (≈43%) in β-arrestin phosphorylation manifested in β-arrestin immunoprecipitate vs. the increased phosphorylation (≈115%) of the β-arrestin that coimmunoprecipitates with G_β suggests that its chronic morphine-induced phosphorylation promotes association with $G_{\beta\gamma}$.

In contrast, phosphorylation of G_β seems to attenuate its association with GRK2/3. This is indicated by (*i*) the significantly smaller chronic morphine-induced G_β phosphorylation increment manifested in GRK2/3 vs. G_β immunoprecipitate; (*ii*) chronic morphine-induced augmented phosphorylation of G_β is associated with decreased coimmunoprecipitation of GRK2/3 protein (Fig. 3*B*); and (*iii*) *in vitro* phosphorylation of purified bovine $G_{\beta\gamma}$ via PKC and PKA, both of which contribute to its chronic morphine-induced phosphorylation *in vivo*, attenuates its interaction with recombinant GRK2 protein (Fig. 4). Because equivalent amounts of G_β protein are contained in anti-G_β immunoprecipitate derived from opioid naïve vs. chronic morphine-treated preparations (Fig. 3*C*), data interpretation is not confounded by differential immunoprecipitation of nonphosphorylated vs. phosphorylated G_β.

The consequences of chronic morphine-induced augmented phosphorylation of G_β and GRK2/3 have substantial functional implications regarding altered opioid receptor-coupled signaling that result from persistent opioid receptor activation. Decreased association of $G_{\beta\gamma}$ with GRK2/3 would increase its availability for interaction with biological effectors such as AC. This could contribute to the augmented $G_{\beta\gamma}$ stimulatory AC signaling demonstrated in chronic morphine-treated LMMP tissue (8). Additionally (or alternatively), the phosphorylated G_β subunit could participate in an enzymatic transfer of a phosphate to GDP at G protein α subunits (26) and thereby prevent reformation of heterotrimeric G proteins. This would maintain the pool of free $G_{\beta\gamma}$ subunits available for signaling. Therefore, increased signaling via $G_{\beta\gamma}$ subunits during opioid tolerance could result not only from effector modification (AC isoform-specific synthesis and phosphorylation) but from chemical modification (phosphorylation) of signaling/transducing molecules as well.

Phosphorylation of GRK2/3 has previously been shown to increase its kinase activity (27, 28). Additionally, it promotes association with $G_{\beta\gamma}$ (as described herein). Thus, the effect of chronic morphine treatment on the interaction between GRK and G_β is the result of two competing processes. Phosphorylation of GRK increases whereas phosphorylation of G_β decreases their interaction. However, the effect of increasing levels of phosphorylated G_β on complex formation predominates. This is evidenced by the observation that in chronic morphine-treated tissue, the total amount of GRK that coimmunoprecipitates with G_β is significantly reduced (Fig. 3*B*). Thus, following chronic morphine, the net effect is a reduction in the association between GRK and G_β. Nevertheless, the membrane content of GRK remains constant. The most plausible explanation is that phosphorylation of G_β effectively reduces the amount of this protein that would be available for interaction with GRK, which is partially compensated for by the enhanced ability of phosphorylated GRK to interact with nonphosphorylated G_β. Following chronic morphine, $G_{\beta\gamma}$ continues to recruit GRK2/3 to the membrane that is then stored in association with charged membrane phospholipids via a pleckstrin homology domain (29). Such interactions are known to be essential for agonist-dependent phosphorylation of GPCRs by GRK2/3 (30).

The coimmunoprecipitation of phosphorylated GRK2/3, β-arrestin, and G_β, using antibodies generated against either GRK2/3 or G_β but not preadsorbed antiserum, indicates that all three proteins exist in the membrane, at least in part, as a multi-

molecular complex. This is consistent with previous demonstrations of interactions between GRK2/3 and $G_{\beta\gamma}$ (12, 31). It is also consistent with (*i*) the ability of β-arrestins to bind to phosphorylated GPCR (32), (*ii*) the extensive colocalization of the β_2-adrenergic receptor and GRK2/3 in intracellular vesicles following receptor stimulation (33), and (*iii*) the formation of a GRK2/β-arrestin/CCR2 receptor complex upon receptor activation (34). The present results demonstrate the existence of steady-state levels of a complex simultaneously consisting of β-arrestin, GRK2/3, and $G_{\beta\gamma}$. The presence of the μ-opioid receptor in GRK2/3 immunoprecipitate, detected by Western analysis using an anti-μ-opioid receptor antibody (ref. 35 and data not shown) could explain, at least in part, the coimmunoprecipitation of β-arrestin with GRK and G_β proteins.

GRK2/3 have been shown to be subject to phosphorylation and regulation by multiple kinases. c-Src can directly phosphorylate GRK2 on tyrosine residues (28). Additionally, a mixture of PKCs, purified from rat brain, phosphorylates purified recombinant β-adrenergic receptor kinase-1 (βARK-1) (24). The current observations that chronic morphine-induced phosphorylation of GRK2/3 in LMMP tissues, as well as β-arrestin and G_β, was significantly reduced (50–80%), but not abolished, by kinase inhibitors at concentrations that enable discrimination between PKC and PKA underscores that the activities of both are required for their chronic morphine-induced phosphorylation. Involvement of PKC in the chronic morphine-induced AC phosphorylation in the LMMP tissue as well as the shift from predominantly opioid receptor inhibitory to stimulatory signaling has previously been reported (4, 36). The imputed role of PKC in opioid tolerance in LMMP tissue is consonant with its up-regulation in this tissue (36) as well as in spinal cord following chronic morphine treatment (37). The current demonstration of the involvement of PKA is consistent with the important role this kinase family plays in other biochemical sequelae of chronic morphine (38) and its up-regulation following chronic opioid administration (39).

In summary, chronic systemic administration of morphine was found to augment the concomitant phosphorylation of key signaling proteins. Targeted immunoprecipitation resulted in the coimmunoprecipitation of GRK2/3, β-arrestin, and G_β. Antibody selectivity in combination with the observed antibody-dependent increment in phosphorylation indicates that phosphorylation of GRK2/3, β-arrestin, and G_β alters their association. As a result, there would be increased availability of $G_{\beta\gamma}$ while maintaining constant membrane levels of GRK.

$G_{\beta\gamma}$ subunits, derived from heterotrimeric G proteins upon activation of GPCRs, play a complex role in signal transduction (16). Potassium channels as well as many signal transduction enzymes, e.g., phospholipase A_2, phospholipase Cβ, AC isoforms, mitogen-activated protein kinase, are regulated by $G_{\beta\gamma}$ (16, 40). The nature of the regulation can be quite complex depending both on the effector and on interactions with G_α subunits. Decreased association of $G_{\beta\gamma}$ subunits with GRK2/3 and its consequent increased availability to interact with other signaling proteins could result in the altered activity of divergent signaling cascades and thus have functional implications that are quite broad. This could underlie, in part, the multiplicity of systems that are affected by chronic morphine and the resulting complexity of opioid tolerance and dependence.

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