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Mechanisms of Agonist-Induced Down-Regulation of the Human κ-Opioid Receptor: Internalization Is Required for Down-Regulation

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ABSTRACT

Previously, we showed that the human κ-opioid receptor (hkor) stably expressed in Chinese hamster ovary (CHO) cells underwent down-regulation after prolonged U50,488H treatment. In the present study, we determined the mechanisms underlying this process. U50,488H caused a significant down-regulation of the hkor, although etorphine did not. Neither U50,488H nor etorphine caused down-regulation of the rat κ-opioid receptor. Thus, similar to internalization, there are agonist and species differences in down-regulation of κ-opioid receptors. Expression of the dominant negative mutants arrestin-2(319-418) or dynamin I-K44A significantly reduced U50,488H-induced down-regulation of the hkor. Coexpression of GRK2 or GRK2 and arrestin-2 permitted etorphine to induce down-regulation of the hkor, although expression of arrestin-2 or dynamin I alone did not. Expression of the dominant negative mutants rab5A-N133I or rab7-N125I blunted U50,488H-induced down-regulation. Pretreatment with lysosomal enzyme inhibitors [(2*S*,3*S*)*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester or chloroquine] or proteasome inhibitors (proteasome inhibitor I, MG-132, or lactacystin) decreased the extent of U50,488H-induced down-regulation. A combination of chloroquine and proteasome inhibitor I abolished U50,488H-induced down-regulation. These results indicate that U50,488H-induced down-regulation of the hkor involves GRK-, arrestin-2-, dynamin-, rab5-, and rab7-dependent mechanisms and receptors seem to be trafficked to lysosomes and proteasomes for degradation. Thus, U50,488H-induced internalization and down-regulation of the hkor share initial common mechanisms. To the best of our knowledge, these results represent the first report on the involvement of both rab5 and rab7 in agonist-induced down-regulation of a G protein-coupled receptor. In addition, this study is among the first to show the involvement of proteasomes in agonist-induced down-regulation of a G protein-coupled receptor.

Opioid receptors have been classified into at least three types (μ, δ, and κ) based on pharmacological (for a review, see Pasternak, 1988), anatomical (for a review, see Mansour et al., 1988), and molecular analysis (for a review, see Knapp et al., 1995). Activation of κ-opioid receptors produces many effects including analgesia (von Voigtlander et al., 1983; Dykstra et al., 1987), dysphoria (Pfeiffer et al., 1986; Dykstra et al., 1987), water diuresis (von Voigtlander et al., 1983; Dykstra et al., 1987) and hypothermia (Adler and Geller, 1993). Chronic use of κ-opioid agonists causes tolerance (Bhargava et al., 1989) that can be partially accounted for at the receptor level (von Voigtlander et al., 1983; Bhargava et al., 1989; Morris and Herz, 1989; Joseph and Bidlack, 1995; Jin et al., 1997). After the cloning of the mouse κ-opioid receptor (Evans et al., 1992; Kieffer et al., 1992), cloning of the κ-opioid receptor from several other species was reported (for a review, see Knapp et al., 1995). Deduced amino acid sequences of these clones display the motif of seven transmembrane helices, characteristic of G protein-coupled receptors (GPCRs).

Many GPCRs show adaptive responses to agonists after prolonged or repeated activation. Three processes are involved in response to agonists occurring over a time scale ranging from seconds to days: desensitization (seconds to hours), internalization (minutes to hours), and down-regulation (hours to days) (for reviews, see Hausdorff et al., 1990; Krupnick and Benovic, 1998; Roth et al., 1998). These processes have been best studied for the β_2-adrenergic receptor

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ABBREVIATIONS: GPCR, G protein-coupled receptors; β_2AR, β_2-adrenergic receptor; DADLE, [D-Ala^2,D-Leu^5]-enkephalin; CHO, Chinese hamster ovary; GRK, G protein-coupled receptor kinases; EST, (2*S*,3*S*)*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; CHO-hkor, CHO cells stably transfected with the cloned human κ-opioid receptor; CHO-rkor, CHO cells stably transfected with the cloned rat κ-opioid receptor; proteasome inhibitor I, Z-Ile-Glu(OtBu)-Ala-Leu-CHO; MG-132, carbobenzoxy-L-leucy-L-leucinal; GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate.

(β_2AR) (for reviews, see Hausdorff et al., 1990; Krupnick and Benovic, 1998).

Internalization of GPCRs is generally envisioned to be a rapid agonist-induced movement of the receptor into endosomes from the plasma membrane where it is unavailable for signal transduction (for reviews, see Hausdorff et al., 1990; Krupnick and Benovic, 1998; Roth et al., 1998). Internalized receptors are thought to have several potential fates. One is dephosphorylation of the receptor in endosomes followed by recycling back to the plasma membrane. Another is that internalized receptors are degraded, which results, in part, in receptor down-regulation. Down-regulation involves a reduction in the number of receptors with or without attenuated responses. For the β_2AR, a multitude of events occurs during down-regulation: enhanced degradation of the receptor (Gagnon et al., 1998; Kallal et al., 1998), reduced transcription of the receptor gene, a decrease in the stability of receptor mRNA, and a corresponding reduction in de novo receptor synthesis (for a review, see Collins et al., 1992).

κ-Opioid receptors have been shown to undergo down-regulation after chronic agonist exposure (Attali and Vogel, 1990; Joseph and Bidlack, 1995; Blake et al., 1997; Zhu et al., 1998). Prolonged agonist treatment led to 30 to 50% reduction in the κ-opioid receptor number. The degree of down-regulation depended on the agonist concentration and incubation time. (−)U50,488H [(−)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide]-induced down-regulation was receptor-mediated and the receptor number returned to normal 24 h after the removal of the agonist (Zhu et al., 1998).

Whether agonist-induced internalization of GPCRs is required for down-regulation is of great interest. Gagnon et al. (1998) demonstrated that arrestin-2 and dynamin-dependent receptor internalization is critical for agonist-induced down-regulation of β_2AR in COS-1, HeLa, and HEK293 cells. In contrast, in L cells and A431 cells, blockade of receptor internalization did not prevent agonist-induced down-regulation of β_2AR (Jockers et al., 1999), demonstrating that in these cells, internalization is not a necessary prerequisite for β_2AR down-regulation. In addition, mutagenesis studies on GPCR have demonstrated that some mutations block agonist-induced internalization without affecting down-regulation (Barak et al., 1994; Cvejic et al., 1996; Trapaidze et al., 1996), whereas other mutations blunt agonist-induced down-regulation, leaving the extent of internalization unaltered (Campbell et al., 1991; Goldman and Nathanson, 1994). These studies, therefore, argue for distinct mechanisms for internalization and down-regulation.

A few studies have addressed the question as to the pathway and the site of receptor degradation during agonist-promoted down-regulation of GPCRs. In NG108-15 cells, chronic [^{3}H][D-Ala^2,D-Leu^5]-enkephalin (DADLE) incubation resulted in internalization of ligand-receptor complexes that were accumulated in the lysosomes in the presence of chloroquine, a lysosomal enzyme inhibitor (Law et al., 1984). In addition, morphological analysis showed that β_2AR was trafficked to early endosomes and then lysosomes (Gagnon et al., 1998; Kallal et al., 1998). These studies point to lysosomes as the site of receptor degradation. In contrast, in L cells, specific blockers of the lysosomal and proteasome-associated degradation pathways were ineffective in preventing β_2AR down-regulation (Jockers et al., 1999). In addition, in A431 cells, inactivation of the lysosomal degradation pathway did not block β_2AR down-regulation, whereas epidermal growth receptor degradation was inhibited (Jockers et al., 1999). These data indicate that in these cells, degradation of β_2AR may occur at the plasma membrane.

We have shown recently that U50,488H, but not etorphine, promoted internalization of the human κ-opioid receptor in Chinese hamster ovary (CHO) cells and GPCR kinases (GRK), arrestin-2 and dynamin I were involved in this process (Li et al., 1999). In contrast, the rat κ-opioid receptor did not undergo internalization when activated by U50,488H or etorphine (Li et al., 1999). In the present study, we investigated whether agonist-induced internalization of κ-opioid receptors was a prerequisite for down-regulation. In addition, we examined the pathway involved in down-regulation of the human κ-opioid receptor by determining the roles of rab5, rab7, proteasomes, and lysosomes in the process.

Experimental Procedures

Materials. [^{3}H]Diprenorphine (58 Ci/mmol) and [^{35}S]GTPγS (1000–1200 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Naloxone and (−)U50,488H were gifts from DuPont/Merck Co.(Wilmington, DE) and Upjohn Co. (Kalamazoo, MI), respectively. The National Institute on Drug Abuse provided diprenorphine and etorphine. (2*S*,3*S*)*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (EST), Z-Ile-Glu(OtBu)-Ala-Leu-CHO (proteasome inhibitor I), carbobenzoxy-L-leucy-Leu-CHO (MG-132), and lactacystin were purchased from Calbiochem (La Jolla, CA) and geneticin (G418 sulfate) from Mediatech Co. (Herndon, VA). Chloroquine and commonly used chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Clones of dynamin I and dynamin I-K44A (clone pUHD10–3) were obtained from Drs. S. Schmid and H. Damke of Scripps Research Institute and cloned into pcDNA3 as described (Gagnon et al., 1998). Expression constructs of rab5A-N133I and rab7-N125I were gifts from Dr. A. Wandinger-Ness of the University of New Mexico.

Stable Expression of Human and Rat κ-Opioid Receptors in CHO Cells. Clonal CHO cells stably transfected with the human or rat κ-opioid receptor (Li et al., 1993; Zhu et al., 1995) (CHO-hkor and CHO-rkor cells, respectively) were established as described previously (Li et al., 1999). CHO-hkor or CHO-rkor cells were cultured in Dulbecco's modified Eagle's medium F12/Ham's medium supplemented with 10% fetal calf serum, 0.1 mg/ml geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2, 95% air at 37°C.

Pretreatment of CHO-hkor Cells with the κ-Opioid Agonist (−)U50,488H. At ~90% confluence, CHO-hkor cells were treated with 1 μM (−)U50,488H in the medium mentioned above for 4 h. In some experiments, cells were preincubated with or without 20 μM EST, 50 μM chloroquine, 5 μM proteasome inhibitor I, 200 μM MG-132, or 20 μM lactacystin for 10 min before U50,488H treatment. Cells were harvested and membranes prepared using a procedure similar to that described previously (Zhu et al., 1998). Briefly, cells were washed twice with 100 mM PBS, pH 7.0, harvested in Versene solution and centrifuged at 500*g* for 3 min and washed once with PBS. The cell pellet was resuspended in 50 mM Tris·HCl buffer containing 1 mM EGTA, 5 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, sonicated, and centrifuged at 46,000*g* for 30 min. The pellet was resuspended in 50 mM Tris, pH 7.0, and centrifuged again. The membrane pellet was resuspended in 50 mM Tris, 0.32 M sucrose, pH 7.0, aliquoted at ~100 μg of protein/ml, frozen in dry ice/ethanol and stored at −70°C until use. All procedures were performed at 4°C.

Expression of GRK2, Arrestin-2 or Dynamin I or the Dominant Negative Mutants of GRK2, Arrestin-2, or Dynamin I, rab5A or rab7. CHO-hkor cells grown in 100-mm dishes were

transiently transfected with 8 μg of bovine GRK2 (Benovic et al., 1989) in pcDNA3.1 Zeo(+); GRK2-K220R (Kong et al., 1994) in pcDNA3.1 Zeo(+); bovine arrestin-2 (Lohse et al., 1990) in pcDNA 3.1 Zeo(+); arrestin-2(319-418) (Krupnick et al., 1997) in pcDNA3; dynamin I or dynamin I-K44A (van der Bliek et al., 1993; Damke et al., 1994) in pcDNA3; rab5A-N133I (Bucci et al., 1992) in pcDNA3; or rab7-N125I (Feng et al., 1995) in pCR3.1 using LipofectAMINE (50 μl) following the manufacturer's instructions. Control cells were transfected with pcDNA 3.1 Zeo(+) or pcDNA3. Transfection efficiency was approximately 60%. Sixty to 72 h later, cells were treated with or without 1 μM U50,488H at 37°C for 4 h, washed extensively and membranes prepared. Saturation [^{3}H]diprenorphine binding was performed and K_d and B_{max} values determined.

κ-Opioid Receptor Binding Assay. Receptor binding was conducted with [^{3}H]diprenorphine in 50 mM Tris·HCl buffer containing 1 mM EGTA and 5 μM leupeptin, pH 7.4, as described previously (Zhu et al., 1998). (−)Naloxone (10 μM) was used to define nonspecific binding. Saturation experiments were performed with various concentrations of [^{3}H]diprenorphine (ranging from 0.02 nM to 2 nM). Competitive inhibition of [^{3}H]diprenorphine binding was performed with [^{3}H]diprenorphine at a concentration close to its K_d value (~0.2 nM) and various concentrations of (−)U50,488H or etorphine. Binding was conducted at 25°C for 60 min in duplicate in a volume of 1 ml with 30 to 40 μg of protein. Bound and free ligands were separated by rapid filtration under reduced pressure over GF/B filters presoaked with 0.2% polyethylenimine and 0.1% BSA in 50 mM Tris·HCl, pH 7.4, for 1 h. Binding data were analyzed with EBDA and LIGAND programs (McPherson, 1983).

[^{35}S]GTPγS Binding Assay. [^{35}S]Guanosine-5′-*O*-(3-thio)-triphosphate (GTPγS) binding assay was performed as described previously (Zhu et al., 1997). Before assay, membranes were thawed at 37°C, chilled on ice, passed through a 22-gauge needle and diluted with 50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA with 1 mM dithiothreitol and 0.1% BSA freshly added (pH 7.4). Membranes (~10 μg of protein) were incubated in the buffer described above containing [^{35}S]GTPγS (100,000–150,000 dpm, ~80 pM), GDP (3 μM) and varying concentrations of the κ-opioid agonist (−)U50,488H (10^{-11} to 10^{-5} M) in a total volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was defined by incubation in the presence of 10 μM GTPγS. Bound and free [^{35}S]GTPγS were separated by filtration through GF/B filters under reduced pressure. Radioactivity on filters was determined by liquid scintillation counting. EC_{50} and maximal response values were calculated by use of the equation $y = [E_{max}/[1 + (x/EC_{50})^s]]$ + background, in which y is the response at the dose x, E_{max} is the maximal response, and s is a slope factor.

Protein Assay. Protein contents of membranes were determined by the bicinchoninic acid method of Smith et al. (1985) with BSA as the standard.

Statistical Analysis. For comparison of multiple groups, data were analyzed with analysis of variance to determine whether there were significant differences among groups. If so, Dunnett's test was performed to determine whether there was significant difference between the control and each treatment group. $P < .05$ was used as the level of significance.

Results and Discussion

Effect of Pretreatment with (−)U50,488H and Etorphine on the Human and Rat κ-opioid Receptor Stably Expressed in CHO Cells. Pretreatment of CHO-hkor cells with (−)U50,488H, but not etorphine, for 4 h caused a significant reduction in the number of human κ-opioid receptors without changing the affinity (Fig. 1). The extent of decrease in receptor number after a 4-h exposure to 1 μM (−)U50,488H at 37°C was ~30%, which is consistent with our previous report (Zhu et al., 1998). We have demonstrated previously that a 24-h preincubation causes a similar extent of down-regulation as a 4-h exposure, whereas a 1-h pretreatment does not promote down-regulation (Zhu et al., 1998). However, unlike the human κ-receptor, neither (−)U50,488H nor etorphine caused a significant down-regulation of the rat κ-opioid receptor (Fig. 1).

To determine whether the inability of etorphine in inducing down-regulation of the human κ-opioid receptor was caused by its inability to activate the receptor, we compared its potency and efficacy in promoting [^{35}S]GTPγS binding and its binding affinity to the receptor. Both U50,488H and etorphine are full agonists at the human κ-opioid receptor in enhancing [^{35}S]GTPγS binding; etorphine has a lower EC_{50} value and, thus, higher potency (Table 1). Etorphine also had high binding affinity for the human κ-opioid receptor (Table 1). Hence, etorphine is able to fully activate the human κ-receptor without causing down-regulation. Thus, there seem to be agonist and species differences in promoting κ-receptor down-regulation.

The extent of down-regulation (~30%) in the present study is similar to that reported by Blake et al. (1997) after a 3-h pretreatment with 1 μM U50,488H. However, this down-regulation was less than the 50% reduction observed by Joseph and Bidlack (1995) after incubation with 0.1 μM U50,488H for 24 h in R1.1 mouse thymoma cells. This difference may be caused by a difference in pretreatment time and a reflection of the difference in the levels of molecules required for the down-regulation process in different cell systems.

The findings that exposure to U50,488H, but not etorphine, caused down-regulation of the human κ-opioid receptor and that the rat κ-opioid receptor did not undergo down-regulation after pretreatment with U50,488H or etorphine are reminiscent of the similar agonist and species differences in agonist-promoted internalization of the κ-receptors. Although U50,488H, but not etorphine, caused internalization of the human κ-receptor, neither agonist promoted internalization of the rat κ-opioid receptor (Li et al., 1999). These

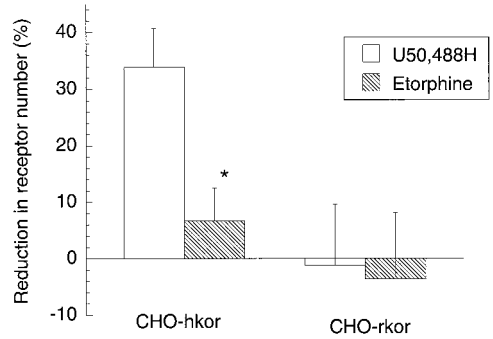

Fig. 1. Effects of pretreatment with U50,488H and etorphine on receptor numbers in membrane of CHO-hkor cells (A) and CHO-rkor cells (B). CHO-hkor cells and CHO-rkor cells were treated with or without 1 μM U50,488H or 1 μM etorphine at 37°C for 4 h, washed extensively, and membranes were prepared. Saturation [^{3}H]diprenorphine binding was performed and K_d and B_{max} values were determined (mean ± S.E.M., n = 3 in duplicate). For the control receptors, K_d and B_{max} values were 0.20 ± 0.01 nM and 1186 ± 34 fmol/mg of protein for CHO-hkor and 0.20 ± 0.02 nM and 1000 ± 114 fmol/mg of protein for CHO-rkor. U50,488H, but not etorphine, promoted down-regulation of the human κ-opioid receptor. Neither U50,488H nor etorphine caused down-regulation of the rat κ-opioid receptor. K_d values were not changed by either treatment. *$P < .05$ compared with U50,488H pretreatment by one-factor ANOVA followed by Dunnett's test.

parallels suggest that internalization and down-regulation are likely to share common initial mechanisms.

Our finding on the differential abilities of U50,488H and etorphine to down-regulate the human κ-opioid receptor in CHO cells is consistent with that of Blake et al. (1997), who showed that U50,488H, but not levorphanol, caused down-regulation of the human κ-receptor in HEK293 cells.

Because both rat and human κ-opioid receptors were expressed in CHO cells, the species difference in agonist-induced down-regulation of the κ-opioid receptors may reflect species difference in the κ-opioid receptor properties. Comparison of the C-terminal domain sequences of the rat and human κ-opioid receptors shows Ser-358 in the human receptor as opposed to Asn-358 in the rat. Whether this amino acid difference contributes to differential internalization and down-regulation is being investigated.

Effects of Expression of the Dominant Negative Mutants Arrestin-2(319-418) and Dynamin I-K44A on (−)U50,488H Induced Down-Regulation of the Human κ-Opioid Receptor. Expression of arrestin-2(319-418), a dominant negative mutant that inhibits receptor internalization by binding constitutively to clathrin (Krupnick et al., 1997), effectively reduced U50,488H-induced down-regulation (Fig. 2). Dynamin I-K44A, a dominant negative mutant that blocks endocytosis at a stage preceding the sequestration into deeply invaginated coated pits (van der Bliek et al., 1993), significantly attenuated (−)U50,488H induced down-regulation of the human κ-opioid receptor (Fig. 2). Neither arrestin-2(319-418) nor dynamin I-K44A affected the expression level of the receptor. The results indicate that down-regulation of the human κ-opioid receptor occurs via arrestin-2- and dynamin I-dependent mechanisms.

Effect of Expression of GRK2, Arrestin-2, and Dynamin on Etorphine-Induced Down-Regulation of the Human κ-Opioid Receptor. Expression of GRK2 plus arrestin-2 or GRK2 promoted etorphine-induced down-regulation of the human κ-opioid receptor, whereas expression of dynamin I alone did not (Fig. 3). Arrestin-2 alone seemed to have a modest effect in promoting etorphine-induced down-regulation, but the effect did not reach statistical significance (Fig. 3). GRK2, arrestin-2, dynamin I, or GRK2 plus arrestin-2 did not affect the expression level of the human κ-opioid receptor in CHO-hkor cells. We reported previously that over-expression of the dominant negative mutant GRK2-K220R, arrestin-2(319-418), or dynamin I-K44A significantly inhibited U50,488H-induced internalization of the human κ-opioid receptor expressed in CHO cells, indicating that (−)U50,488H-induced internalization of the human κ-opioid receptor occurred via GRK-, arrestin-2- or dynamin I-dependent pathways (Li et al., 1999). These results further support the notion that GRK-promoted phosphorylation of the human κ-opioid receptor followed by arrestin-2-mediated internalization is essential for down-regulation of the human κ-opioid receptor.

Our results are similar to those of Gagnon et al. (1998). They found that nonvisual arrestins promoted agonist-induced internalization and down-regulation of the β_2AR,

TABLE 1

K_i values of U50, 488H and etorphine in inhibiting [^{3}H] diprenorphine binding to the human κ-opioid receptor in membranes of CHO-hkor cells and their EC_{50} and E_{max} values in enhancing [^{35}S] GTPγS binding to membranes

Each value represents the mean ± S.E.M. of three experiments in duplicate.

Agonist	[^{3}H] Diprenorphine Binding	[^{35}S] GTPγS Binding	
	K_i	EC_{50}	E_{max}
	nM	*nM*	*fmol / mg protein*
U50,488H	0.73 ± 0.05	1.98 ± 0.46	102 ± 6.6
Etorphine	0.045 ± 0.005	0.12 ± 0.01	101 ± 2.6

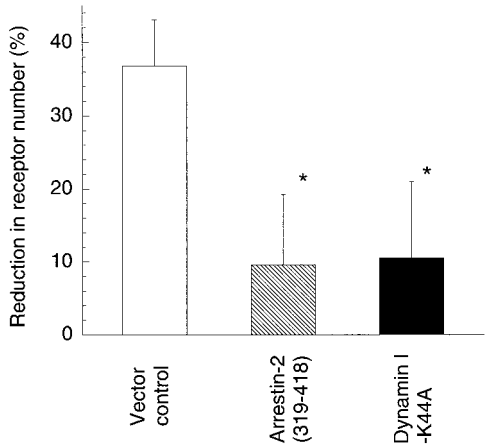

Fig. 2. Effects of expression of the dominant negative mutants arrestin-2(319-418) and dynamin I-K44A on U50,488H-induced down-regulation of the human κ-opioid receptor in CHO-hkor cells. CHO-hkor cells were transfected transiently with expression constructs of arrestin-2(319-418) or dynamin I-K44A or the vector pcDNA3. Sixty to 72 h later, cells were treated with or without 1 μM U50,488H at 37°C for 4 h, washed extensively, and membranes prepared. Saturation [^{3}H]diprenorphine binding was performed and K_d and B_{max} values determined (mean ± S.E.M., n = 3 or 4 in duplicate). For the control receptors, K_d and B_{max} values were 0.19 ± 0.03 nM and 1174 ± 47 fmol/mg of protein. Expression of arrestin-2(319-418) or dynamin I-K44A, which did not affect the expression level of the receptor in the untreated groups, decreased the extent of down-regulation. K_d values were unchanged by all treatments. *$P < .05$ compared with control by one-factor ANOVA followed by Dunnett's test.

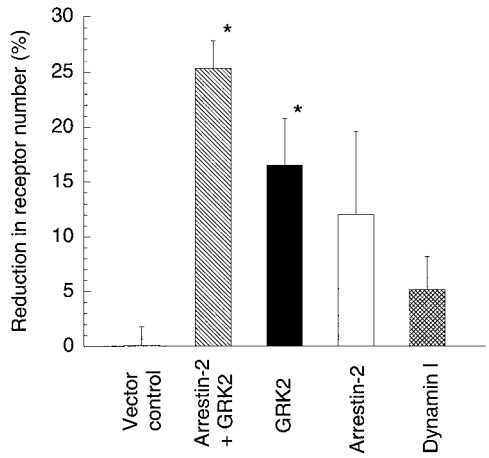

Fig. 3. Effects of expression of GRK2, arrestin-2, or dynamin on etorphine-induced down-regulation of the human κ-opioid receptor in CHO-hkor cells. CHO-hkor cells were transfected transiently with expression constructs of GRK2, arrestin-2, GRK2, and arrestin-2, dynamin I, or the vector pcDNA3. Sixty to 72 h later, cells were treated with or without 1 μM etorphine at 37°C for 4 h, washed extensively, and membranes prepared. Saturation [^{3}H]diprenorphine binding was performed and K_d and B_{max} values determined (mean ± S.E.M., n = 3 or 4 in duplicate). The control has K_d and B_{max} values of 0.11 ± 0.03 nM and 1140 ± 40 fmol/mg of protein. None of the treatments changed the K_d value. Expression of GRK2, arrestin-2, GRK2, and arrestin-2 or dynamin I did not affect the receptor numbers in the control receptors. GRK2 or GRK2 and arrestin-2 permitted etorphine to promote down-regulation. *$P < .05$ compared with control by one-factor ANOVA followed by Dunnett's test.

whereas dominant-negative mutants arrestin-2(319-418) and dynamin I-K44A inhibited agonist-induced internalization and down-regulation of the β_2AR, indicating that arrestin-2 and dynamin-dependent receptor internalization is critical for down-regulation of the β_2AR in HEK293 cells.

However, our results are different from those of Jockers et al. (1999). These researchers found that in L cells stably transfected with β_2AR, blockade of agonist-promoted internalization of β_2AR by coexpression of dynamin I-K44A or chemical treatment (hypertonic sucrose, concanavalin A, potassium depletion, cytosolic acidification) did not affect receptor down-regulation. In addition, in A431 cells, which endogenously express β_2AR and epidermal growth factor receptor, inhibition of receptor internalization by the above-mentioned chemical treatments blocked down-regulation of the epidermal growth factor receptor, but not that of that β_2AR (Jockers et al., 1999). This difference may be attributed to cell type and/or receptor type differences. Moreover, several GPCR mutants have been shown to have greatly reduced agonist-mediated receptor internalization, yet still retain the ability to undergo down-regulation in response to agonists. A Y326A mutation in the β_2AR completely abolished agonist-mediated receptor internalization without affecting the ability of the receptor to down-regulate (Barak et al., 1994). The κ-opioid receptor mutant lacking the C-terminal 15 amino acids exhibited a substantially slower rate of receptor internalization (Trapaidze et al., 1996), whereas this truncation did not affect down-regulation (Cvejic et al., 1996). Conversely, some GPCR mutants showed blunted receptor down-regulation in response to agonists, yet exhibited receptor internalization equivalent to that of the wildtype. Several mutants of the β_2AR with substitutions in the third intracellular loop and C-terminal domain displayed substantially lower levels of down-regulation, with no change in receptor internalization (Campbell et al., 1991). Mutation of Tyr-459 in the C-terminal domain of the m_2 mAChR significantly attenuated agonist-induced down-regulation without affecting agonist-induced internalization (Goldman and Nathanson, 1994). These observations led the authors to suggest that internalization and down-regulation may be mediated by distinct mechanisms. One possibility is that GPCR mutants may not be trafficked in the same manner as the wild-type receptors. Another likely explanation is that internalization was determined after a short period of agonist treatment, whereas down-regulation was measured after a longer treatment period. An alteration in the rate or extent of internalization may not affect the degree of down-regulation.

Effects of Expression of the Dominant Negative rab5A-N133I and rab7-N125I Mutants on (−)U50,488H Induced Down-Regulation of the Human κ-Opioid Receptor. Rab proteins are a family of more than 40 mammalian proteins. They are ras-related GTPases of ~25 kDa that are associated with distinct intracellular membranes where they control vesicle trafficking between intracellular compartments (for reviews see Simons and Zerial, 1993; Olkkonen and Stenmark, 1997). Rab5 is mainly involved in early endosome transport and the fusion of endocytic vesicles with endosomes (Bucci et al., 1992; Stenmark et al., 1994). Rab7 has been implicated in membrane transport from early endosomes to late endosomes (Feng et al., 1995; Vitelli et al., 1997) or late endosomes to lysosomes (Meresse et al., 1995). N133I mutation of rab5A, one of the isoforms of rab5, and N125I mutation of rab7 result in guanine nucleotide binding defective forms that are dominant inhibitors of the endogenous rab5 and rab7, respectively (Bucci et al., 1992; Feng et al., 1995; Meresse et al., 1995; Vitelli et al., 1997). To further investigate the mechanism of κ-opioid receptor down-regulation, we transiently transfected CHO-hkor cells with the expression construct of rab5A-N133I, rab7-N125I, or vector. Expression of rab5A-N133I or rab7-N125I significantly attenuated U50,488H-induced down-regulation of the human κ-opioid receptor (Fig. 4). This result indicates that down-regulation of the human κ-opioid receptor involves rab5- and rab7-dependent vesicle fusion processes. However, direct morphological evidence for trafficking of the human κ-opioid receptor from early endosomes to late endosomes and late endosomes to lysosomes requires further investigation.

To the best of our knowledge, our results represent the first report on the involvement of both rab5 and rab7 in agonist-induced down-regulation of a GPCR. Rab5 has been demonstrated to be involved in internalization of GPCRs. Iwata et al. (1999) reported that a constitutively active mutant of rab5A facilitated dopamine-induced internalization of D_2 dopamine receptors, whereas a dominant negative mutant of rab5A suppressed it. In addition, agonist activation of the β_2AR induces receptor internalization into rab5-containing endosomes (Moore et al., 1995).

Effect of Lysosome Inhibitors and Proteasome Inhibitors on (−)U50,488H Induced Down-Regulation of the Human κ-Opioid Receptor. Studies have demonstrated that degradation of some GPCRs during agonist-induced down-regulation seems to occur in lysosomes (Law et al., 1984, 1985; Gagnon et al., 1998; Kallal et al., 1998; Ko et al., 1999). We examined effects of the lysosomal enzyme inhibitors EST and chloroquine on U50,488H-promoted down-regulation of the human κ-opioid receptor. Pretreat-

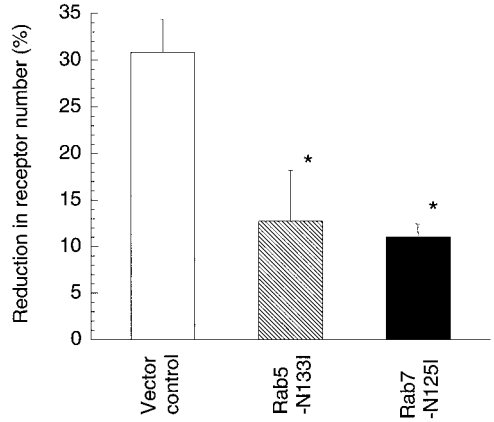

Fig. 4. Effects of expression of the dominant negative mutants rab5A-N133I and rab7-N125I on U50,488H-induced down-regulation of the human κ-opioid receptor in CHO-hkor cells. CHO-hkor cells were transfected transiently with the expression constructs of rab5A-N133I, rab7-N125I, or the vector pcDNA3. Sixty to 72 h later, cells were treated with or without 1 μM U50,488H at 37°C for 4 h, washed extensively and membranes prepared. Saturation [^{3}H]diprenorphine binding was performed and K_d and B_{max} values determined (mean ± S.E.M., $n = 3$ in duplicate). The control group had K_d and B_{max} values of 0.06 ± 0.01 nM and 1117 ± 45 fmol/mg of protein. Expression of rab5A-N133I, rab7-N125I, which did not affect the receptor numbers in the controls, reduced the extent to U50,488H-induced down-regulation. K_d values were not changed by any treatment. $^*P < .05$ compared with control by one-factor ANOVA followed by Dunnett's test.

ment of cells with chloroquine, which did not affect U50,488H-promoted internalization (data not shown), reduced the degree of U50,488H-induced down-regulation (Fig. 5). Treatment with EST similarly decreased the extent of down-regulation (Fig. 5).

Proteasomes are large protein complexes made of multisubunit proteolytic enzymes. Most of the proteins that are degraded in the cytosol are delivered to proteasomes, which are present in many copies and are dispersed throughout the cell. To determine whether proteasomes are involved in degradation of the κ-opioid receptor during down-regulation, we examined effects of three cell-permeable proteasome inhibitors on agonist-induced down-regulation of the human κ-opioid receptor. Pretreatment of CHO-hkor cells with proteasome inhibitor I, MG132, or lactacystin significantly decreased the extent of U50,488H-induced down-regulation (Fig. 5), indicating the involvement of proteasomes in κ-opioid receptor degradation during agonist-induced down-regulation. Although proteasome inhibitor I reduced U50,488H-induced down-regulation, it had no effect on U50,488H-promoted internalization (data not shown).

We then determined whether the combination of a lysosomal inhibitor and a proteasome inhibitor had additive effects on U50,488H-induced down-regulation of the human κ-opioid receptor. Pretreatment of CHO-hkor with chloroquine and proteasome inhibitor I completely blocked U50,488H-induced hkor down-regulation (Fig. 5).

These results indicate that both lysosomes and proteasomes are involved in the degradation of the human κ-opioid receptor during agonist-induced down-regulation. A possible scenario is that a fraction of the receptors was degraded in lysosomes, whereas others were degraded in proteasomes. Another possibility is that proteasome degradation of a protein or proteins other than the receptor is required for targeting and transport of the receptor to and degradation of the receptor by lysosomes, as suggested by Hicke (1999).

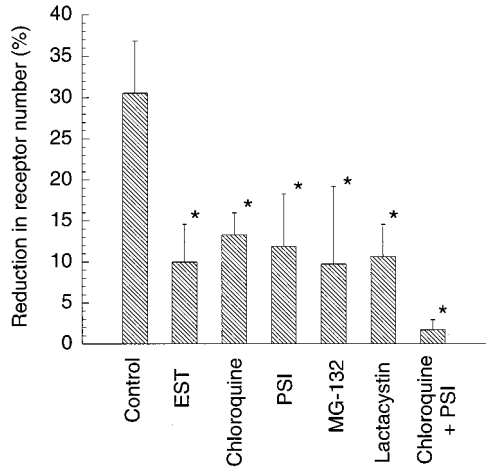

Fig. 5. Effects of pretreatment with lysosome inhibitors (EST or chloroquine) and proteasome inhibitors (proteasome inhibitor I, MG-132, or lactacystin) on U50,488H-induced down-regulation of human κ-opioid receptor. CHO-hkor cells were treated with or without 20 μM EST, 50 μM chloroquine, 5 μM proteasome inhibitor I, 200 μM MG-132, 20 μM lactacystin, or 50 μM chloroquine, and 5 μM proteasome inhibitor I for 10 min and then incubated together with 1 μM U50,488H at 37°C for 4 h, washed extensively and membranes prepared. Saturation [^{3}H]diprenorphine binding was performed and K_d and B_{max} values determined (mean ± S.E.M., n = 3–5 in duplicate). The control group had K_d and B_{max} values of 0.12 ± 0.05 nM and 1137 ± 47 fmol/mg of protein. Each inhibitor decreased the degree of U50,488H-induced down-regulation, whereas a combination of chloroquine and proteasome inhibitor I abolished down-regulation. None of the treatments affected the expression level of the receptor. K_d values were not changed by any treatment. $^*P <$ .05 compared with control by one-factor ANOVA followed by Dunnett's test.

Our finding that lysosomes are involved in the degradation of the human κ-receptor during agonist-induced down-regulation is consistent with published reports (Law et al., 1984, 1985; Hein et al., 1994; Gagnon et al., 1998; Kallal et al., 1998; Ko et al., 1999). Law et al. (1984, 1985) found that [^{3}H]DADLE-bound to the κ-opioid receptor in NG108-15 cells is translocated from plasma membrane fractions to the lysosomal fractions after prolonged exposure to [^{3}H]DADLE. The κ-opioid receptor stably expressed in neuro2A cells was colocalized with transferrin and lysosome-associated membrane protein (LAMP-2) after short-term and prolonged agonist exposure, indicating that the receptor was distributed to early endosomes and then to lysosomes for degradation (down-regulation), respectively (Ko et al., 1999). Kallal et al. found that after agonist treatment, β_2AR tagged with green fluorescence protein was first colocalized with rhodamine-labeled transferrin, a marker for early endosomes, and later with rhodamine-labeled dextran, a marker for lysosomes (Gagnon et al., 1998; Kallal et al., 1998). Presumably, degradation of the receptor occurred in lysosomes. Most activated thrombin receptors were internalized and targeted to lysosomes (Hein et al., 1994).

Some membrane-bound receptors have been shown to be degraded by proteasomes (Mori et al., 1995; Jeffers et al., 1997). Proteasome inhibitors greatly inhibited agonist-induced degradation of the platelet-derived growth factor β-receptor (Mori et al., 1995) and the Met tyrosine kinase receptor (Jeffers et al., 1997). These receptors undergo rapid agonist-promoted polyubiquitination, which targets them for recognition and degradation by proteasomes. Because of the low degree of agonist-induced down-regulation (~30%) of the human κ-opioid receptor, it is not feasible to examine whether the human κ-opioid receptor is polyubiquitinated in the system used in this study. Our result that proteasomes participated in degradation of the κ-opioid receptor shows the complexity of mechanisms underlying GPCR degradation during down-regulation. Indeed, in HeLa cells, the dynamin-K44A mutant had only a modest effect in blocking agonist-induced down-regulation of β_2AR, prompting Benovic and colleagues (Gagnon et al., 1998) to suggest that there may be other pathways involved in the degradation of β_2AR during down-regulation.

In conclusion, our results show that there are agonist and species differences in agonist-induced down-regulation of κ-opioid receptors, similar to what we found for internalization. Based on the results in the present study, we propose the following scheme. U50,488H treatment enhances GRK phosphorylation of the receptor. Subsequent binding of arrestin-2 to the phosphorylated receptors, in turn, initiates the internalization process by binding to clathrin and the receptor-arrestin-2 complex is then sequestered in clathrin-coated pits. By the action of dynamin, the clathrin-coated pits are pinched off to become clathrin-coated vesicles. The rab5- and rab7-dependent vesicle fusion processes are involved in U50,488H-induced κ-receptor down-regulation, possibly trafficking from early endosomes to late endosomes to lysosomes. After prolonged U50,488H treatment, the receptors are traf-

ficked to lysosomes for degradation. In addition, a fraction of the receptors may be degraded in proteasomes by a yet undefined pathway.

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