U50,488H-induced Internalization of the Human \(\kappa\) Opioid Receptor Involves a \(\beta\)- Arrestin- and Dynamin-dependent Mechanism

\(\kappa\) RECEPTOR INTERNALIZATION IS NOT REQUIRED FOR MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION

(Received for publication, November 9, 1998, and in revised form, February 5, 1999)

Jian-Guo Li, Lai-Yi Luo, Jason G. Krupnick‡, Jeffrey L. Benovic‡§, and Lee-Yuan Liu-Chen¶

From the Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140 and the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Agonist-promoted internalization of some G protein-coupled receptors has been shown to mediate receptor desensitization, resensitization, and down-regulation. In this study, we investigated whether opioids induced internalization of the human and rat \(\kappa\) opioid receptors stably expressed in Chinese hamster ovary cells, the potential mechanisms involved in this process and its possible role in activation of mitogen-activated protein (MAP) kinase. Exposure of the human \(\kappa\) opioid receptor to the agonists U50,488H, U69,593, ethylketocyclazocine, or tifluadom, but not etorphine, promoted receptor internalization. However, none of these agonists induced significant internalization of the rat \(\kappa\) opioid receptor. U50,488H-induced human \(\kappa\) receptor internalization was time- and concentration-dependent, with 30–40% of the receptors internalized following a 30-min exposure to 1 \(\mu\)M U50,488H. Agonist removal resulted in the receptors gradually returning to the cell surface over a 60-min period. The antagonist naloxone blocked U50,488H-induced internalization without affecting internalization itself, while pretreatment with pertussis toxin had no effect on U50,488H-induced internalization. In contrast, incubation with sucrose (0.4–0.8 M) significantly reduced U50,488H-induced internalization of the \(\kappa\) receptor. While co-expression of the wild type GRK2, \(\beta\)-arrestin, or dynamin I had no effect on \(\kappa\) receptor internalization, co-expression of the dominant negative mutants GRK2-K220R, \(\beta\)-arrestin (319–418), or dynamin I-K44A significantly inhibited receptor internalization. Whether receptor internalization is critical for MAP kinase activation was next investigated. Co-expression of dominant negative mutants of \(\beta\)-arrestin or dynamin I, which greatly reduced U50,488H-induced internalization, did not affect MAP kinase activation by the agonist. In addition, etorphine, which did not promote human \(\kappa\) receptor internalization, was able to fully activate MAP kinase. Moreover, U50,488H or etorphine stimulation of the rat \(\kappa\) receptor, which did not undergo internalization, also effectively activated MAP kinase. Thus, U50,488H-induced internalization of the human \(\kappa\) opioid receptor in Chinese hamster ovary cells occurs via a GRK-\(\beta\)-arrestin- and dynamin I-dependent process that likely involves clathrin-coated pits. In addition, internalization of the \(\kappa\) receptor is not required for activation of MAP kinase.

Opioid receptors are members of the G protein-coupled receptor (GPCR) family and can be classified into at least three types, \(\mu\), \(\delta\), and \(\kappa\), based on pharmacological (for review, see Ref. 1), anatomical (2), and molecular analysis (for reviews, see Refs. 3 and 4). Activation of \(\kappa\) opioid receptors produces many effects including analgesia (5, 6), dysphoria (6, 7), water diuresis (5, 6), hypothermia (8), and modulation of immune responses (9). \(\kappa\) opioid receptors are coupled through G proteins to affect a variety of effectors, which include adenylate cyclase, potassium channels, calcium channels (for review, see Ref. 10), and mitogen-activated protein kinase pathways (11).

Most GPCRs show an attenuated responsiveness to agonists following prolonged or repeated activation. Three temporally distinct processes that occur over a time scale of seconds to days have been implicated: desensitization (seconds to hours), internalization (minutes to hours), and down-regulation (hours to days). These processes have been best studied for the \(\beta\)-adrenergic receptor (\(\beta\)-AR) (for reviews, see Refs. 12–14). Chronic use of \(\kappa\) opioid agonists causes tolerance (15) that can be partially accounted for at the receptor level (5, 15–17).

Internalization or sequestration is generally envisioned to be a rapid agonist-induced movement of the receptor into a cell compartment distinct from the plasma membrane, where it is unavailable for binding hydrophilic ligands but remains detectable by hydrophobic ligands (18–21). GPCR internalization has been widely studied and has been shown to be important for resensitization of the \(\beta\)-AR (18, 22) as well as desensitization of m3 muscarinic receptors (m3AchR) (23). Agonist-induced \(\beta\)-AR internalization also appears to be involved in receptor down-regulation (24) and activation of mitogen-activated protein kinase pathways (25). While the mechanisms have not been completely elucidated, there appear to be at least two distinct pathways involved in agonist-induced internalization of GPCRs. The first one, for which the \(\beta\)-AR has been best characterized, is mediated by agonist-promoted phosphorylation of the receptor by G protein-coupled receptor kinases.

The abbreviations used are: GPCR, G protein-coupled receptor; hkor, human \(\kappa\) opioid receptor; GRK, G protein-coupled receptor kinase; CHO, Chinese hamster ovary cells; CHO-hkor cells, Chinese hamster ovary cells stably transfected with the cloned human \(\kappa\) opioid receptor; CHO-rkor, CHO cell line stably transfected with the rat \(\kappa\) opioid receptor; rkor, the rat \(\kappa\) opioid receptor; \(\beta\)-AR, \(\beta\)-adrenergic receptor; mAchR, muscarinic cholinergic receptor; MAP kinase, mitogen-activated protein kinase; U50,488H, 2-(1-pyrrolidinyl)cyclohexylbenzeneacetamide; GTP\(\gamma\)S, guanosine 5\'-3-(thio)-triphosphate.
(GRKs), binding of β-arrestin, binding of the phosphorylated receptor-β-arrestin complex to clathrin, and subsequent endocytosis of the receptor in a process dependent on dynamin (26–30). The other, for which the angiotensin II type 1A receptor is prototypic, appears to be β-arrestin and dynamin-independent (29). Some GPCRs, such as the cholecystokinin receptor, appear to be internalized by both pathways (31).

Agorist-induced internalization of μ and δ opioid receptors has been extensively studied (20, 32–42). Endorphine and various peptide agonists promote internalization of both μ and δ opioid receptors to transferrin-containing endosomes, while morphine and levorphan do not (32, 33, 37, 39–42). Hypertonic sucrose solutions block agonist-induced internalization of μ and δ opioid receptors (33, 34, 37). Overexpression of GRK2 enhanced receptor phosphorylation by morphine and facilitated morphine-induced μ opioid receptor internalization (43, 44), whereas expression of the dominant negative mutant GRK2-K220M substantially reduced etorphine-induced μ opioid receptor internalization (43). While co-expression of β-arrestin enhanced morphine-induced μ opioid receptor internalization (44), expression of its dominant negative mutant β-arrestin-V53D reduced etorphine-induced μ opioid receptor internalization (43). Dominant negative mutants of dynamin also inhibited etorphine-induced internalization of μ and δ opioid receptors (38, 43, 44). While opioid receptor internalization has not been extensively investigated, the potent non-selective opioid agonist etorphine did not induce internalization of the mouse κ opioid receptor expressed in HEK293 cells (38).

We have previously established a CHO cell line stably transfected with the human κ opioid receptor (CHO-hkor) (45, 46). These cells exhibit the expected binding affinity and specificity for opioid ligands, and activation by κ opioid agonists enhances [35S]GTPyS binding to pertussis toxin-sensitive G proteins and inhibits forskolin-stimulated adenylate cyclase (45, 46). In addition, we observed that after exposure of CHO-hkor cells to U50,488H, a selective κ opioid agonist, the human κ opioid receptor underwent desensitization and down-regulation (46). A CHO cell line stably expressing the rat κ opioid receptor (47) was also established. In the present study, we investigated whether agonists promoted internalization of the human and rat κ opioid receptors in CHO cells and, if so, whether GRK, β-arrestin, and dynamin I were involved in this process. In addition, since Daaka et al. (25) demonstrated that β-arrestin- and dynamin-mediated internalization of the β2-AR and lysophosphatidic acid receptor was essential for activation of the MAP kinase pathway, we also examined whether agonist-promoted κ receptor internalization played a critical role in activation of MAP kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Diprenorphine (58 Ci/mmol) was purchased from NEN Life Science Products Inc. (Boston, MA). (–)U50,488H and U69,593 were generous gifts from Upjohn Co. (Kalamazoo, MI). Diprenorphine, etorphine, ethylketocyclazocine, and ifufudan were provided by the National Institute on Drug Abuse. Reagents were purchased from the indicated companies: naloxone, RBI (Natick, MA); dynorphin A (1–17), Peninsula Laboratories (Belmont, CA); LipofectAMINE, penicillin, and streptomycin, Life Technologies, Inc. Co. (Manassas, VA); PhosphoPlus p44/p42 MAP Kinase Antibody Kit, New England BioLabs (Beverly, MA); Dulbecco’s modified Eagle’s medium/ Ham’s F-12, pertussis toxin, and sucrose, Sigma; genetin (G418 sulfate), Mediatech Co. (Herndon, VA); fetal calf serum, HyClone Co. (Logan, UT); Constitutively active chemiluminescence was obtained from Amersham. Clones of dynamin I and dynamin I-K44A (clone pHUH10–3) (48, 49) were obtained from Drs. S. Schmid and H. Damke and cloned into pcDNA3 as described (24).

**Stable Expression of Human and Rat κ Opioid Receptors in CHO Cells**—CHO cells were transfected with the hkor cDNA in the vector pBK-CMV (50) or the rkor cDNA in pcDNA3 (47) and cloned cell lines stably expressing hkor or rkor were established with genetin selection (0.5 mg/ml) as described previously (45). CHO-hkor cells and CHO-rkor cells express approximately 1.2 pmol of the human κ receptor and 1.0 pmol of the rat κ receptor per mg of membrane protein, respectively, as determined by [3H]diprenorphine binding (46). CHO-hkor and CHO-rkor cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 0.1 mg/ml gentamicin, 10 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO2 and 95% air at 37 °C.

**Internalization of the κ Receptor following Agonist Exposure**—Opioid receptor binding on intact cells was conducted according to Ref. 51. Briefly, binding of CHO-hkor cells to CHO-hor receptor internalization with [3H]diprenorphine in Krebs-Ringer HEPES buffer solution (110 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.4). Binding of 2 nM [3H]diprenorphine to untreated CHO-hkor cells in the presence or absence of 1 μM diprenorphine was compared at three different temperatures: 0 °C, 12 °C, and room temperature (–22 °C). Binding reached equilibrium in 4 h at 0 °C, 3 h at 12 °C, and 1 h at room temperature. At equilibrium, similar binding levels were attained at the three temperatures. Saturation [3H]diprenorphine binding to intact cells was performed at room temperature for 1 h and the Kd of [3H]diprenorphine was determined to be ~2 nM, which is much higher than the Kd of [3H]diprenorphine binding to κ receptors in membranes conducted in 50 mM Tris/HCl buffer (pH 7.4) (0.2 nM) (45).

CHO-hkor cells cultured in 24-well plates were incubated with U50,488H for the indicated intervals (up to 60 min) at 37 °C. Culture medium was removed and the cells were washed three times on ice with ice-cold phosphate-buffered saline (pH 7.0). Total receptor levels were assessed by binding with 2 nM [3H]diprenorphine in the presence or absence of 1 μM diprenorphine, while surface receptors were measured by binding with 2 nM [3H]diprenorphine in the presence or absence of 1 μM dynorphin A(1–17). Typically, binding was performed at room temperature for 60 min. Diprenorphine, a hydrophobic ligand, can bind to both cell surface and intracellular receptors, whereas dynorphin A(1–17), a hydrophilic ligand, binds only to the cell surface receptors. Thus, the difference between total receptor binding and cell surface receptor binding represents the binding to the intracellular receptor pool. An increase in intracellular [3H]diprenorphine binding over the basal level following agonist exposure provides a quantitative measure of internalized receptors.

**Expression of GRK2, β-Arrestin, and Dynamin I and Their Dominant Negative Mutants**—CHO-hkor cells grown in 100-mm dishes were transiently transfected with 8 μg of bovine GRK2 (52) in pcDNA3.1 Zeo+, GRK2-R220R (53) in pcDNA3.1 Zeo+, bovine β-arrestin (54) in pcDNA3.1 Zeo(+) or pcDNA3 or dynamin I or dynamin I-K44A (48, 49) in pcDNA3 following the manufacturer’s instructions. Control cells were transfected with pcDNA3.1 Zeo(+) or pcDNA3 and exhibited no opioid receptor binding (data not shown). Following transfection (~18 h) the cells were incubated with fresh medium and allowed to recover 24–28 h before being reseeded in 24-well dishes and allowed to grow an additional 24 h. The cells were then analyzed for agonist-induced receptor internalization.

**MAP Kinase Phosphorylation**—CHO-hkor or CHO-rkor cells were transferred to 24-well plates and changed to serum-free medium for 2 h to overnight to reduce basal MAP kinase phosphorylation. Cells were treated with or without an agonist at 37 °C for 10 min and then lysed by addition of Laemmli sample buffer. Aliquots of the lysates were separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Phosphorylated MAP kinase was detected by Western blot using phosphospecific MAP kinase antibodies and enzyme-linked chemiluminescence with the PhosphoPlus p44/p42 MAP Kinase (Thr202/Tyr204) Antibody Kit according to the manufacturer’s instructions.

**Statistical Analysis**—For comparison of multiple groups, data were analyzed by analysis of variance to determine if there were significant differences among groups. If so, Scheffe F test was performed to determine whether there were significant differences between control and treatment groups. For comparison of two groups, Student’s t test was performed. p < 0.05 was the level of significance in all statistical analyses.

**RESULTS AND DISCUSSION**

**Effects of Opioid Agonists on Internalization of the Human κ Opioid Receptor**—We first assessed whether exposure to the selective κ agonist U50,488H (1 μM) promoted internalization...
of the human \( \kappa \) opioid receptor. CHO-hkor cells were treated with or without U50,488H at 37 °C for 30 min and internalized receptors were determined by \[^{3}H\]diprenorphine binding as described under "Experimental Procedures." A, 1 \( \mu \)M U50,488H pretreatment and \[^{3}H\]diprenorphine binding at three different temperatures; B, pretreatment with different concentrations of U50,488H and \[^{3}H\]diprenorphine binding at room temp. Each point represents mean ± S.E. of three independent experiments. *, \( p < 0.05 \) compared with the basal level by analysis of variance followed by Scheffe F-test.

**Fig. 1. Effects of U50,488H on internalization of the human \( \kappa \) opioid receptor.** CHO-hkor cells were treated with U50,488H at 37 °C for 30 min and internalized receptors were determined by \[^{3}H\]diprenorphine binding as described under “Experimental Procedures.” A, 1 \( \mu \)M U50,488H pretreatment and \[^{3}H\]diprenorphine binding at three different temperatures; B, pretreatment with different concentrations of U50,488H and \[^{3}H\]diprenorphine binding at room temp. Each point represents mean ± S.E. of three independent experiments. *, \( p < 0.05 \) compared with the basal level by analysis of variance followed by Scheffe F-test.

**Fig. 2. Agonist and species differences of the \( \kappa \) opioid receptor internalization.** CHO-hkor cells and CHO-rkor cells were treated with U50,488H, U69,593, ethylketocyclazocine (EKC), tifluadom, or etorphine at 1 \( \mu \)M each at 37 °C for 30 min and internalized receptors were determined by \[^{3}H\]diprenorphine binding at room temperature as described under “Experimental Procedures.” A, agonist-induced internalization of human \( \kappa \) opioid receptor; B, agonist-induced internalization of rat \( \kappa \) opioid receptor. Each point represents mean ± S.E. of three to five independent experiments. *, \( p < 0.05 \) compared with the basal level by analysis of variance followed by Scheffe F-test.

**Agonist and Species Differences in the \( \kappa \) Opioid Receptor Internalization**—We next tested whether other full agonists also promoted internalization of human \( \kappa \) opioid receptors. Etorphine, a potent nonselective opioid agonist, did not induce internalization of the mouse \( \kappa \) opioid receptor (38) and indeed it also did not promote internalization of the human \( \kappa \) opioid receptor (Fig. 2A). Since etorphine was previously shown to promote internalization of \( \mu \) and \( \delta \) opioid receptors (33, 38, 40, 41), these results confirm a type-specific difference among opioid receptors in their response to this agonist observed by Chu and colleagues (38). In contrast, incubation with 1 \( \mu \)M of several full agonists, U69,593, ethylketocyclazocine, or tifluadom, effectively promoted \( \kappa \) receptor internalization (Fig. 2A). Thus, opioid agonists appear to have differential abilities to promote \( \kappa \) receptor internalization. Interestingly, similar observations have been reported for \( \mu \) and \( \delta \) opioid receptors (32, 33, 39–42). Peptide ligands and etorphine caused internalization of \( \mu \) and \( \delta \) receptors in HEK293 cells while morphine and levorphanol did not (32, 33, 39, 40, 42). These differences have also been observed in vivo. Etorphine injected intraperitoneally induced \( \mu \) receptor internalization into endosomes in the guinea pig myenteric plexus (41) and the rat brain (42). However, morphine did not cause detectable internalization, and it partially inhibited the etorphine-induced \( \mu \) receptor endocytosis (41, 42).

We also examined whether the rat \( \kappa \) opioid receptor stably expressed in CHO cells underwent agonist-induced internalization. To our surprise, in contrast to the human \( \kappa \) opioid receptor, the rat \( \kappa \) opioid receptor was not internalized to a significant extent following incubation with U50,488H, U69,593, ethylketocyclazocine, tifluadom, or etorphine at 1 \( \mu \)M (Fig. 2B). Thus, there appears to be a species difference between human and rat in agonist-induced internalization of the \( \kappa \) opioid receptor in CHO cells. Whether the rat \( \kappa \) opioid receptor undergoes agonist-induced internalization has not been examined in vivo. It seems likely that the inability of agonists to promote internalization of the rat \( \kappa \) opioid receptor may be due to the cell system used. Interestingly, Murray et al. (56) reported recently that a truncated \( \delta \) opioid receptor underwent agonist-induced internalization in HEK293 cells, but not in CHO cells. However, since both rat and human \( \kappa \) opioid receptors were expressed in CHO cells, this difference provides a
unique opportunity for molecular and biochemical analysis. Comparison of the third intracellular loop and C-terminal domain sequences of the rat and human kappa opioid receptors reveals several differences. One difference, which may be of relevance to receptor internalization, is that Ser358 in the C-terminal domain of the human receptor is replaced by Asn 358 in the rat receptor. Serine residues can be phosphorylated, while Asn cannot. Whether this difference contributes to differential internalization is being investigated.

Time Courses of U50,488H-induced Internalization and Recycling of the Human Kappa Opioid Receptor—Treatment of CHO-hkor cells with 1 μM U50,488H at 37 °C resulted in a time-dependent increase in internalized kappa opioid receptors (Fig. 3A). Internalization was initially rapid with a half-maximal increase at ~10 min and then gradually slowed approaching a plateau at ~60 min.

To assess whether the internalized receptors could return to the cell surface, CHO-hkor cells were initially treated with 1 μM U50,488H at 37 °C for 30 min, washed extensively, and then allowed to recover at 37 °C in the absence of agonist. Internalized receptors were found to gradually return to the cell surface over a 60-min period (Fig. 3B). The rate of receptor return was initially rapid with a half-maximum at ~20 min and then gradually slowed. These results indicate that U50,488H-induced kappa receptor internalization is time-dependent and reversible after agonist removal.

Effects of Naloxone and Pertussis Toxin on Internalization of the Human Kappa Opioid Receptor—Naloxone (10 μM), a non-selective opioid antagonist, had no direct effect on receptor internalization, but effectively blocked U50,488H-induced internalization (Fig. 4A). Thus, U50,488H-induced kappa receptor internalization requires receptor activation, although in the case of etorphine, receptor activation does not appear to be sufficient to promote internalization.

We also examined whether coupling to pertussis toxin-sensitive G proteins was required for internalization of the kappa receptor. Pretreatment with pertussis toxin (100 ng/ml) for 18 h, which abolished U50,488H-promoted MAP kinase phosphorylation (data not shown), did not affect basal or U50,488H-induced internalization (Fig. 4B). This demonstrates that activation of pertussis toxin-sensitive G proteins is not necessary.

**Fig. 3.** Time courses of (A) U50,488H-induced internalization of the human kappa opioid receptor and (B) return of internalized kappa receptors to the cell surface. A, CHO-hkor cells were treated with 1 μM U50,488H at 37 °C for different periods of time. B, CHO-hkor cells were treated with 1 μM U50,488H at 37 °C for 30 min, washed extensively and allowed to recover in culture medium at 37 °C for different intervals. Internalized receptors were determined as described under “Experimental Procedures.” Each point represents mean ± S.E. of three or four (A) or three to six (B) independent experiments.

**Fig. 4.** Effects of naloxone (A), pertussis toxin (B), and hypertonic sucrose (C) solutions on internalization of the human kappa opioid receptor. A, CHO-hkor cells were treated with or without 1 μM U50,488H at 37 °C for 30 min in the presence or absence of 10 μM naloxone. B, CHO-hkor cells were treated with or without 1 μM U50,488H at 37 °C for 30 min after pretreatment with pertussis toxin (100 ng/ml) for 18 h. C, CHO-hkor cells were treated with or without 1 μM U50,488H at 37 °C for 30 min in the presence of different concentrations of sucrose. Internalized receptors were determined as described under “Experimental Procedures.” Each point represents mean ± S.E. of three independent experiments. *, p < 0.05 compared with the basal level by analysis of variance followed by Scheffe F-test.
for κ receptor internalization. In this regard, the κ receptor is similar to the δ opioid receptor where DADLE- promoted internalization of the δ receptor in Neuro2A cells was not affected by pertussis toxin treatment (36). In contrast, DAMGO- induced internalization of the μ opioid receptor in Neuro2A cells was completely abolished by pertussis toxin pretreatment (36).

Effect of Hypertonic Sucrose Solutions on Internalization of the Human κ Opioid Receptor—Hypertonic solutions, such as sucrose, have been shown to inhibit receptor-mediated endocytosis by blocking the formation of clathrin-coated pits (57). Sucrose (0.4–0.8 M) reduced U50,488H-induced internalization of the human κ opioid receptor by about 90%, without affecting the basal level (Fig. 4C). This suggests that clathrin-coated pits are likely involved in κ receptor internalization. Indeed, similar results have been obtained with the μ and δ receptors where hyperosmolar sucrose inhibited agonist-induced internalization of μ (33, 37) and δ opioid receptors (33, 34) expressed in HEK293 cells.

Effect of GRK2 and GRK2-K220R on Internalization of the Human κ Opioid Receptor—GRK2-mediated phosphorylation of the β2-AR and the m2AchR has been shown to play an important role in agonist-induced internalization (27, 58). Thus, we next investigated whether GRK2 was also involved in human κ opioid receptor internalization. CHO-hkor cells were transiently transfected with wild type GRK2, the dominant negative mutant GRK2-K220R, or vector. While expression of GRK2 had no effect on either basal or agonist-induced internalization, GRK2-K220R expression substantially reduced U50,488H-induced internalization (Fig. 5). This effect is quite significant since GRK2-K220R was transiently transfected and thus only ~50% of the cells are expressing GRK2-K220R, while 100% of the cells are expressing the stably transfected κ receptor. These observations suggest that GRK-mediated phosphorylation of the κ receptor may be involved in mediating κ receptor internalization. While we have not directly investigated U50,488H-induced phosphorylation of the human κ receptor in CHO cells, Appleyard and co-workers (59) recently demonstrated that the κ opioid receptor in guinea pig hippocampal slices was phosphorylated to a higher degree in U50,488H-tolerant animals than in control animals.

The finding that dominant negative GRK2 attenuated U50,488H-induced κ receptor internalization is similar to results obtained with the β2-AR, m2AchR, and μ opioid receptors. Co-expression of dominant negative GRK2 mutant was shown to attenuate agonist-promoted phosphorylation and internalization of both the β2-AR (27) and m2AchR (58, 60) and internalization of the μ opioid receptor (43). That wild type GRK2 had no effect on U50,488H-induced κ receptor internalization may be due to the relatively high endogenous level of GRK2 present in CHO cells (61). Indeed, expression of GRK2 had a minimal effect on agonist-promoted internalization of the wild type β2-AR in HEK 293 cells, which also have high endogenous levels of GRK2 (61), while having a significant effect on internalization of β2-AR-Y326A, an internalization-defective mutant (27). Wild type GRK2 also enhanced morphine-induced phosphorylation and internalization of the μ opioid receptor in HEK293 cells (43).

Effect of β-Arrestin and β-Arrestin (319–418) on Internalization of the Human κ Opioid Receptor—The non-visual arrestins, β-arrestin and arrestin3, play an integral role in agonist-promoted internalization of the β2-AR (28, 30). This appears to be due to their ability to function as adapter proteins, being capable of binding to both GRK-phosphorylated receptors (26) and clathrin (30), the major protein component of clathrin-coated pits. To assess the potential involvement of β-arrestin in human κ opioid receptor internalization, we transiently transfected CHO-hkor cells with β-arrestin, β-arrestin (319–418), or vector. Wild type β-arrestin had no apparent effect on κ receptor internalization (Fig. 6). However, β-arrestin (319–418), a dominant negative mutant that inhibits receptor internalization by binding constitutively to clathrin (55), effectively reduced both basal and U50,488H-induced internalization (Fig. 6). These results suggest that β-arrestin plays a significant role in U50,488H-induced internalization of the κ opioid receptor in CHO cells. These results are similar to recent observations on the μ opioid receptor (43, 44).

Recent studies by Menard and co-workers (61) have correlated agonist-promoted internalization of the β2-AR with the endogenous cellular levels of GRK2 and arrestins. They demonstrated that in cell lines that have high GRK and arrestin levels, such as HEK293 and CHO, expression of wild type arrestins has a minimal effect on β2-AR internalization (28, 29, 55, 61). However, these cells tend to be good models for studying the effects of dominant negative mutants of GRKs and arrestins. Thus, dominant negative arrestin mutants such as β-arrestin (319–418) and β-arrestin-V53D, which have a reduced ability to bind to GPCRs, are effective inhibitors of agonist-promoted internalization of the β2-AR in HEK293 cells.
Scheffe F-test. Compared with the vector control by analysis of variance followed by represents mean in a dose-dependent manner (Figs. 8 and 9). Expression of hkor cells by U50,488H increased MAP kinase phosphorylation effectively reduced the basal level of I-K44A did not completely block observation that internalized receptors, although fewer in number, might still be sufficient to fully activate MAP kinase.

Effect of Dynamin I and Dynamin I-K44A on Internalization of the Human κ Opioid Receptor—Dynamin I is a GTPase that regulates the formation of clathrin-coated vesicles (62). Dynamin I mutants that are defective in GTP binding, such as dynamin I-K44A, effectively block endocytosis at a stage after the initiation of coat assembly and preceding the sequestration of ligands into deeply invaginated coated pits (48). To further investigate the mechanism of κ opioid receptor internalization, we transiently transfected CHO-hkor cells with dynamin I, dynamin I-K44A, or vector. Dynamin I-K44A significantly reduced both basal and U50,488H-induced internalization, while wild type dynamin I had no effect on receptor internalization (Fig. 7). These results are similar to those previously observed for the β2-AR (29) and μ and δ opioid receptors (38, 43), and further implicate a role for clathrin-coated pits in κ opioid receptor internalization.

One potentially intriguing aspect of these results was the observation that β-arrestin (319–418) and dynamin I-K44A effectively reduced the basal level of κ receptor internalization (Figs. 6 and 7). These results suggest that the human κ receptor may be constitutively internalized (in the absence of agonist) and recycled back to the cell surface in a dynamic process that results in a steady state level of internalized receptor. β-Arrestin (319–418) and dynamin I-K44A effectively attenuated this process resulting in a lower steady state level of internalized κ receptors while U50,488H effectively stimulated this process.

Role of κ Opioid Receptor Internalization in MAP Kinase Activation—Activation of human κ opioid receptor in CHO-hkor cells by U50,488H increased MAP kinase phosphorylation in a dose-dependent manner (Figs. 8 and 9). Expression of β-arrestin (319–418) or dynamin I-K44A in CHO-hkor cells, which effectively reduced the κ receptor internalization, did not have any effect on U50,488H-induced increase in MAP kinase phosphorylation (Fig. 8). This result suggests that internalization of the human κ receptor is not essential for MAP kinase activation. However, since β-arrestin (319–418) or dynamin I-K44A did not completely block κ receptor internalization, it is possible that the internalized receptors, although fewer in number, might still be sufficient to fully activate MAP kinase.

We next investigated whether etorphine, which did not promote internalization of the human κ receptor, increased MAP kinase phosphorylation. Etorphine was able to stimulate the human κ receptor to activate MAP kinase to a similar extent as U50,488H (Fig. 9A). In addition, activation by U50,488H or etorphine of the rat κ receptor, which did not undergo internalization, effectively activated MAP kinase (Fig. 9B). The finding is
similar to those of Fukuda et al. (11) that activation of the rat κ opioid receptor expressed in CHO cells increased MAP kinase phosphorylation. These results indicate that internalization of the κ opioid receptor is not required for activation of MAP kinase. Indeed, a similar dissociation between receptor internalization and MAP kinase activation was observed for α2-adrenergic receptors expressed in COS-1 cells.² These findings are different from those of Daaka et al. (25) that internalization of β2-adrenergic and lysophosphatidic acid receptors is essential for MAP kinase activation. The reason for these differences between the κ opioid receptor and β2-adrenergic and lysophosphatidic acid receptors is not clear. Since the lysophosphatidic acid receptor is a Gαq-coupled receptor, like the κ opioid receptor, the discrepancy cannot be attributed to differences in G protein coupling. Different cell systems may contribute in part to the observed difference. While Daaka et al. (25) conducted their studies in HEK 293 cells, we did it in CHO cells. In addition, the κ opioid receptor may activate MAP kinase via a pathway distinct from those used by β2-adrenergic and lysophosphatidic acid receptors.

Methods for Assessing GPCR Internalization—The two major methods that have been used to examine GPCR internalization are radiolabeled ligand binding (18–23, 27, 34, 60, 63) and fluorescence microscopy primarily using either immunofluorescence of epitope-tagged receptors (32–34, 36–38, 40) or fluorescence of green fluorescent protein-tagged receptors (64, 65). In the present study, we employed radiolabeled ligand binding to circumvent the need to express and characterize an epitope-tagged receptor. This technique is dependent on the ability of hydrophobic ligands to freely pass through the plasma membrane and bind to both cell surface and intracellular receptors, while hydrophilic ligands will only bind to cell surface receptors. While this method is simple and enables an accurate quantitation of cell surface and internalized receptors, it does have potential limitations. Perhaps the main limitation for most GPCRs is the availability of effective hydrophobic and hydrophilic ligands. While such compounds are available to study the β2-AR, mAChR, and opioid receptors, there are many GPCRs where this method cannot be used. Another limitation is the requirement for effective removal of the pretreatment ligand. For ligands that are difficult to wash out, such as dynorphin peptides and norbinaltorphimine, it is impossible to determine their effects on receptor internalization using this method. We nevertheless feel that this is an appropriate method for the present study. Indeed results obtained with similar binding methods (18, 19, 22, 27, 34, 63) were often confirmed using immunofluorescence or fluorescence methods (30, 34, 38, 64, 65).

In conclusion, we demonstrated that agonist induced internalization of κ opioid receptors. There appears to be agonist and species differences. Exposure of the human κ receptor to U50,488H, U69,593, ethylketocyclazocine, or tifluadom, but not etorphine, promotes a time- and concentration-dependent increase in intracellular receptors. In contrast, the rat κ opioid receptor was not internalized significantly following exposure to the same agonists. U50,488H-induced human κ receptor internalization is reversible and requires receptor activation, but not receptor/G protein coupling. U50,488H-induced internalization of the human κ opioid receptor is mediated by a GRK-, β-arrestin-, and dynamin I-dependent process that likely involves clathrin-coated pits. Finally, agonist-induced receptor internalization of the κ receptor is not essential for MAP kinase activation.

²DeGraff, J. L., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) J. Biol. Chem., in press.
Internalization of κ Opioid Receptor

47. Li, S., Zhu, J., Chen, C., Chen, Y.-W., de Riel, J. K., Ashby, B., and Liu-Chen, L.-Y. (1993) Biochem. J. 285, 629–633
48. van der Bliek, A. M., Redelmeier, T. E., Danke, H., Tisdale, E. J., Meyerowitz, E. M., and Schmid, S. L. (1993) J. Cell Biol. 122, 553–563
49. Danke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
50. Zhu, J., Chen, C., Xue, J.-C., Kunapuli, S., de Riel, J. K., and Liu-Chen, L.-Y. (1995) Life Sci. 56, 201–207
51. Toll, L. (1992) J. Pharmacol. Exp. Ther. 260, 9–15
52. Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989) Science 246, 235–240
53. Kong, G., Penn, R., and Benovic, J. (1994) J. Biol. Chem. 269, 13084–13087
54. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) Science 248, 1547–1550
55. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 32567–32572
56. Murray, S. R., Evans, C. J., and von Zastrow, M. (1998) J. Biol. Chem. 273, 24987–24991
57. Heuser, J. E., and Anderson, R. G. (1989) J. Cell Biol. 108, 389–400
58. Tsuga, H., Kameyama, K., Haga, T., Karose, H., and Nagao, T. (1994) J. Biol. Chem. 269, 32522–32527
59. Appleyard, S. M., Patterson, T. A., Jin, W. Z., and Chavkin, C. (1997) J. Neurochem. 69, 2405–2412
60. Pals-Rylaarsdam, R., Xu, Y., Witt-Endersby, P., Benovic, J. L., and Hosey, M. M. (1995) J. Biol. Chem. 270, 29004–29011
61. Menard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) Mol. Pharmacol. 51, 800–808
62. Schmid, S. L. (1997) Annu. Rev. Biochem. 66, 511–548
63. Barak, L. S., Tiberi, M., Freedman, N. J., Kwastra, M. M., Lefkowitz, R. J., and Caron, M. G. (1994) J. Biol. Chem. 269, 2790–2795
64. Kallal, L., Gagnon, A. W., Penn, R. B., and Benovic, J. L. (1998) J. Biol. Chem. 273, 322–328
65. Barak, L. S., Ferguson, S. S., Zhang, J., Martenson, C., Meyer, T., and Caron, M. G. (1997) Mol. Pharmacol. 51, 177–184