It is generally accepted that the internalization and desensitization of \(\mu\)-opioid receptor (MOR) involves receptor phosphorylation and \(\beta\)-arrestin recruitment. However, a mutant MOR, which is truncated after the amino acid residue Ser\(^{363}\) (MOR363D), was found to undergo phosphorylation-independent internalization and desensitization. As expected, MOR363D, missing the putative agonist-induced phosphorylation sites, did not exhibit detectable agonist-induced phosphorylation. MOR363D underwent slower internalization as reflected in the attenuation of membrane translocation of \(\beta\)-arrestin 2 when compared with wild type MOR, but the level of receptor being internalized was similar to that of wild type MOR after 4 h of etorphine treatment. Furthermore, MOR363D was observed to desensitize faster than that of wild type MOR upon agonist activation. Surface biotinylation assay demonstrated that the wild type receptors recycled back to membrane after agonist-induced internalization, which contributed to the receptor resensitization and thus partially reversed the receptor desensitization. On the contrary, MOR363D did not recycle after internalization. Hence, MOR desensitization is controlled by the receptor internalization and the recycling of internalized receptor to cell surface in an active state. Taken together, our data indicated that receptor phosphorylation is not absolutely required in the internalization, but receptor phosphorylation and subsequent \(\beta\)-arrestin recruitment play important roles in the resensitization of internalized receptors.

The regulation of G protein-coupled receptors (GPCRs)\(^1\) signaling highlights the complex relationship between multiple mechanisms acting at different levels of signal propagation. Following the agonist activation of the receptor, G protein-coupled receptor kinases (GRKs) are recruited to the membrane and phosphorylate the agonist-activated GPCRs. GRK-mediated receptor phosphorylation enhances the interaction between GPCRs and cytoplasmic proteins, arrestins, which uncouple the GPCRs from G proteins and also target the GPCRs to clathrin-coated pits for internalization (endocytosis). The internalized receptors can subsequently be dephosphorylated and recycled back to the plasma membrane, which contributes to resensitization. Alternatively, the internalized receptors can be targeted to lysosomes for degradation (1–4). Receptor desensitization, the progressive loss of receptor function under continued exposure to an agonist, occurs in many GPCRs. Desensitization can be a consequence of multiple processes of receptor uncoupling, internalization, degradation, and recycling. Therefore, phosphorylation of agonist-activated receptors and subsequent arrestin recruitment are important processes in the modulation of GPCRs responsiveness.

However, multiple studies challenge the critical role phosphorylation played in desensitization and internalization of many GPCRs. Desensitization and internalization of human parathyroid hormone receptor (5), \(\alpha_{1A}\) angiotensin receptor (6), and metabotropic glutamate receptor (7) are independent of agonist-induced phosphorylation of the receptor. C-terminally truncated \(\delta\)-opioid receptor (8) and human substance P receptor (9) without any detectable phosphorylation undergo normal, dynamin-dependent endocytosis. Internalization of \(\alpha_2\) muscarinic acetylcholine receptor (10) and somatostatin receptor 2A (11) is reported to be independent of \(\beta\)-arrestin. Thus, these observations suggest a more complicated picture in the regulation of GPCRs.

The \(\mu\)-opioid receptor (MOR) belongs to the rhodopsin subfamily of GPCRs. Similar to other members of this family, MOR undergoes receptor phosphorylation, desensitization, internalization, and recycling upon agonist activation. The roles of phosphorylation and the subsequent events in MOR desensitization and internalization have been extensively studied. Overexpression of GRK2 increases agonist-induced phosphorylation and promotes the processes of internalization and desensitization (12). The time course and dose-response relationships between phosphorylation and agonist-induced desensitization display parallels in CHO cells stably expressing MOR (13). The absence of agonist-induced phosphorylation by the mutation of putative phosphorylation sites to alanine completely blocks or significantly attenuates the agonist-induced receptor desensitization (14, 15) and internalization (16). The enhanced phosphorylation of MOR has been correlated with the desensitization of MOR in thalamus of rats chronically treated with morphine (17). All these results support the hypothesis that receptor phosphorylation leading to subsequent cellular events is necessary for the functional regulation of MOR. However, other reports have suggested the role of MOR desensitization and internalization is controlled by cellular events other than receptor phosphorylation. There is a discrepancy between the rate of MOR phosphorylation and desensitization (18). A recep-
α-Opioid Receptor Phosphorylation and Desensitization

The potency of etorphine to inhibit forskolin-stimulated intracellular cAMP levels (K<sub>d</sub> 0.16 nM) and the ability of the α-opioid receptor agonist, etorphine, to induce receptor desensitization and internalization was examined.

EXPERIMENTAL PROCEDURES

Materials—β-arrestin 2 tagged with GFP at the C terminus (in pEGFP-N1) was kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Cell culture reagents, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml sodium penicillin were purchased from Life Technologies Inc. laboratories (South San Francisco, CA). Alexa<sup>TM</sup>-488 goat anti-mouse IgG was purchased from Zymed Laboratories (South San Francisco, CA). Mouse monoclonal anti-hemagglutinin protein (HA) antibody (HA.11, 1:2000) and goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000). Blots were developed using enhanced chemiluminescence substrate (Amersham Biosciences) and scanned with Storm 840.

Surface Biotinylation Assay—Stably transfected N2A cells expressing wild type MOR or MOR363D receptors were biotinylated at 4 °C with 900 μg/ml sulfo-NHS-SS-biotin in phosphate-buffered saline for 30 min. Biotinylation reaction was quenched by rinsing with Tris-buffered saline (154 mM NaCl, 10 mM Tris, pH 7.4). Then the cells were washed to 37 °C and incubated with 50 μM monensin for 1 h or not, followed by incubation with 1 μM etorphine for 30 min or 2 h. The remaining cell surface biotin was cleaved with a stripping buffer (50 mM glutathione, 0.3 mM NaCl, 75 mM NaOH, and 1% Triton X-100) in a 10% CO<sub>2</sub> incubator at 37 °C. Determination of Receptor Desensitization and Resensitization by Measurement of Intracellular cAMP Levels—Approximately 4 × 10<sup>5</sup> cells/well were seeded in 96-well plates the day before experiment. For desensitization assays, cells were exposed to 1 μM etorphine, morphine, or DAMGO for 0, 0.5, 1, 2, 4, or 6 h. For resensitization experiments, the cells were washed after 4 h of etorphine exposure and incubated at 37 °C for different periods (0, 10, 20, 30, or 60 min) in the absence of etorphine. Then the medium was removed and replaced with 100 μl treatment buffer with or without agonist. The treatment buffer consisted of 0.5 mM isobutylmethyloxanthine (IBMX) and 10 μM forskolin in Krebs-Ringer-HEPES buffer (KRHi; 110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, pH 7.4). The cells were then incubated for 15 min at 37 °C. The reaction was terminated by heating cells at 90 °C for 4 min. Then cells were cooled on ice for 10 min and stored at 4 °C until CAMP assay. The measurement of CAMP level in supernatant was performed by using AlphaScreen<sup>TM</sup> cAMP detection kit (BioSignal, Montreal, Canada).

Determination of Receptor Internalization by FACS Analysis—N2A cells stably expressing MOR or MOR363D were incubated with 1 μM etorphine for the indicated time intervals. Cells were then chilled on ice to stop membrane trafficking, and all subsequent incubations were performed at 4 °C. Residual cell surface receptors were monitored by incubating cells with anti-HA antibody (1:500) in serum-free DMEM for 1 h followed by an incubation with the Alexa 488-conjugated anti-mouse IgG antibody (1:500) for 1 h. Surface receptor staining intensity of antibody-labeled cells was analyzed using fluorescence flow cytometry (FACScan, BD Biosciences). Fluorescence intensity of 10,000 cells was collected for each sample. All experiments were conducted at least three times with triplicate samples.

Receptor Phosphorylation—Cells cultured in 150-mm dishes were incubated with 1 μM etorphine for 30 min at 37 °C. The reactions were terminated on ice at which point the cells were washed with ice-cold phosphate-buffered saline and subsequently lysed in 3 ml lysis buffer (1% Triton X-100, 100 mM NaCl, 25 mM HEPES, pH 7.4, with 0.1 mM phenylmethanesulfonfyl fluoride, 40 μg/ml Complete<sup>®</sup> protease inhibitors mixture (Roche Applied Science), and with 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.1 mM sodium vanadate as phosphatase inhibitors). After solubilization, insoluble debris was removed by centrifugation at 14,000 × g for 15 min at 4 °C. Then the samples were incubated in the presence of 10 μg of rabbit anti-phosphorylated protein antibody, mouse mono- and polyclonal antibodies to β-arrestin 2 (Pierce) overnight at 4 °C. The final samples were eluted from the agarose beads with 2× SDS sample buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8) and separated on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The immunoprecipitated receptors were detected by incubation with mouse anti-HA (HA.11, 1:2000) and goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000). Blots were developed using enhanced chemiluminescence substrate (Amersham Biosciences) and scanned with Storm 840.

Confocal Imaging—N2A cells stably expressing wild type or mutant MOR were transiently transfected with GFP-tagged β-arrestin 2 using the transfection reagent effectene (Qiagen). Briefly, cells cultured in 6-well plates were transfected with 0.8 μg GFP-tagged β-arrestin 2. 24 h after transfection, the cells were harvested and plated onto poly-t-lysine coated coverslips. Treatments were carried out 48 h post-transfection and the cells fixed in 3.7% formaldehyde. The cellular location of GFP-tagged β-arrestin 2 was visualized with a Bio-Rad confocal microscope (MRC 1024).

Statistical Analysis—Data are presented as mean ± S.E. Either Student’s t test (two-tailed) or a one-way analysis of variance (ANOVA) was performed for statistical comparisons. When ANOVA was used and when this analysis indicated significance (p < 0.05), Dunnett’s multiple comparison test was used to determine which conditions were significantly different from controls.

RESULTS

Phosphorylation of MOR and MOR363D and the Translocation of β-Arrestin—Several studies report that the C terminus of MOR is involved in receptor phosphorylation (14–16, 21). Two research groups (14, 21) report that Thr<sup>180</sup> is a crucial residue for DAMGO-induced phosphorylation and desensitization of MOR in CHO cells. Our studies (16) using mutagenesis analysis have indicated three Ser/Thr residues (Ser<sup>363</sup>, Thr<sup>370</sup>,...
and Ser375) in the C terminus of MOR as putative phosphorylation sites in HEK 293 cells. Basal phosphorylation is observed at Ser363 and Thr370, whereas the agonist-induced phosphorylation sites in HEK 293 cells. Basal phosphorylation is observed 24 h after transfection, cells were treated with 1 μM etorphine for 30 min. Phosphorylated receptor was analyzed by immunoprecipitation with anti-phosphorylated proteins antibody. Arrow indicates the phosphorylated receptor. B, cells stably expressing MOR or MOR363D were transiently transfected with GFP-tagged β-arrestin 2. 48 h after transfection, cells were treated with 1 μM etorphine for 2 or 15 min. Cells were fixed by 3.7% formaldehyde to stop the reaction. The arrestin localization was examined by confocal microscopy.

MOR363D were transiently transfected with GFP-tagged β-arrestin 2 because MOR is shown to belong to class A GPCR that binds β-arrestin 2 with higher affinity than β-arrestin 1 and does not interact with visual arrestin (22). The GFP-arrestin recruited to the membrane was detected visually by confocal microscopy. Consistent with previous studies (23), activation of wild type MOR induced by 1 μM etorphine strongly promoted recruitment of β-arrestin 2 to plasma membrane as reflected by the punctuate formation at the membrane (Fig. 1B). This process occurred rapidly after receptor activation (~2 min) with less β-arrestin 2 being translocated after 15 min of agonist treatment. On the contrary, agonist activation of MOR363D did not significantly enhance the translocation of β-arrestin 2 from the cytosol to the plasma membrane (Fig. 1B). This result is consistent with the deficiency of agonist-induced phosphorylation of the MOR363D.

**Internalization of MOR and MOR363D**—To investigate the capacity of MOR363D to undergo agonist-induced internalization, the time course of receptor internalization was evaluated by FACS analysis. MOR363D still internalized in the presence of 1 μM etorphine, although the process of internalization was slower than that observed with MOR. MOR363D internalized with half-life (t 1/2) = 2.2 ± 0.34 h, whereas MOR internalized with t 1/2 = 0.22 ± 0.13 h (Fig. 2). However, the levels of receptor being internalized for MOR and MOR363D were similar after 4 h treatment with etorphine, reaching the maximum of 62 ± 11% and 65 ± 5.0% after 6 h of etorphine treatment, respectively (Fig. 2). To exclude the clonal variation, a different N2A clone (clone number 15), which expressed 0.58 pmol/mg protein of MOR363D receptor, was employed and similar internalization kinetics was obtained with t 1/2 = 2.6 ± 0.30 h and maximal internalization rate was 74 ± 10.2% after 6 h of etorphine treatment (data not shown). Thus, it is evident that phosphorylation of the receptor and subsequent β-arrestin recruitment are not obligatory for agonist-induced internalization of MOR363D. But the rapid internalization rate observed with MOR is consistent with the phosphorylation and significant β-arrestin recruitment, suggesting that phosphorylation is involved in and facilitated the internalization of MOR.

**Desensitization of MOR and MOR363D**—We next examined whether the observed differences in receptor internalization would affect the rate of agonist-induced desensitization. N2A cells expressing MOR or MOR363D were pre-incubated with 1 μM etorphine for various time periods followed by the measurement of agonist-induced inhibition of forskolin-stimulated
cAMP production. Wild type MOR exhibited substantial desensitization with 32 ± 6.3% loss in etorphine inhibition of forskolin-stimulated cAMP production after 6 h of agonist exposure. Unexpectedly, a more pronounced desensitization was observed with MOR363D, with 57 ± 8.8% loss in etorphine activity after 6 h agonist exposure (Fig. 3). Similar pronounced desensitization rate was obtained in another MOR363D-expressing N2A clone (clone number 15), which showed 60 ± 9.3% loss in etorphine activity after 6 h of agonist exposure (data not shown). Opioid agonists with different efficacy exhibit different capacities to induce desensitization (24, 25), and andaloid and peptide agonists induce differential desensitization kinetics on δ-opioid receptors (26). Thus the ability of morphine, an agonist with lower efficacy than etorphine and DAMGO, a peptide μ-opioid agonist, to induce receptor desensitization were also tested. Similarly, MOR363D-expressing cells exhibited faster desensitization rates following morphine or DAMGO pretreatment (data not shown). These results suggest that phosphorylation is not required for agonist-induced desensitization of MOR363D.

Phosphorylation-deficient MOR363D exhibited a lower agonist-induced internalization rate but a markedly faster receptor desensitization rate. Internalization can enhance desensitization by reducing functional receptors at the cellular membrane or can reduce desensitization by promoting receptor recycling to cell surface. μ-Opioid receptor is reported to recycle back to membrane and resensitize after internalization (27, 28). Thus, the slower desensitization rate of wild type MOR could be attributed to its faster recycling, which is associated with resensitization. The truncated carboxyl tail sequence missing in MOR363D could prevent recycling and resensitization. To test this hypothesis, we carried out desensitization assays in the presence of 50 μM monensin, which prevents receptor recycling by trapping the internalized receptors in endosomes. As shown in Fig. 3, upon monensin pretreatment, wild type MOR desensitized in a faster rate, which was significantly different from that observed in the absence of monensin. This rate was comparable similar to the desensitization rate of MOR363D observed in the absence of monensin. The addition of monensin did not significantly alter the agonist-induced desensitization rate of MOR363D. Again, the effects of monensin on morphine- or DAMGO-induced receptor desensitization were also studied, and similar increases in the desensitization kinetics were observed (data not shown).

**The Fate of Internalized Receptors**—To further characterize the fate of internalized receptor, surface biotinylation assays were carried out. Cells expressing wild type MOR or MOR363D were biotinylated with a cleavable biotin label as described under “Experimental Procedures.” After agonist treatment, biotinylated receptors were either recycled and resensitized, or degraded. The internalized receptors in MOR363D-expressing cells after 30 h of agonist exposure (Fig. 3). Similar pronounced desensitization rate was obtained in another MOR363D-expressing N2A clone (clone number 15), which showed 60 ± 9.3% loss in etorphine activity after 6 h of agonist exposure (data not shown). Opioid agonists with different efficacy exhibit different capacities to induce desensitization (24, 25), and andaloid and peptide agonists induce differential desensitization kinetics on δ-opioid receptors (26). Thus the ability of morphine, an agonist with lower efficacy than etorphine and DAMGO, a peptide μ-opioid agonist, to induce receptor desensitization were also tested. Similarly, MOR363D-expressing cells exhibited faster desensitization rates following morphine or DAMGO pretreatment (data not shown). These results suggest that phosphorylation is not required for agonist-induced desensitization of MOR363D.

**Phosphorylation-deficient MOR363D exhibited a lower agonist-induced internalization rate but a markedly faster receptor desensitization rate. Internalization can enhance desensitization by reducing functional receptors at the cellular membrane or can reduce desensitization by promoting receptor recycling to cell surface. μ-Opioid receptor is reported to recycle back to membrane and resensitize after internalization (27, 28). Thus, the slower desensitization rate of wild type MOR could be attributed to its faster recycling, which is associated with resensitization. The truncated carboxyl tail sequence missing in MOR363D could prevent recycling and resensitization. To test this hypothesis, we carried out desensitization assays in the presence of 50 μM monensin, which prevents receptor recycling by trapping the internalized receptors in endosomes. As shown in Fig. 3, upon monensin pretreatment, wild type MOR desensitized in a faster rate, which was significantly different from that observed in the absence of monensin. This rate was comparable similar to the desensitization rate of MOR363D observed in the absence of monensin. The addition of monensin did not significantly alter the agonist-induced desensitization rate of MOR363D. Again, the effects of monensin on morphine- or DAMGO-induced receptor desensitization were also studied, and similar increases in the desensitization kinetics were observed (data not shown).

**The Fate of Internalized Receptors**—To further characterize the fate of internalized receptor, surface biotinylation assays were carried out. Cells expressing wild type MOR or MOR363D were biotinylated with a cleavable biotin label as described under “Experimental Procedures.” After agonist treatment, biotinylated receptors were either recycled and resensitized, or degraded. The internalized receptors in MOR363D-expressing cells after 30 h of agonist exposure (Fig. 3). Similar pronounced desensitization rate was obtained in another MOR363D-expressing N2A clone (clone number 15), which showed 60 ± 9.3% loss in etorphine activity after 6 h of agonist exposure (data not shown). Opioid agonists with different efficacy exhibit different capacities to induce desensitization (24, 25), and andaloid and peptide agonists induce differential desensitization kinetics on δ-opioid receptors (26). Thus the ability of morphine, an agonist with lower efficacy than etorphine and DAMGO, a peptide μ-opioid agonist, to induce receptor desensitization were also tested. Similarly, MOR363D-expressing cells exhibited faster desensitization rates following morphine or DAMGO pretreatment (data not shown). These results suggest that phosphorylation is not required for agonist-induced desensitization of MOR363D.

**Phosphorylation-deficient MOR363D exhibited a lower agonist-induced internalization rate but a markedly faster receptor desensitization rate. Internalization can enhance desensitization by reducing functional receptors at the cellular membrane or can reduce desensitization by promoting receptor recycling to cell surface. μ-Opioid receptor is reported to recycle back to membrane and resensitize after internalization (27, 28). Thus, the slower desensitization rate of wild type MOR could be attributed to its faster recycling, which is associated with resensitization. The truncated carboxyl tail sequence missing in MOR363D could prevent recycling and resensitization. To test this hypothesis, we carried out desensitization assays in the presence of 50 μM monensin, which prevents receptor recycling by trapping the internalized receptors in endosomes. As shown in Fig. 3, upon monensin pretreatment, wild type MOR desensitized in a faster rate, which was significantly different from that observed in the absence of monensin. This rate was comparable similar to the desensitization rate of MOR363D observed in the absence of monensin. The addition of monensin did not significantly alter the agonist-induced desensitization rate of MOR363D. Again, the effects of monensin on morphine- or DAMGO-induced receptor desensitization were also studied, and similar increases in the desensitization kinetics were observed (data not shown).

**The Fate of Internalized Receptors**—To further characterize the fate of internalized receptor, surface biotinylation assays were carried out. Cells expressing wild type MOR or MOR363D were biotinylated with a cleavable biotin label as described under “Experimental Procedures.” After agonist treatment, biotinylated receptors were either recycled and resensitized, or degraded. The internalized receptors in MOR363D-expressing cells after 30 h of agonist exposure (Fig. 3). Similar pronounced desensitization rate was obtained in another MOR363D-expressing N2A clone (clone number 15), which showed 60 ± 9.3% loss in etorphine activity after 6 h of agonist exposure (data not shown). Opioid agonists with different efficacy exhibit different capacities to induce desensitization (24, 25), and andaloid and peptide agonists induce differential desensitization kinetics on δ-opioid receptors (26). Thus the ability of morphine, an agonist with lower efficacy than etorphine and DAMGO, a peptide μ-opioid agonist, to induce receptor desensitization were also tested. Similarly, MOR363D-expressing cells exhibited faster desensitization rates following morphine or DAMGO pretreatment (data not shown). These results suggest that phosphorylation is not required for agonist-induced desensitization of MOR363D.
min treatment of 1 μM etorphine was ~20% of total receptors (Fig. 4, A and C), consistent with the results obtained with FACS analysis (Fig. 2), where ~80% receptors were observed at cell surface. After 2 h of etorphine treatment, ~35% MOR363D were observed in the internal compartments of the N2A cells (Fig. 4, A and C). This level was also quite similar to that observed in the FACS analysis (Fig. 2). Monensin did not significantly increase the amount of MOR363D detected intracellularly (Fig. 4, A and C). These observations indicated that the internalized MOR363D did not undergo any detectable degradation or recycling during the 2 h of etorphine treatment. For wild type MOR, ~40% receptors were present intracellularly after 30 min of etorphine treatment (Fig. 4, A and B). This level was also similar to that observed in FACS analysis (Fig. 2). However, only ~20% MOR were observed after 2 h of etorphine treatment (Fig. 4, A and B). This level was significantly less than that observed after 30 min of etorphine treatment (p < 0.05). Pre-incubation with monensin dramatically maintained the intracellular level of the internalized receptors (Fig. 4, A and B), suggesting that the internalized receptors disappeared in the absence of monensin were recycled back to the cell surface, where the biotin labels can be stripped.

To further confirm that internalized MOR363D receptor does not recycle back to cell surface, we examined the fate of the internalized receptor. After biotinylation of cell surface receptors, MOR- or MOR363D-expressing N2A cells were treated with 1 μM etorphine for 30 min in the presence of monensin, thus trapping the internalized receptors in endosomes. Then etorphine and monensin were removed by repeated washings, and the cells were incubated at 37 °C for various times. Then the total and intracellularly located biotinylated receptors were determined. As shown in Fig. 5 A, B (lower panel), and C, there was no difference in the total biotinylated MOR and MOR363D during the incubation periods, demonstrating that there was no detectable degradation. For MOR, the amount of internalized receptors decreased with time (Fig. 5, A, upper panel, and D). After 3 h, a 36 ± 5.6% decrease of internal receptors was observed. Conversely, there was no significant change in the amount of internalized MOR363D after prolonged incubation (Fig. 5, B, upper panel, and D). After 3 h of incubation, 105 ± 13% of biotinylated MOR363D remained intracellularly. These observations provide substantial evidence that wild type MOR recycled back to the surface after agonist-induced internalization, whereas MOR363D did not.

Resensitization of MOR and MOR363D—To determine whether receptor recycling contributes to resensitization, the ability of the previously desensitized receptor to inhibit forskolin-stimulated adenylate cyclase activity was measured. Receptor desensitization was first induced by 4 h exposure to 1 μM etorphine, and the cells were further incubated for 10, 20, 30, or 60 min in an agonist-free medium at 37 °C. As shown in Fig. 6, 20 min after agonist withdrawal, wild type MOR resensitized significantly. 1 μM etorphine could inhibit the forskolin-stimulated cAMP production by 92 ± 3.1% of the maximal inhibition rate observed in controlled cells. The process of resensitization was almost complete during 60 min of incubation after etor-
phine withdrawal. In contrast, no obvious resensitization was observed after 60 min of agonist withdrawal for the MOR363D receptors (Fig. 6, B), corresponding with a poor recycling process. These findings suggest that receptor recycling resulted in the resensitization of the µ-opioid receptor.

DISCUSSION

Truncation of the carboxyl tail sequence after Ser^363, which removes the putative phosphorylation sites, resulted in the failure of etorphine to induce receptor phosphorylation. Absence of receptor phosphorylation was accompanied by the attenuation in the receptor ability to recruit β-arrestin, subsequently the rate but not the magnitude of etorphine-induced receptor internalization was reduced. Our current observation is in agreement with the model that phosphorylation of GPCR enhances the affinity of the receptor for β-arrestin (3). However, the fact that the magnitude of MOR363D internalization was unchanged when compared with wild type MOR demonstrated that phosphorylation is not an absolute requirement for agonist-induced receptor internalization, and rather, is a facilitatory factor for internalization of MOR. This observation is consistent with a previous study that a mutant MOR with all major phosphorylation sites mutated to alanine still shows around 20% internalization after 1 h of agonist exposure (16). Another mutant MOR that exhibits receptor phosphorylation upon agonist activation does not undergo endocytosis (19). These studies suggest that receptor phosphorylation is not essential for agonist-induced internalization of MOR.

Our data did not preclude the critical role of C terminus in internalization that has been suggested in multiple studies (16, 19, 29). MOR363D contains two important residues, Ser^356 and Ser^363, which are identified to be involved in the endocytosis of MOR (19), explaining why MOR363D undergoes internalization, and also suggesting that the certain residues in the C terminus trigger internalization through unphosphorylated pathway(s).

Our current studies could not exclude definitely the interaction between MOR363D and β-arrestin. Previous studies showed that β-arrestin is required to terminate the GPCR signaling by uncoupling the receptor from G proteins and by targeting the desensitized receptor to clathrin-coated pits for endocytosis (12, 30, 31). And β-arrestin can still interact with agonist-occupied unphosphorylated receptor, just with less efficiency (32). Thus, we suggest that the truncation after Ser^363 reduces the affinity of arrestin for receptor and β-arrestin still binds to unphosphorylated MOR363D and is involved in the internalization. This suggestion is supported by the observation that the truncated MOR363D underwent a slower internalization. With this hypothesis, our results are not in conflict with the fact that chronic morphine treatment fails to elicit desensitization in β-arrestin 2 knockout mice in which β-arrestin 2 is totally depleted (33).

Consistent with a previous report (34) that MOR binds poorly to G protein-coupled receptor-associated sorting protein, a newly found postendocytic sorting protein that directs receptors to lysosome for degradation, MOR did not exhibit detectable degradation after internalization. But the internalized MOR363D with the unphosphorylated tail did not recycle, either, indicating depletion of a sorting process that directs receptors to the recycling endosomes. Several studies suggest a dual role of β-arrestin in the regulation of GPCR function (30, 35, 36). On one hand, as mentioned above, β-arrestin terminates the GPCR signaling. On the other hand, several studies suggest a role of β-arrestin in dictating receptor recycling. Overexpression of a dominant-negative mutant β-arrestin 1-V53D impairs dephosphorylation and resensitization of β2-adrenergic receptor (β2AR) (35). The ability of a β2AR mutant (Y326A), which does not resensitize after desensitization, to resensitize can be rescued by overexpression of β-arrestin 1 (35), demonstrating that β-arrestin is a critical determinant for resensitization. This is further supported by the observation that a specific arrestin interacting motif in the GPCR carboxyl-terminal tail dictates the rate of receptor dephosphorylation, recycling, and resensitization (36). Moreover, the carboxyl-terminal tails of some GPCRs have a great influence on the sorting of these internalized GPCRs to recycling pathways (37, 38), and even two distinct structural motifs have been identified to direct the sorting of internalized hormone receptor from degradation to recycling (39). Taken together, we propose that the mutual interaction between specific motif(s) in the C terminus of the wild type MOR and β-arrestin, which is promoted by phosphorylation, directs the internalized receptors to recycling pathway. We favor this hypothesis by the fact that MOR363D receptors, missing the distal portion of C terminus and binding poorly with β-arrestin, did not undergo recycling. Our data also suggest that the specific motif(s) dictating the intracellular fate of receptor is/are probably located in the distal portion of the C-terminal tail after Ser^363.

In the present study, we measured the long-term desensitization that is affected greatly by the receptor internalization and its intracellular sorting. In a previous study (27), trapping of the internalized receptors at endosomes with monensin enhances the rate and magnitude of agonist-induced receptor desensitization. Current studies verify that the agonist-induced MOR desensitization was partially reversed by the recycling and activation of internalized receptor to cell surface. Unphosphorylation and subsequently poor recruitment of β-arrestin resulted in the inability of MOR363D to resensitize and consequently enhanced the agonist-induced desensitization. Thus, the rate of desensitization is affected greatly by the rate of resensitization. These results suggest that the dynamic nature of receptor internalization, recycling, and resensitization, not the uncoupling of the receptor after phosphorylation, is critical for desensitization development.

In conclusion, the truncation of carboxyl tail blocked the receptor phosphorylation and reduced the β-arrestin-receptor interaction, and subsequently the rate of receptor internalization, but did not reduce the magnitude of internalization. The failure to phosphorylate in the presence of agonist and the
reduction in arrestin recruitment result in the abolishment of the ability of receptors to recycle and resensitize, thus increasing the rate of desensitization. However, the molecular mechanism of receptor phosphorylation and β-arrestin recruitment in receptor recycling needs to be clarified. Nevertheless, this study provides new insights into how MOR phosphorylation might regulate desensitization differentially in opioid receptors.

REFERENCES

1. Krupnick, J. G., and Benovic, J. L. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 289–319
2. Ferguson, S. S. (2001) Pharmacol. Rev. 53, 1–24
3. Claing, A., Laporte, S. A., Caron, M. G., and Lefkowitz, R. J. (2002) Prog. Neurobiol. 66, 61–79
4. Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Nat. Rev. Mol. Cell Biol. 3, 639–650
5. Dicker, F., Quitterer, U., Winstel, R., Honold, K., and Lohse, M. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5476–5481
6. Olivares-Reyes, J. A., Smith, R. D., Hunyady, L., Shah, B. H., and Catt, K. J. (2001) J. Biol. Chem. 276, 37761–37768
7. Dhami, G. K., Anborgh, P. H., Dale, L. B., Sterne-Marr, R., and Ferguson, S. S. (2002) J. Biol. Chem. 277, 25206–25212
8. Murray, S. R., Evans, C. J., and von Zastrow, M. (1998) J. Biol. Chem. 273, 24987–24991
9. Richardson, M. D., Balus, A. M., Yamaguchi, K., Frelich, E. R., Barak, L. S., and Kwatra, M. M. (2003) J. Neurochem. 84, 854–863
10. Vagler, O., Noite, B., Voss, M., Schmidt, M., Jakobs, K. H., and van Koppen, C. J. (1999) J. Biol. Chem. 274, 12333–12338
11. Brasselet, S., Guilen, S., Vincent, J. P., and Mazella, J. (2002) J. Biol. Chem. 277, 37761–37768
12. Ferguson, S. S. (2001) J. Biol. Chem. 276, 34488–34495
13. Baratti-Elbaz, C., Ghinea, N., Lahuna, O., Loosfelt, H., Pichon, C., and Milgrom, E. (1999) Mol. Pharmacol. 54, 704–711
14. Alouache, S., Rousset, M., Marie, N., and Jouzae, P. (1999) Eur. J. Pharmacol. 371, 235–240
15. Law, P. Y., Erickson, L. J., El-Kouhen, O. M., Dicker, L., Solberg, J., Wang, W., Miller, E., Burd, A. L., and Loh, H. H. (2000) Mol. Pharmacol. 58, 388–398
16. Koch, T., Schulz, S., Pfeiffer, M., Kliauga, M., Schroder, H., Kahl, E., and Holti, Y. (2001) J. Biol. Chem. 276, 31408–31414
17. Afify, E. A., Law, P. Y., Riedl, M., Elde, R., and Loh, H. H. (1998) Brain Res. Mol. Brain Res. 54, 24–34
18. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1998) Science 271, 363–366
19. Nakamura, K., Krupnick, J. G., Benovic, J. L., and Ascoli, M. (1998) J. Biol. Chem. 273, 24346–24354
20. Gurevich, V. V., Dion, S. B., Onorato, J. J., Gurevich, V. V., Dion, S. B., Onorato, J. J., Spasienski, J., Kim, C. M., Sterne-Marr, R., Hossy, M. M., and Benovic, J. L. (1995) J. Biol. Chem. 270, 720–731
21. Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J., and Caron, M. G. (2000) Nature 408, 720–723
22. Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., Murray, S. R., and von Zastrow, M. (2002) Science 297, 615–620
23. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. (1997) J. Biol. Chem. 272, 27005–27014
24. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) J. Biol. Chem. 274, 32248–32257
25. Trejo, J., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 23577–23584
26. Baratti-Elbaz, C., Ghinea, N., Lahuna, O., Loosfelt, H., Pichon, C., and Milgrom, E. (1999) Mol. Endocrinol. 13, 1751–1765
27. Kishi, M., Liu, X., Hirakawa, T., Recek, D., Bretscher, A., and Ascoli, M. (2001) Mol. Endocrinol. 15, 1624–1635
