Phosphorylation of a Carboxyl-terminal Serine within the κ-Opioid Receptor Produces Desensitization and Internalization*

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G-protein receptor kinase and β-arrestin mediated desensitization of the rat κ-opioid receptor (KOR) was previously shown using Xenopus oocyte expression to require serine 369 within the C terminus of KOR. To define the effects of phosphorylation of this residue in desensitization and internalization processes in mammalian expression systems, wild-type KOR-green fluorescent protein (KOR-GFP) and KOR(S369A)-GFP were stably expressed in AtT-20 and HEK293 cells. Using whole-cell patch clamp recording in transfected AtT-20 cells, agonist activation of either κ receptor form produced equivalent activation of the intrinsic G-protein-gated inwardly rectifying potassium channel. Incubation for 60 min with the κ agonist U50,488 (100 nM) desensitized the response in cells expressing wild-type KOR-GFP by 86% but had no effect on KOR(S369A)-GFP-expressing cells. Phosphorylation of serine 369 was detected using a phosphospecific antibody (KOR-P) able to distinguish the phosphorylated form of the receptor. The agonist-induced increase in KOR-P labeling was dose-dependent, blocked by co-treatment with the κ antagonist norbinaltorphimine, and prevented by co-expression of the dominant negative form of the G-protein receptor kinase, GRK2(K220R). In contrast, agonist-induced increase in KOR-P labeling was not evident in KOR(S369A) expressing cells. Prolonged activation resulted in receptor internalization that was also blocked by KOR(S369A) substitution, but interestingly, KOR-P labeling was evident at lower agonist concentrations than required to induce internalization. Following the removal of agonist, receptor dephosphorylation detected by loss of KOR-P labeling was complete within 60 min, could be blocked by okadaic acid, and was not blocked by surcrose inhibition of receptor internalization. These results demonstrate that GRK-mediated phosphorylation of serine 369 mediates rat KOR desensitization and internalization.

The use of opioid agonists to produce clinical analgesia is limited by their propensity to induce drug tolerance and dependence (1). Therefore, regulatory mechanisms responsible for opioid tolerance are of therapeutic interest. Opioid receptor desensitization and internalization are likely to play significant roles in the control of receptor signaling, but the biochemical steps underlying these mechanisms are uncertain. Agonist-induced receptor phosphorylation is thought to mediate both G-protein coupled receptor desensitization and internalization (for a recent review, see Ref. 2). However, establishing a direct link between these receptor mechanisms and the phosphorylation of individual amino acid residues on the opioid receptors has been confounded by the large number of potential phosphorylation sites and the practical difficulty of identifying specific phosphorylated sites within the opioid receptors.

Direct evidence of κ-opioid receptor (KOR) phosphorylation was obtained by immunoprecipitation of 32P-labeled KOR from guinea pig hippocampal slices treated with a KOR-selective agonist (3). This increase in KOR phosphorylation after agonist treatment coincided with an equivalent reduction in electrophysiological response to the KOR agonist and a significant reduction in the amount of immunoprecipitated protein detected, possible examples of agonist-induced receptor desensitization and internalization, respectively. However, determining the site of 32P incorporation into the KOR was not possible with this ex vivo model because of the low abundance of receptor in brain and uncertain stoichiometry of 32P labeling.

Further evidence correlating KOR control mechanisms to agonist-induced phosphorylation of specific amino acids has come from site-directed mutagenesis and subsequent expression of receptors lacking potential phosphorylation sites in cell lines and heterologous gene expression systems, but this work has yielded contradictory results. For example, electrophysiological studies of Xenopus oocytes expressing wild-type rat KOR (rKOR) demonstrated a G-protein receptor kinase (GRK)- and β-arrestin-2-mediated desensitization of the κ agonist response that was blocked by substitution of KOR(S369A) but not KOR(T363A) or KOR(S356A,T357A) (4). These results suggest that rKOR desensitization results from an agonist-induced phosphorylation of serine 369. Consistent with this finding Joseph and Bidelack (5) reported that prolonged exposure to κ agonists can produce a substantial reduction in KOR expression in thymoma cells. However, others have reported that κ agonist treatment can produce rKOR desensitization without an increase in phosphorylation or receptor internalization (6, 7). Taken together, the studies cited above suggest that the relationship between agonist-induced phosphorylation of specific sites within KOR and either receptor desensitization or internalization remains unclear. In addition, the direct identification of agonist-induced phosphorylation sites responsible

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‡ The abbreviations used are: KOR, κ-opioid receptor; GFP, green fluorescent protein; KOR-P, affinity-purified rabbit polyclonal antibody directed to page chasrine 369 in the rodent κ-opioid receptor; KT-2, affinity-purified rabbit anti-rat/mouse κ-opioid receptor polyclonal antibody; KE-4, affinity-purified rabbit anti-rat/mouse κ-opioid receptor polyclonal antibody; MOR, μ-opioid receptor; GRK, G-protein receptor kinase; nor-BNI, norbinaltorphimine; U50,488, (±)-trans-3,4-dichloro-N-methyl-N-isopropyl-3,4-dihydro-2H-1,2-benzisoxazolecarboxamide methane sulfonate hydrate.
for the desensitization or internalization of KOR has not been achieved.

To address this question, we expressed rat KOR-GFP and KOR(S369A)-GFP fusion proteins in AtT-20 cells for electrophysiology studies of receptor desensitization and in HEK293 cells to examine receptor internalization under confocal microscopy. Moreover, we used immunocytochemistry and a newly generated antibody (KOR-ρ) selective for the phosphorylated form of serine 369 in the KOR to directly determine whether agonist-induced phosphorylation of rKOR(S369) mediates either K receptor desensitization or internalization.

EXPERIMENTAL PROCEDURES

Chemicals—(+)–US50,488 was obtained from the National Institute on Drug Abuse drug supply program (National Institutes of Health, Bethesda, MD) and Sigma. Dynorphin A1–13 was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA). All other drugs were purchased from Sigma.

Peptides and Antibody Production—A phosphorylated peptide corresponding to the carboxyl-terminal domain of KOR designated KOR-P (RVRNTVQDPAS(P04)MRD; matching amino acids 359–372) was synthesized and purified by PeptidoGenic Research and Co. (Livermore, CA). This peptide sequence was compared with known proteins using the blast search function and no other proteins were found having >50% homology or four identical amino acids in a row. Purified peptide was coupled to keyhole limpet hemocyanin through the N-terminal lysine, and KOR-P antibody was then produced and affinity-purified as described earlier (3). The two antibodies KT2 (raised against the C-terminal tail of the KOR) and KE-4 (raised against the predicted fourth extracellular loop of the rat KOR—300–312), GSTSH-STAALSSY) were characterized as previously described (3, 8).

Enzyme-linked Immunosorbent Assay—Sterile 96-well plates were coated overnight at 4 °C with 0.5 μg/well of either KOR-P peptide, KOR-Non-P peptide (a nonphosphorylated peptide otherwise identical to KOR-P), or peptides KE-4 or MOR-P (an unrelated peptide from the predicted sequence of the μ-opioid receptor, MOR-(123–184), HPVKALDFR(P04)IPRNA in 0.1 μl phosphate-buffered saline (PBS), pH 7.4. Unbound sites were blocked with 1% nonfat dry milk in PBS for 2 h at 37 °C, and then the wells were washed three times with PBS-T (0.1% PBS with 0.05% Tween 20). Affinity-purified KOR-P antibody, serially diluted to 0.05 from 10 μg/ml, was added to each well and incubated overnight at 4 °C under gentle agitation. Each concentration was tested in triplicate. Wells were washed three times with PBS-T and then incubated for 2 h in alkaline phosphatase-conjugated anti-rabbit IgG, diluted 1:3000 in PBS plus 1% bovine serum albumin. After washing the wells six times with PBS-T, the enzyme substrate p-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine, 10% MgCl2, and 0.02% sodium azide) was added and allowed to react for 30 min in the dark. The reaction was stopped with the addition of 2.5 M NaOH, and absorbance was promptly read at 410 nm for each well.

Complementary DNA Clones, Mutagenesis of KOR to Produce KOR-GFP and KOR(S369A)-GFP, and Production of Complementary RNA—The cDNA for GFP-37 (GenBank accession number U57624) was subcloned into the EcoRI site of pcDNA3 (Invitrogen) vector to produce pcDNA3–GFP37. Restriction sites for KpnI and BstXI were added to the 5′- and 3′-ends of the rat KOR sequence (thereby replacing the stop codon with the BstXI site) via PCR amplification. This KOR cDNA was digested and subcloned into the KpnI and BstXI sites of the pcDNA3–GFP37 vector with T4 DNA ligase as described elsewhere (9). The resultant KOR-GFP construct fused the GFP to the carboxyl-terminal tail of KOR. Point mutations were made in KOR-GFP to produce KOR(S369A)-GFP, using techniques described previously (10). All mutations were confirmed by DNA sequencing. cDNA encoding for the K receptors was used to make RNA as described previously (10).

Cell Culture and Construction of Cell Lines Expressing KOR-GFP or KOR(S369A)-GFP—HEK293 cells were grown in Dulbecco’s modified Eagle’s media/F-12 media supplemented with 10% fetal bovine serum (Invitrogen), and AtT-20 cells were grown in Dulbecco’s modified Eagle’s media high glucose medium supplemented with 10% horse serum and 2.5% fetal bovine serum (Invitrogen). Stable cell lines expressing KOR-GFP or KOR(S369A)-GFP protein in both types of cells were constructed by transfecting both cell lines with 10 μg of cDNA coding for KOR-GFP or KOR(S369A)-GFP proteins/well for 2–3 h using Superfect (Qiagen) reagent and the manufacturer’s recommended protocol and then placing expressing cultures under selection pressure with G418 (0.4 μg/ml for HEK293 cultures; 1.5 μg/ml for AtT-20 cultures). Cells expressing GFP were identified under fluorescence, isolated, and maintained under selection pressure for a minimum of 3 more weeks. Additionally, a small set of Xenopus laevis oocytes were microinjected with RNA coding for the wild type rKOR, KOR-GFP, or KOR(S369A)-GFP receptors (4 ng/oocyte) along with RNA expressing the heteromeric inwardly rectifying potassium channels, Kgs 3.1/3.2 (0.02 ng/oocyte) as described previously (10).

Electrode Voltage Clamp and Whole-cell Patch Clamp Electrochemistry—To confirm the function of KOR-GFP and KOR(S369A)-GFP as κ-opioid receptors, Xenopus oocytes expressing either fusion protein or wild type rKOR with Kgs 3.1/3.2 were used in two-electrode voltage clamp recordings as described previously (10). Otherwise, AtT-20 cells stably expressing KOR-GFP or KOR(S369A)-GFP were grown on polystyrene-coated cover slips for experimental use. Cells were used in patch clamp experiments in the whole-cell configuration with a pipette containing 130 mM KCl, 20 mM HEPES, 10 mM EGTA, 5 mM MgCl2, 3 mM Na-ATP, and 0.6 mM GTP, pH 7.25, in an external solution containing 40 mM KCl, 130 mM NaCl, 1 mM CaCl2, 25 mM HEPES, and 10 mM glucose, pH 7.35. Whole-cell potassium currents were acquired with an Axopatch 200 and Axotape (Axon Instruments) and then analyzed with Clampfit software (Axon Instruments). In the whole-cell configuration of the patch clamp, cells were held at −45 mV and hyperpolarized to −100 mV for 50 ms every 5 s. Recorded traces represent average current during the hyperpolarization, plotted over time. Desensitization during agonist treatment was measured as the percent change in response to agonist after a 60-min exposure to 100 μM US50,488.

KOR-labeling Immunocytochemistry and Confocal Microscopy—Cells stably expressing KOR-GFP or KOR(S369A)-GFP were split onto polystyrene-coated cover slips in 24-well plates for experimental use. Note that for experiments using the dominant negative mutant of GRK2, GRK(K220R)2, HEK293 cells were transiently transfected with both 10 μg of KOR-GFP and 2 μg of GRK220R2 cDNA, using the Superfect reagent as described above. After 48 h of protein expression, cells were treated at 37 °C as noted and prepared for immunocytochemistry and confocal microscopy as described previously (11). Either KT-2 (15 μg/ml) or KOR-P (4.5 μg/ml) was used as the primary antibody with fixed cells, incubated up to 70 h at 4 °C in 0.3% glycera/PBS with 0.025% Triton-X. Lissamine-rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used as a secondary antibody to label the bound antibody. Both fluorochromes (GFP from the expressed protein and Rhodamine Red-bound KOR-P labeling) were viewed separately or simultaneously using a Bio-Rad confocal microscope (model MRC600) with COMOS 6.01 software.

Data Analysis—To quantify the degree of internalization induced by agonist treatment of HEK293 cells, images collected with the confocal microscope were analyzed with NIH Image version 1.62 software (National Institutes of Health, Bethesda, MD) for the intensity of pixels within the cytoplasm of cells expressing KOR-GFP or KOR(S369A)-GFP following medium or agonist treatment. To quantify the ratio of red and green pixels labeled by the KOR-P antibody, confocal microscopy were separated into green pixel-only images (showing GFP signaling) and red pixel-only images (showing antibody labeling) using Photoshop software (Adobe Systems Inc., San Jose, CA). Each image was analyzed for number of nonblank pixels, and a ratio of red to green pixels was generated for each original image taken (therefore, a value of 1.0 denotes complete and precise overlap of KOR/P- Rhodamine Red antibody labeling and green GFP signaling). Statistical significance throughout this study was defined by p < 0.05 as determined by Student’s t test.

RESULTS

Whole-cell AtT20 Electrophysiology Demonstrates the Involvement of Serine 369 in KOR Agonist Desensitization—Previous work with rat KOR expressed in Xenopus oocytes demonstrated the importance of serine 369 in desensitization of the wild type receptor (4). However, the role of this residue in a mammalian expression system was not determined, and the analysis of cellular tolerance in Xenopus oocytes is limited by a lack of robust internalization of agonist-activated receptor in this expression system (data not shown). Therefore, we constructed GFP-tagged rat KOR and KOR(S369A) receptors and first checked whether the GFP tag adversely affected receptor functioning. When equal amounts of receptor cRNA were expressed in Xenopus oocytes, maximal activation (2–3 μM
Phosphorylation of KOR Ser^{369} Mediates Desensitization

Fig. 1. Prolonged \( \kappa \)-opioid agonist incubation induces KOR-GFP desensitization that is mediated by phosphorylation of KOR-GFP serine 369. A, representative whole-cell trace of potassium channel currents induced by the \( \kappa \)-opioid agonist, U50,488. AtT-20 cells stably expressing KOR-GFP were clamped at \( -45 \) mV and hyperpolarized to \( -100 \) mV using patch-clamped cells in the whole-cell configuration with a pipette containing 30 mM KCl saline solution and bathed in saline buffer containing 40 mM KCl as described under “Experimental Procedures” for single-cell recordings. Cells stably expressing the KOR-GFP showed an increase in current when treated with the \( \kappa \)-opioid agonist, U50,488 (1 \( \mu \)M), represented by the downward deflection of the trace, indicating an increase in outward current. The upward deflection in the trace followed the addition of the KOR-selective antagonist nor-BNI (10 \( \mu \)M) to the chamber. B, data summarized shows that wild type (WT) KOR-GFP exposed to U50,488 (100 \( \mu \)M, 1 h) resulted in the desensitization of the opioid-induced potassium channel response by 86%. However, although the mutant KOR(S369A)-GFP functioned normally to induce potassium current upon agonist stimulation, this mutant \( \kappa \) receptor did not show a significant agonist-induced desensitization, \( p < 0.01 \) versus untreated KOR-GFP/AtT-20 cells; \( n = 4–5 \) cells/br. C, representative confocal images of AtT-20 cells stably expressing KOR-GFP fusion proteins and exposed at \( 37^\circ \)C for 1 h to media (a), 100 \( \mu \)M U50,488 (b), or 10 \( \mu \)M U50,488 (c). No significant internalization of KOR-GFP was observed after treatment with the low concentration of U50,488 as compared with the medium-treated cells, whereas considerable receptor clustering and internalization resulted from treatment with high concentrations of U50,488.

U50,488) of wild-type KOR, KOR-GFP, and KOR(S369A)-GFP increased \( K_p^{3.1 / 3.2} \) current and produced 1190 \( \pm 645, 750 \pm 378, \) and 1006 \( \pm 1044 \) nA, respectively (\( n = 7–13 \) oocytes), for cells voltage clamped at \( -80 \) mV as previously described (10).

Fig. 2. Development of KOR-P, an antibody specific to the phosphorylated serine 369 form of the \( \kappa \)-opioid receptor. A, schematic representing the \( \kappa \)-opioid receptor and the location of three antibodies raised to it. The KOR-P antibody was raised as described under “Experimental Procedures” to the region of the \( \kappa \) receptor corresponding to KOR 359–372, a 14- amino acid sequence containing the phosphorylated serine 369. The KOR-selective antibodies KE-4 (raised to the putative third extracellular loop, corresponding to KOR 390–312) and KT-2 (raised to the putative intracellular tail, corresponding to KOR 371–380) were developed previously and are not phosphoselective. B, enzyme-linked immunosorbent assay results using affinity-purified KOR-P antibody. Different concentrations of KOR-P antibody (0.005–10 \( \mu \)g/ml) were incubated overnight with separate peptides (0.5 \( \mu \)g/ml) corresponding to KOR 359–372, either with serine 369 phosphorylated (○) or nonphosphorylated (□). KOR-P antibody was also incubated with separate peptides KE-4 (□; corresponding to KOR 300–312) and MOR-P (●; corresponding to MOR-170–184) and containing a phosphorylated threonine 180) to further estimate nonspecific KOR-P antibody binding. Samples were then processed as described under “Experimental Procedures,” and absorbance was measured at 405 nm. Results displayed are plotted with background, nonspecific signal (as measured by a lack of antibody) subtracted. Points represent average absorbance from three experiments, performed in triplicate.

These responses were not significantly different, and the results suggest that neither point mutation nor the addition of GFP to the C terminus of the KOR significantly affected receptor activation of \( K_H^{3.1 / 3.2} \) currents.

Moreover, the KOR-GFP expressed in AtT20 cells showed robust receptor expression and cell surface distribution (Fig. 1C, a). KOR-GFP expressed in AtT20 cells that were patch-clamped in high potassium buffer as described under “Experimental Procedures” produced a strong potassium current in response to stimulation with the \( \kappa \) agonist, U50,488 that was antagonized by the \( \kappa \)-selective antagonist, nor-BNI (Fig. 1A). Likewise, activation of KOR(S369A)-GFP produced an equivalent potassium influx in response to U50,488 and at a similar magnitude to that of the wild-type receptor (Fig. 1B). After prolonged agonist incubation (100 \( \mu \)M U50,488, 1 h), the potassium channel response to 1 \( \mu \)M U50,488 by KOR-GFP was significantly reduced by 86%, whereas the KOR(S369A)-GFP
response following the same treatment was unchanged (Fig. 1B). This result suggests that the KOR(S369A) residue was also important for receptor desensitization in a mammalian cell system. Whereas robust internalization of KOR-GFP was evident following stimulation with higher concentrations of U50,488 (10 μM, 1 h; Fig. 1C, c), the lower concentration (100 nM, 1-h treatment) that produced receptor desensitization did not increase receptor internalization (Fig. 1C, b), as compared with untreated cells (Fig. 1C, a), suggesting that desensitization was not due to internalization.

Development of κ-Opioid Receptor Antibodies Specific to the Serine 369-Phosphorylated Receptor—To develop a probe to detect phosphorylation of serine 369 in KOR, we generated and affinity-purified a polyclonal antibody (KOR-P) against a 14-amino acid peptide corresponding to rat KOR-(359–372) containing a phosphorylated serine 369 (Fig. 2A). Affinity-purified KOR-P antibody reacted up to 80 times more strongly in an enzyme-linked immunosorbent assay against the phosphopeptide than the equivalent, nonphosphorylated sequence (Fig. 2B). The KOR-P antibody also failed to recognize peptides corresponding to the other regions of the κ receptor and unrelated, phosphorylated peptides (Fig. 2B).

Demonstration of Immunocytochemical Labeling of KOR-GFP and KOR(S369A)-GFP by Two κ-Opioid Receptor-selective Antibodies—High levels of background antibody labeling in the cytosol of KOR-GFP or KOR(S369A)-GFP expressing AtT-20 cells made quantitation of receptor trafficking difficult. Therefore, HEK293 cells stably expressing either KOR-GFP or KOR(S369A)-GFP were used in immunocytochemistry experiments with either KT-2 (not phosphoselective) or KOR-P (phosphoselective) as the primary antibody. The lower cytosolic background staining evident in KOR-transfected HEK293 cells facilitated quantitation of receptor trafficking. Confocal microscopy revealed that KT-2 selectively immunolabeled both KOR-GFP and KOR(S369A)-GFP as the red KT-2 antibody labeling overlapped the green GFP fluorescent signal (Fig. 3, A–D). Incubation of HEK cells with 10 μM U50,488 produced clear internalization of KOR-GFP but not KOR(S369A)-GFP, as detected by KT-2 and localization of GFP fluorescence (Fig. 3, B and D). In contrast, untreated HEK cells expressing KOR-GFP were unlabeled by KOR-P (Fig. 4A), whereas KOR-P labeling was evident in cells treated with U50,488 (0.1–10 μM, 30 min at 37 °C) (Fig. 4, B–D). KOR-P labeling remained on the cell surface following treatment (30 min, 37 °C) with 0.1 and 1.0 μM U50,488. However, whereas these concentrations of U50,488 failed to produce characteristic signs of KOR-GFP internalization, incubation with 10 μM U50,488 (Fig. 4D) resulted in punctate structures and internalization of KOR-GFP evident by both KOR-P antibody labeling and GFP fluorescence. These effects were activation-dependent, since incubation with the KOR-selective antagonist nor-BNI did not produce either KOR-P antibody labeling or evidence of receptor trafficking (Fig. 4E). In addition, coincubation of nor-BNI with 10 μM U50,488 prevented both receptor trafficking and KOR-P antibody labeling (Fig. 4F). Agonist-induced increases in KOR-P labeling and receptor internalization were also evident after treatment with the κ-selective opioid peptide, dynorphin A(1–13) (Fig. 4G), and labeling was again blocked by coincubation with nor-BNI (Fig. 4H). Preabsorbing the KOR-P antibody with the corresponding KOR-P peptide prevented antibody labeling following 10 μM U50,488 (Fig. 4I). In addition, HEK293 cells stably expressing KOR(S369A)-GFP failed to demonstrate KOR-P antibody labeling, either when untreated (Fig. 4J) or after incubation with 10 μM U50,488 (Fig. 4K). Finally, KOR-P antibody labeling was not evident in untransfected HEK293 cells treated with 10 μM U50,488 (Fig. 4L). The results suggest that KOR-P antibody could specifically detect the Ser369-phosphorylated form of KOR and that phosphorylated receptor

Fig. 3. Recognition and localization of KOR-GFP and KOR(S369A)-GFP by the KT-2 antibody. HEK293 cells were stably transfected with KOR-GFP or KOR(S369A)-GFP cDNA as described under “Experimental Procedures” and then grown on coverslips. After 2 days of expression, cells were treated either with media (A and C) or 10 μM U50,488 (B and D) for 30 min at 37 °C. Cells were then fixed, labeled with affinity-purified KT-2 antibody (15 μg/ml), and visualized with a rabbit IgG-rhodamine Red secondary antibody using the confocal microscope. Larger panels represent the overlap of dual fluorescent GFP signals (excited at 488 nm) and fluorescent rhodamine-KT-2 antibody complex signals (at 568 nm); insets show only the Rhodamine Red KT-2 antibody labeling. Yellow colocalization of signal within dual-labeled panels demonstrates U50,488-induced internalization of the wild type, but not serine 369 mutant, KOR-GFP. However, KT-2 labeled both receptors selectively and with equal intensity regardless of pretreatment.
**FIG. 4.** KOR serine 369 is phosphorylated in a concentration-dependent manner by κ-opioid agonists to mediate receptor internalization. HEK293 cells were stably transfected with KOR-GFP or KOR(S369A)-GFP cDNA and were pretreated for 30 min at 37 °C with the drugs listed above each image. Cells were fixed, labeled with affinity-purified KOR-P antibody (4.5 μg/ml), and visualized with a rabbit IgG-Rhodamine Red secondary antibody using the confocal microscope. Panels represent the overlap of dual fluorescent GFP signals (excited at 488 nm) and fluorescent rhodamine-KOR-P antibody complex signals (at 568 nm); insets show only the Rhodamine Red KOR-P antibody labeling. KOR-P antibody did not label untreated cells (A). However, incubation with U50,488 (0.1–10 μM; B–D) produced a concentration-dependent increase in KOR-P antibody labeling that colocalized with the KOR-GFP receptor signaling. A high (10 μM) concentration of U50,488 also induced KOR-GFP receptor internalization (D). In contrast, incubation with the KOR-selective antagonist nor-BNI (10 μM) did not result in KOR-P labeling (E) but blocked the KOR-P antibody labeling and receptor trafficking induced by 10 μM U50,488 (F). Similar to the effects of U50,488, pretreatment with 10 μM dynorphin A-(1–13) resulted in strong KOR-P antibody labeling of KOR-GFP, receptor trafficking, and internalization (G). Coincubation of dynorphin A with the KOR-selective antagonist nor-BNI also blocked agonist-induced receptor trafficking and the increase in KOR-P antibody labeling of KOR-GFP (10 μM; H). U50,488 (10 μM)-induced KOR-P labeling of KOR-GFP was abolished by preblocking the KOR-P antibody with the serine-phosphorylated peptide corresponding to KOR-(359–372) prior to incubation with the cells, but agonist-induced internalization of KOR-GFP was unaffected (I). Notably, agonist-induced increases in KOR-P antibody labeling of the mutated receptor KOR(S369A)-GFP were not evident in samples either untreated (J) or exposed to 10 μM U50,488 pretreatment (K). Moreover, KOR-P antibody labeling was not detected in HEK cells not transfected with KOR-GFP, even after U50,488 treatment (L). All experiments were performed on 2–5 separate occasions, with images shown representative of multiple images taken from different areas of the coverslips.
could be detected at both the plasma membrane and in cytoplasmic structures following agonist treatment.

**Agonist-induced Phosphorylation of KOR-GFP in HEK293 Cells Is Mediated by a GRK**—HEK293 cells stably expressing KOR-GFP cDNA were pretreated with the inhibitors listed above the image for 15 min at 37 °C, and then 10 μM U50,488 was added to all cells for an additional 30 min. Cells in C were also transiently transfected with GRK2(K220R). Following incubation, cells were washed, fixed, labeled with affinity-purified KOR-P antibody (4.5 μg/ml), and visualized with a rabbit IgG-Rhodamine Red secondary antibody using the confocal microscope. Panels represent the overlap of dual fluorescent GFP signals (excited at 488 nm) and fluorescent rhodamine-KOR-P antibody complex signals (at 568 nm); insets show only the Rhodamine Red KOR-P antibody labeling. Pretreatment with media alone resulted in robust KOR-P antibody labeling and receptor internalization after agonist treatment (A). Agonist-induced KOR-P labeling and receptor internalization were not blocked by preincubation with the protein kinase A/protein kinase C inhibitor staurosporine (B), the MEK1/2 inhibitor PD98,059 (D), or the phospholipase C inhibitor U73,122 (E). However, coexpression of the dominant negative G-protein receptor kinase, GRK2(K220R), in HEK cells with KOR-GFP greatly reduced the agonist-induced increase in KOR-P antibody labeling and prevented receptor internalization (C). Experiments were performed on 2–3 separate occasions, with images shown representative of multiple images taken from different areas of the coverslips.

**Agonist-induced Receptor Internalization Is Mediated by KOR Serine-369 in HEK293 Cells**—Since previous results suggested that receptor internalization required the serine 369 phosphorylation site (Fig. 4K) and phosphorylation by GRK (Fig 5C), receptor internalization was quantified for both KOR-GFP (Fig. 6A) and the mutant KOR(S369A)-GFP (Fig. 6C). Treatment with 10 μM U50,488 induced clear receptor trafficking, with punctate, clustered KOR-GFP structures identified on the cell surface and internalized within the cytoplasm (Fig. 6B). Image quantitation showed a significant increase in the cytosolic pixel intensity following agonist treatment of cells expressing KOR-GFP (Fig. 6E). In contrast, KOR(S369A)-GFP did not show agonist-induced receptor trafficking (Fig. 6D), and a uniform distribution of the mutant receptor continued to be primarily localized in the plasma membrane (Fig. 6D). Quantitation of KOR(S369A)-GFP images revealed no increase in the cytoplasmic pixel intensity following U50,488 treatment (Fig. 6E).
Rapid Reversal of Agonist-induced KOR Serine 369 Phosphorylation Is Mediated by Phosphatases Both in the Cytosplasm and Directly at the Plasma Membrane—Agonist-induced phosphorylation of G-protein-coupled receptors is thought to be quickly reversed upon removal of the agonist by receptor internalization and dephosphorylation (12, 13). To test this, HEK293 cells stably expressing KOR-GFP were treated with 10 μM U50,488 at 37 °C, rinsed free of agonist after 30 min, and then allowed to recover in media for times up to 60 min prior to KOR-P antibody labeling. Similar to previous results (e.g. Figs. 5A and 6B), U50,488-induced increases in KOR-P labeling and KOR-GFP internalization were evident 1 min after agonist removal (Fig. 7A). KOR-P antibody labeling was still evident 10 min after agonist removal, although it was significantly diminished (Fig. 7B). KOR-P antibody labeling of U50,488-treated KOR-GFP/HEK293 cells was barely detectable 30 min after agonist removal (Fig. 7C) and was undetectable 60 min after agonist removal (Fig. 7D). Quantitation of total red and green pixel intensities verified that KOR-P labeling declined steadily during the 60 min following U50,488 washout (Fig. 8, open circles). To determine whether receptor internalization was required for dephosphorylation, cells were treated with 0.4 M sucrose to prevent receptor internalization, and the kinetics of KOR-P labeling decline was assessed following washout of 10 μM U50,488. Inclusion of sucrose did not prevent U50,488-induced increase in KOR-P antibody labeling, although KOR-GFP internalization was blocked (Fig. 7E). In addition, KOR-GFP dephosphorylation was not blocked by sucrose, since KOR-P labeling still declined in cell culture media containing 0.4 M sucrose (Fig. 7F). However, the rate of loss of KOR-P labeling was significantly delayed by sucrose treatment, suggesting that dephosphorylation of KOR may be facilitated by receptor internalization (Fig. 8, squares).

To characterize the phosphatase mediating dephosphorylation, HEK293 cells stably expressing KOR-GFP and stimulated with 10 μM U50,488 were treated with 100 nM okadaic acid to inhibit the protein serine/threonine phosphatases 1 and 2A or with a combination of 0.4 M sucrose and 100 nM okadaic acid. Neither okadaic acid alone nor the combination of sucrose and okadaic acid treatment impaired the U50,488-induced increase in KOR-P antibody labeling observed immediately after the removal of agonist (Fig. 7, G and I, respectively). Okadaic acid alone slowed, but did not prevent, the decline in KOR-P labeling (Figs. 7H and 8 (triangles)). In contrast, the combination of sucrose and okadaic acid prevented the loss of KOR-P antibody labeling as long as 60 min after agonist removal (Figs. 7J and 8 (diamonds)). Together, these results suggest that receptor internalization may accelerate the dephosphorylation of KOR serine 369 and that receptor dephosphorylation by either protein phosphatase 1 or 2A may occur at the plasma membrane without internalization.

**DISCUSSION**

The principal finding of this study is that the rat κ opioid receptor was desensitized by phosphorylation of serine 369 by a GRK-dependent mechanism. Receptor phosphorylation was detected by a novel phosphospecific antibody, KOR-P that enabled immunocytochemical localization of the κ-opioid receptor. Substitution of alanine for serine 369 blocked both receptor uncoupling and receptor internalization, two processes that underlie desensitization. Moreover, KOR dephosphorylation was facilitated by receptor internalization but could occur at the plasma membrane. This study directly demonstrated phosphorylation at a specific site within the opioid receptor and helps to resolve the key steps in the desensitization and resensitization process regulating KOR functioning in mammalian cells. These findings are consistent with other reports implicating an agonist-induced phosphorylation event underlying the desensitization of human KOR (7) and μ-opioid receptors (14–16).

Agonist-induced receptor desensitization is thought to be a necessary prerequisite to the internalization and down-regulation of opioid receptors (17, 18). However, the correlation between agonist-induced KOR desensitization and internalization is at best unclear. For example, a chronic incubation of cultured rat spinal cord-dorsal root ganglia neurons with 10 μM U50,488 produced complete desensitization and KOR-induced inhibition of adenylyl cyclase activity (19). A subsequent study demonstrated that a 24-h pretreatment with 10 μM U50,488 or 1 μM etorphine failed to produce a down-regulation of KOR number in dorsal root ganglia cultures (20). Likewise, epitope-tagged KOR expressed in HEK293 cells failed to internalize after acute treatment with 10 μM etorphine (21). In contrast, 0.1 μM U50,488 treatment produced a 50% reduction in KOR number on R1.1 mouse thymoma cells without significant receptor desensitization (5). Curiously, in another study using epitope-tagged KOR expressed in Chinese hamster ovary cells, dynorphin peptides readily induced KOR internalization and down-regulation, whereas nonpeptidic agonists such as
FIG. 7. Serine 369 dephosphorylation of the agonist-incubated KOR-GFP is rapid and does not require receptor internalization. Cells were pretreated with media (A–D), 0.4 M sucrose alone for 60 min (E and F), 100 nM okadaic acid for 45 min (G and H), or 0.4 M sucrose for 60 min in conjunction with 100 nM okadaic acid for 45 min (I and J). Incubation was then continued for an additional 30 min with 10 μM U50,488, and then, cells were washed three times with media and allowed to recover in their respective media conditions in the absence of U50488 for the time specified. Following these incubations, all cells were washed, fixed, labeled with affinity-purified KOR-P antibody (4.5 μg/ml), and visualized with a rabbit IgG-Rhodamine Red secondary antibody using the confocal microscope. Panels represent the overlap of dual fluorescent GFP signals (excited at 488 nm) and fluorescent rhodamine-KOR-P antibody complex signals (at 568 nm); insets show only the Rhodamine Red KOR-P antibody labeling. Agonist-induced receptor trafficking, internalization, and phosphorylation of serine 369, identified by an increase in KOR-P antibody labeling, was pronounced 1 min after agonist removal (A). Ten minutes after agonist removal, the number of punctate receptor structures and KOR-P antibody labeling was reduced, suggesting that a KOR-GFP dephosphorylation was under way (B). This KOR-P antibody labeling returned to base-line labeling after 30 min of recovery time, suggesting that dephosphorylation was complete by this time (C). Moreover, KOR-GFP internalization and punctate formations were not observed 30 min after the removal of agonist, suggesting that the receptor had returned to the
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Fig. 8. Dephosphorylation of KOR-GFP is accelerated by, but does not require, receptor internalization. The graph represents summarized image analysis of treatment sets represented in Fig. 7. Cells were pretreated up to 1 h in media (C), 0.4 M sucrose (D), 0.8 M okadaic acid (E), or sucrose and okadaic acid (F) and then treated with 10 μM U50,488 and allowed to recover up to 60 min in the same conditions as detailed in the legend to Fig. 7. Points represent the summarized ratio of red KOR-Prbodanilabeled pixels to green fluorescent protein-labeled pixels analyzed from each dual image as described under “Experimental Procedures.” Untreated cells recovering from U50,488 stimulation demonstrated peak ratios of red/green pixel count values in the first 5 min following agonist removal, indicating a high number of KOR-P-labeled \(\kappa\)-opioid receptors. This ratio of red/green pixels declined rapidly, returning to base-line values within 60 min. Likewise, cells pretreated with 0.4 M sucrose and exposed to U50,488 demonstrated an initial high ratio of red/green counted pixels following agonist removal that returned to base-line values within 60 min. In contrast, U50,488 treatment of cells pretreated with 100 nM okadaic acid induced a similar elevation of the ratio of red/green counted pixels after agonist removal, but the ratio did not return to base-line values in the course of the 60-min recovery period. Moreover, combined okadaic acid and sucrose pretreatment prevented the expected decrease in KOR-P antibody labeling measured as a reduction in the ratio of red/green pixels. The continued labeling of KOR-GFP by KOR-P antibody up to 60 min after agonist stimulation in the presence of both sucrose and okadaic acid suggests that receptor dephosphorylation may have also occurred at the cell surface, independent of receptor internalization. All experiments were performed on 2–5 separate occasions, with analysis of 3–14 images from different areas of assorted coverslips.

U50,488 did not prevent receptor internalization (22). Subsequent work with Chinese hamster ovary cells expressing epitope-tagged KOR demonstrated that 1 μM U50,488 induced an increase in \(^{32}\)P incorporation that corresponded with desensitization of the human, but not rat, KOR (7). A parallel study demonstrated that 1 μM U50,488 induced internalization of the human, but not rat, KOR (23), although the involvement of KOR phosphorylation in agonist-induced internalization was not directly demonstrated. The authors suggested that the lack of agonist-induced rKOR regulation was due to the presence of Aman585 in the cytoplasmic tail of the rKOR, preventing agonist-induced phosphorylation in Chinese hamster ovary cells (7, 23). However, direct \(^{32}\)P incorporation into specific phosphorylation sites was not demonstrated, and the substitution of KOR(N358S) did not restore an agonist-induced desensitization as might be predicted, suggesting that another phosphorylation site may underlie receptor desensitization and down-regulation.

The results of the present study show reasonable agreement with the majority of studies on the topic; pretreatment with lower (≤1 μM) concentrations of U50,488 failed to produce KOR internalization but still induced significant wild type \(\kappa\) receptor desensitization in the intact cell. Only higher concentrations of U50,488 (10 μM) induced KOR internalization. Admittedly, these data contain two important caveats. First, receptor internalization was not quantified in the agonist exposure experiments, making a direct comparison with previous studies difficult. Second, the receptor constructs examined in this study were by necessity GFP tail fusion proteins, which are not conclusively determined to faithfully mimic the internalization properties of the wild type KOR. Still, the present study extends previous findings, as the KOR-P antibody labeling intensity increased in an agonist concentration-dependent manner that was specific for the GFP-tagged KOR and reversed with receptor recycling to the cell surface. These results suggest that agonist-induced phosphorylation of KOR serine 369 underlies agonist-induced receptor regulation events, as detected by relative KOR-P antibody labeling, consistent with earlier observations in cell lines (17, 18).

The agonist-induced phosphorylation of KOR detected by KOR-P labeling was prevented by coexpression of KOR-GFP with the dominant negative mutant GRK2(K220R), which is thought to impede native GRK in host cells (24). Inhibitors selective for the kinases protein kinase A, protein kinase C, phospholipase C, or MEK1/2 were ineffective in blocking agonist-induced KOR-GFP phosphorylation. Whereas the possible interaction of other kinases with the KOR cannot be discounted, the ability of both overexpression of GRK2(K220R) and substitution of KOR(S369A) to prevent agonist-induced receptor internalization suggests that agonist-induced phosphorylation of KOR-GFP serine 369 in HEK293 cells was mediated by a GRK. These results further support the hypothesis that a GRK-mediated, agonist-induced phosphorylation of KOR(S369) is required for receptor internalization.

Similarly, dephosphorylation of agonist-phosphorylated KOR(S369) was suggested by the loss of KOR-P labeling correlated with the recycling of internalized KOR-GFP to the cell surface. Relatively little is known about the role or rate of receptor dephosphorylation in reversing the internalization of opioid receptors, perhaps due to the difficulty in specific identification of dephosphorylated, recycled receptors with \(^{32}\)P incorporation methods. Studies using transfected MOR indicate that in cell lines, dephosphorylation occurs quickly following cessation of agonist stimulation (25). Recycling of \(\beta\)-adrenergic-2 or cannabinoid receptors internalized after short agonist exposure was shown to require dephosphorylation, since recycling was prevented by okadaic acid-sensitive phosphatases (12, 13). The present results suggest that a similar dephosphorylation event at serine 369 was required for the recycling of internalized KOR-GFP. Intriguingly, a dephosphorylation of serine-369 following agonist stimulation of KOR-GFP occurred,

cell surface (C). Supporting this possibility, receptors remained primarily on the cell surface and free of KOR-P antibody labeling 60 min after the removal of U50,488 (D). Likewise, sucrose treatment prevented receptor internalization but not an increase in KOR-P antibody labeling (E). However, despite the prevention of internalization, sucrose pretreatment delayed but did not prevent the reduction in KOR-P antibody labeling observed after the removal of agonist (F). Okadaic acid pretreatment also did not prevent KOR-P antibody labeling after U50,488 stimulation and did not prevent receptor trafficking and internalization (G). Okadaic acid alone delayed, but did not prevent, the reduction in KOR-P antibody labeling observed after the removal of agonist (H). In contrast, pretreatment combining okadaic acid and sucrose prevented both agonist-induced receptor internalization (I) and the expected decrease in KOR-P antibody labeling characterized previously following agonist removal (J). The continued labeling of KOR-GFP by KOR-P antibody up to 60 min after agonist stimulation in the presence of both sucrose and okadaic acid suggests that receptor dephosphorylation may occur at the cell surface, independent of receptor internalization. All experiments were performed on 2–5 separate occasions, with images shown representative of multiple images taken from different areas of the coverslips.
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Although delayed, even after the prevention of KOR internalization with 0.4 M sucrose treatment, suggesting that serine phosphatase activity may restore receptor function at the cell membrane.

A major conclusion of this study is that efficient dephosphorylation can occur without internalization of the KOR. This contrasts with current views on this issue based on studies of several other G-protein-coupled receptors. For example, agonist-induced desensitization of \(\beta\)-adrenergic-2 receptors was reversed by rapid receptor sequestration and the return of the receptor to the cell surface within minutes of agonist removal (26). These events were shown to be dependent on agonist-induced receptor phosphorylation and thought to involve subsequent binding with cytosolic proteins, such as the \(\beta\)-arrestins, to induce receptor internalization (27). Likewise, regulation of receptor internalization and recycling have been demonstrated to depend on agonist-induced phosphorylation of specific receptor residues, with receptor resensitization requiring subsequent receptor dephosphorylation in studies examining bradykinin receptors (28), \(\delta\)-opioid receptors (29, 30), and \(\mu\)-opioid receptors (25, 31–33). However, at least in the case of the MOR, this effect may prove to be agonist-dependent, since etorphine, morphine, and the enkephalins were shown to induce rapid DOR desensitization and internalization, whereas morphine could not induce rapid MOR internalization (32). These results may suggest that alternative mechanisms to receptor internalization exist in the process of receptor resensitization with 0.4 M sucrose treatment, suggesting that serine phosphorylation of KOR(S369) is implicated in the recycling of the internalized receptor to the cell surface. Taken together with previous findings, these results suggest that the state of agonist-induced receptor phosphorylation determines the regulatory response mechanism to agonist stimulation that may result in the development of cellular opioid tolerance.

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