THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 19, Issue of May 10, pp. 11449–11454, 1996
Printed in U.S.A.

Differential μ Opiate Receptor Phosphorylation and Desensitization Induced by Agonists and Phorbol Esters*

(Received for publication, January 16, 1996, and in revised form, February 22, 1996)

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μ opiate receptors, the principal sites for opiate analgesia and reward, can display compensatory responses to opiate agonist drug administration. Agonist-induced K^+ channel responses mediated by these receptors desensitize when examined in *Xenopus* oocyte expression systems. Mechanisms underlying such processes could include phosphorylation events similar to those reported to desensitize other G-protein-linked receptors. We used C-terminally directed anti-μ receptor antibodies to immunoprecipitate a phosphoprotein with size appropriate for the μ receptor from stably expressing Chinese hamster ovary cells. Phosphorylation of this μ opiate receptor protein was enhanced approximately 5-fold by treatment with the μ agonist morphine. The time course and dose-response relationships between μ receptor phosphorylation and agonist-induced desensitization display interesting parallels. Phosphorylation of μ opiate receptor protein is also enhanced ~5-fold by treatment with the protein kinase C activator phorbol 12-myristate 13-acetate. The protein kinase inhibitor staurosporine blocked the effect of phorbol 12-myristate 13-acetate on μ receptor phosphorylation. However, staurosporine failed to block morphine-induced phosphorylation. These observations suggest that several biochemical pathways can lead to μ receptor phosphorylation events that may include mechanisms involved in μ receptor desensitization.

Opioid receptors are G-protein-coupled, seven transmembrane domain receptors. These receptors mediate the actions of opiate drugs and endogenous opioid neuropeptides in producing euphoria, modulating pain perception, and altering other important functions in the central and peripheral nervous systems (Herz, 1993). Pharmacological characterization and cDNA cloning have focused attention on the μ opiate receptor subtype, the site at which opiate drugs such as morphine and its derivatives exert most of their analgesic and euphoric effects (Chen *et al.*, 1993; Fukuda *et al.*, 1993; Wang *et al.*, 1993; Thompson *et al.*, 1993). G-protein cascades activated by μ receptors can reduce adenylyl cyclase activity, alter inositol trisphosphate turnover, activate G-protein-linked, inward potassium channels, and close calcium channels (Childers, 1991; Smart *et al.*, 1994; North *et al.*, 1987).

A prominent characteristic of morphine-like drugs is their ability to induce drug tolerance and dependence in humans. Another feature of these drugs' actions is the rate-sensitivity.[1] Rapidly acting opiates such as heroin induce much more striking euphoria than more slowly acting opiates such as methadone.[1] Potential μ opiate receptor mechanisms that might contribute to opiate tolerance, dependence, and rate-sensitivity have thus been of interest. Receptor desensitization is one of the cellular mechanisms that could play possibly significant roles in these neuroadaptive processes.

Phosphorylation is a post-translational modification used to regulate the functions of a wide variety of proteins, including G-protein-linked neurotransmitter receptors (Walaas and Greengard, 1991). Studies of adrenergic and other receptors demonstrate that agonist-dependent phosphorylation of the receptors contribute to mechanisms of receptor desensitization, although receptor internalization and altered rates of receptor gene expression can also play roles (*e.g.* Kobilka, 1992). Such studies also point to the possibilities that mechanisms of homologous desensitization occurring as a result of agonist occupancy of the receptor can be different from heterologous desensitization mechanisms produced by the consequences of occupation of other G-protein-linked receptors coexpressed on the same cell.

Xenopus oocytes co-expressing cDNAs encoding the μ receptor and GIRK1, an inwardly rectifying potassium channel that can be activated by μ receptor-activated G-proteins, have allowed detection of agonist-mediated desensitization of μ receptor-mediated K^+ channel responses in two laboratories (Chen and Yu, 1994; Kovoor *et al.*, 1995). These two reports, however, disagree on the effects of stimulators of protein kinase A and/or protein kinase C (PKC)[2] in this desensitization.

The possibility that the μ opiate receptor might undergo phosphorylation was suggested by the presence of sequences with homology to consensus sites for protein kinase A and C phosphorylation in putative intracellular receptor domains when the receptor's sequence was elucidated by cDNA cloning (Wang *et al.*, 1993). Antibodies recognizing epitope-tagged opiate receptors have immunoprecipitated apparent phosphorylated tagged μ receptor protein from expressing HEK293 cells

* This work was supported in part by National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism Intramural Research Programs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] G. R. Uhl, D. Gorelick, and M. J. Kreek, submitted for publication.

[2] The abbreviations used are: PKC, protein kinase C; DAMGO, [D-Ala^2,MePhe^4, Gly-ol^5]enkephalin; DMSO, dimethyl sulfoxide; CHO, Chinese hamster ovary; hμCHO, human μ opiate receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

(Arden *et al.*, 1995). However, no current work has established the biochemical pathways for such phosphorylation or examined the parallels between biochemical evidence for μ receptor phosphorylation and the receptor desensitization noted electrophysiologically.

In order to approach this problem, we have studied agonist-dependent and protein kinase-mediated μ receptor phosphorylation events and sought parallels with patterns of desensitization of μ receptor-mediated potassium channel responses. We have documented agonist-induced and PKC-induced μ receptor phosphorylation and agonist- and PKC-mediated receptor desensitization. Observed patterns of phosphorylation, desensitization, and PKC-inhibitor effects provide evidence for at least two pathways for μ receptor phosphorylation events, some of which could be involved in receptor desensitization.

EXPERIMENTAL PROCEDURES

Preparation of Xenopus Oocytes Coexpressing the μ Opiate Receptor and G-protein-activated K^+ Channels—cDNAs encoding the human opioid μ receptor (Wang *et al.*, 1994) and GIRK1 (kindly provided by Dr. Henry Lester; Dascal *et al.*, 1993) were subcloned into the expression vector pcDNAI, plasmids linearized with *Xba*I, and capped mRNA-sense RNAs prepared by *in vitro* transcription using T7 polymerase (mMACHIEN kit; Ambion). RNA quality and sizes were assessed after separations using 1.2% formaldehyde agarose gels, and full-length RNAs were stored in 75% ethanol at −70 °C.

Oocytes were isolated from mature female *Xenopus laevis* (*Xenopus* I, Ann Arbor, MI), defolliculated by treatment with 0.2% collagenase A, injected with 16–20 ng of RNAs encoding the μ opiate receptor and GIRK1 in molar ratios of 3:1, and incubated for 2–3 days at 19–20 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 5 mM HEPES, pH 7.5) supplemented with 2 mM sodium pyruvate, 10,000 units/liter penicillin, 10 mg/liter streptomycin, and 0.5 mM theophylline.

Electrophysiological Recording—Whole cell currents in the expressing oocytes were measured at 22 °C under two-electrode voltage-clamped conditions at −70 mV, using a GeneClamp 500 amplifier (Axon Instrument). Oocytes were placed on a nylon mesh in a 90-μl bath chamber and continuously superfused at 6 ml/min with either ND96 or "hK" medium (ND96 medium with 96 mM KCl and 2 mM NaCl). Oocytes were superfused with ND96 between applications of hK solution alone, hK solution during which opiate agonists were transiently applied with or without opiate antagonists, or ND96 containing phorbol esters (Sigma) in dimethyl sulfoxide (DMSO) concentrations less than 0.01%. Values presented are mean ± S.E., with statistical significance analyzed using Student's *t* test or analysis of variance, as appropriate. Concentration-response curves were obtained using the program NFIT by fitting data to the logistic equation, $y = \{(E_{max} - E_{min})/(1 + (x/EC_{50})^{-n})\} + E_{min}$, where x represents concentration, y represents response, E_{max} the maximal response, E_{min} the minimal response, EC_{50} the half-maximal concentration, and n the apparent Hill coefficient.

μ Opiate Receptor Phosphorylation—Phosphorylation of the μ opiate receptor in Chinese hamster ovary cells stably expressing human μ opiate receptor (hμCHO, Wang *et al.*, 1994; Rothman *et al.*, 1995) was examined by modifications of the method described by Pei *et al.* (1995). hμCHO and nontransfected Chinese hamster ovary (CHO) cells were plated at 80% confluence in six-well plates, grown for 24 h in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 100 mM penicillin, and 100 mg/liter streptomycin, washed twice with phosphate-free DMEM, and incubated at 37 °C for 2 h with 300 μCi/ml [^{32}P]orthophosphate (8500 Ci/mmol; DuPont NEN) in phosphate-free DMEM. Labeled cells then were exposed to morphine (1 μM; 20 min) with or without coincubation with naloxone (1 μM) or staurosporine (2 μM) or to PMA or 4α-PMA (1 μM; 20 min) with or without coincubation with staurosporine (2 μM), or to other treatment times and concentrations as indicated in figure legends. Cells were cooled to 4 °C by washing with ice-cold phosphate-buffered saline (PBS); all subsequent procedures were carried out at this temperature. Proteins were extracted for 60 min with 0.8 ml of RIPA$^+$ buffer (1% Nonidet P-40, 0.5% Na_2deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM Na_2pyrophosphate, 1 μM okadaic acid, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A in PBS buffer). Supernatant from a 15-min, 150,000 × *g* centrifugation was assayed for protein concentration by the Bradford method (Bio-Rad) and preadsorbed by incubation with 100 μl of presoaked protein A-Sepharose beads (Pharmacia Biotech), followed by microcentrifugation. For immunoprecipitation, 700 μl of the supernatant from the preadsorption step was incubated for 2 h with 100 μl of the protein A-Sepharose bead slurry and a 1:500 final dilution of an antiserum directed against the μ opiate receptor's C-terminal 18 amino acids. Beads were washed three times by resuspension with 1 ml of RIPA$^+$ followed by microcentrifugation, and immunoprecipitated proteins were then dissociated from beads by extraction with 60 μl of SDS-PAGE gel loading buffer (4% SDS, 25 mM Tris-HCl, pH 6.8, 5% glycerol, 0.5% 2-mercaptoethanol, and 0.005% bromphenol blue). 20 μl of the immunoprecipitated proteins were separated on 8% SDS-PAGE gels with prestained molecular mass standards (Amersham Corp.), and radiolabeled proteins were identified by autoradiography using Hyperfilm-MP (Amersham Corp.) with intensifying screens. Densities of bands of interest were quantified by scanning densitometry and normalized to the amounts of extracted cell protein subjected to immunoprecipitation.

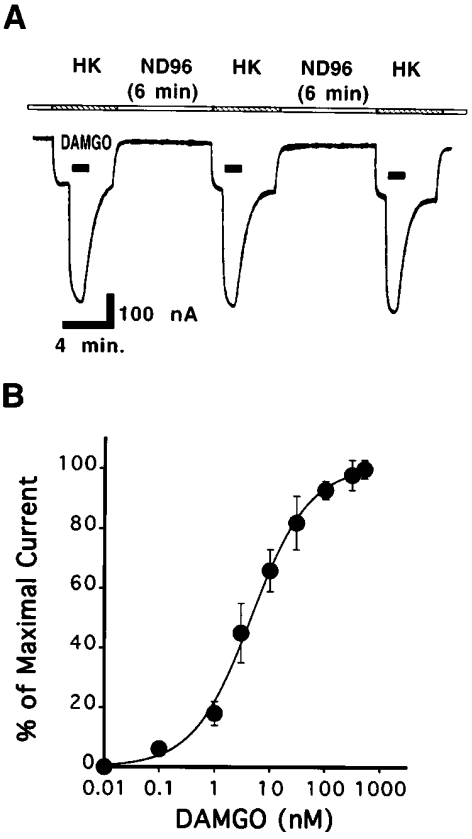

FIG. 1. *A*, currents induced in *Xenopus* oocytes coexpressing μ opiate receptors and GIRK1 during superfusion with ND96 (*open bars*), hK solution (*cross-hatched bars*), or 10 nM DAMGO in hK solution (*solid bars*). *B*, dose-response relationship for DAMGO in inducing inward currents greater than those found in hK medium alone. Peak currents during DAMGO/hK superfusion were corrected for currents found during superfusion of hK alone and reported as mean ± S.E. of maximal currents determined for each of 5–7 individual expressing oocytes determined with 1 μM DAMGO. The EC_{50} of DAMGO is 4.6 nM.

RESULTS

μ Opiate Agonist-activated K^+ Currents, Agonist-induced Desensitization, and PMA Effects—Both sham-injected *Xenopus* oocytes and those coexpressing the μ opiate receptor and GIRK1 displayed inward currents that reached plateaus in 30–40 s after superfusion with hK solution under two-electrode voltage clamp conditions (noted in expressing oocytes in Fig. 1*A* as "shoulders" of the traces; see also Kovoor *et al.*, 1995). Sham-injected oocytes showed no further responses to peptide or nonpeptide opiate agonists (data not shown). By contrast, oocytes coinjected with *in vitro* transcribed μ opiate receptor and GIRK1 RNAs revealed 200–600 nA inward currents when exposed to 10 nM DAMGO that were 2–6-fold greater than the currents induced by perfusion with hK me-

dium alone (Fig. 1*A*). DAMGO displayed an EC_{50} of 4.6 nM (Fig. 1*B*) in producing these additional currents; this effect was blocked by cotreatment with 1 μM naloxone (data not shown). The amplitude of the currents recovered fully after 6 min of oocyte washing with ND96 medium (Fig. 1*A*).

Agonist-induced desensitization was observed, manifested as a gradual decline in the amplitude of current responses to short test applications of 1 μM DAMGO applied in hK solution to oocytes previously exposed to 1 μM DAMGO superfused in ND96 medium for varying periods of time. hK-elicited currents were assessed during washout periods using hK medium alone (Fig. 2*A*). This paradigm reveals agonist-induced desensitization after as little as 3-min oocyte exposures to 1 μM DAMGO; the desensitization reaches maximal values by 20 min and is reversible. Short test doses of 1 μM DAMGO administered following as little as 10 min of washing with ND96 medium reveal recovery to near-normal function. The magnitude of observed desensitization depends on both the exposure time and concentration of agonist (Fig. 2, *B* and *C*). In experiments in which oocyte responses to test DAMGO administrations were recorded after 30 min of continuous DAMGO exposure, maximum desensitization was observed at 10 μM DAMGO (Fig. 1*C*).

In order to seek evidence for involvement of protein kinase C activation on desensitization, we examined effects of bath-application pretreatments with the active phorbol ester PMA and the inactive control compound 4α-PMA on opiate agonist-activated K^+ currents. Although these agents required DMSO for solubilization, control experiments revealed that the 0.01% DMSO concentrations that resulted in the superfusate produced no detectable effects on DAMGO-activated currents (data not shown). To avoid concealing PMA effects by agonist-induced desensitization manifested at greater magnitude, experiments were conducted using 10 nM DAMGO exposures for 1 min. These DAMGO concentration and exposure times did not lead to desensitization (*e.g.* Fig. 2*D*). Currents activated by 10 nM DAMGO were significantly attenuated after application of 100 nM PMA for 10 min (Fig. 2*E*). This inhibition was maximal by 40 min after PMA pretreatment. In initial experiments, the effect persisted for as much as 2 h after the end of PMA treatment (data not shown). By contrast, the amplitude of the current activated by 10 nM DAMGO in oocytes pretreated for 60 min with the inactive 4α-PMA was indistinguishable from that induced in oocytes not pretreated (Fig. 2, *D* and *E*). The effect of PMA was concentration-dependent, with an EC_{50} of 10.4 ± 3 nM (Fig. 2*F*). Maximal inhibition to 68 ± 6% of control values was reached at ~300 nM PMA ($p < 0.001$, $n = 5$).

μ Opiate Receptor Phosphorylation, Agonist- and PMA-induced Changes—Both hμCHO and nontransfected CHO cells incubated for 2 h with $^{32}P_i$ revealed a number of common phosphoprotein bands that could be noted following immunoprecipitation with specific polyclonal anti-μ receptor antisera, separation on SDS-PAGE, and autoradiography. However, phosphoproteins extracted from the hμCHO displayed an ~70-kDa immunoprecipitable phosphoprotein not found in extracts of nontransfected control CHO cells (Fig. 3*A*). The protein selectively presented in the receptor-expressing cells was thus of the size anticipated for the μ opiate receptor protein purified from brain by ligand affinity and other nonimmunological methods (Eppler *et al.*, 1993; Surratt *et al.*, 1994). Several other lines of evidence also supported the identification of this protein as the μ opiate receptor. The modest levels of basal receptor phosphorylation in cells without drug treatment were dramatically stimulated ~5-fold by treatment of cells for 20 min with 1 μM morphine or PMA. Naloxone (10 μM) cotreatment blocked this morphine-induced phosphorylation. The PMA an-

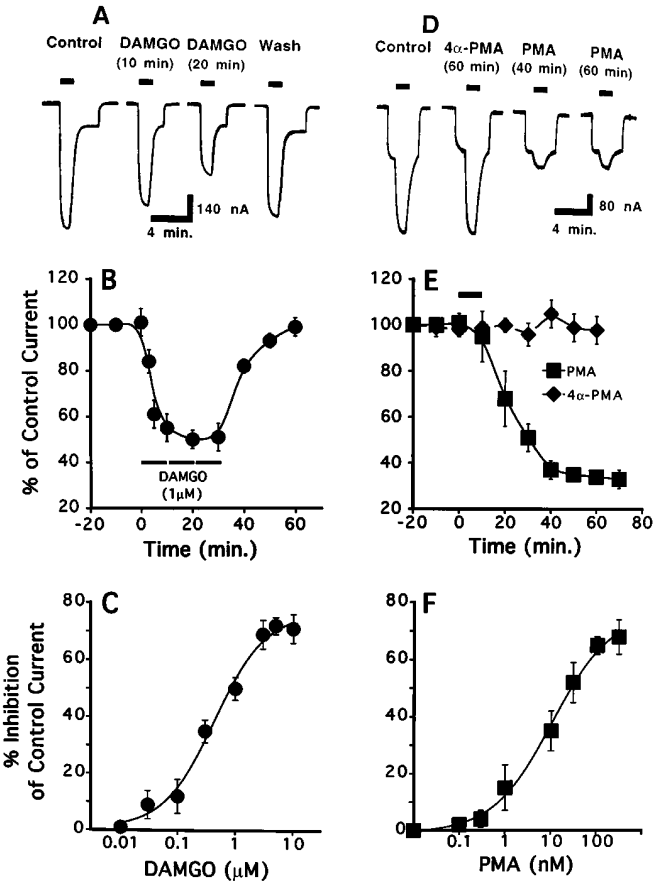

FIG. 2. **Currents induced in *Xenopus* oocytes coexpressing opiate receptors and GIRK1.** *A*, recordings of responses to superfusion with ND96 or test superfusions with 1 μM DAMGO in hK (*solid bars*) followed by hK solution superfusion for 2 min in oocytes previously exposed for 10 or 20 min to superfusion of 1 μM DAMGO in ND96. *B*, time course of desensitization of μ opiate receptor/GIRK1 responses in oocytes pretreated with opiate agonist for various periods. Peak currents during test DAMGO/hK superfusions following 0–30-min treatment with 1 μM DAMGO in ND96 were corrected for currents found during superfusion of hK alone and reported as mean ± S.E. of maximal currents determined for DAMGO/hK responses obtained before DAMGO pretreatments (times −20 to 0 min). Recovery of peak currents obtained during test DAMGO/hK superfusions performed during DAMGO washout (times 30–60 min). *C*, dose-response relationship for DAMGO in inducing desensitization of μ opiate receptor/GIRK1 responses. Oocytes were pretreated for 30 min with varying concentrations of DAMGO in ND96 and then tested by DAMGO/hK superfusions. Values obtained during test DAMGO/hK superfusions were corrected for currents found during superfusion of hK alone and reported as mean ± S.E. of maximal currents determined for DAMGO/hK responses obtained before DAMGO/ND96 pretreatments. Maximal inhibition of control currents peaks at ~70%; half of this effect is reached at DAMGO concentrations ~0.4 μM. *D*, recordings of responses to test superfusions with 10 nM DAMGO in hK (*solid bars*) followed by hK solution superfusion for 2 min in oocytes pretreated for 40 min with ND96 control superfusions alone (*left*), 60 min with 100 nM4α-PMA (*second from left*), 40 min with 10 nM PMA (*third from left*), and 60 min with 10 nM PMA (*right trace*). *E*, time course of effects of pretreatments with 100 nM PMA or 4α-PMA (*solid bar*) on test responses to 10 nM DAMGO in hK medium. Peak currents during test DAMGO/hK superfusions performed at various times before or after 10-min superfusions of phorbol esters in ND96 are reported as mean ± S.E. of maximal currents determined for DAMGO/hK responses obtained before phorbol ester treatment. *F*, dose-response relationships for the effects of 10-min PMA treatments in inhibiting responses to 10 nM DAMGO obtained during test DAMGO/hK superfusions performed 40 min later. Currents are corrected for currents found during superfusion of hK alone and reported as mean ± S.E. of maximal currents determined for DAMGO/hK responses obtained before PMA treatments. Maximal inhibition of control currents peaks at ~67%; half of this effect is reached at PMA concentrations of 10.4 ± 3 nM (n = 5–7).

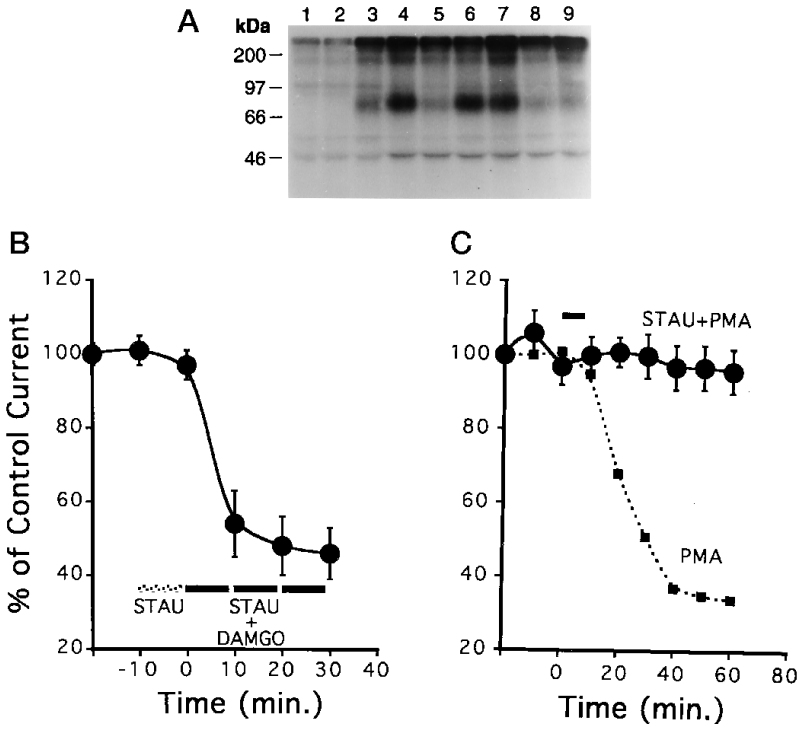

FIG. 3. *A*, autoradiogram of phosphoproteins extracted from nonexpressing CHO cells (*lanes 1* and *2*) and hμCHO cells (*lanes 3-9*) immunoprecipitated with anti-μ receptor antibodies and separated on SDS-PAGE. Cells were pretreated for 20 min at 37 °C with 1 μM concentrations of morphine (*lanes 2* and *4*), morphine and naloxone (*lane 5*), morphine and 2 μM staurosporine (*lane 6*), PMA (*lane 7*), PMA and 2 μM staurosporine (*lane 8*), and 4α-PMA (*lane 9*). Autoradiogram was exposed for 2 days, and size standards were derived from prestained markers electrophoresed in adjacent lanes. *B*, effect of staurosporine on DAMGO-induced desensitization. Expressing oocytes were pretreated with 2 μM staurosporine for 10 min (*dotted bar*) and then exposed to desensitizing treatments with 1 μM DAMGO and 2 μM staurosporine in ND96 for various times (*solid bar*). Responses to test DAMGO/hK superfusions performed at various times were corrected for currents found during superfusion of hK alone and reported as mean ± S.E. of maximal currents determined for DAMGO/hK responses obtained during staurosporine pretreatments. *C*, the effects of staurosporine on PMA-induced desensitization. Expressing oocytes were pretreated (*solid bar*) with 2 μM staurosporine and 100 nM PMA or with 100 nM PMA alone. Responses to test DAMGO/hK superfusions performed at various times were corrected for currents found during superfusion of hK alone and reported as mean ± S.E. of maximal currents determined for DAMGO/hK responses obtained prior to or during PMA or staurosporine and PMA pretreatments.

alog 4α-PMA lacked activity in PKC stimulation and had no effect on apparent μ opiate receptor phosphorylation levels.

The time course of receptor phosphorylation induced by morphine appeared to be rapid; it reached near-peak levels after 5 min of morphine exposure (Fig. 4*A*). This pattern differed slightly from that observed with PMA (Fig. 4*C*), which manifested stimulation of phosphorylation that began at least as early but was maintained perhaps more poorly with prolonged incubations. μ receptor phosphorylation was concentration-dependent for both receptor agonist and PMA (Fig. 4, *B* and *D*). Morphine-induced receptor phosphorylation was detectable after 10-min exposures to 10 nM morphine, and maximum phosphorylation was found after exposure to 1 μM morphine (Fig. 4*B*). Concentrations of PMA as low as 1 nM enhanced μ opiate receptor phosphorylation, whereas maximum effects were observed at 100 nM PMA (Fig. 4*D*).

Differential Staurosporine Effects on Agonist and PMA-induced μ Receptor Phosphorylation and μ Receptor Desensitization—As anticipated, PMA influences on μ receptor opiate receptor phosphorylation were totally blocked by treatment with staurosporine, a relatively selective inhibitor of PKC. Interestingly, however, staurosporine failed to alter morphine-induced μ receptor phosphorylation (Fig. 3*A*) in either of two replicate experiments.

Electrophysiological experiments also suggested that staurosporine exerts differential effects on PMA and on opiate agonist-induced alterations in inward K^+ currents. Staurosporine pretreatments virtually abolished PMA effects on inward K^+ currents induced by nondesensitizing opiate agonist treatments but failed to alter agonist-induced desensitization (Fig. 3, *B* and *C*).

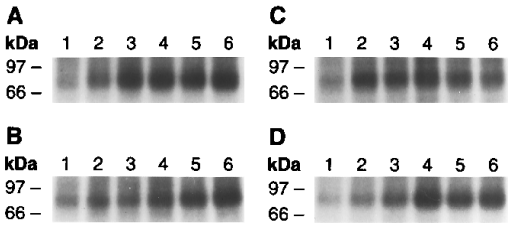

FIG. 4. **Autoradiograms displaying the time course (*A* and *C*) and concentration dependence (*B* and *D*) of morphine (*A* and *B*) and PMA (*C* and *D*) effects on μ opiate receptor phosphoprotein densities in proteins extracted from hμCHO cells.** *A*, μ opiate receptor phosphoproteins in hμCHO cells incubated with 1 μM morphine for 0 min (*lane 1*), 1 min (*lane 2*), 2 min (*lane 3*), 5 min (*lane 4*), 10 min (*lane 5*), and 20 min (*lane 6*). *B*, μ opiate receptor phosphoproteins in hμCHO cells incubated for 10 min with morphine at 0 nM (*lane 1*), 1 nM (*lane 2*), 10 nM (*lane 3*), 100 nM (*lane 4*), 1000 nM (*lane 5*), and 5000 nM (*lane 6*). *C*, μ opiate receptor phosphoproteins in hμCHO cells incubated with 1 μM PMA for 0 min (*lane 1*), 2 min (*lane 2*), 5 min (*lane 3*), 10 min (*lane 4*), 20 min (*lane 5*), and 40 min (*lane 6*). *D*, μ opiate receptor phosphoproteins in hμCHO cells incubated for 10 min with PMA at 0 nM (*lane 1*), 1 nM (*lane 2*), 10 nM (*lane 3*), 100 nM (*lane 4*), 1000 nM (*lane 5*), and 10,000 nM (*lane 6*).

DISCUSSION

The current results provide data that add to the identification of the μ opiate receptor as a potentially rapidly regulated phosphoprotein. It also provides evidence for parallels between the effects of time of opiate agonist exposure and agonist dose

on the phosphorylation and desensitization of a major μ receptor function, opening inwardly rectifying potassium channels. Studies of the effects of agonists and PKC on μ receptor phosphorylation and function provide some of the first evidence that μ receptor phosphorylation is likely to be heterogeneous and that different phosphorylation patterns may contribute to μ receptor desensitization.

Several lines of biochemical evidence support the likelihood that the immunoprecipitated phosphoproteins examined in these studies include authentic phosphorylated μ opiate receptors. The immunoprecipitable phosphoproteins isolated from CHO cells expressing the μ opiate receptor include a 70-kDa band of the size found for the mature μ receptor purified from striatum but not found in protein extracted from CHO cells that do not express the μ receptor. The specificities of the anti-μ receptor C-terminal antibodies used here for immunoprecipitation have been well documented in Western blotting and immunohistochemical experiments (Surratt *et al.*, 1994; Cheng *et al.*, 1995). The morphine-specific patterns of regulation also support detection of authentic μ receptor in this protein band.

Our studies in *Xenopus* oocytes coexpressing μ receptor and potassium channel cDNAs indicated initially brisk DAMGO-induced alteration of potassium currents that desensitized to ~50% of basal values with repeated DAMGO applications and recovered with repeated washing over periods of ~30 min. These desensitizing currents displayed several other characteristics anticipated to μ receptor-mediated events. They were also noted with repeated morphine administration, were blocked by coadministration of the opiate antagonist naloxone, and were not seen in oocytes that did not express the μ receptor. Interestingly, two preliminary experiments each document that μ receptor-coupled inhibition of adenylyl cyclase also displays desensitization. cAMP levels in hμCHO cells decline 30–40% less following a second challenge with DAMGO than after initial DAMGO application (data not shown). Physiological desensitization of μ receptor responses can thus occur in two distinct cellular systems and in two distinct physiological responses.

Several observations suggest that protein kinase C can induce μ receptor desensitization in the *Xenopus* expression system. The ability of the active phorbol PMA to produce this effect, the failure of the inactive phorbol 4α-PMA to reproduce it, and the ability of staurosporine to inhibit it each support its mediation through protein kinase C. These results are also in accord with those reported by Yu and co-workers (Chen and Yu, 1994) in a similar expression system, although they differ from those obtained by Chavkin and colleagues (Kovoor *et al.*, 1995). These substantial effects of PKC stimulation contrast markedly with our failure to detect any effects of the protein kinase A stimulator forskolin on this desensitization (data not shown).

PMA and μ receptor agonists both stimulate μ receptor phosphorylation by ~5-fold. Since experiments assessing phosphorylation are performed in the presence of a phosphatase inhibitor mixture, this magnitude of phosphate accumulation may not provide an accurate estimate of the magnitude of the effects of receptor or protein kinase C stimulation in systems with normal balances between phosphatase and kinase activities. Conceivably, differences in balances between phosphatase and kinase activities in HEK293 cells could account for the stronger levels of basal phosphorylation and weaker magnitudes of agonist-induced phosphorylation recently reported in this expression system by Sadee and co-workers (Arden *et al.*, 1995). The magnitude of the ~5-fold changes in phosphorylation found in our CHO cell expression system is nevertheless equivalent to that found in studies of other G-protein-coupled receptors *e.g.* (Pei *et al.*, 1995; Tobin and Nahorski, 1993). Our μ receptor phosphorylation results could also represent underestimates of the extent of this receptor phosphorylation if the antibody used to immunoprecipitate the receptor recognized the phosphorylated μ receptor less avidly than the nonphosphorylated receptor.

Comparisons between the time course and dose-response relationships for agonist-mediated μ receptor phosphorylation and agonist-mediated μ receptor desensitization reveal remarkable parallels. Each can be detected as early as 3 min following application of modest agonist concentrations. Each is reversible but persists for minutes following agonist washout. These observations are consistent with a role for phosphorylation in agonist-induced desensitization, although neither of these parallels by itself provides proof for causal relationships.

The differential effects of staurosporine on agonist-induced and PKC-mediated responses, however, provide striking evidence that these two means of receptor modulation employ different biochemical pathways. While this PKC inhibitor eliminated PMA influences on desensitization, as anticipated, it failed to influence desensitizations induced by repeated administration of agonist alone. These results point to the likelihood that PKC activation may alter μ receptor phosphorylation in a fashion different from that induced by agonists, even though agonist activity at μ receptors can increase inositol trisphosphate turnover in a fashion that may well stimulate PKC (Johnson *et al.*, 1994; Smart *et al.*, 1994). Conceivably, these results could also point toward utilization of different μ receptor phosphorylation sites in different modes of receptor regulation. Ongoing experiments with μ receptors mutated in these sites will help to define such distinctions. They could argue as well for participation of different members of the growing family of G-protein-coupled receptor kinases in such distinct paths (Lefkowitz, 1993). Indeed, recent studies of agonist-dependent phosphorylation of the δ opiate receptor suggest involvement of one or more G-protein-coupled receptor kinases in this receptor's desensitization (Pei *et al.*, 1995).

Many brain neurons normally express several types of receptors. Effects of agonists at one receptor in desensitizing the same receptor are termed homologous desensitization, whereas effects of agonists at coexpressed receptors are termed heterologous desensitization. The presence of staurosporine-sensitive and staurosporine-insensitive pathways for stimulation of μ receptor phosphorylation makes more plausible the idea that μ receptor function could be modulated by prior exposure of cells to both agonists at the μ receptor and agonists at other coexpressed receptors. Conceivably, for example, heterologous desensitization could result from actions induced at coexpressed receptors by drugs often coadministered with opiates. Elucidating how dopamine/μ receptor costimulation as a result of psychostimulant/opiate coadministration or cannabanoid CB1/μ receptor costimulation with marijuana/opiate coadministration could alter the function of the μ receptor would help to understand the interactions between these commonly coadministered drug classes.

The expression systems used in current experiments rely on endogenous complements of G-proteins to interconnect μ receptors, cellular effectors, kinases, and G-protein-linked ion channels. Studies in normally receptor-expressing neurons may be required for accurate determination of the roles actually played by receptor phosphorylation events *in vivo*.

Agonist-induced desensitization, documented in functional assays, could be caused through a variety of mechanisms. Phosphoreceptor function could be altered while μ receptor was still expressed on cell surfaces, phosphoreceptor internalization could be speeded and/or phosphoreceptor degradation could be accelerated. Our data document recovery of receptor function within less than 3 min after beginning agonist washout in the

Xenopus system. This time course is poorly compatible with that expected for reversal of receptor internalization or degradation but fits well with the rapid time course with which phosphorylation/dephosphorylation cycles can be observed for a number of cellular signaling proteins. Indeed, μ receptor phosphorylation could be noted at above background levels within less than 2 min of agonist or PMA treatment. Functional and biochemical analyses of μ receptor phosphorylation thus make this receptor's phosphorylation a plausible candidate to contribute to the mechanisms of the physiologically observed acute agonist-induced desensitization. However, other cellular proteins, including the GIRK1 and G-proteins that are necessary for the responses measured here, could also represent plausible phosphorylation targets and possible sites for agonist-induced desensitization. Studies of the magnitudes, time courses, and physiological effects of GIRK1, G-protein, and other important protein phosphorylation events will allow comparisons with the results presented here for the μ receptor and help to place their roles in physiological desensitization processes into the appropriate context.

It will be of interest to localize the sites of phosphorylation on the receptor, to determine which protein kinases are responsible for agonist-induced desensitization, and to understand how different receptor phosphorylation events could interact with each other. Each of these results should help to elucidate the nature of the regulatory mechanisms employed by this important brain receptor for pain and pleasure.

Acknowledgments—We thank Dr. Henry Lester for providing the GIRK1 cDNA and Hui Sun for technical assistance.

REFERENCES

Arden, J. R., Segredo, V., Wang, Z., Lameh, J., and Sadee, W. (1995) *J. Neurochem.* **65,** 1636–1645
Chen, Y., and Yu, L. (1994) *J. Biol. Chem.* **269,** 7839–7842
Chen, Y., Mestek, A., Liu, J., Hurley, J. A., and Yu, L. (1993) *Mol. Pharmacol.* **44,** 8–12
Cheng, P. Y., Moriwaki, A., Wang, J. B., Uhl, G. R., and Pickel, V. M. (1995) *Analgesia* **1,** 363–366
Childers, S. R. (1991) *Life Sci.* **48,** 1191–2003
Dascal, N., Lin, N. F., Schreibmayer, W., Wang, W., Davidson, N., and Lester, H. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6596–6600
Eppler, M. C., Hulmes, J. D., Wang, J. B., Johnson, B., Corbett, M., Luthin, D. R., Uhl, G. R., and Linden, J. (1993) *J. Biol. Chem.* **268,** 26447–26451
Fukuda, K., Kato, S., Mori, K., Nishi, M., and Takeshima, H. (1993) *FEBS Lett.* **327,** 311–314
Herz, A. (1993) *Opioids, Handbook of Experimental Pharmacology,* Vol. 104, Springer-Verlag, New York
Johnson, P. S., Wang, J. B., and Uhl, G. R. (1994) *Regul. Pept.* **54,** 139–140
Kobilka, B. (1992) *Annu. Rev. Neurosci.* **15,** 87–114
Kovoor, A., Henry, D. J., and Chavkin, C. (1995) *J. Biol. Chem.* **270,** 589–595
Lefkowitz, R. J. (1993) *Cell* **74,** 409–412
North, R. A., Williams, J. T., Surprenant, A., and Christie, M. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5487–5491
Pei, G., Kieffer, B. L., Lefkowitz, R. J., and Freedman, N. J. (1995) *Mol. Pharmacol.* **48,** 173–177
Rothman, R. B., Xu, H., Wang, J. B., Partilla, J. S., Kayakiri, H., Rice, K. C., and Uhl, G. R. (1995) *Synapse* **21,** 60–64
Smart, D., Smith, G., and Lambert, D. G. (1994) *J. Neurochem.* **62,** 1009–1014
Surratt, C. K., Johnson, P. S., Moriwaki, A., Seidleck, B. K., Blaschak, C. J., Wang, J. B., and Uhl, G. R. (1994) *J. Biol. Chem.* **269,** 20548–20553
Thompson, R. C., Mansour, A., Akil, H., and Watson, S. J. (1993) *Neuron* **11,** 903–914
Tobin, A. B., and Nahorski, S. R. (1993) *J. Biol. Chem.* **268,** 9817–9823
Walaas, S. I., and Greengard, P. (1991) *Pharmacol. Rev.* **43,** 299–349
Wang, J. B., Imai, Y., Eppler, M. C., Gregor, P., Spivak, C., and Uhl, G. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10230–10234
Wang, J. B., Johnson, P. S., Persico, A. M., Hawkins, A. L., Griffin, C. A., and Uhl, G. R. (1994) *FEBS Lett.* **338,** 217–222 .