**μ** Opioid Receptor Phosphorylation, Desensitization, and Ligand Efficacy*

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μ opioid receptors are subject to phosphorylation and desensitization through actions of at least two distinct biochemical pathways: agonist-dependent μ receptor phosphorylation and desensitization induced by a biochemically distinct second pathway dependent on protein kinase C activation (1). To better understand the nature of the agonist-induced μ receptor phosphorylation events, we have investigated the effects of a variety of opiate ligands of varying potencies and intrinsic activities on μ receptor phosphorylation and desensitization. Exposure to the potent full agonists sufentanil, dibaudeorphanine, etorphine, etonitazene, and [d-Ala², MePhe⁴, Glyol⁵]enkephalin (DAMGO) led to strong receptor phosphorylation, while methadone, l-α-acetyl-methadone (LAAM), morphine, meperidine, DADL, β-endorphin(1–31) enkephalins, and dynorphin A(1–17) produced intermediate effects. The partial agonist buprenorphine minimally enhanced receptor phosphorylation while antagonists failed to alter phosphorylation. Buprenorphine and full antagonists each antagonized the enhanced μ receptor phosphorylation induced by morphine or DAMGO. The rank order of opiate ligand efficacies in producing μ receptor-mediated functional desensitization generally paralleled their rank order of efficacies in producing receptor phosphorylation. Interestingly, the desensitization and phosphorylation mediated by methadone and LAAM were disproportionate to their efficacies in two distinct test systems. This generally good fit between the efficacies of opiates in μ receptor activation, phosphorylation, and desensitization supports the idea that activated receptor/agonist/G-protein complexes and/or receptor conformational changes induced by agonists are required for agonist-induced μ receptor phosphorylation. Data for methadone and LAAM suggest possible contribution from their enhanced desensitizing abilities to their therapeutic efficacies.

Opioid receptors are G-protein coupled receptors that mediate the potent analgesic actions and addictive properties of morphine-derived compounds. Under physiological conditions, these receptors interact with endogenous opioid peptides to modulate pain-controlling pathways and circuits that modulate behaviors including mood and reward (2). μ opioid receptors interact with rapidly acting opioid drugs, such as heroin, to produce marked euphoria and behavioral reward. They are also primary targets of the slower and longer acting opioids, such as methadone and LAAM,† that represent the best current substitution therapeutics for opiate addiction (3).

μ receptors desensitize after repeated stimulation by opioid agonists, in fashions that display similarities to desensitizing events noted for other G-protein coupled receptors. Agonist-induced μ receptor desensitization can be correlated with receptor phosphorylation. μ receptors display naloxone-reversible phosphorylation and desensitize after morphine or DAMGO treatments (1). Both of these agonist-induced events are insensitive to pretreatments with the protein kinase C inhibitor staurosporine, which inhibits phorbol ester-induced μ receptor phosphorylation and desensitization.

Many opiates and opioid ligands can recognize μ receptors with high affinities. Plant-derived alkaloids, synthetic compounds of several classes, and endogenous opioid peptides can function as agonists, partial agonists, or antagonists with a range of abilities to induce or block induction of analgesia and euphoria. However, mutagenesis studies and studies with receptor chimeras support the idea that different receptor features could be involved in recognition of these different ligand classes (4–6). Mutations that change naloxone from a full antagonist to a partial agonist can alter the intrinsic activity of opioid peptides unchanged, for example see Claude et al. (7). These differences raise the possibility that μ receptor occupancies by opioid drugs of different classes could alter the conformation of the μ receptor in distinct fashions. Some of these differences could render the receptor an improved or a worse substrate for kinases and phosphatases and thus directly confer different susceptibilities to phosphorylation or dephosphorylation. Alternatively, selective activation of different G-protein classes by different μ agonists could trigger different μ receptor phosphorylation and desensitization events.

The current study thus investigates the effects of opioid ligands of various classes and varying intrinsic activities on μ receptor activation, phosphorylation, and desensitization using human μ receptors expressed in CHO cells (hμCHO), and the receptors coexpressed in Xenopus oocytes with a G-protein linked K⁺ channel. The results support striking parallels between opioid efficacies in opening ion channels and inhibiting

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‡The abbreviations used are: LAAM, l-α-acetyl-methadone; DAMGO, [d-Ala², MePhe⁴, Glyol⁵]enkephalin; CHO, Chinese hamster ovary cells; hμCHO, CHO cells stably expressing human μ receptor; PAGE, polyacrylamide gel electrophoresis.
adenyl cyclase activity and their efficacies in agonist-induced µ receptor phosphorylation and desensitization. The data also reveal that methadone and LAAM provide phosphorylation and desensitization disproportionate to their efficacies in mediating µ receptor-mediated ion channel activation or adenyl cyclase inhibition, differences that could conceivably contribute to their efficacies as principal current agonist-substitution anti-addiction therapeutics.

MATERIALS AND METHODS

Chemicals—Opioid peptides and naloxone were purchased from Research Biochemicals Inc. (Natick, MA); morphine and buprenorphine were purchased from Mallinckrodt Chemical Co (St. Louis, MO); and dihydroethorphine was a gift from Dr. Xiongqi Gong (China). Other opioid ligands were kindly provided by Dr. Richard Rothman and Dr. Heng Xu, (National Institute on Drug Abuse-IRP, Baltimore). All other chemicals and reagents were purchased from Sigma or as indicated in methods specifically.

µ Opioid Receptor Phosphorylation—Phosphorylation of the µ opioid receptor in hµCHO (8) was described as (1). Briefly, hµCHO and non-transfected Chinese hamster ovary (CHO) cells were plated at 80% confluence in 6-well plates and grown for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37 °C for 2 h with 300 µM of [32P]orthophosphate (8500 Ci/mmol; NEN Life Science Products) in phosphate-free Dulbecco's modified Eagle's medium. Labeled cells were then exposed to various opioid ligands at 1 µM for 20 min or to other treatment times and concentrations as indicated in the figure legends. Ligands and free 32P were removed from cells by washing with ice-cold phosphate-buffered saline; subsequent procedures were carried out at 4 °C. Cells were solubilized for 60 min with 0.8 ml of RIPA* buffer (1% IGEPA CA-630, 0.5% Na2 deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM Na2 pyrophosphate, 1 µM okadaic acid, 0.1% N-methylsulfonyl fluoride, 10 µM benzamidine, 10 µg/ml leupeptin, and 1 µg/ml pepstatin A in phosphate-buffered saline buffer). Proteins were separated on 8% SDS-PAGE gels with prestained molecular mass standards (Amersham), and radiolabeled proteins were identified by autoradiography using Hyperfilm-MP (Amersham) with intensifying screens. Autoradiographic densities of bands of interest were quantified by scanning densitometry and normalized to the amounts of extracted cell protein subjected to immunoprecipitation.

Xenopus Oocyte µ Opioid Receptor/G-Protein-activated K Channel Coexpression—cDNAs encoding the human opioid µ receptor (8) and GIRK1 (9) were subcloned into the expression vector pcDNAI, plasmids containing bound cAMP was assessed by liquid scintillation counting. n=3, values are mean ± S.E.

Electrophysiological Recording—Whole cell currents in expressing oocytes were measured at 22 °C under 2-electrode voltage clamped 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 NaCl, 2.5 mM CaCl2, 25 mM sodium pyruvate, 10,000 units/liter penicillin, 10 mg/ml streptomycin, and 0.5 µg/ml theophylline). Buprenorphine

| Drug       | Maximum inhibition | EC50  |
|------------|--------------------|-------|
| DAMGO      | 88%                | 20.5 ± 4.9 |
| Morphine   | 75%                | 5.3 ± 0.14 |
| Methadone  | 75%                | 16.0 ± 1.7 |
| LAAM       | 44%                | 10.3 ± 4.6 |
| Buprenorphine | 42%             | 0.02 ± 0.01 |

During which opioid agonists were transiently applied with opioid antagonists, or ND96 containing phorbol esters (Sigma) in di- methyl sulfoxide (Me2SO) concentrations less than 0.01%. Values presented are mean ± standard error (S.E.). Concentration-response curves were obtained using the program NFTIF by fitting data to the logistic equation, y = (Emax - Emin)/(1 + [EC50]^-1) + Emin, where x represents concentration, y represents response, Emax represents the maximal response, Emin represents the minimal response, EC50 represents the half-maximal concentration, and n represents the apparent Hill coefficient.

Determination of Adenyl Cyclase Activity in hµCHO Cells—hµCHO cells were cultured as described above, harvested, washed with 2.0 mM Tris-HCl, pH 7.4, 2.0 mM EDTA buffer, and suspended in the same Tris buffer. Cell suspension corresponding to 30 µg of protein/sample was added on ice to assay tubes containing 10 µM forskolin, assay buffer (80 mM Tris, pH 7.4, 10 mM theophylline, 1 mM MgSO4, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 0.01 mM GTP) and tested drugs. Triplicate samples for each treatment were incubated at 37 °C for 10 min, adenyl cyclase activity was terminated by boiling for 2 min, and the amounts of CAMP formed were determined by a cAMP protein binding assay as described (10, 11). Briefly, 4 mM [3H]cAMP (Amersham, 39 Ci/mmol), and bovine adrenal binding protein preparations were incubated with samples at 4 °C for 90 min. Assays were terminated by adding charcoal and centrifuging, and supernatant radioactivity containing bound cAMP was assessed by liquid scintillation counting. Dose-response curves were obtained by nonlinear regression analyses using PRISM (GraphPad Software, Inc. San Diego, CA).

RESULTS

22 compounds, representing each of the major opiate and opioid classes, produced substantially different effects on µ receptor phosphorylation when tested in receptor phosphorylation assays at screening dose concentrations (1 µM) that were significantly above their previously reported 0.1–10 nM K values (Table 1) (12). When drugs were incubated with hµCHO cells at this concentration for 20 min, dihydroethorphine, sufentanil, etorphine, etonitazaine, and DAMGO caused increased µ receptor phosphorylation producing values 6–8-fold above basal levels. Methadone, morphine, meperidine, DADL, β- endorphin(1–31), Met-enkephalin, Leu-enkephalin, and dynorphin A1–17, produced intermediate effects, ~2–5 fold above basal levels. Neither the partial agonist buprenorphine, the largely µ agonists ketocyclazocine and α-neoendorphin, the mixed agonist/antagonist pentazocine, the mixed κ/µ agonist butorphanol, the weak µ agonists meperidine and 3-hydroxy-5-phenylmorphin, or the µ antagonists naloxonazine and LY2555582 had any robust effect on µ receptor phosphorylation. Each stimulated phosphorylation levels less than two basal levels (Fig. 1A–D). Phosphorylation was reversible. Addition of the oxo analog without incubation with reduced µ receptor phosphorylation levels to close to basal values by 30 min (Fig. 2). The partial agonist properties of buprenorphine extended to its effects on µ receptor phosphorylation. 1 µM buprenorphine blocked µ receptor phosphorylation when co- incubated with 1 µM concentrations of the fuller µ agonists morphine or DAMGO (Fig. 3).

Since these results indicated that different opioid ligands

TABLE I

Inhibition of forskolin-stimulated adenyl cyclase activity in hµCHO cells

hµCHO cells were treated with 10 µM forskolin and varying opiate concentrations for 10 min. Data points represent mean ± S.E. from four independent experiments, each performed in triplicate.
could display differential influence on μ receptor phosphorylation, we selected eight representative opioid ligands for further study. Potencies and efficacies of etorphine, sufentanil, DAMGO, methadone, LAAM, morphine, buprenorphine, and 3-m-hydroxy-5-phenylmorphan were assessed in opening a G-linked K\(^+\) channel coexpressed with the μ receptor in Xenopus oocytes and in inhibiting adenylyl cyclase in h\(\mu\) CHO cells. These studies provided assessments of the efficacies of these compounds, with rank order etorphine > sufentanil > DAMGO > morphine > methadone > LAAM > 3-m-hydroxy-5-phenylmorphan > buprenorphine (Fig. 4, Table I). The profound desensitization exerted by sufentanil and etorphine did not allow us to accurately assess their potencies in adenyl cyclase inhibition.

Each of these opioid ligands also exerts a concentration-dependent effect on receptor phosphorylation (Fig. 5). Significant agonist-induced μ receptor phosphorylation enhancements were observed at concentrations as low as 10 pM for etorphine, 100 pM for sufentanil, more than 10 nM for DAMGO, 100 nM for methadone and LAAM and more than 100 nM for morphine. Concentrations of buprenorphine yielding robust receptor phosphorylation were 100,000 times higher than effective concentrations of etorphine, whereas even 100 μM phenylmorphan treatments barely altered μ receptor phosphorylation.

Desensitization, examined in both the Xenopus oocyte system and h\(\mu\) CHO (Fig. 6 and 7), was mediated by etorphine > sufentanil > DAMGO > methadone > LAAM > morphine > buprenorphine (in h\(\mu\) CHO) or 3-m-hydroxy-5-phenylmorphan (in Xenopus oocyte). The rank order of the desensitizing abilities of these opioid ligands was thus similar to their rank order for efficacies, with two exceptions. Methadone and LAAM each led to more dramatic receptor desensitization than morphine, despite efficacies and potencies in producing μ receptor-mediated K\(^+\) channel opening and adenylyl cyclase inhibition that
Effects of Opioid Ligands on \( \mu \) Receptor Phosphorylation

Drugs were added to HK and the amplitude of current activated by the HK vehicle alone. The current findings substantially expand data documenting relationships among the effects of different opioid ligands on \( \mu \) opioid receptor activation, phosphorylation, and desensitization (1, 13, 14). The parallels between ligand efficacy and desensitization in the current data are consistent with the relationships between the properties of these opioid ligands in neurally derived cell lines that endogenously express \( \delta \) opioid receptors (15). Correlations between the efficacies of these opioid ligands in receptor activation and their abilities to cause \( \mu \) receptor phosphorylation, relationships between receptor desensitization and phosphorylation, and parallels between time course and dose-response relationships for \( \mu \) receptor phosphorylation and desensitization (1) each support the possibility that causal relationships could exist among agonist efficacy in ion channel opening, adenylyl cyclase inhibition, receptor phosphorylation and desensitization.

Studies from several laboratories, but not all, have demonstrated desensitization observed in agonist-treated \( \mu \) receptor/GIRK coexpressing Xenopus oocytes by treatments that alter levels of kinases including calcium/calcmodulin-dependent protein kinase and protein kinase C (1, 16, 17). Only one report (1) separated a kinase-dependent pathway from an agonist-induced pathway. Kovoor et al. (18), however, interpreted data from a similar system to suggest that desensitization may not involve direct receptor phosphorylation. Although the results from the current study do not document direct evidence for \( \mu \) receptor phosphorylation in Xenopus oocytes, the similar patterns of opioid ligand efficacies observed in the Xenopus oocyte and the \( \mu \)CHO systems are totally consistent with the idea that the mechanisms of receptor desensitization found in these two distinct test systems are likely to be similar. Studies of phosphorylation and desensitization patterns in receptor mutants with systematic alanine substitutions for individual candidate phosphoacceptor sites will help to add evidence to the increasingly strong current hypothesis that \( \mu \) receptor phosphorylation is targeted by agonist activation and leads to desensitization.

The parallels between higher ligand efficacy and enhanced receptor phosphorylation that suggest that an activated receptor conformation may enhance \( \mu \) receptor phosphorylation have also been described for \( \beta 2 \) adrenergic receptors (19). The data from methadone and LAAM, on the other hand, provide highly interesting exceptions to this general rule. Methadone and LAAM represent some of the current most effective anti-addiction substitution pharmacotherapeutics (3, 20). Although their long-acting human pharmacologies have been considered important for their therapeutic efficacies (21), detailed studies of these mechanisms of action have not been carried out. More prominent receptor phosphorylation and desensitization could enhance the abilities of both methadone and LAAM to block effects of subsequent doses of addictive opiates, possibly

![Concentration-response relationships for opioid ligands (log, M)](image)

**Fig. 4.** Concentration-response relationships for opioid ligand-activated \( K^+ \) currents in Xenopus oocytes coexpressing \( \mu \) opioid receptors and \( G \)-protein activated inward rectifier potassium channels (GIRK1). Coexpressing oocytes had membrane potentials clamped at \(-70\) mV, drugs were applied, and the amplitude of each current response amplitude was determined as the difference between the amplitude of peak current activated by that opioid ligand in HK and the amplitude of current activated by the HK vehicle alone. Opioid activated currents are shown as responses normalized to that of 10 \( \mu M \) morphine. Each data point represents mean \( \pm \) S.E. of the recordings from 5–7 oocytes. The curves shown were fitted to the data using the logistic equation described in the methods. Some error bars not visible since they are smaller than the symbol sizes. The order of maximal response obtained are: etorphine \((241 \pm 10\%, n = 5) > \) sufentanil \((216 \pm 14\%, n = 7) > \) AMGO \((157 \pm 14\%, n = 7) > \) morphine \((100 \pm 7\%, n = 7) > \) methadone \((80 \pm 13\%, n = 5) > \) LAAM \((72 \pm 3\%) > \) phenylmorphan \((35 \pm 3\%, n = 6) > \) buprenorphine \((13 \pm 2\%, n = 7)\).

![Receptor phosphorylation autoradiography](image)

**Fig. 5.** Concentration-dependent effects of opioid ligands on \( \mu \)-receptor phosphorylation. Drugs were added to expressing cells as noted, and \( \mu \) receptor phosphorylation assessed as noted above. The autoradiography shows results representative of three independent experiments.
contributing to their pharmacological blockade properties and therapeutic efficacies.

Studies of ligand interactions with mutant µ receptors suggest that different ligand classes recognize amino acids lying in different µ receptor domains. Receptor occupancies by opioid drugs of different classes might thus alter µ receptor conformations in distinct fashions. The ligand-to-ligand differences in µ receptor phosphorylation observed in the present studies could reflect ligand-to-ligand differences in receptor/ligand conformations that could improve, or worsen its ability to serve as a substrate for activated kinases or phosphatases. If methadone and LAAM occupancies produced a µ receptor conformation that resulted in better access to kinases without enhanced efficacy in G-protein activation, for example, its observed differences from morphine could be explained. Distinct ligand/µ receptor conformations could also differentially activate different G-proteins. More prominent activation of selected G-proteins by µ receptors occupied by methadone or LAAM could also conceivably yield more strongly enhanced efficacies in activating the kinases that lead to receptor phosphorylation than those that activate ion channels or inhibit adenylyl cyclase, providing another explanation for the observed methadone/morphine differences described here. Further experiments that test effects of µ receptor mutants and chimeras on methadone, LAAM, and morphine affinities and efficacies, as well as association and dissociation rates for receptor binding, may provide further evidence to support the relative contributions of each of these mechanisms.

Desensitization could represent the inability of µ receptors still expressed on cell surfaces to activate appropriate G-proteins when they bind agonist ligands. Removal of µ receptors from cell surfaces to sites where they can no longer transduce ligand recognition to G-protein activation could also contribute to the observed patterns of desensitization. There is some cor-

![Figure 6](image1.png) **FIG. 6.** Opioid ligand-induced desensitization of µ opioid receptor mediated responses in Xenopus oocytes. Oocytes were preincubated for 20 min with indicated drugs in ND96 and then tested for µ receptor-mediated K+ channel responses following application of the same ligands. A, records of currents activated by opioid ligand before and after 20-min agonist preincubations. Open bar, period of HK solution superfusion; solid bar, period of opioid ligand application in HK. B, average decreases in opioid receptor-mediated K+ channel responses as compared with no preincubation controls. Each data point represents mean ± S.E. of the recordings from 5–7 oocytes. Decreases in current amplitude induced each opioid ligand after preincubation are: etorphine, 79 ± 6%; sufentanil, 73 ± 6%; LAAM, 52 ± 4%; DAMGO, 50 ± 7%; methadone, 49 ± 4%; morphine, 19 ± 9%, and phenylmorphan, 0% of control currents.

![Figure 7](image2.png) **FIG. 7.** Opioid ligand-induced desensitization of µ opioid receptor mediated responses in hµCHO cells. hµCHO cells were preincubated for 20 min with indicated drugs in culture medium; each drug concentration was 100 times of its EC50 (Table I) and then was tested for µ receptor mediated adenylyl cyclase inhibition as described under “Materials and Methods,” with application of the ligands at the same concentration. Percentile changes in opioid receptor mediated cAMP production as compared with no preincubation controls are shown. Each data point represents mean ± S.E. of two independent experiments, each performed in triplicate.
relation between desensitization results and the loss of membrane receptors in some expressing cell systems (22). Ligand-dependent opioid receptor internalization has been reported (23). μ receptor internalization in CHO cells can be mediated by opiate agonists in the rank order etorphine > DAMGO > methadone in initial studies. However, morphine treatments provoke no internalization at concentrations up to 10 μM.2 The time courses for agonist-induced μ receptor phosphorylation are also faster than the reported losses of membrane receptors (1, 22). Receptor dephosphorylation and resensitization both take place within minutes of agonist removal (Fig. 2). Each of these lines of evidence suggests significant differences between the internalization results and the results obtained for phosphorylation and for desensitization. It thus seems unlikely that internalization contributes to all of the desensitizing effect observed in the current studies. Further studies, including those using phosphoacceptor site mutants, may well document which contributions internalization may make to agonist-induced desensitization and the role that phosphorylation may play in each event.

G-protein receptor kinases (GRKs) readily phosphorylate the ligand-activated forms of several G-protein coupled receptors but can find unoccupied or antagonist-occupied receptors poor substrates (24). Desensitization of μ receptors, observed in systems including CHO cells, Xenopus oocytes, SH-SY5Y cells, and locus coeruleus neurons (1, 25–28), appears unlikely to require a kinase of limited distribution. It is conceivable that a widely distributed member of the G-linked receptor kinase family, or another broadly-distributed kinase, could be responsible for agonist-induced μ receptor phosphorylation. Regulation of dephosphorylation could also play a significant role in the accumulation of phosphorylated receptor species.

The pharmacological profile of μ opioid receptor efficacy, phosphorylation, and desensitization that these data define documents that phosphorylation and desensitization are widespread consequences of opioid receptor occupancy by agonists that include opioid peptides. Responses to both exogenous opioids and endogenous opiates are thus both likely to desensitize through broadly distributed biochemical mechanisms. Conceivably, these data will also help to explain the especial therapeutic efficacies of methadone and LAAM through enhanced abilities to phosphorylate and desensitize the μ opioid receptors that contribute so substantially to opioid-mediated reward.

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