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A Select Set of Opioid Ligands Induce Up-Regulation by Promoting the Maturation and Stability of the Rat κ-Opioid Receptor in Human Embryonic Kidney 293 Cells

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ABSTRACT

Ligand-induced regulation of the rat κ-opioid receptor (rKOR) was investigated in human embryonic kidney 293 cells stably expressing the FLAG-tagged rKOR. Incubation of rKOR cells with naltrexone for 24 h increased the B_{max} >3-fold, with no change in the affinity of [^{3}H]diprenorphine. Two immunoreactive receptor species were present in cell lysates: naltrexone treatment caused a >3-fold increase in the 52-kDa species while decreasing the level of the 42-kDa species. Dynorphin(1–13), U69,593 [(5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide], or salvinorin A [2*S*,4*aR*,6*aR*,7*R*,9*S*,10*aS*,10*bR*)-9-(acetyloxy)-2-(3-furanyl)dodecahydro-6*a*,10*b*-dimethyl-4,10-dioxo-2*H*-naphtho[2,1*c*]pyran-7-carboxylic acid methyl ester] treatment did not alter the level of immunoreactive rKOR protein, whereas etorphine, cyclazocine, naloxone, and naloxone methiodide increased the 52-kDa and decreased the 42-kDa rKOR bands. Receptor up-regulation was associated with an increase in the number of cell surface receptors and a 2-fold increase in the E_{max} for guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding. Glycosidase digestion indicated that the 52- and 42-kDa receptors contained complex and high-mannose *N*-glycans, respectively, Pulse-chase analysis and glycosidase digestion sensitivities suggested that the 42-kDa rKOR species was a precursor of the 52-kDa species. Naltrexone did not alter rKOR mRNA levels or translational efficiency, and rKOR up-regulation was not inhibited by cycloheximide. Brefeldin A caused accumulation of intracellular rKOR intermediates, and coincubation with naltrexone increased the levels of the brefeldin-induced species significantly. These results suggest that select opioid ligands up-regulate rKOR by enhancing the rate of receptor folding and maturation and by protecting the receptor from degradation, resulting in an increase in the number of rKOR binding sites, immunoreactive protein, and functional receptors.

Chronic administration of morphine or other opioid agonists for pain relief results in tolerance to the analgesic effects and physical dependence (McQuay, 1999). In contrast, chronic blockade of opioid receptors with antagonists such as naloxone and naltrexone does not result in physical dependence and was shown nearly 30 years ago to be associated with supersensitivity to the analgesic actions of morphine (Tang and Collins, 1978). The enhanced analgesic effects of morphine following infusion of naloxone were correlated with an increase in the number of opioid receptor binding sites (Lahti and Collins, 1978). Antagonist-mediated opioid receptor up-regulation has been shown to occur in vivo for μ-opioid receptors (MORs), δ-opioid receptors, and κ-opioid receptors (KORs) (Morris et al., 1988; Lesscher et al., 2003), highlighting its biological relevance; however, the underlying mechanisms remain unclear. The steady-state level of opioid receptor mRNAs did not change in response to chronic in vivo antagonist treatment; therefore, post-transcriptional mechanisms are apparently involved (for review, see Unterwald and Howells, 2008).

A large number of human diseases, for example cystic fibrosis, are due to genetic mutations that cause protein misfolding (Thomas et al., 1995; Welch and Howard, 2000).

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ABBREVIATIONS: MOR, μ-opioid receptor; KOR, κ-opioid receptor; HEK, human embryonic kidney; salvinorin A, (2*S*,4*aR*,6*aR*,7*R*,9*S*,10*aS*,10*bR*)-9-(acetyloxy)-2-(3-furanyl)dodecahydro-6*a*,10*b*-dimethyl-4,10-dioxo-2*H*-naphtho[2,1*c*]pyran-7-carboxylic acid methyl ester; ZLLL, carbobenzoxy-leucyl-leucyl-leucinal; rKOR, the rat κ-opioid receptor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide: *N*-glycosidase F; Endo H, endoglycosidase H; WGA, wheat germ agglutinin; BFA, brefeldin A; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; U69,593, (5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; ANOVA, analysis of variance.

Various mutations of G protein-coupled receptors also result in improper folding and loss of function. Mutant rhodopsins cause retinitis pigmentosa (Sung et al., 1991), mutated forms of the luteinizing hormone receptor result in endocrine disorders in males and females (Latronico and Segaloff, 1999), and mutations at various loci in the vasopressin V2 receptor are responsible for X-linked nephrogenic diabetes insipidus (Oksche and Rosenthal, 1998). These genetic mutations cause improper folding during protein synthesis, and the misfolded receptors are deglycosylated, ubiquitinated, and degraded by the 26S proteasome under the surveillance of the endoplasmic reticulum quality control system (Ellgaard and Helenius, 2003).

Morello et al. (2000) reported that nonpeptidic vasopressin receptor antagonists increased cell surface expression and rescued the function of several mutant forms of the V2 receptor that cause human X-linked nephrogenic diabetes insipidus. They proposed that the antagonists acted intracellularly as pharmacological chaperones by binding to and stabilizing the newly synthesized mutant receptors, thereby promoting proper folding, maturation, exit from the endoplasmic reticulum, and trafficking to the cell surface. The antagonists, however, did not increase the cell surface expression of the wild-type V2 vasopressin receptor. It was also reported that pharmacological chaperones rescued mutant misfolded forms of gonadotropin-releasing hormone receptors that cause hypogonadotropic hypogonadism (Leanos-Miranda et al., 2005).

In the opioid field, substitution of the aspartic acid in the DRY sequence of the MOR reduced receptor expression to undetectable levels in transfected cells; however, inclusion of naloxone in the cell culture medium greatly enhanced the binding activity and expression level of the mutated receptor (Li et al., 2001). Naloxone, however, had minimal effects on wild-type MOR binding and immunoreactivity. It was concluded that naloxone up-regulated the mutant D164Q MOR receptor by stabilizing the active conformation of the binding site, thereby inhibiting constitutive internalization and proteolysis.

Chaipatikul et al. (2003) reported that deletion of the sequence RLSKV within the third intracellular loop or KRCFR from the proximal C terminus of the rat MOR led to low levels of receptor expression in transfected HEK 293 cells, and incubation of cells with hydrophobic antagonists and agonists increased the cell surface expression of the mutant MORs. Again, the active ligands had no effect on the cell surface expression of the wild-type MOR. Deletion of either the RLSKV or KRCFR sequences from the MOR did not cause the mutants to become constitutively active; hence, the mechanism for the mutant receptor up-regulation differed from that proposed by Li et al. (2001), and it was suggested that the hydrophobic MOR ligands were promoting the intracellular trafficking of the mutant receptors.

It has also been reported that a D95A substitution in the δ-opioid receptor resulted in significant retention of incompletely processed receptor precursors in the endoplasmic reticulum, and cell-permeable antagonists increased the maturation and exit of the receptor from the endoplasmic reticulum and increased the cell surface expression of the mutant receptor (Petaja-Repo et al., 2002). In this instance, cell-permeable antagonists also acted as pharmacological chaperones for the wild-type receptor, and it was proposed that the chaperone effect was due to ligand engagement of intracellular misfolded receptors (Petaja-Repo et al., 2002). In the present study, we used complementary biochemical and pharmacological approaches to investigate the regulation by agonists and antagonists of the KOR stably expressed in HEK 293 cells and explored the mechanism of the observed up-regulation induced by a select set of ligands.

Materials and Methods

Materials. Dynorphin A(1–13) was a product of Multiple Peptide Systems (San Diego, CA). Naloxone methiodide was purchased from Sigma-Aldrich (St. Louis, MO), and salvinorin A was from Tocris Bioscience (Ellisville, MO). All other opioid ligands were obtained from the National Institute on Drug Abuse (Bethesda, MD). *n*-Dodecyl-β-D-maltopyranoside was purchased from Anatrace (Maumee, OH). Carbobenzoxy-leucyl-leucyl-leucinal (ZLLL) was obtained from Peptides International (Louisville, KY). All other chemicals and reagents were from Sigma-Aldrich.

Cell Culture and Transfection. HEK 293 cells were transfected with an expression plasmid encoding the rat κ-opioid receptor tagged with an N-terminal FLAG epitope (kindly provided by Dr. Lakshmi Devi, Mt. Sinai Medical Center, New York, NY) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). A clonal cell line stably expressing the FLAG-tagged rat κ-opioid receptor (rKOR) cells was obtained following transfected cell selection in media containing 1 mg/ml G418 (Invitrogen). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 mg/ml G418. rKOR cells were incubated with ligands, reagents, or vehicle in serum-free Dulbecco's modified Eagle's medium for various times as indicated at 37°C.

Membrane Preparation and Radioligand Binding Assays. rKOR cells were harvested in phosphate-buffered saline (PBS) and centrifuged at 500*g* for 5 min. Cell pellets were resuspended in PBS and centrifuged as above. The cell pellets were homogenized with a Tekmar tissuemizer (Cincinnati, OH) in 50 mM Tris HCl, pH 7.5, and a membrane fraction was prepared by ultracentrifugation of the homogenate at 100,000*g* for 30 min. The membrane pellet was washed with 50 mM Tris HCl, pH 7.5, and resuspended by homogenization in 0.32 mM sucrose and 50 mM Tris HCl, pH 7.5. Membrane preparations were stored at −80°C if not used immediately. The protein concentration of the membrane preparations was determined using the D_c protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Radioligand binding assays were conducted in a final volume of 0.25 ml, using rKOR cell membrane preparations diluted with 50 mM Tris HCl, pH 7.5, to contain 15 to 20 μg of protein/0.25 ml. Saturation binding assays were conducted in duplicate at room temperature using concentrations of [15,16-^{3}H](−)diprenorphine (specific activity, 50.0 Ci/mmol; Perkin Elmer, Boston, MA) ranging from 0.05 to 7 nM. Samples containing tritiated diprenorphine in the presence of excess unlabeled cyclazocine (1 μM) were assayed to define nonspecific binding, which was subtracted from total binding to obtain specific binding. After incubation for 30 min to reach equilibrium, binding assays were terminated by filtration through Whatman GF/B filters (VWR International, Buffalo Grove, IL). Filters were immersed in Ecoscint H liquid scintillation cocktail (National Diagnostics, Somerville, NJ) before determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter (Beckman Coulter, Fullerton, CA). Saturation curves were analyzed by nonlinear regression using Prism 3.0 (GraphPad Software, San Diego, CA) to determine B_{max} and K_d values.

Western Blotting. rKOR whole-cell lysates were prepared by solubilization of cells with 1% *n*-dodecyl-β-D-maltopyranoside, 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10%

glycerol, and a protease inhibitor cocktail (1:100; Sigma-Aldrich). The detergent lysate was centrifuged at 16,000*g* for 20 min, and the supernatant was recovered for immunoblot analysis. SDS-PAGE and Western blotting, using the anti-FLAG M1 monoclonal antibody (Sigma-Aldrich) for detection of the epitope-tagged rKOR, was done as described previously (Chaturvedi et al., 2001), and levels were normalized to α-tubulin as a loading control (detected with a monoclonal antibody to α-tubulin from Sigma-Aldrich). Several exposure times were developed to ensure that the signal on the film had not reached saturation, and band intensities were quantified using SynGene Software (Synoptics Ltd., Cambridge, UK).

Characterization of rKOR *N*-Linked Glycosylation. Cell lysates were prepared as described above for Western blot analysis. Peptide: *N*-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA) was added to a final concentration of 625 U/ml and incubated for 3 h at 37°C. For digestion with endoglycosidase H (Endo H; Roche Diagnostics, Indianapolis, IN), sodium acetate, pH 6.0, was added to the cell lysate to bring the concentration to 100 mM, and samples were incubated with the enzyme (500 mU/ml) for 3 h at 37°C. Digests were resolved using 10% SDS-PAGE and transferred to polyvinylidene difluoride. Receptor species were identified using the anti-FLAG M1 antibody as described above.

For wheat germ agglutinin (WGA)-agarose chromatography, cells were solubilized with 1% *n*-dodecyl-β-D-maltopyranoside lysis buffer and incubated for 6 h with WGA-agarose (Vector Laboratories, Burlingame, CA). The receptor-bound WGA beads were washed five times with column wash buffer containing 0.5% Triton X-100, 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Proteins bound to the resin were eluted with 500 mM *N*-acetylglucosamine in column wash buffer. Eluted proteins were resolved using 10% SDS-PAGE, transferred to polyvinylidene diflouride, and rKOR was detected using anti-FLAG M1 antibody as described above.

Involvement of the Proteasome in Turnover of the rKOR. rKOR cells were incubated in serum-free medium for 6 h in the absence or presence of 10 μM ZLLL, a cell-permeable proteasome inhibitor, and whole-cell lysates were analyzed by Western blotting.

Effect of Cycloheximide and Brefeldin A on Naltrexone-Induced κ-Receptor Up-Regulation. rKOR cells were preincubated at 37°C for 30 min in the absence or presence of 120 μg/ml cycloheximide or with 3 μg/ml brefeldin A (BFA), then incubated for an additional 2 or 6 h as indicated in the absence and presence of KOR ligands (1 μM). Cell lysates were prepared and subjected to SDS-PAGE and Western blot analysis using the anti-FLAG M1 antibody for rKOR detection and a tubulin antibody for normalization of protein loading.

Reverse Transcriptase-Polymerase Chain Reaction (PCR). rKOR cells were incubated in the absence or presence of 1 μM naltrexone for 2 or 24 h. Cells were harvested in PBS and centrifuged at 500*g* for 5 min. Cell pellets were washed with PBS and centrifuged as above. Cells were lysed with TRI reagent (Sigma-Aldrich) to extract RNA, mixed with 0.2 volumes of chloroform, and centrifuged at 12,000*g* for 15 min. Total RNA was precipitated from the upper aqueous layer with isopropanol and centrifuged for 12,000*g* for 15 min. RNA pellets were washed with chilled 70% ethanol and resuspended in diethylpyrocarbonate-treated water. cDNA was prepared using random hexamer primers and ThermoScript reverse transcriptase (Invitrogen). Ten percent of the cDNA synthesis reaction was added to SYBR green master mix (Applied Biosystems, Foster City, CA) and amplified for 40 cycles on a 7500 Real Time PCR System (Applied Biosystems) using an rKOR sense-strand primer, 5′-TTGGCTACTGGCATCATCTG-3′, derived from transmembrane domain 4-encoding sequence in exon 3 of the rKOR transcript, and a rKOR antisense-strand primer, 5′-GACTGCAACCACTACCAGCA-3′, derived from transmembrane domain 6-encoding sequence in exon 4 of the rKOR transcript. This choice of primers ensured that any possible contaminating genomic DNA in the RNA preparation would not lead to false-positive PCR products, due to the long intervening intron between exons 3 and 4 of the rKOR gene. The rKOR PCR product is 301 bp in length. The level of actin mRNA in each sample was determined for normalization of the rKOR mRNA. The actin sense-strand primer, 5′-TTGGCCTTAGGGTTCAGGGGGG-3′, and the antisense-strand primer, 5′-CGTGGGGCGCCCCAGGCACCA-3′, yield an actin PCR product 243 bp long. Analysis of the PCR reaction products was done using 7500 System SDS software (Applied Biosystems).

Comparison of the Rate of de Novo rKOR mRNA Translation by Metabolic Labeling with [^{35}S]Methionine and [^{35}S]Cysteine after Incubation of rKOR Cells in the Absence and Presence of Naltrexone. rKOR cells were preincubated in the absence or presence of 1 μM naltrexone for 2 h. The culture medium was removed and replaced with methionine- and cysteine-free DMEM in the absence and presence of naltrexone and incubated further for 1 h. [^{35}S]Methionine and [^{35}S]cysteine (Translabel; MP Biomedicals, Irvine, CA) were added at 100 μCi/ml, and rKOR cells were incubated in the absence and presence of naltrexone for an additional 1 h at 37°C to allow incorporation of the radiolabeled amino acids into newly synthesized protein. Whole-cell lysates were prepared as described above, and rKOR was immunoprecipitated by overnight incubation at 4°C with anti-FLAG M1 monoclonal antibody conjugated to agarose beads. At the end of the incubation, the beads were washed three times with column wash buffer (0.5% Triton X-100, 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM $CaCl_2$, and 1 mM $MgCl_2$), followed by two additional washes with column buffer containing 300 mM NaCl. The M1 antibody-antigen complex is Ca^{2+}-dependent; therefore, rKOR was eluted from the beads with 20 mM EDTA in SDS sample loading buffer. The eluted protein was resolved using 10% SDS-PAGE and visualized by autoradiography.

Comparison of the Rate of Maturation of rKOR by Pulse-Chase Metabolic Labeling in the Absence and Presence of Naltrexone. rKOR cells were incubated in methionine- and cysteine-free DMEM for 1 h at 37°C. [^{35}S]Methionine and [^{35}S]cysteine were added at 150 μCi/ml, and rKOR cells were incubated for an additional 30 min at 37°C. Cells were washed with serum-free DMEM containing 120 μg/ml cycloheximide to block further protein synthesis and incubated in the absence or presence of 1 μM naltrexone for 1, 2, 3, or 4 h in the continued presence of cycloheximide. Whole-cell lysates were prepared, and rKOR immunoprecipitations were performed as described above. The ^{35}S-labeled rKOR was resolved using 10% SDS-PAGE and visualized by autoradiography.

Analysis of rKOR Up-Regulation by Flow Cytometry. rKOR cells were treated with or without naloxone (1 μM) or naloxone methiodide (20 μM) for 24 h. Cells were harvested and dissociated in chilled PBS, pH 7.2, supplemented with 2 mM EDTA, then fixed with 3.7% paraformaldehyde for 5 min at room temperature. Cells were washed with PBS to remove the paraformaldehyde, then incubated with mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) for 1 h in the presence of 1% (v/v) normal goat serum in PBS to block nonspecific binding. Cells were washed with PBS, then incubated with goat anti-mouse IgG conjugated with Alexafluor 488 (Molecular Probes, Carlsbad, CA) for 30 min. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by acquiring 10,000 cells from each sample, and the geometric mean of fluorescence intensity was calculated using CellQuest Pro 5.2 software (BD Biosciences, San Jose, CA).

Evaluation of Naloxone and U69,593 Cell Permeability. HEK 293 cells (25 million cells/ml) were incubated in serum-free DMEM with 10 nM [*N*-allyl-2,3-^{3}H](−) naloxone (specific activity, 67.0 Ci/mmol; Perkin Elmer) or 10 nM [phenyl-3,4-^{3}H](−) U69,593 (specific activity, 41.7 Ci/mmol; Perkin Elmer) in a total volume of 1 ml for 1, 2, or 4 h at 37°C. Cells were harvested and centrifuged at 160*g* for 3 min. Cell pellets were washed four times with serum-free DMEM to removed residual [^{3}H] ligands, and aliquots were taken for scintillation counting. The thoroughly washed cell pellet was resuspended in H_2O, and cells were broken by hypotonic lysis. Membranes

were removed by centrifugation at 100,000*g* for 30 min at 4°C. The presence of [^{3}H]naloxone and [^{3}H]U69,593 in the resulting cytoplasmic and membrane pellets was determined by liquid scintillation counting.

Agonist-Induced Stimulation of [^{35}S]GTPγS Binding. rKOR cell membrane fractions were prepared, and [^{35}S]GTPγS binding assays were conducted as described previously (Yadav et al., 2007). rKOR membrane fractions (7.5 μg of protein) were incubated with 0.3 nM [^{35}S]GTPγS (specific activity, 1117 Ci/mmol; Amersham Bioscience, Piscataway, NJ) and 10 μM GDP (Calbiochem, La Jolla, CA) in the absence or presence of varying concentrations of U69,593 (ranging from 1 nM to 100 μM) in 1 ml of 50 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 0.1% bovine serum albumin, 1 mM dithiothreitol, and 0.025% digitonin. Reactions were incubated at 30°C for 90 min. Nonspecific binding was determined by incubation of samples in the presence of 15 μM unlabeled GTPγS and was subtracted from total basal and total agonist-stimulated binding. Reactions were terminated by filtration through Whatman GF/B filters. Filters were immersed in Ecoscint H liquid scintillation cocktail before determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter. Dose-response curves were analyzed by nonlinear regression using Prism 3.0 (GraphPad Software) to determine E_{max} and EC_{50} values.

Results

Differential Up-Regulation of rKOR by Select κ-Receptor Ligands. A clonal HEK 293 cell line was established following transfection and G418 selection that stably expressed the rKOR. The apparent dissociation constant (K_d) for [^{3}H]diprenorphine was 0.9 ± 0.3 nM, and the maximum number of binding sites (B_{max}) was 1.6 ± 0.3 pmol/mg protein (mean ± S.E.M., n = 10). To investigate ligand-dependent regulation of the receptor, rKOR cells were incubated in the absence and presence of 1 μM naltrexone for 2, 8, or 24 h, and membrane fractions derived from these cells were assayed by saturation analysis using [^{3}H]diprenorphine. The K_d for [^{3}H]diprenorphine was not changed significantly at any time point following naltrexone treatment ($p > 0.05$, ANOVA, Newman-Keuls multiple comparison test); however, the B_{max} increased 2-fold after 2 h of incubation with naltrexone and increased more than 3-fold following 8- and 24-h exposure to the antagonist (Fig. 1, A and C). The differences in B_{max} values between naltrexone-treated samples and the saline control were all statistically significant at all time points ($p < 0.05$, ANOVA, Newman-Keuls multiple comparison test).

Western blot analysis of rKOR cell lysates revealed that two immunoreactive rKOR species were expressed, with apparent molecular masses of 42 and 52 kDa (Fig. 1B). These bands were not observed in control, nontransfected HEK 293 cell lysates, confirming that the 42- and 52-kDa species were different forms of the rKOR expressed in transfected cells. The 42-kDa receptor species was more abundant than the 52-kDa form under basal conditions. After exposure of rKOR cells to naltrexone, the levels of the 52-kDa rKOR increased in parallel with the time-dependent increase in KOR binding sites (Fig. 1, B and C), whereas the level of the 42-kDa band decreased 2-fold at 2 h and decreased 3-fold relative to the tubulin control following naltrexone treatment for 8 and 24 h (Fig. 1, B and C).

We next sought to characterize the efficacy of other ligands to induce up-regulation of the κ receptor. Incubation of rKOR cells for 24 h with 1 μM dynorphin A(1–13), a potent endogenous peptide agonist, did not alter the levels of the 52- or

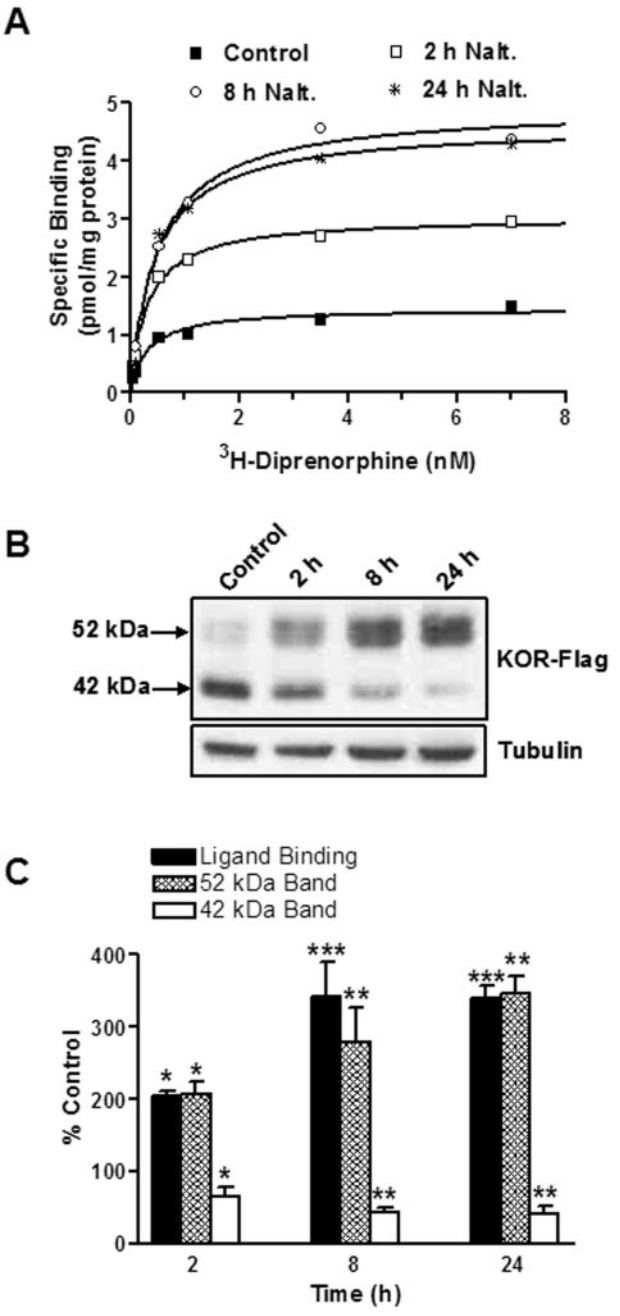

Fig. 1. Kinetics of naltrexone-induced up-regulation of rKOR. Culture medium for rKOR cells was replaced with serum-free media at zero time, and cells were incubated at 37°C in the absence or presence of 1 μM naltrexone for 2, 8, or 24 h. A, membrane fractions were prepared from each treatment group and assayed by saturation analysis using [^{3}H]diprenorphine (0.05–7 nM). B, whole-cell lysates were prepared from each sample and subjected to SDS-PAGE and Western blot analysis using the anti-FLAG M1 antibody to detect rKOR or α-tubulin antibody to control for the amount of protein loaded in each lane and for normalization of quantitative analysis. C, quantitative analysis of the naltrexone-induced changes in B_{max} (n = 3) and levels of the 52- and 42-kDa rKOR immunoreactive species (n = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ANOVA, Newman-Keuls multiple comparison test for naltrexone-treated samples versus saline controls.

42-kDa rKOR species, in marked contrast to the effect of naltrexone under the same conditions (Fig. 2A). Dynorphin was still inactive when assayed at 10 μM (data not shown). In addition, treatment of rKOR cells with dynorphin did not alter the K_d of [^{3}H]diprenorphine or the rKOR B_{max} com-

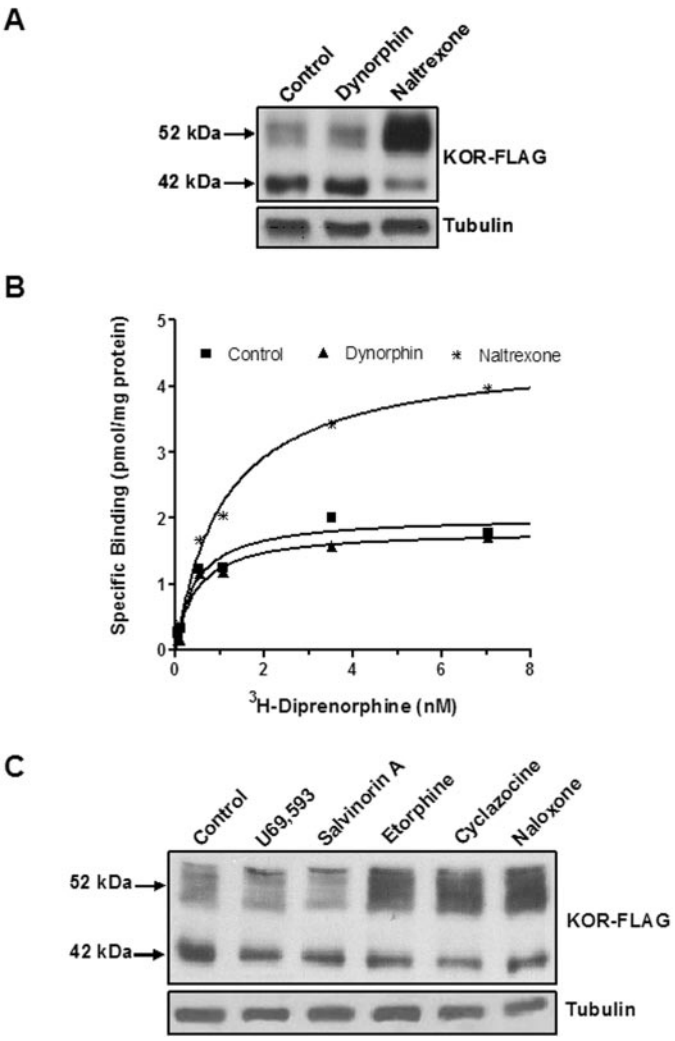

Fig. 2. Ligand-dependent up-regulation of KOR. rKOR cells were incubated for 24 h with either saline (control), 1 μM dynorphin A(1–13), or 1 μM naltrexone. A, whole-cell lysates were analyzed by Western blotting using the anti-FLAG M1 antibody to detect rKOR or α-tubulin antibody. B, membrane fractions were prepared and assayed by saturation analysis using [^{3}H]diprenorphine (0.05–7 nM). C, rKOR cells were incubated in serum-free medium for 24 h with either saline (control) or with various opioid ligands (each at 1 μM). Cell lysates were subjected to SDS-PAGE and Western blot analysis. Data are representative of three independent experiments.

pared with controls as determined by binding assays, unlike the marked increase in B_{max} induced by naltrexone (Fig. 2B). The endogenous KOR peptide agonist, β-neoendorphin, was similarly inactive regarding KOR up-regulation when assayed at 1 and 10 μM (data not shown). The possibility that dynorphin was degraded during the incubation period was considered; however, incubation of dynorphin in the cell culture media for 24 h did not significantly alter its IC_{50} when subsequently assayed for displacement of [^{3}H]diprenorphine binding (data not shown), suggesting that there was minimal degradation during the incubation period.

Analysis of other κ ligands revealed that the efficacy of a ligand to up-regulate the rKOR was not simply related to whether the ligand was an agonist or antagonist. Incubation of rKOR cells for 24 h with U69,593, a nonpeptide arylacetamide κ agonist, or salvinorin A, a naturally occurring, nonnitrogenous κ agonist, similarly had no effect on the level of the 52-kDa rKOR, although both compounds decreased the level of the 42-kDa band relative to the vehicle-treated control (Fig. 2C). Incubation of salvinorin in the cell culture media for 24 h did not significantly alter its IC_{50} when subsequently assayed for displacement of [^{3}H]diprenorphine binding (data not shown), suggesting that there was minimal degradation of salvinorin during the incubation period. Furthermore, when U69,593, salvinorin, or β-neoendorphin were coincubated at 10 μM with naltrexone at 1 μM, the inactive compounds did not inhibit naltrexone-induced κ-receptor up-regulation (data not shown). To our knowledge, this is a novel finding that select nonpeptidic κ ligands are unable to up-regulate the κ-opioid receptor. In contrast, the potent oripavine agonist etorphine and the benzomorphan agonist cyclazocine significantly increased the level of the 52-kDa rKOR immunoreactive band relative to the control (Fig. 2C) and decreased the 42-kDa species. Incubation of rKOR cells with naloxone, an antagonist that is structurally similar to naltrexone, also up-regulated the rKOR 52-kDa band and decreased the level of the 42-kDa band (Fig. 2C).

We next investigated whether the quaternary analog of naloxone, naloxone methiodide, which is generally assumed to be unable to diffuse across the plasma membrane due to its permanent positive charge, was capable of inducing rKOR up-regulation. Because naloxone methiodide has been shown to have a lower affinity for the guinea pig brain KOR compared with naloxone (Magnan et al., 1982), we first compared the affinities of naloxone methiodide and naloxone for the rKOR expressed in HEK 293 cells. As shown in Fig. 3A, naloxone methiodide competitively inhibited [^{3}H]diprenorphine binding to rKOR membranes; however, its affinity was more than 20-fold lower than that of naloxone (K_is were 480 $\pm$ 85 and 21 $\pm$ 2 nM for naloxone methiodide and naloxone, respectively). Given the greater than 20-fold difference in affinity between naloxone and naloxone methiodide, the efficacy of naloxone methiodide to up-regulate rKOR binding sites was tested at 1 and 20 μM and compared with the effect of 1 μM naloxone. After 24-h incubation with 1 μM naloxone, the B_{max} for [^{3}H]diprenorphine increased 2-fold relative to controls (Fig. 3B, $p < 0.05$, ANOVA, Newman-Keuls multiple comparison test). At an equimolar (1 μM) concentration, naloxone methiodide increased the B_{max} by approximately 30%; however, the difference was not statistically significant compared with the control ($p > 0.05$). Incubation of rKOR cells with 20 μM naloxone methiodide, however, proved to be equally as effective as 1 μM naloxone at increasing the B_{max} ($p < 0.05$, ANOVA, Newman-Keuls multiple comparison test). Western blot analysis confirmed that incubation of rKOR cells with 20 μM naloxone methiodide significantly increased the level of the 52-kDa receptor species and decreased the 42-kDa species to an extent similar to 1 μM naloxone (Fig. 3C). Up-regulation of rKOR by naloxone and naloxone methiodide was also investigated using fluorescence-activated cell sorting to determine whether antagonist treatment led to an increased number of κ receptors on the cell surface. Treatment of cells for 24 h with either 1 or 20 μM naloxone methiodide caused a significant 60% increase in the number of cell surface κ receptors (Fig. 3D, $p < 0.05$, ANOVA, Newman-Keuls multiple comparison test). The magnitude of the increase in the number of cell surface receptors determined by fluorescence-activated cell sorting was not statistically different from the increase in B_{max} de-

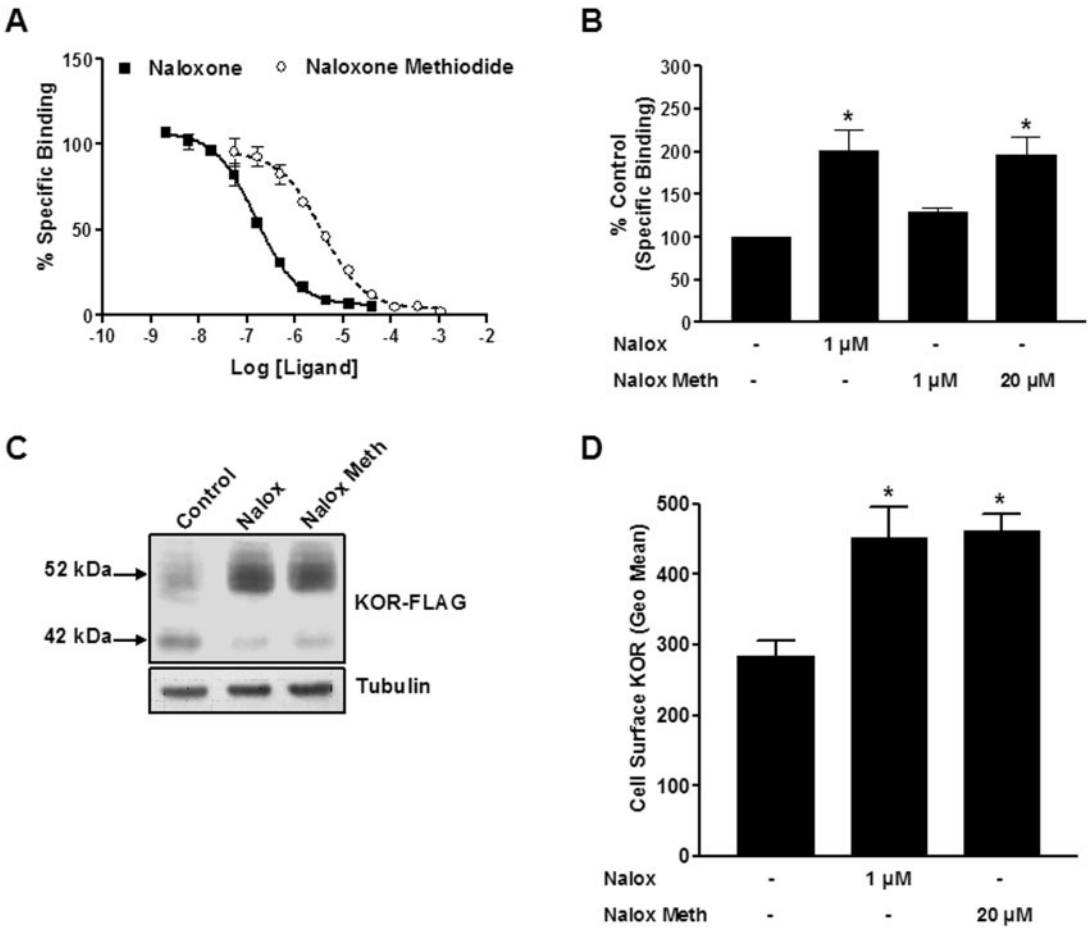

Fig. 3. Comparison of rKOR up-regulation by naloxone and naloxone methiodide. A, rKOR membrane fractions were used to measure the apparent dissociation constant (K_i) of naloxone and naloxone methiodide. Ten concentrations (between 1 nM and 1 mM) of each ligand were assayed for displacement of [^{3}H]diprenorphine binding (7 nM). IC_{50} values were determined by nonlinear regression analysis of the displacement curves using Prism 3.0, and K_i values were calculated using the Cheng-Prusoff equation. The graph is representative of three independent experiments. B, rKOR cells were incubated at 37°C with 1 μM naloxone or naloxone methiodide at 1 or 20 μM for 24 h, and membrane fractions were prepared for saturation analysis using [^{3}H]diprenorphine. The increases in B_{max} of the treated samples are plotted relative to the vehicle control, which was normalized to 100%. Neither ligand affected the K_d of [^{3}H]diprenorphine. Values represent the means of three independent experiments. *, $p < 0.05$, ANOVA followed by Newman-Keuls multiple comparison test for treated samples compared with controls. C, Western blot analysis of cell lysates following incubation of rKOR cells for 24 h with saline (control), 1 μM naloxone, or 20 μM naloxone methiodide. The immunoblot shown is representative of three independent experiments. D, rKOR cells were incubated at 37°C with saline, naloxone (1 μM), or naloxone methiodide (20 μM) for 24 h. Cells were then dissociated, fixed with paraformaldehyde, washed, incubated with anti-FLAG M2 monoclonal antibody for 1 h, and then incubated with anti-mouse IgG conjugated with Alexafluor 488 for 30 min. Cell surface receptors were quantified with a FACScan flow cytometer by acquiring 10,000 cells from each sample, and the geometric mean of fluorescence intensity was calculated using CellQuest Pro 5.2 software. Data are the means of four independent experiments. *, $p < 0.05$, ANOVA, Newman-Keuls multiple comparison test for treated samples versus saline controls.

termined by radioligand binding ($p > 0.05$, ANOVA, Newman-Keuls multiple comparison test).

Biochemical Characterization of the rKOR Immunoreactive Species. To investigate the glycoprotein nature of the receptor, rKOR cell lysates were digested with two glycosidases, PNGase F and Endo H. Digestion with PNGase F resulted in a decrease of the apparent molecular mass of both the 52- and the 42-kDa KOR species, which was indicative that both forms contain extensive complex *N*-linked glycosylation (Fig. 4A). In contrast, only the 42-kDa KOR species was sensitive to Endo H digestion, which was converted to a form with an apparent molecular mass of 35 kDa (Fig. 4A). It is unclear why the apparent molecular masses of the PNGase F and Endo H products differ. The theoretical molecular weight of the unmodified epitope-tagged rat κ receptor is 43,600, indicating that the Endo H digestion product, which should have a single *N*-acetylglucosamine moiety on each of two putative asparagines within *N*-linked glycosylation consensus sites in the amino-terminal domain of the receptor, migrates anomalously on SDS-PAGE. Putative differential *O*-linked glycosylation of the 52- and 42-kDa forms further complicates the expected migration patterns on SDS-PAGE.

Further biochemical characterization of the KOR expressed in rKOR cells was accomplished using WGA-agarose lectin affinity chromatography. It was observed that only the 52-kDa receptor was bound and subsequently eluted from the WGA-agarose matrix, whereas the 42-kDa species did not interact avidly with the matrix and was recovered in the flow-through fraction (Fig. 4B).

The cell-permeable proteasome inhibitor, ZLLL, was utilized to determine whether the proteasome was involved in the turnover of the 42- and 52-kDa κ receptors. rKOR cells were incubated for 6 h in the absence and presence of 10 μM ZLLL, and then whole-cell lysates were analyzed by Western blotting. Incubation of rKOR cells with the proteasome inhibitor caused a robust 5-fold increase in the level of the

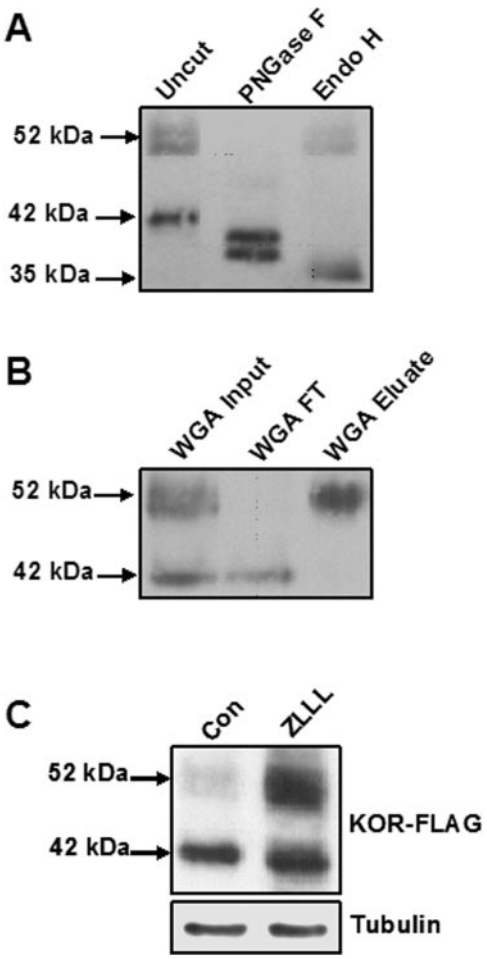

Fig. 4. Biochemical characterization of the rKOR immunoreactive species. A, rKOR cell lysates were incubated with PNGase F (625 U/ml) or Endo H (500 mU/ml) for 3 h at 37°C. Digests were resolved using 10% SDS-PAGE, and receptor species were identified using the anti-FLAG M1 antibody. B, rKOR cells were solubilized with dodecylmaltoside and incubated for 6 h with WGA-agarose. The flow-through (FT) fraction was collected, and proteins bound to the resin were eluted with 500 mM *N*-acetylglucosamine in column wash buffer. Samples were assayed by Western blotting using anti-FLAG M1 antibody. C, rKOR cells were incubated for 6 h in the absence and presence of 10 mM ZLLL, and then whole-cell lysates were assayed by Western blotting using the anti-FLAG M1 and α-tubulin antibodies. Data are representative of three independent experiments.

52-kDa KOR species and a smaller 2-fold increase in the 42-kDa form, whereas α-tubulin levels remained unchanged (Fig. 4C). It is apparent that the proteasome is involved in the basal turnover rate of the κ-opioid receptor.

Effect of Naltrexone on KOR mRNA Levels and Translational Efficiency of KOR mRNA and Sensitivity of Naltrexone-Induced rKOR Up-Regulation to Cycloheximide. Reverse transcriptase-PCR was performed to determine whether the naltrexone-induced up-regulation of KOR binding sites and immunoreactivity were associated with an increase in the level of rKOR mRNA. Cells that had been treated in the absence and presence of 1 μM naltrexone for 2 or 24 h showed no change in the steady-state levels of rKOR mRNA after normalization to actin mRNA (Fig. 5, A and B).

To determine whether naltrexone-induced up-regulation of rKOR was due to an increase in the translational efficiency of its mRNA, rKOR cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine in the presence or absence of naltrexone. After the 1-h pulse period, the 42-kDa band was the predominant KOR receptor species, and a significantly lower amount of the label was incorporated into the 52-kDa form of the receptor. As seen in Fig. 5C, naltrexone treatment did not alter the incorporation of radiolabeled amino acids into either the 42- or 52-kDa species, suggesting that naltrexone did not alter the rate of de novo rKOR protein synthesis.

The effect of the protein synthesis inhibitor, cycloheximide, on ligand-induced κ-receptor up-regulation was also investigated. For these experiments, a 2-h incubation time was used to limit the cellular toxicity of the inhibitor. As shown earlier in Fig. 1, naltrexone treatment alone for 2 h increased the level of the 52-kDa κ-receptor band by 2-fold and reduced the level of the 42-kDa form by 50%. Incubation of rKOR cells with cycloheximide alone caused a 50% decrease in the steady-state level of the 52-kDa species and a 90% decrease in the 42-kDa species, whereas α-tubulin levels remained unchanged. When coincubated with the antagonist, cycloheximide had no effect on naltrexone-induced KOR up-regulation (Fig. 5D). The 52-kDa KOR band was increased 2-fold by naltrexone relative to the sample incubated with cycloheximide alone, and naltrexone also induced a further reduction in the level of the faint 42-kDa band in the presence of cycloheximide. The lack of change in the level of α-tubulin following inhibition of protein synthesis indicated that α-tubulin has a significantly longer half-life than the κ receptor and provided evidence that cycloheximide was not overtly toxic to the cell following short-term (2 h) exposure.

Effect of BFA on κ-Receptor Maturation and Naltrexone-Induced Up-Regulation. We next tested the effect of disrupting the intracellular membrane secretory pathway with brefeldin A on ligand-induced receptor up-regulation. Treatment of rKOR cells for 6 h with naltrexone caused an approximate 3-fold increase in the 52-kDa KOR species and a concomitant 2-fold decrease in the 42-kDa species (Fig. 6A), which was similar to the results obtained after 8 h of exposure (Fig. 1B). After incubation of the cells for 6 h with BFA alone (at a concentration slightly lower than that used previously by Petaja-Repo et al., 2002 and Chen et al., 2006), the pattern of rKOR immunoreactivity was altered. After BFA treatment, the predominant receptor species in rKOR cells was a diffuse band with an apparent molecular mass of approximately 44 kDa, and a faint 48-kDa form was also present (Fig. 6A). The 42- and 52-kDa receptors observed under basal conditions were absent. Coincubation of rKOR cells with BFA and naltrexone for 6 h increased the accumulation of the brefeldin-induced species by more than 1.5-fold (Fig. 6A). To confirm that the BFA-induced receptor species were localized on intracellular membranes, intact rKOR cells were digested with trypsin. Because there is an arginine in the extracellular amino-terminal domain of the rat κ receptor, and a lysine within the FLAG epitope at the amino terminus of the receptor, digestion with trypsin will release amino-terminal peptides rendering rKOR undetectable with anti-FLAG antibodies if the receptor is present on the cell surface. After trypsin digestion, the 52-kDa KOR species disappeared, confirming its presence on the cell plasma membrane, whereas the 42-kDa species remained intact (Fig. 6B). α-Tubulin immunoreactivity was unaltered, verifying that the trypsin digestion did not disrupt the integrity of the plasma membrane. Treatment of intact live cells with trypsin also failed to alter the KOR species that were present after BFA treatment, confirm-

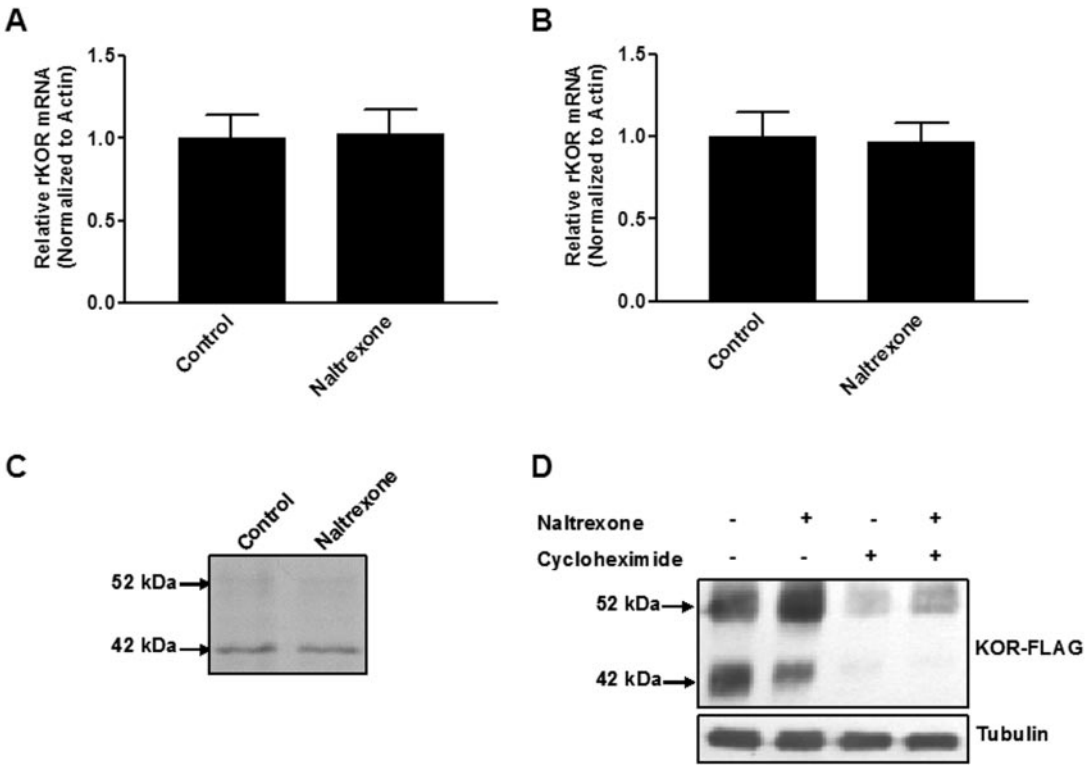

Fig. 5. Effect of naltrexone on KOR mRNA levels and translational efficiency and insensitivity of naltrexone-induced rKOR up-regulation to cycloheximide. rKOR cells were incubated at 37°C in the absence and presence of 1 μM naltrexone for 2 (A) or 24 (B) h. Total RNA was isolated, and real-time PCR was performed with the rKOR and actin primer pairs. Relative rKOR/actin mRNA levels are displayed (n = 9). C, rKOR cells were preincubated in the absence or presence of 1 μM naltrexone for 2 h. Culture medium replaced with methionine- and cysteine-free DMEM in the absence and presence of naltrexone and incubated further for 1 h. [^{35}S]Methionine and [^{35}S]cysteine were added at 100 μCi/ml, and rKOR cells were incubated with and without naltrexone for an additional 1 h at 37°C. rKOR was immunoprecipitated from cell lysates with anti-FLAG M1 antibody-agarose beads, resolved using 10% SDS-PAGE, and visualized by autoradiography. Data are representative of three independent experiments. D, rKOR cells were preincubated at 37°C for 30 min in the absence and presence of cycloheximide (120 μg/ml), then incubated for an additional 2 h in the absence and presence of naltrexone (1 μM). Cell lysates were prepared and subjected to SDS-PAGE and Western blot analysis using the anti-FLAG M1 and α-tubulin antibodies. Data are representative of three independent experiments.

ing that they were localized intracellularly (Fig. 6B). Similar to naltrexone, coincubation with etorphine, cyclazocine, naloxone, and naloxone methiodide increased the abundance of the BFA-dependent species, whereas dynorphin, U69,593, and salvinorin A had no effect (Fig. 6C). It is significant that select nonpeptidic κ ligands were incapable of up-regulating the BFA-dependent rKOR species, whereas other ligands, including naloxone methiodide, stabilized the intracellular, BFA-induced receptor species.

Pulse-Chase Analysis of the κ Receptor in the Absence and Presence of Naltrexone. rKOR cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min, then chased with unlabeled amino acids in the presence or absence of 1 μM naltrexone for various times. After the 30-min labeling period, the 42-kDa KOR species was predominant (Fig. 7A). A maximal 40% conversion of the 42-kDa receptor to the 52-kDa species was observed after a 1-h chase in the absence of naltrexone (Fig. 7A). The extent of conversion increased maximally to 80 to 90% after a 2-h chase in the presence of naltrexone. Naltrexone treatment caused an increase in the ratio of the 52- to 42-kDa species relative to the control samples at all chase times (Fig. 7A).

Cellular Uptake of [^{3}H]Naloxone and [^{3}H]U69,593. To gain further insight into the mechanism and cellular compartment involved in ligand-dependent receptor up-regulation, we sought to determine whether the active ligands were cell permeable. To assess cell permeability, we incubated untransfected HEK 293 cells with [^{3}H]naloxone (which induces κ-receptor up-regulation, Fig. 2C) or [^{3}H]U69,593 (which does not induce κ-receptor up-regulation, Fig. 2C). After incubation of cells with either tritiated ligand for 1, 2, or 4 h, followed by extensive washing to remove any extracellular ligand, the cells were subjected to hypotonic lysis and ultracentrifugation to separate the cytoplasmic fraction from the lysed cell membranes. Both [^{3}H]naloxone and [^{3}H]U69,593 were present in the cytoplasmic fraction, as evident from the increased ligand content of the cytoplasmic fraction relative to the last cell wash (Fig. 8, A and B). These results provide direct evidence that select nonpeptidic opioid ligands are cell permeable. The results also imply that the cell permeability of a ligand is not, in itself, sufficient for inducing κ-receptor up-regulation, given the inactivity of U69,593.

Functional Consequences of Naltrexone-Induced rKOR Up-Regulation. [^{35}S]GTPγS binding was assayed to determine whether the naltrexone-induced up-regulation of rKOR was associated with an increase in agonist-dependent G protein activation. rKOR cells were incubated for 24 h in the absence or presence of 1 μM naltrexone, and stimulation of [^{35}S]GTPγS binding induced by U69,593 was assayed using membrane preparations from each group (Fig. 9). Naltrexone pretreatment caused a significant 2.2-fold increase in the E_{max} of U69,593 activation, from 250 ± 30% of basal levels in the controls to 540 ± 35% of basal levels in the

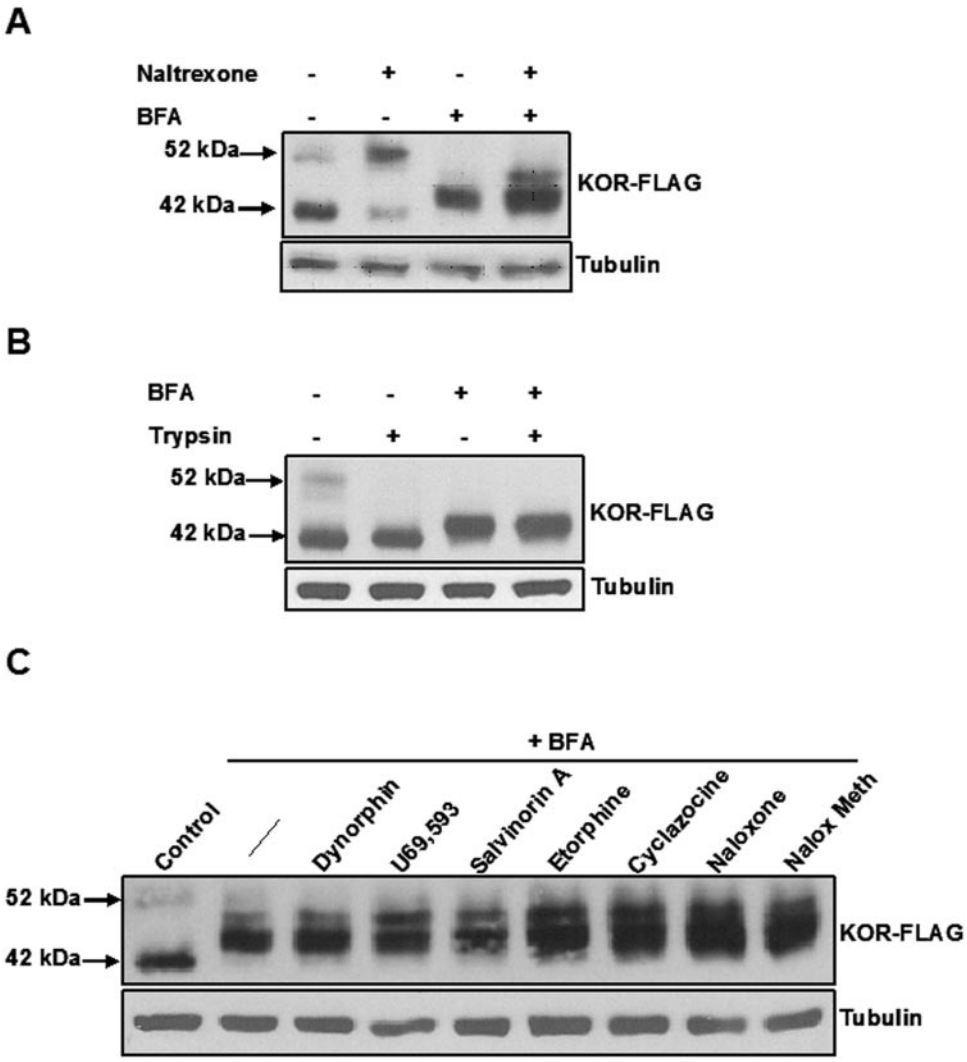

Fig. 6. Effect of brefeldin A on κ-receptor processing and ligand-induced up-regulation. A, rKOR cells were preincubated at 37°C for 30 min in the absence and presence of 3 μg/ml BFA, then incubated with and without 1 μM naltrexone for 6 h, and cell lysates were assayed by Western blotting. B, rKOR cells were treated with or without 3 μg/ml BFA for 6 h at 37°C, then 0.25% trypsin was added and incubated for 5 min at 37°C. Cells were harvested and washed, and whole-cell lysates were analyzed by Western blotting using the anti-FLAG M1 and α-tubulin antibodies. C, rKOR cells were preincubated at 37°C for 30 min in the absence and presence of 3 μg/ml BFA, then incubated with and without opioid ligands for 6 h, and cell lysates were assayed by Western blotting. Ligands were tested at 1 μM, except naloxone methiodide, which was used at 20 μM. Whole-cell lysates were prepared and subjected to Western blot analysis. Data are representative of three independent experiments.

naltrexone-treated samples (mean ± S.E.M., $p < 0.05$, two-tailed Student's t test). Naltrexone pretreatment did not significantly alter the EC_{50} of U69,593, which was 330 ± 60 and 170 ± 25 nM in vehicle- and naltrexone-treated samples, respectively (mean ± S.E.M., $p = 0.06$, two-tailed Student's t test).

Discussion

Although the observation of antagonist-mediated opioid receptor up-regulation was made many years ago (Lahti and Collins, 1978), the mechanism has remained enigmatic. In this study, the abundance of rKOR mRNA did not change following naltrexone, indicating that receptor up-regulation occurred at a post-transcriptional step. Previous studies also found no change in μ- and δ-receptor mRNA levels after antagonist treatment (Jenab et al., 1995; Unterwald et al., 1995; Castelli et al., 1997). Naltrexone did not alter amino acid incorporation into rKOR during a 1-h pulse, suggesting that up-regulation occurred post-translationally. Consistent with this observation, ligand-induced rKOR up-regulation was insensitive to inhibition of protein synthesis.

rKOR up-regulation was associated with a decrease in the level of the 42-kDa precursor and a concomitant increase in the mature 52-kDa receptor, and the increase in immunoreactivity occurred in parallel with the increase in B_{max}. The 3- to 4-fold up-regulation observed herein was significantly greater than the maximal 30% up-regulation of the human KOR induced by naloxone (Chen et al., 2006). The reason for the discrepant magnitudes of up-regulation may be due to species differences or to the use of different cell lines to express the receptors.

We found that rKOR did not down-regulate following incubation of rKOR cells with agonists, suggesting that ligand-induced up-regulation was not simply the reversal of processes involved in down-regulation. Jordan et al. (2000) also reported that treatment with dynorphin A(1–13) and U69,593 for 24 to 72 h did not down-regulate the rKOR expressed in Chinese hamster ovary cells. It has been observed that prolonged treatment with U50,488H led to down-regulation of the human, but not rat, KOR expressed in Chinese hamster ovary cells (Zhang et al., 2002). This species-dependent difference is remarkable given that the human and rat KORs are identical in 94% of amino acid positions. Chen et al. (2006) reported that alkaloid agonists (with the exception of etorphine) caused a modest reduction (15–30%) in the level of the mature 55-kDa human KOR, whereas etorphine, pentazocine, naloxone, norbinaltorphimine, and naloxonazine increased the level by 10 to 30%.

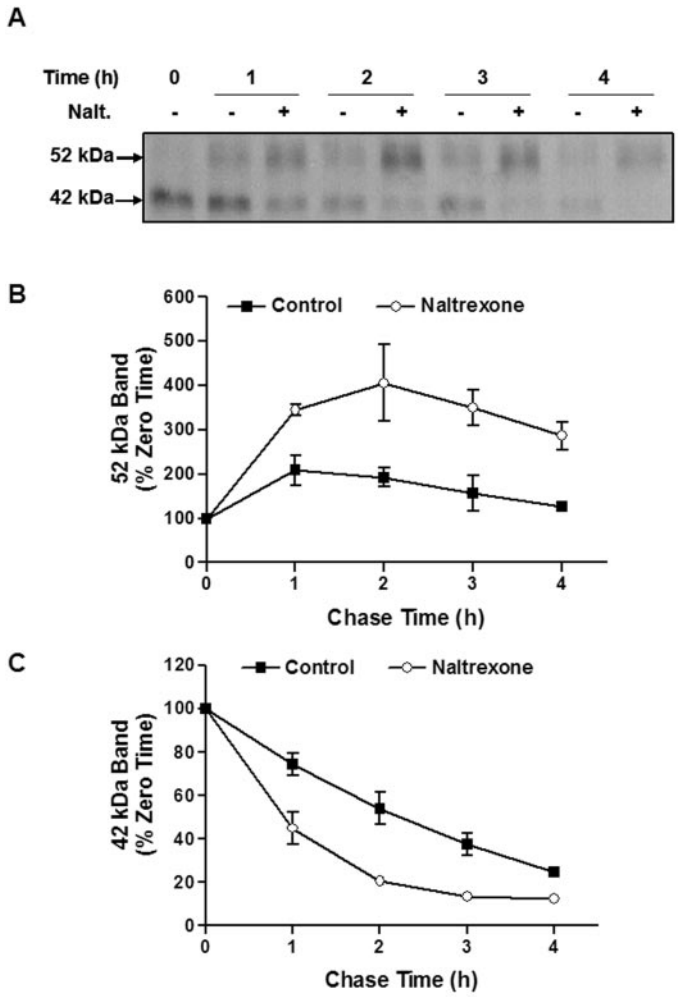

Fig. 7. Pulse-chase analysis of κ-receptor expression following naltrexone treatment. rKOR cells were incubated in methionine- and cysteine-free DMEM for 1 h at 37°C. [^{35}S]Methionine and [^{35}S]cysteine were added at 150 μCi/ml, and cells were pulse-labeled for 30 min at 37°C. Cells were washed with serum-free DMEM containing 120 μg/ml cycloheximide to block further incorporation and incubated in the absence or presence of 1 μM naltrexone for various times in the continued presence of cycloheximide. rKOR was immunoprecipitated from cell lysates with anti-FLAG M1 antibody-agarose beads, and the eluted protein was resolved using 10% SDS-PAGE and visualized by autoradiography (A). B, quantitative analysis of the 52-kDa species over time. C, quantitative analysis of the 42-kDa species over time.

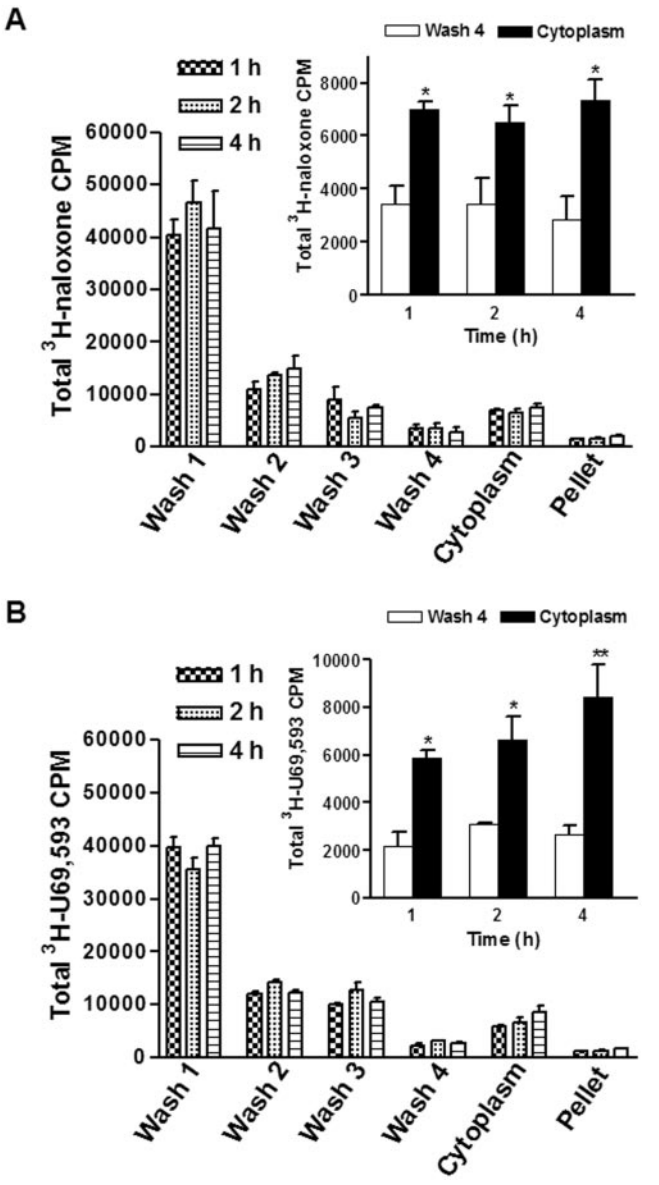

Fig. 8. Cellular uptake of [^{3}H]naloxone and [^{3}H]U69,593. Untransfected HEK 293 cells were incubated in serum-free DMEM with 10 nM [^{3}H]naloxone (A) or [^{3}H]U69,593 (B) for 1, 2, or 4 h at 37°C. Cells were harvested, washed four times, then lysed in hypotonic buffer. The cell cytoplasm was separated from the membrane pellet by centrifugation at 100,000*g* for 30 min at 4°C. The presence of tritiated ligand in the resulting fractions was determined by liquid scintillation counting. Inset, tritiated ligand content in the last wash and cytoplasmic fractions. Data are the means of three independent experiments. *, $p < 0.05$; **, $p < 0.01$, ANOVA, Newman-Keuls multiple comparison test for cytoplasm versus last wash at each time point.

In our study, dynorphin(1–13), β-neoendorphin, salvinorin A, and U69,593 did not induce up-regulation of rKOR; however, other agonists (etorphine and cyclazocine) did. All antagonists that were tested (including naloxone methiodide) up-regulated rKOR. It is obvious that neither relative ligand affinity nor pharmacological profile (agonist or antagonist) correlated with the capability to induce up-regulation. At the concentrations used, all ligands tested would saturate the rKOR binding sites at the cell surface; therefore, engagement of the receptor on the plasma membrane was not sufficient for induction of up-regulation. Up-regulation was associated with more efficient processing of the 42-kDa intermediate to the 52-kDa mature receptor, so it is possible that the active ligands exert their activity by entering the cell and acting as pharmacological chaperones, as proposed for the vasopressin receptor (Morello et al., 2000). It is also possible that agonists and antagonists capable of inducing up-regulation do so by activating a signal transduction pathway that is initiated by receptor binding at the cell surface that regulates intracellular receptor maturation. Pertussis toxin does not inhibit ligand-induced rKOR up-regulation, however, and ligands that did not induce up-regulation did not block the up-regulation induced by active ligands (K. M. Wannemacher and R. D. Howells, unpublished data).

One might speculate that the inactivity of dynorphin(1–13) was due to its peptidic nature, which would disfavor membrane permeability; however, it has been reported that dynorphin(1–17) was membrane permeable (Marinova et al., 2005). If dynorphin(1–13) is cell-permeable, it is possible that

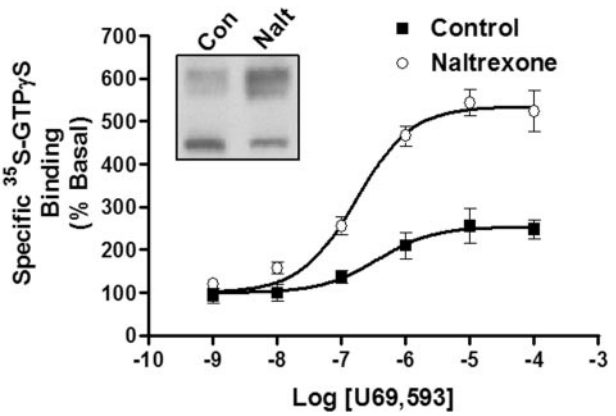

Fig. 9. Increased agonist-induced stimulation of [^{35}S]GTPγS binding following naltrexone-induced rKOR up-regulation. rKOR cells were incubated at 37°C for 24 h in the absence and presence of 1 μM naltrexone. Membrane fractions (7.5 μg of protein) were incubated with 0.3 nM [^{35}S]GTPγS and 10 μM GDP in the absence or presence of varying concentrations of U69,593 at 30°C for 90 min. Reactions were terminated by filtration, and filter-bound radioactivity was determined by liquid scintillation counting. Dose-response curves were analyzed by nonlinear regression using Prism 3.0 (GraphPad Software) to determine E_{max} and EC_{50} values. Data are representative of three independent experiments. Inset, Western blot analysis of rKOR in cell lysates derived from the vehicle- and naltrexone-treated cells.

it lacks the activity of a pharmacological chaperone. The same argument may be applicable to explain the inactivity of U69,593 because we have shown that it is cell-permeable (Fig. 8B). It is also likely that salvinorin A is cell-permeable because it is uncharged, hydrophobic, and orally active. If these ligands can penetrate the cell, their inactivity may be due to a lack of intrinsic activity as pharmacological chaperones. It is widely assumed that quaternary opioid analogs, like naloxone methiodide, are not cell permeable; however, subcutaneous injection of quaternary naloxone and quaternary nalorphine precipitated withdrawal in morphine-dependent rhesus monkeys (Valentino et al., 1983), supporting the notion that naloxone methiodide can enter the cell because it is able to cross the blood-brain barrier.

In transfected HEK 293 cells, the predominant 42-kDa receptor species and the less abundant 52-kDa κ receptor species contained asparagine-linked oligosaccharides, based on their susceptibility to digestion with PNGase F. The two PNGase F-digestion products migrated differently, presumably due to other post-translational modifications, such as *O*-glycosylation, that occur on the larger PNGase product. In contrast, only the 42-kDa receptor was cleaved by endoglycosidase H, indicating that it contained high mannose-type oligosaccharides. This suggested that the 42-kDa form was a biosynthetic intermediate of the 52-kDa species. Pulse-chase analysis also strongly supported this proposal (Fig. 7). In addition, the 52-kDa receptor species bound to and was eluted from WGA-agarose, whereas the 42-kDa form did not. WGA binds to outer chain *N*-acetylglucosamine and terminal sialic acid residues in complex-type oligosaccharides (Bhavanandan and Katlic, 1979), and peroxidase-conjugated WGA was shown to stain the *trans*-Golgi cisternae, *trans*-Golgi network, associated vesicles, and the cell membrane, but not the nuclear envelope, rough endoplasmic reticulum, or *cis*-Golgi cisternae (Tartakoff and Vassalli, 1983). These observations provide further support that the 52-kDa KOR receptor species is located in a membrane compartment distal to the *trans*-Golgi in the secretory pathway, and the 42-kDa species is localized in an intracellular membrane compartment somewhere between the endoplasmic reticulum and the medial-Golgi cisternae. Trypsin treatment of intact cells completely eliminated the 52-kDa KOR but did not affect the 42-kDa KOR, suggesting that the 52-kDa receptor was located on the plasma membrane, whereas the 42-kDa form was intracellular.

A large fraction (30%) of newly synthesized proteins are degraded by the proteasome as a result of misfolding and targeting by the endoplasmic reticulum quality control system (Schubert et al., 2000). Incubation of rKOR cells with a proteasome inhibitor increased the abundance of both KOR species, although the effect was greater for the 52-kDa form. Therefore, the proteasome is also involved in the turnover of the rKOR, as reported previously for μ- and δ-opioid receptors (Chaturvedi et al., 2001). Proteasome inhibition blocked the degradation of both KOR species, but stabilization of the smaller form presumably also promotes its processing to the 52-kDa receptor.

BFA disrupts the secretory pathway by inhibiting anterograde vesicular transport through the Golgi apparatus and causes the collapse of Golgi cisternae and the redistribution of Golgi enzymes into the endoplasmic reticulum (Lippincott-Schwartz et al., 1989). BFA acts as an uncompetitive inhibitor of the Arf1 guanine nucleotide exchange factor, GBF1, by binding to an Arf1-GDP/GBF1 complex (Niu et al., 2005). After incubation of rKOR cells with BFA, the apparent molecular mass of the predominant rKOR species increased to 44 kDa. This shift was probably due to the mixing of enzymes from the Golgi cisternae with those of the endoplasmic reticulum, and the 44-kDa intermediate was unable to traffic out of the endoplasmic reticulum/Golgi apparatus in the presence of BFA. Treatment of whole cells with trypsin failed to alter the abundance of the BFA-induced species, providing evidence that the 44-kDa protein was located intracellularly (in the BFA-induced, merged endoplasmic reticulum/Golgi complex). Processing was obviously incomplete after BFA because the mature 52-kDa receptor species was absent. Acquisition of sialic acid is one of the last steps in *N*-linked oligosaccharide processing, and the sialyltransferase resides in the *trans*-Golgi network that does not redistribute back into the endoplasmic reticulum in the presence of BFA (Chege and Pfeffer, 1990; Lippincott-Schwartz et al., 1991). Coincubation of BFA with naltrexone, naloxone, naloxone methiodide, etorphine, or cyclazocine further increased the accumulation of the 44- to 48-kDa KOR immunoreactivity by 1.5-fold relative to BFA treatment alone. This subset of ligands was the same that up-regulated the 52-kDa KOR species in the absence of BFA (Figs. 2C and 3C), providing support for the hypothesis that these ligands act intracellularly by binding to the κ-receptor population trapped in the BFA-induced ER/Golgi tubules, stimulating proper folding and inhibiting proteolysis mediated by the endoplasmic reticulum-associated degradation system that degrades misfolded proteins.

We propose that the molecular mechanism responsible for KOR up-regulation involves a pharmacological chaperone action of a select subset of KOR ligands mediated by ligand engagement of intracellular receptors, thereby enhancing the folding and rate of receptor maturation through the secretory pathway and by protecting receptor intermediates from deg-

radation by the proteasome. The outcome is an increase in the number of KOR binding sites, an increase in the level of KOR protein at the cell surface, and supersensitivity to agonist-induced G protein activation. It appears that cellular permeability is necessary but not sufficient for a ligand to induce KOR up-regulation. The basis of the differential chaperone activity of select KOR ligands will require further investigation.

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References

Bhavanandan V and Katlic A (1979) The interaction of wheat germ agglutinin with sialoglycoproteins. *J Biol Chem* **254:**4000–4007.

Castelli MP, Melis M, Mameli M, Fadda P, Diaz G, and Gessa GL (1997) Chronic morphine and naltrexone fail to modify μ-opioid receptor mRNA levels in the rat brain. *Mol Brain Res* **45:**149–153.

Chaipatikul V, Erickson-Herbrandson LJ, Loh HH, and Law PY (2003) Rescuing the traffic-deficient mutants of rat μ-opioid receptors with hydrophobic ligands. *Mol Pharmacol* **64:**32–41.

Chaturvedi K, Bandari P, Chinen N, and Howells RD (2001) Proteasome involvement in agonist-induced down regulation of μ and δ opioid receptors. *J Biol Chem* **276:**12345–12355.

Chege NW and Pfeffer SR (1990) Compartmentation of the Golgi complex: brefeldin A distinguishes trans-Golgi cisternae from the trans-Golgi network. *J Cell Biol* **111:**893–899.

Chen Y, Chen C, Wang Y, and Liu-Chen LY (2006) Ligands regulate cell surface level of the human κ opioid receptor by activation-induced down-regulation and pharmacological chaperone-mediated enhancement: differential effects of nonpeptide and peptide agonists. *J Pharmacol Exp Ther* **319:**765–775.

Ellgaard L and Helenius A (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4:**181–191.

Jenab S, Kest B, and Inturrisi CE (1995) Assessment of delta opioid antinociception and receptor mRNA levels in mouse after chronic naltrexone treatment. *Brain Res* **691:**69–75.

Jordan BA, Cvejic S, and Devi LA (2000) Kappa opioid receptor endocytosis by dynorphin peptides. *DNA Cell Biol* **19:**19–27.

Lahti R and Collins R (1978) Chronic naloxone results in prolonged increases in opiate binding sites in brain. *Eur J Pharmacol* **51:**185–186.

Latronico AC and Segaloff DL (1999) Naturally occurring mutations of the luteinizing hormone receptor: lessons learned about reproductive physiology and G protein-coupled receptors. *Am J Hum Genet* 65:949–958.

Leanos-Miranda A, Ulloa-Aguirre A, Janovick JA, and Conn PM (2005) In vitro coexpression and pharmacological rescue of mutant gonadotropin-releasing hormone receptors causing hypogonadotropic hypogonadism in humans expressing compound heterozygous alleles. *J Clin Endocrinol Metab* **90:**3001–3008.

Lesscher HM, Bailey A, Burbach JP, Van Ree JM, Kitchen I, and Gerrits MA (2003) Receptor-selective changes in mu-, delta-, and kappa-opioid receptors after chronic naltrexone treatment in mice. *Eur J Neurosci* **17:**1006–1012.

Li J, Chen C, Huang P, and Liu-Chen L (2001) Inverse agonist up-regulates the constitutively active D3.49(164)Q mutant of the rat μ-opioid receptor by stabilizing the structure and blocking constitutive internalization and down-regulation. *Mol Pharmacol* **60:**1064–1075.

Lippincott-Schwartz J, Yuan LC, Bonifacino JS, and Klausner RD (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56:**801–813.

Lippincott-Schwartz J, Yuan LC, Tipper C, Amherdt M, Orci L, and Klausner RD (1991) Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell* **67:**601–616.

Magnan J, Paterson SJ, Tavani A, and Kosterlitz HW (1982) The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn Schmiedebergs Arch Pharmacol* **319:**197–205.

Marinova Z, Vukojevic V, Surcheva S, Yakovleva T, Cebers G, Pasikova N, Usynin I, Hugonin L, Fang W, Hallberg M, et al. (2005) Translocation of dynorphin neuropeptides across the plasma membrane. *J Biol Chem* **280:**26360–26370.

McQuay H (1999) Opioids in pain management. *Lancet* **353:**2229–2232.

Morello J-P, Salahpour A, Laperriere A, Bernier V, Arthus M-F, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG, et al. (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J Clin Invest* **105:**887–895.

Morris BJ, Millan MJ, and Herz A (1988) Antagonist-induced opioid receptor up-regulation: II. Regionally specific modulation of mu, delta and kappa binding sites in rat brain revealed by quantitative autoradiography. *J Pharmacol Exp Ther* **247:**729–736.

Niu T-K, Pfeifer AC, Lippincott-Schwartz J, and Jackson CL (2005) Dynamics of GBF1, a brefeldin A-sensitive Arf1 exchange factor at the Golgi. *Mol Biol Cell* **16:**1213–1222.

Oksche A and Rosenthal W (1998) The molecular basis of nephrogenic diabetes insipidus. *J Mol Med* **76:**327–337.

Petaja-Repo UE, Hogue M, Bhalla S, Laperriere A, Morello JP, and Bouvier M (2002) Ligands act as pharmacological chaperones and increase the efficiency of δ opioid receptor maturation. *EMBO J* **21:**1628–1637.

Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR (2000) Rapid degradation of a large fraction of newly synthesized proteins by the proteasome. *Nature* **404:**770–774.

Sung C-H, Schneider BG, Agarwal N, Papermaster DS, and Nathans J (1991) Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. *Proc Natl Acad Sci U S A* **88:**8840–8844.

Tang AH and Collins RJ (1978) Enhanced analgesic effects of morphine after chronic administration of naloxone in the rat. *Eur J Pharmacol* **47:**473–474.

Tartakoff AM and Vassalli P (1983) Lectin-binding sites as markers of Golgi subcompartments: proximal-to-distal maturation of oligosaccharides. *J Cell Biol* **97:**1243–1248.

Thomas PJ, Qu B, and Pederson PI (1995) Defective protein folding as a basis of human disease. *Trends Biochem Sci* **20:**456–459.

Unterwald EM, Rubenfeld JM, Imai Y, Wang JB, Uhl GR, and Kreek MJ (1995) Chronic opioid antagonist administration up-regulates mu opioid receptor binding without altering mu opioid receptor mRNA levels. *Mol Brain Res* **33:**351–355.

Unterwald EM and Howells RD (2008) Up-regulation of opioid receptors, in *Opioid Receptors and Antagonists: From Bench to Clinic: Contemporary Neuroscience Series* (Dean RL III, Bilsky EJ, and Negus SS III eds), Humana Press, Totowa, NJ, in press.

Valentino RJ, Katz JL, Medzhiradsky F, and Woods JH (1983) Receptor binding, antagonist, and withdrawal precipitating properties of opiate antagonists. *Life Sci* **32:**2887–2896.

Welch WJ and Howard M (2000) Antagonists to the rescue. *J Clin Invest* **105:**853–854.

Yadav PN, Chaturvedi K, and Howells RD (2007) Inhibition of agonist-induced down regulation of the δ-opioid receptor with a proteasome inhibitor attenuates opioid tolerance in human embryonic kidney 293 cells. *J Pharmacol Exp Ther* **320:**1–9.

Zhang F, Li J, Li J-G, and Liu-Chen L-Y (2002) (−)U50,488H [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide] induces internalization and down-regulation of the human, but not the rat, κ-opioid receptor: structural basis for the differential regulation. *J Pharmacol Exp Ther* **302:**1184–1192.

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