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Rescuing the Traffic-Deficient Mutants of Rat μ-Opioid Receptors with Hydrophobic Ligands

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ABSTRACT

Deletion of a sequence near the fifth transmembrane domain (^{258}RLSKV^{262}, i3-1 mutant) and a motif residing at the proximal carboxyl tail (^{344}KFCTR^{348}, C-2 mutant) resulted in μ-opioid receptor mutants that were poorly expressed on the surface of transfected human embryonic kidney 293 cells. Treatment with the opioid antagonist naloxone, the agonist etorphine, and other hydrophobic ligands enhanced cell surface expression of i3-1 and C-2 mutants. The observed enhancement was time- and concentration-dependent, required the ligands to be membrane permeable, and was not the result of the reversal of the constitutive activities of the mutant receptors. The binding of the ligands resulted in the trafficking of the mutant receptors retained in the endoplasmic reticulum to the cell surface. The cell surface-expressed mutant C-2, but not i3-1, fully retained ability to mediate inhibition of adenylyl cyclase activity. Furthermore, the Golgi-disturbing agents brefeldin A and monensin completely blocked naloxone-enhanced expression of i3-1 and C-2 mutants. Results of these studies suggest that intracellular interactions of agonist and antagonist with mutant receptors can serve as chaperones in the trafficking of the mutants to the cell surface.

The G protein-coupled receptors (GPCRs), with more than 1000 members, are one of the largest superfamilies of membrane proteins (Wess, 1998). A large body of evidence has revealed that transmembrane and extracellular loop determine selectivity of agonist binding, whereas the intracellular loops are responsible for G-protein coupling (Wess, 1998). Mutation or deletion of the transmembrane domains and intracellular loops resulted in the gain or loss of function in several GPCRs. Gain-of-function receptor mutants are characterized by constitutive activities with agonist-independent activation. The constitutive activities can be suppressed by binding of the negative antagonist (inverse agonist). In addition, inverse agonists are able to increase the expression of the constitutively active mutant (Pei et al., 1994; MacEwan and Milligan, 1996; Gether et al., 1997; McLean et al., 1999; Stevens et al., 2000). Inverse agonist-induced up-regulation and agonist-independent phosphorylation of receptor mutants suggest the existence of constitutive down-regulation of the constitutively active receptor mutants. The down-regulation of the receptor could be the mechanism for the lower expression level of many constitutively active receptor mutants observed when they are expressed in cell lines.

In addition to the constitutively active mutants, mutations in any portion of the GPCRs have resulted in the intracellular retention of the mutants at the ER. This retention has no apparent dependence on sequence motif. In particular, the deletion or mutation of the third intracellular (i3) loop or carboxyl tail of many GPCRs has been reported to result in low receptor expression in transfected cells (Cheung et al., 1992; Rozzell et al., 1995; Unson et al., 1995; Bradbury et al., 1997; Chicchi et al., 1997; Ray et al., 1997; Oksche et al., 1998; Schulein et al., 1998; Wonerow et al., 1998). The i3 loop and carboxyl tail have been proposed to have a role in folding and trafficking of receptors to the cell surface (Cheung et al., 1992; Unson et al., 1995; Ray et al., 1997; Schulein et al., 1998; Wonerow et al., 1998).

The molecular cloning of the μ-, δ-, and κ-opioid receptor types has shown that these receptors are members of the superfamily of GPCR. Similar to other GPCRs, the transmembrane domains and the extracellular loops determine selectivity of agonist binding, whereas the intracellular loops are responsible for G-protein coupling (Law et al., 1999). In our attempts to identify residues within the third intracellular (i3) loop and the carboxyl tail that determine the struc-

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ABBREVIATIONS: GPCR, G protein-coupled receptors; μOR, μ-opioid receptor; HEK, human embryonic kidney; HA, hemagglutinin; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; PCR, polymerase chain reaction; MEM, minimal essential medium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; U50,488, (±)-*trans*-U-50-*trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrrodinyl)-cyclohexyl]benzene acetamide methasulfonate; ER, endoplasmic reticulum; rLHR, rat lutropin/chorionic gonadotropin; mAb, monoclonal antibody.

ture and function of the rat μ-opioid receptor (μOR), we generated receptor mutants in which the putative G protein interacting and activating domains were deleted. During the course of the studies, two of these mutants, one with a deletion of five amino acids at the NH_2-terminal of the i3 loop (Δ^{258}RLSKV^{262}, i3-1) and another with a deletion at the proximal carboxyl tail (Δ^{344}KFCTR^{348}, C-2), resulted in poor expression of these receptors in transfected HEK293 cells. Because previous studies have suggested that these two receptor domains are involved in the coupling to the G proteins (Georgoussi et al., 1997), the deletion of these motifs could generate constitutively active receptors and account for the poor expression levels. To determine the mechanism that was responsible for the low expression of the receptor mutants i3-1 and C-2, the contributions of the agonist-independent constitutive activation and the intracellular retention of mutant receptors were examined. Similar to a recent report on the ability of ligands to enhance the trafficking of wild-type δ-opioid receptor to the cell surface (Petaja-Repo et al., 2002), hydrophobic opioid ligands such as naloxone and etorphine could rescue the defective expression of these two mutants. The mechanism for such rescue is mainly a result of the ability of the hydrophobic ligands to act as chaperones in the intracellular trafficking of these receptors and not as antagonist for the mutant receptor putative constitutive activity.

Materials and Methods

Materials. Oligonucleotides were synthesized by an automated DNA synthesizer (model 8905; Millipore, Bedford, MA). *Taq* polymerase and restriction enzymes were obtained from Roche Applied Science (Indianapolis, IN). Expression vector pCDNA3 was purchased from Invitrogen (San Diego, CA). QiaPrep 500 was purchased from QIAGEN Inc. (Valencia, CA). Cell culture reagents, minimum essential medium, fetal calf serum, and G418 were supplied by Invitrogen. Sequenase version 2.0 DNA sequencing kit and [^{3}H]diprenorphine were purchased from Amersham Biosciences (Piscataway, NJ) and ^{125}I-labeled acetylated cAMP was purchased from Linco Research (St. Charles, MO). Polyclonal antibodies for acetylated cAMP were generated in rabbits as described previously (Law et al., 2000). Mouse monoclonal anti-hemagglutinin (HA) antibody (HA.11) was purchased from Covance (Richmond, CA). Rat monoclonal anti-HA antibody (3F10) was purchased from Roche Applied Science. Alexa 488 goat anti-mouse, Alexa 488 goat anti-rat, and Alexa Fluor 594 goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR). [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), etorphine, and naloxone were supplied by the National Institute on Drug Abuse. Other chemicals were purchased from Sigma (St. Louis, MO).

Construction of the μ-Opioid Receptor Mutants. Mutant μORs were generated by site-directed mutagenesis following the methods described by the manufacturer (QuikChange; Stratagene, La Jolla, CA). The expression vector pCDNA3 containing an HA epitope-tagged μOR receptor (MORTAG) cDNA served as the template in the polymerase chain reaction (PCR). Each 50 μl of PCR reaction contained 40 ng of template, 44 nM of each primer, 2.5 mM concentrations of dATP, dGTP, dTTP, and dCTP, and 2.5 U of *Pfu* DNA polymerase. For the generation of the i3-1 deletion mutant, the following primer sequences, with a *Bam*HI restriction site added, were used: CGCCCTGATGATCTTACGCATGCTATCGGGATCCAAAGAAAAGGACA GG. For the generation of the C-1 deletion mutants, the following primer sequences, with an *Eco*RI restriction site added, were used: GGATGAAAACTTCAAGGAATTCTGTATCCCAACCTCGTCCACG. Amplification was carried out at 95°C for 30 s for 1 cycle and 95°C for 30 s, 58°C for 1 min, and 68°C for 14 min for 18 cycles. PCR products were incubated at 37°C for 1 h with 10 U of *Dpn*I to digest the methylated double-stranded DNA template. Nicked circular plasmids containing the mutated receptors were transformed into XL-1 Blue *Escherichia coli*. The receptor mutants were first identified by the restriction enzyme analysis and verified by nucleotide sequencing using Sequenase version 2.0 DNA sequencing kit.

Cell Culture and Transfection. HEK 293 cells were transfected using the $CaPO_4$ precipitation method, as described previously (Chen and Okayama, 1988). After 10 to 14 days of selection in the presence of 1 mg/ml of G418, HEK293 colonies with stable expression of wild-type or mutant receptors were isolated. Positive clones expressing μOR were detected by whole-cell binding assays. Cells were incubated for 90 min with 1 nM [^{3}H]diprenorphine in Krebs-Ringer-HEPES buffer (110 nM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$), pH 7.6, at 22°C. Nonspecific binding was determined in the presence of 10 μM naloxone. Binding reactions were terminated by harvesting the cells on GF/B filter paper, washed three times with 5 ml of 25 mM HEPES, pH 7.6, at 4°C. Radioactivity was determined using a Beckman 5000 scintillation counter (Beckman Coulter, Fullerton, CA). Positive clones were cultured in MEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2.5 μg/ml G418 at 37°C in the humidified atmosphere containing 5% carbon dioxide.

Measurement of Intracellular cAMP in Whole Cells. Intracellular cyclic AMP accumulation in HEK293 cells stably expressed the wild-type or mutant μOR was measured by radioimmunoassay using ^{125}I-labeled acetylated cAMP and polyclonal antibodies for acetylated cAMP. Inhibition of forskolin-stimulated adenylyl cyclase activity was carried out in the presence of various concentrations of the μ-opioid receptor agonist DAMGO. Briefly, cells were cultured in 17 mm-diameter wells of a 24-well plate. After culturing the cells to confluence, the medium was removed and replaced with 0.5 ml of treatment buffer with or without the agonist. Treatment buffer consisted of 0.5 mM isobutylmethylxanthine and 10 μM forskolin in Krebs-Ringer-HEPES buffer. Cells were incubated for 15 min at 37°C. The treatment was terminated by the addition of 75 μl of 3.3 N perchloric acid and neutralized with 150 μl of the mixture of 2 M KOH, 1 M Tris, and 60 mM EDTA. The supernatant was collected for measurement of cAMP using the radioimmunoassay method as described previously (Law et al., 2000). Radioactivity was measured using a Beckman Gamma 5500 counter. The amount of cAMP was calculated from the standard curve. The EC_{50} values of DAMGO were obtained by curve-fitting the dose-response curves using Prism software (GraphPad, San Diego, CA).

Confocal Microscopy. For determining cellular location of the receptors, cells were cultured on polylysine-coated cover slips. Immunocytochemical staining was carried out at room temperature. After treating the cells with various agents, they were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 30 min followed by treatment with 0.3% Triton X-100 for 20 min. Permeabilized cells were incubated with mouse monoclonal anti-HA antibody (HA.11 clone 16B12) in PBS (1:500) for 1 h. After washes with bovine serum albumin-PBS buffer (0.1% bovine serum albumin in PBS), cells were incubated with Alexa Fluor 594 goat anti-mouse IgG (1:200) for 2 h. For colocalization studies, the alternative staining was carried out to avoid the cross reaction of the fluorescent-conjugated secondary antibodies. Permeabilized cells were incubated with rat monoclonal anti-HA antibody 3F10 in PBS (1:300) for 1 h and then incubated with Alexa Fluor 488 goat anti-rat IgG (1:200) for 2 h. After washing with bovine serum albumin-PBS buffer, cells were incubated with monoclonal anti-calnexin antibody (1:500) for 1 h and then with Alexa Fluor 594 goat anti-mouse IgG (1:200) for 2 h. Cells were viewed with a Bio-Rad MRC1000 confocal microscope.

Quantitation of Receptors on Cell Surface with Flow Cytometry. Cells were grown to confluence in 35-mm dishes. Cell surface receptors were labeled with mouse monoclonal anti-HA an-

tibody in MEM (1:500) at 4°C for 1 h. After washing away the excess primary antibody, cells were treated with goat Alexa 488 anti-mouse IgG in MEM (1:400) at 4°C for 2 h. After washings to remove excess secondary antibody, cells were fixed with 3.7% formaldehyde in PBS and then suspended in PBS-EDTA (0.4%). Fluorescence intensity of 10,000 cells in suspension was measured by fluorescence flow cytometry (FACScan; BD Biosciences, Palo Alto, CA).

Results

To investigate the functional role of the i3 loop and carboxyl tail of the rat μOR, a series of receptor deletion mutations (Fig. 1) were generated and transfected into HEK293 cells. HEK293 cells surviving the antibiotic G418 selection and stably expressing the wild-type or mutated receptors were examined. Notably, low specific [^{3}H]diprenorphine binding was detected in HEK293 cells expressing receptor mutants i3-1 and C-2. Mutant i3-1 lacked the ^{258}RLSKV^{262} motif, located adjacent to the fifth transmembrane domain and mutant C-2 lacked the ^{344}KRCFR^{348} motif preceding the palmitoylation site of the carboxyl tail (Fig. 1). The low expression of these receptors could be the result of the agonist-independent down-regulation of the constitutively active receptor. If this were the case, the use of a negative antagonist (inverse agonist) such as naloxone could block the constitutive activity of the receptor and increase the expression of the receptor mutants at the cell surface. As shown in Fig. 2, there was a time-dependent increase in the cell surface expression of i3-1 and C-2 mutants as detected by FACS analyses when these cells were treated with 1 μM of naloxone. The time to produce 50% of the maximal increase in the cell surface receptors was 8.0 ± 1.0 h and 5.2 ± 0.4 h for i3-1 and C-2 receptor mutants, respectively. The maximum expression of

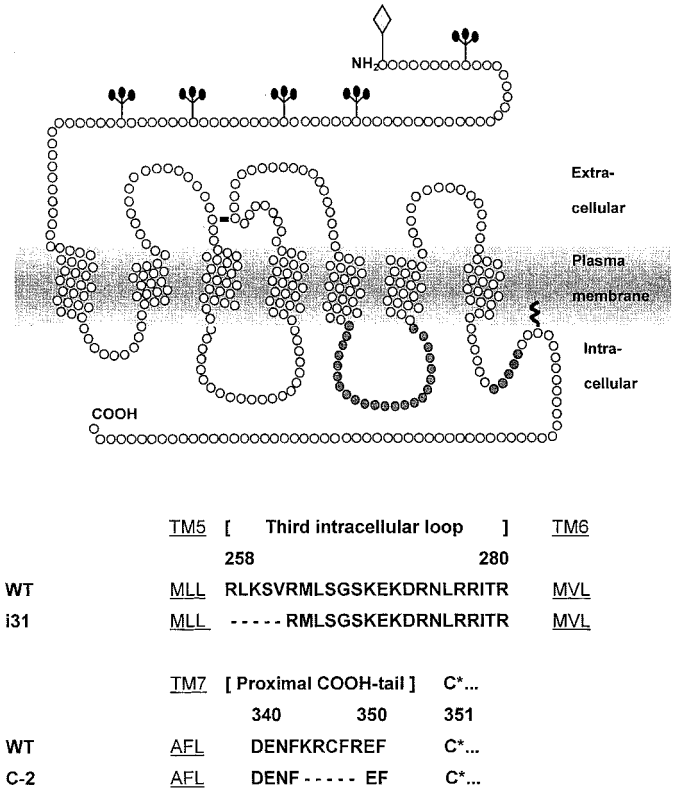

Fig. 1. Schematic structure of the proposed topology of the rat μ-opioid receptor and mutations. The top figure represents the predicted heptahelical topology of the receptor. Branch and zigzag lines indicate sites of glycosylation and palmitoylation, respectively. The third intracellular loop and carboxyl tail proximal to the palmitoylation shown as filled circles and were consecutively deleted as marked. The site of the HA epitope tag is indicated by the diamond (see *Materials And Methods*). The bottom of the figure shows the one-letter amino acid representation for the wild type (WT) and deletion mutants i3-1 and C-2 with the deleted amino acids shown as dashes. The transmembrane regions (TM) and amino acid positions are shown above the sequence. *, palmitoylated cysteine of the carboxyl-tail.

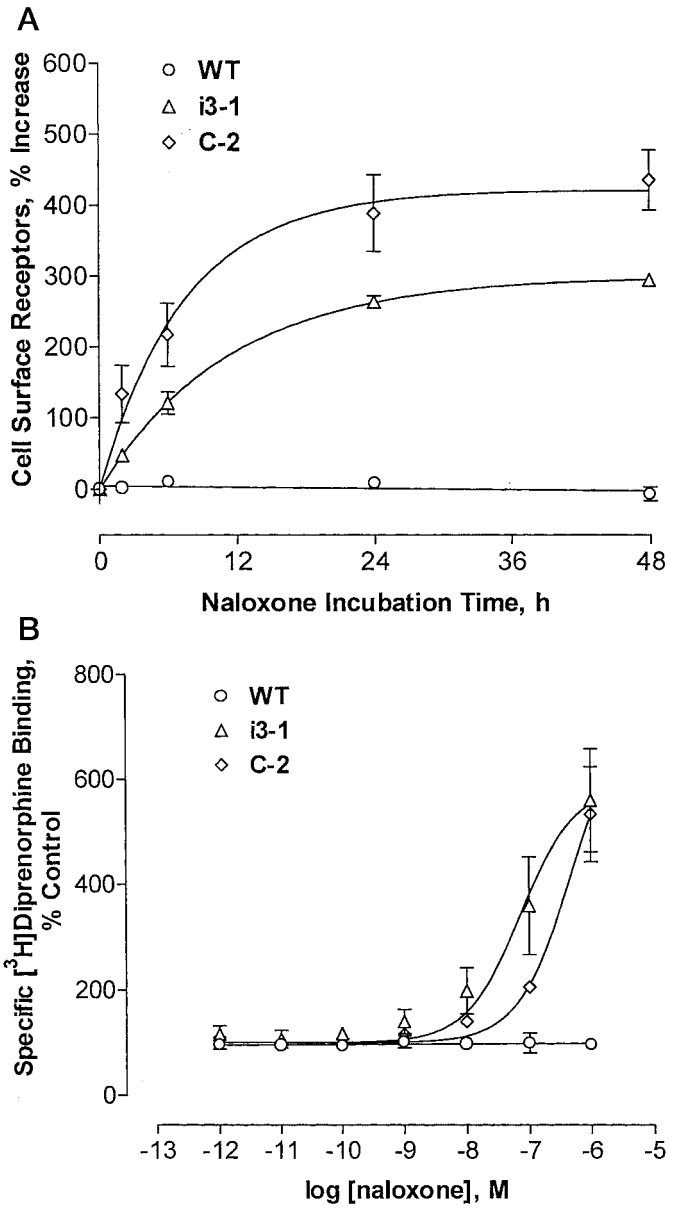

Fig. 2. Naloxone promotes expression of mutant rat μ-opioid receptor in a concentration- and time-dependent manner. HEK293 cells stably expressing wild-type (WT) or mutant i3-1 or C-2 receptors were treated with naloxone. The expression of WT receptor or mutants was measured by FACS analysis (A) or by the specific binding of [^{3}H]diprenorphine (B). A, surface expression of μ-opioid receptor was determined by specific antibody staining and FACS analyses as described under *Materials and Methods*. Each point is the mean of the percentage increase in cell surface receptor from control ± S.E.M. of two separate experiments performed in triplicate. The concentration-dependent enhancement of expression by naloxone is shown in Fig. 2B. Specific binding of [^{3}H]diprenorphine in untreated cells (control) and cells treated with 1×10^{-12} to 10^{-6} M naloxone for 48 h was compared. Each point is the mean ± S.E.M. of two separate experiments performed in triplicate.

i3-1and C-2 was observed after 24 h of naloxone treatment and persisted for at least 48 h after the initiation of the treatment. Similar naloxone treatment did not alter the cell surface expression of the wild-type μ-opioid receptor (Fig. 2A). Furthermore, the amount of i3-1 and C-2 mutants expressed on the cell surface after naloxone treatment was comparable with that of the wild-type μ-opioid receptor. Specific [^{3}H]diprenorphine binding increased from 0.31 ± 0.00 to 1.76 ± 0.41 pmol/mg in mutant i3-1 and from 0.46 ± 0.02 to 2.38 ± 0.41 pmol/mg in mutant C-2 after naloxone treatment. In contrast, similar to the results obtained with FACS analyses, the specific [^{3}H]diprenorphine binding of the untreated or naloxone-treated HEK293 cells expressing the wild-type receptor remained unchanged (2.07 ± 0.62 and 1.96 ± 0.35 pmol/mg, respectively). In addition, sustained incubation of naloxone was required to maintain the steady-state number of receptors on the cell surface. Removal of naloxone from the culturing medium after 48 h of naloxone treatment resulted in a time-dependent decrease in the number of cell surface receptors for i3-1 and C-2 mutants. The $t_{1/2}$ for decrease in cell surface receptors of i3-1 and C-2 mutants was determined to be 21.8 ± 1.5 h and 19.7 ± 5.8 h, respectively. These rates were comparable with the turnover rate reported for the wild-type δ-opioid receptor (Petaja-Repo et al., 2000).

The observed increase in the cell surface expression of these two mutant μ-opioid receptors exhibited naloxone concentration dependence as well. Monitoring the cell surface expression either by FACS analyses or by [^{3}H]diprenorphine binding assays, the EC_{50} values of naloxone for the increased expression of receptors were 0.11 ± 0.08 and 0.80 ± 0.73 μM for i3-1 and C-2, respectively (Fig. 2B). Although the EC_{50} values were relative higher than the reported affinity value (K_i) of naloxone (nanomolar range), it could be demonstrated that the naloxone effect resulted from the ligand binding to the mutant receptors. When the HEK293 cells expressing the i3-1 and C-2 mutant receptors were treated with (+)-naloxone, the inactive stereoisomer of naloxone, there was no measurable increase in the cell surface expression of the mutant receptors (Fig. 3). Meanwhile, parallel experiments using (±)-naloxone resulted in the expected increase in the cell surface receptor (Fig. 3). Furthermore, the observed naloxone effect required the antagonist to diffuse into the intracellular compartments. When HEK293 cells expressing wild-type μ-opioid receptor, i3-1, or C-2 mutants were treated with naloxone methiodide, the quaternary salt of naloxone did not cross the membrane but had similar antagonist properties; a slight but significantly smaller increase in the cell surface mutant i3-1 and C-2 receptor expression were observed (Fig. 3). These results suggested that naloxone increased the cell surface expression of the mutant receptor by binding to the intracellularly located receptors.

If the naloxone-induced increase in cell surface expression of the mutants required the translocation of the ligand across the plasma membrane, then treatment of the HEK293 cells with any membrane-impermeable opioid ligand would not cause any measurable increase in the cell surface receptor content. Hence, the abilities of various opioid agonists and antagonists to induce the cell surface expression of these mutant receptors were determined. Fig. 4 summarizes the inability of membrane-impermeable peptide ligands CTOP (μ-selective antagonist) and DAMGO (μ-selective agonist) to promote receptor expression, whereas membrane permeable μ-opioid agonists, etorphine, and morphine were able to induce the increase in receptor expression. An expected decrease in the cell surface wild-type receptor level was observed after DAMGO, etorphine, and morphine pretreatment. The magnitude of increased i3-1 mutant expression by naloxone or etorphine was statistically indistinguishable ($p > 0.05$). Unlike the i3-1 mutant, however, the degree of increased expression of C-2 was greater in the presence of naloxone than in the presence of etorphine ($p <$ 005).

The binding of the opioid ligands to the intracellularly located i3-1 and C2 as a prerequisite for the rescuing of these trafficking deficient mutants could be demonstrated further with ligands that exhibit high affinity for the μ-opioid receptor. As summarized in Table 1, the stereoactive isomers of naloxone and methadone, but not their inactive isomers, could induce the cell surface expression of i3-1 and C2. Opioid antagonists that exhibit high affinity for the μ-opioid receptor (naltrexone, naloxone, naltrindole, and diprenorphine)

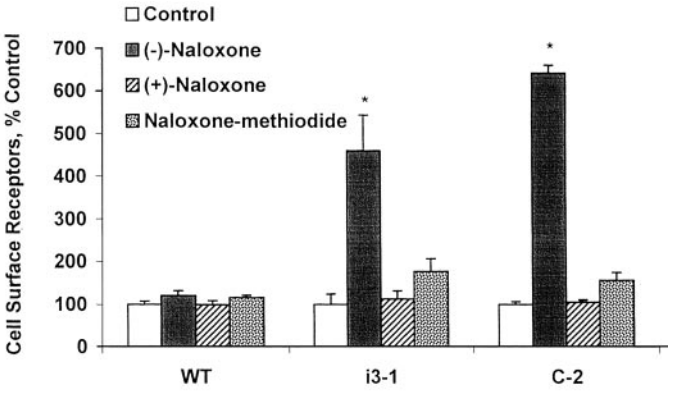

Fig. 3. The naloxone-enhanced expression of mutated receptors was mediated by specific binding of naloxone to intracellular mutated receptors. HEK293 cells treated with (−)-naloxone, the inactive stereoisomer (+)-naloxone, or cell nonpermeable naloxone methiodide for 48 h before cell surface receptor number was determined by FACS analysis as described under *Materials and Methods*. The bars represent the mean and error bars represent the S.E.M. of two separated experiments in triplicate. *, $p < 0.05$ significantly different from untreated receptors.

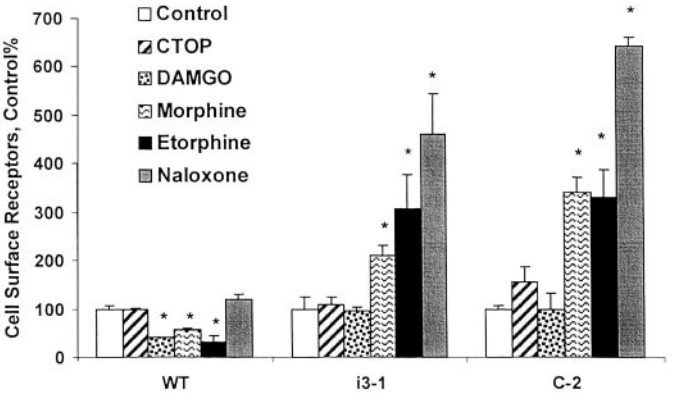

Fig. 4. Etorphine and morphine, but not DAMGO or CTOP, promote expression of mutant rat μ-opioid receptor. HEK293 cells expressing wild-type (WT), mutant i3-1 or C-2 receptors treated with CTOP, DAMGO, morphine, etorphine, or naloxone for 48 h. Cell surface receptor number was determined by FACS analysis as described under *Materials and Methods*. The bars represent the mean and error bars represent the S.E.M. of two separated experiments in triplicate. *, $p < 0.05$ significantly different from untreated receptors.

could induce the increase, whereas the κ-opioid selective antagonist nor-binaltorphimine could not. Partial agonists such as buprenorphine and nalorphine induced cell surface expression of these mutant receptors in levels greater than those observed with membrane permeable agonists, such as etorphine, morphine, oxymorphone, levorphanol, and methadone (Table 1). Pretreatment of HEK293 cells with μ-opioid receptor peptide agonists, such as DAMGO, Tyr-Pro-Trp-Phe-NH_2, and Tyr-Pro-Phe-Phe-NH_2, that do not cross the membrane, or opioid agonists that exhibit κ-opioid receptor selectivity, U50,488 or pentazocine, did not result in measurable increases in the cell surface mutant receptor expression (Table 1). Hence, binding of the ligands to the mutant receptor has to be the initiation step in eliciting the cell surface expression of these two mutant opioid receptors.

If the ability of naloxone, etorphine, or other opioid ligands to enhance the expression of i3-1 and C-2 mutants in HEK293 cell surface is caused by their binding to the intracellular located receptors, the presence of the mutant receptors within intracellular compartments must be demonstrated. When the receptor was visualized with the confocal immunofluorescence microscopy, specific staining of the receptor on the cell surface was observed in untreated or naloxone-treated HEK293 cells expressing wild-type μ-opioid receptor (Fig. 5, A and B). As expected, etorphine induced down-regulation of the wild-type μ-opioid receptor, resulting in the weak immunofluorescence on the cell surface after agonist treatment (Fig. 5C). In contrast, weak staining of the receptor was observed on the cell surface of HEK293 cells expressing i3-1 or C-2 mutant receptors. Diffuse staining was observed within the cytoplasm of HEK293 cells expressing i3-1 mutant (Fig. 5D), whereas receptor staining was detected at the region of the perinucleus of the HEK293 cells expressing the C-2 mutant (Fig. 5G). Treatment with naloxone or etorphine resulted in the receptor staining on the cell surface but the absence of staining in the cytoplasm of HEK293 expressing i3-1 (Fig. 5, E and F) or the perinuclear region of HEK293 cells expressing the C-2 receptor (Fig. 5, H and I).

TABLE 1

Relative percent increase in surface μ-opioid receptor in HEK293 cells expressing the C-2 or i3-1 mutants after agonist or antagonist treatment

HEK293 cells stably expressing the C-2 or the i3-1 mutants of the μ-opioid receptor were treated with 1 μM of various opioid agonists and antagonists for 48 h. Afterward, the amount of the receptor expressed on the cell surface was determined by FACS analyses as described under *Materials and Methods*. The amount of receptor expressed on the cell surface after drug treatment was compared with that expressed in cells cultured in the absence of opioid ligands. The values represent the average ± S.D. of FACS analyses from cells cultured in three different plates.

	i3-1	C-2
Nonselective Hydrophobic Antagonists		
(−)-Naloxone	460 ± 84	642 ± 18
Naltrindole	430 ± 6	760 ± 25
Naltrexone	480 ± 1	770 ± 63
Diprenorphine	430 ± 2	800 ± 18
Partial Agonists		
Buprenorphine	450 ± 8	750 ± 9
Nalorphine	400 ± 13	590 ± 35
Nonselective Hydrophobic Agonists		
Etorphine	320 ± 2	410 ± 32
Morphine	210 ± 6	340 ± 64
Oxymorphone	290 + 2	470 ± 28
Levorphanol	200 + 0	340 ± 31
L-Methadone	240 + 0	410 ± 24
Inactive Isomers		
(+)-Naloxone	113 ± 19	106 ± 5
D-Methadone	100 ± 0	140 ± 8
μ-Selective Peptide Agonists		
DAMGO	97 ± 6	98 ± 35
Tyr-Pro-Trp-Phe-NH_2	120 ± 1	120 ± 16
Tyr-Pro-Phe-Phe-NH_2	95 ± 0	110 ± 10
μ-Selective Peptide Antagonists		
CTOP	130 ± 2	100 ± 7
κ-Selective Agonists		
U50-488H	120 ± 0	120 ± 7
Pentazocine	97 ± 2	120 ± 5
κ-Selective Antagonists		
nor-Binaltorphimine	130 ± 4	130 ± 7

The intracellular location of the i3-1 and C-2 mutant receptors could be demonstrated further by immunofluorescence colocalization studies. A large number of studies have suggested that GPCR mutants with defective trafficking to the plasma membrane are retained in the ER. When the HEK293 cells expressing the wild-type μ-opioid receptor were stained with both the rat anti-HA monoclonal antibodies and the mouse monoclonal antibody against the ER chaperone calnexin, separating staining of the μ-opioid receptor expressed on the cell surface and the intracellularly located calnexin was observed (Fig. 6A). In contrast, a majority of the i3-1 mutant receptor was colocalized with calnexin staining (Fig. 6B). Similar colocalization of C-2 and calnexin was observed (data not shown). These results suggested that, in the absence of naloxone or etorphine, both i3-1 and C-2 μ-opioid receptor mutants were localized in the intracellular compartment of HEK293 cells enriched with calnexin, probably the ER compartment. Treatment with naloxone or etorphine resulted in increased trafficking of mutated receptors to cell surface and the separation of the calnexin and receptor staining similar to that of wild-type receptor.

It has been shown that antagonists can induce up-regulation of the constitutively active β-adrenergic receptor (Gether et al., 1997; Samama et al., 1997). Thus, the observed naloxone effects could be caused by the ligand's chaperone activity or by constitutive activities of i3-1 and C-2 mutants. Because opioid receptors inhibit adenylyl cyclase activity, the presence of putative constitutively active i3-1 and C-2 mutant receptors should lower the basal cAMP levels in the HEK293 cells. Furthermore, if the i3-1 and C-2 mutants were constitutively active, naloxone should reverse the constitutive activities of the receptors, resulting in an elevation of basal cAMP level. When the intracellular cAMP levels were determined in HEK293 cells expressing wild-type, i3-1, or C-2 μ-opioid receptors before and after pretreatment with 1 μM naloxone for 48 h, there was no significant difference in the basal intracellular cAMP levels in these cells (Fig. 7A). Because there was a ~5-fold increase in the mutant receptor levels and no change in the wild-type receptor level after naloxone pretreatment, these results suggested that the i3-1 and C-2 mutant μ-opioid receptors are not constitutively active.

The absence of constitutive activity in these receptor mutants could be demonstrated further by examining the ability of opioid agonist DAMGO to activate these receptors. DAMGO inhibited the forskolin-stimulated cAMP accumulation in a concentration-dependent manner with same magnitude of maximum response in control or naloxone-treated HEK293 cells expressing the wild-type μ-opioid receptor (Fig. 7B). Although there was an apparent decrease of DAMGO potency after naloxone pretreatment, the EC_{50} values of DAMGO in untreated and naloxone-treated HEK293

cells (2.5 ± 0.63 and 9.7 ± 4.4 nM, respectively) were not statistically different. On the other hand, DAMGO did not inhibit the forskolin-stimulated cAMP production until at the highest concentration tested in control or naloxone-treated HEK293 cells expressing the i3-1 mutant receptors (Fig. 7B). At 10 μM DAMGO, forskolin-stimulated cAMP production was inhibited by 29 ± 4 and 30 ± 12%, respectively, in untreated and naloxone-treated cells. Clearly, the i3-1 mutants do not efficiently activate the Gi/Go proteins, even in the presence of an agonist such as DAMGO.

In contrast, DAMGO inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent fashion in both control and naloxone-treated HEK293 cells expressing the C-2 mutant μ-opioid receptor (Fig. 7B). The EC_{50} values of DAMGO in control and treated C-2 cells were significantly different (49 ± 14 and 6.6 ± 3.1 nM, respectively). Similarly, a significant increase in the maximum response was observed (46 ± 4 and 87 ± 4% for control and treated C-2 cells, respectively). In addition, with equivalent levels of receptor expressed on the cell surface, the EC_{50} values and the magnitude of maximum response in naloxone-treated HEK293 cells expressing C-2 or wild-type receptor were similar. Hence, the C-2 mutant receptor activated the Gi/o proteins similarly to the wild-type μ-opioid receptor when trafficked to the cell surface. Because the trafficking of both i3-1 and C-2 mutants to cell surface were enhanced by naloxone, and i3-1 could not efficiently activate the Gi/Go proteins, these studies clearly suggested that the inhibition of the mutant receptors constitutive activities was not the mechanism responsible for the up-regulation of the receptor. Rather, naloxone and other hydrophobic ligands bind to the intracellular located i3-1 and C-2 mutants and act as chaperones in their intracellular trafficking.

If naloxone or etorphine could serve as chaperones for the mutant receptors in their maturation processes through the ER and Golgi apparatus, then agents such as brefeldin A or monensin that could block the protein transport from ER to *cis*-Golgi apparatus or protein maturation in Golgi apparatus should inhibit the action of the opiate ligands. Hence, cells expressing the i3-1 or C-2 mutants were pretreated for 1 h with 5 μM brefeldin A or 50 μM monensin before treatment with naloxone for 2 h. The cells were treated with naloxone for a short time so as to eliminate any toxicity effects of brefeldin A and monensin during the long-term treatment. In the absence of naloxone, treatment with brefeldin A or monensin reduced slightly the number of wild-type receptor, i3-1, or C-2 receptors expressed on the surface of HEK293 cells (Fig. 8). The presence of brefeldin A, however, completely reversed the ability of naloxone to increase the expression of i3-1 and C-2 mutants at the cell surface as shown in Fig. 8. Similar results were observed when HEK293 expressing i3-1 or C-2 mutants were incubated with 50 μM monensin for 1 h before naloxone treatment (Fig. 8). The presence of monensin completely reversed the ability of naloxone to enhance the expression of i3-1 and C-2 mutants. Because monensin blocks human δ-opioid receptor maturation in the Golgi apparatus by inhibiting the glycogen transferase activity (Petaja-Repo et al., 2000), the increased expression of i3-1 and C-2 in the presence of naloxone might depend on both transport of receptors from the ER to the *cis*-Golgi apparatus and the subsequent glycosylation of the receptor within the Golgi apparatus.

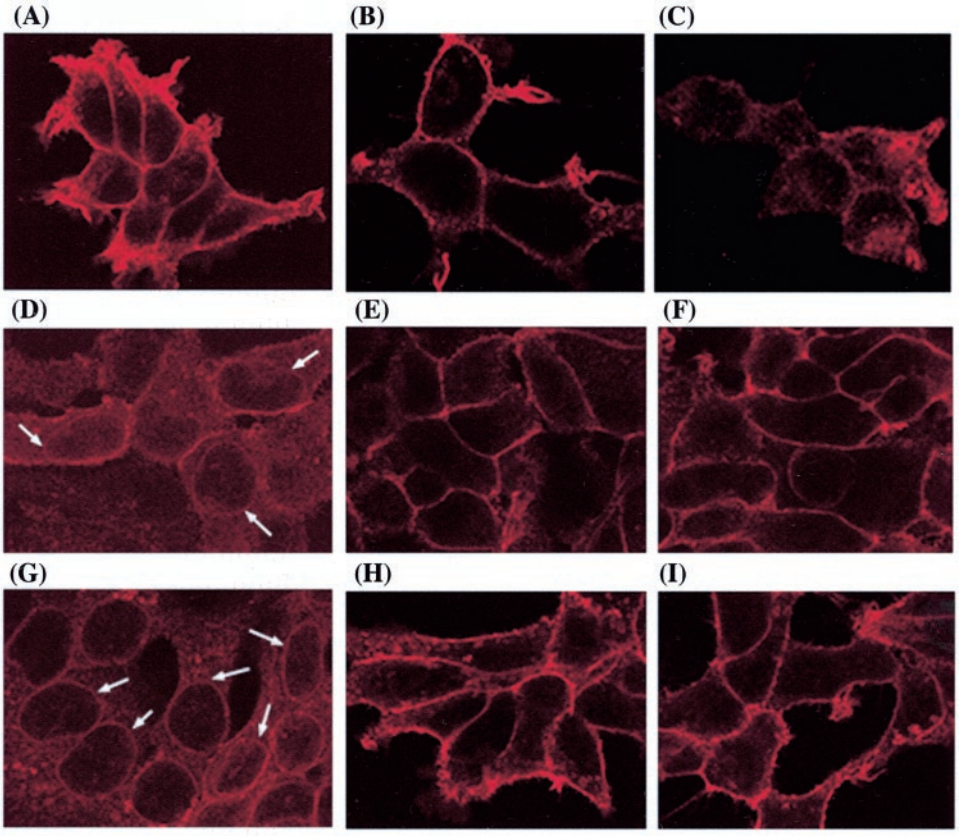

Fig. 5. Confocal microscopy reveals localization of receptors before and after treatment with naloxone or etorphine. Permeable HEK293 cells expressing wild-type μOR (A–C), i3-1 mutants (D–F), or C-2 mutants (G–I) were stained with primary mouse mAb anti-HA antibody as described under *Materials and Methods*. Receptors at the cell surface or in the intracellular compartment were revealed after staining with anti-mouse Alexa Fluor 594 secondary antibody. A, D, and G show the localization of receptor in the nontreated cells expressing wild-type, i3-1 mutant, or C-2 mutant μ-opioid receptors, respectively. B, E, and H represent cells that were treated with 1 μM naloxone for 48 h; C, F, and I represent cells that were treated with 1 μM etorphine for 48 h. Arrows indicated the staining of receptor surrounding the nucleus.

Discussion

Numerous GPCR mutations have resulted in the low cell surface expression of these receptors. The decrease in the cell surface receptor content has been attributed to constitutive activities of the receptors resulting in the constitutive receptor down-regulation (Pei et al., 1994; Heinflink et al., 1995). Stabilization of the labile receptors resulting from ligand receptor interaction has been suggested to cause the up-regulation of constitutively active mutant of β-adrenergic receptors by antagonist (Gether et al., 1997; Samama et al., 1997). Thus, a possible mechanism for our current observation, in which naloxone up-regulated the i31 and C2 mutant receptors is that these receptors are constitutively active. The μ-opioid receptor has been reported to be constitutively active (Huang et al., 2001; Li et al., 2001). The binding of the naloxone to the mutant receptors antagonized the constitutive activity, thus preventing the constitutive receptor down-regulation. However, our results with the adenylyl cyclase measurements did not indicate that the two mutant receptors possess constitutive activity. With different receptor levels expressed in the same clonal cell line, the intracellular cAMP levels remained similar (Fig. 7). Furthermore, treatment of agonist etorphine also resulted in an increase of these two mutant receptor levels. Hence, the mechanism of up-regulation of receptor number of these two μ-opioid receptor mutants by naloxone is not caused by reversal of the constitutive activity, as described in the constitutive active β-adrenergic receptors.

Another observation that did not support the mechanism of antagonist reversal of constitutive activity is the measured half-life of the mutant receptors. Biosynthesis of human δ-opioid receptor using pulse-chase metabolic study indicates that wild-type receptors have a half-life of 19.6 h on the cell surface (Petaja-Repo et al., 2000). Similarly, in the present study, after removing naloxone the time required for 50% decrease in the receptor i3-1 and C-2 mutant on the cell surface was determined as 22 and 19 h, respectively. This similarity in the half-life values between wild-type and mutant receptors suggests that naloxone promotes synthesis or trafficking of the receptor mutant rather than stabilizing the putative constitutive active receptor mutant on the cell surface. If the stabilization of the constitutively active receptors on the cell surface is the mechanism, removal of naloxone would not prevent the continued trafficking of the newly synthesized receptors to the cell surface. The rate of disappearance should be slower, or at least different from the degradation rate observed with pulse-chase studies (Petaja-Repo et al., 2000). Studies with naloxone methiodide and peptide antagonists showing that naloxone acted by binding to the intracellular receptor are consistent with the explanation that the half-life of receptor mutant during naloxone-withdrawal reflects the degradation of the cell surface receptors. Furthermore, the ability of brefeldin A and monensin to block the naloxone effect (Fig. 8) also suggested the antagonist action was at the intracellular trafficking of these mutant receptors. Brefeldin A and monensin were shown to interrupt the maturation process of the wild-type δ-opioid

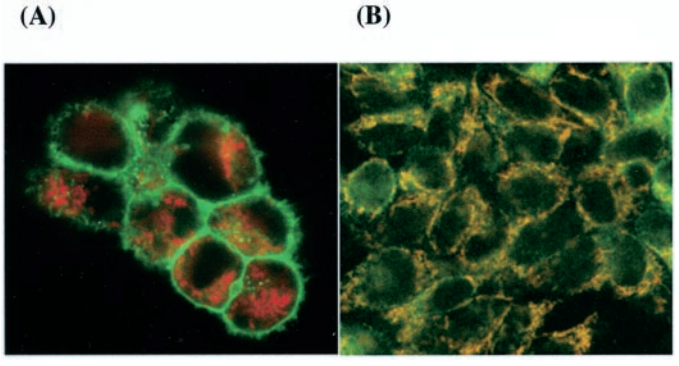

Fig. 6. Confocal microscopy reveals colocalization of i3-1 mutant with an ER chaperone calnexin. Permeable HEK293 cells expressing wild-type or i3-1 mutant μ-opioid receptors were stained with rat mAb anti-HA antibody and mouse mAb anti-calnexin. The receptors were revealed after staining with anti-rat Alexa Fluor 488, whereas the ER marker calnexin was revealed after staining with anti-mouse Alexa Fluor 594. A, the merged image of the receptor and calnexin staining revealing no accumulation of wild-type μ-opioid receptor in the ER. Colocalization of the ER chaperone calnexin with i3-1 was indicated in yellow in the merged images (B).

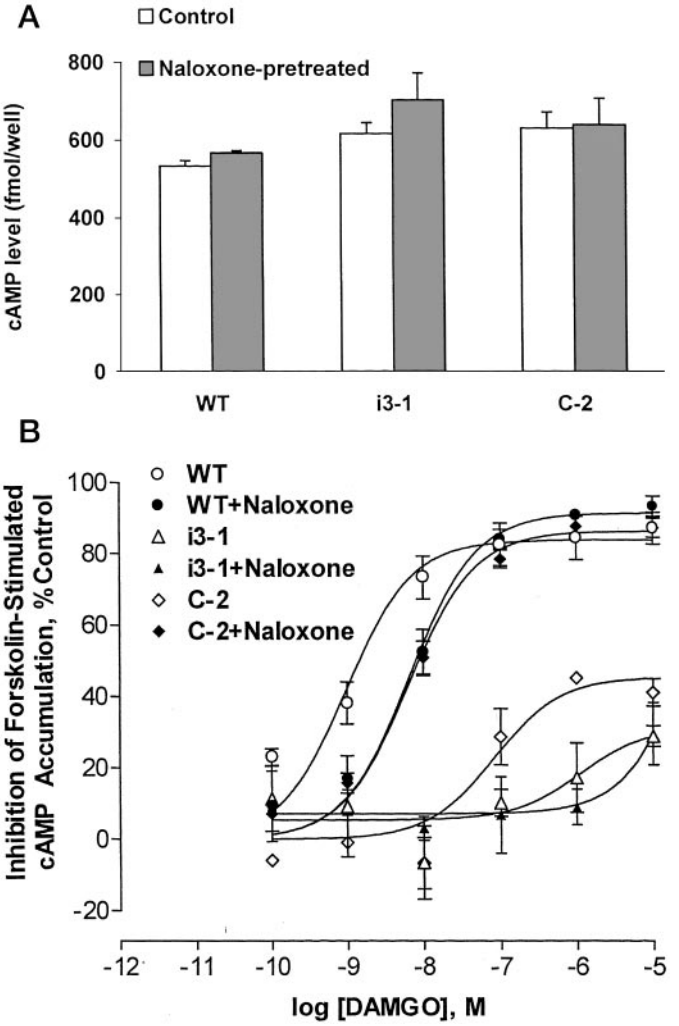

Fig. 7. Ability of rescued i3-1 and C-2 mutants to mediate agonist-inhibited adenylyl cyclase activity. HEK293 cells were treated with 1 μM naloxone for 48 h after removal of naloxone, the amount of cAMP was measured as described under *Materials and Methods*. A, basal cAMP accumulation in untreated (□) or naloxone-treated cells (▨). The effect of DAMGO on function of mutants in the inhibition of forskolin-stimulated cAMP accumulation was shown to be concentration-dependent (B). Each point is mean ± S.E.M. for two separate experiments performed in triplicate.

receptor, resulting in a decrease in the cell surface receptor level (Petaja-Repo et al., 2000).

Although the naloxone rescuing the i3-1 and C-2 mutants require the intracellular binding of the ligand to the mutant receptors as demonstrated by the (+) naloxone, *d*-methadone, and naloxone methiodide studies (Fig. 3, Table 1), the trafficking of the mutant receptors do not require the preexisted interaction with the heterotrimeric G proteins. The i3-1 mutant does not interact with G proteins, as demonstrated by the loss of high-affinity DAMGO binding and by the inability of DAMGO to inhibit the adenylyl cyclase activity after i3-1 mutant is trafficked to the cell surface (Fig. 7B). In contrast, the C-2 mutant can mediate the DAMGO inhibition of the adenylyl cyclase activity when trafficked to the cell surface (Fig. 7B), and DAMGO high-affinity binding was observed. Thus, regardless of G protein coupling, naloxone could serve as a chaperone for the intracellular trafficking of these mutant μ-opioid receptors.

The colocalization of the ER marker calnexin and the mutant opioid receptors suggested that these μ-opioid receptors were retained in the ER of the HEK293 cells (Fig. 6). The ER is a site for protein synthesis and modification, such as glycosylation of proteins. It is also a site of processing the conformation-dependent molecular sorting of newly synthesized proteins, generally known as "quality control". In the biogenesis of many transmembrane glycoproteins, the newly synthesized protein undergoes several processes, including glycosylation and transient interaction with the chaperone for properly folding. Proteins are subsequently exported to the Golgi apparatus for the complete maturation. The immature proteins are the high-mannose glycosylated forms and are associated with the chaperone, whereas the mature proteins are the complex-oligosaccharide glycosylated form and are dissociated from the chaperone. The high-mannose and complex-oligosaccharide glycosylated forms of the wild-type human δ-opioid receptor also were detected in the ER fraction and the plasma membrane, respectively (Petaja-Repo et al., 2000). In the studies of several GPCRs such as V_2 vasopressin (Heinflink et al., 1995), human luteinizing hormone/chorionic gonadotropin (Couvineau et al., 1996), rat lutropin/chorionic gonadotropin (rLHR) (Cheung et al., 1992), and human calcium receptors (Ray et al., 1997), receptor mutants are the immature high-mannose glycosylated proteins and

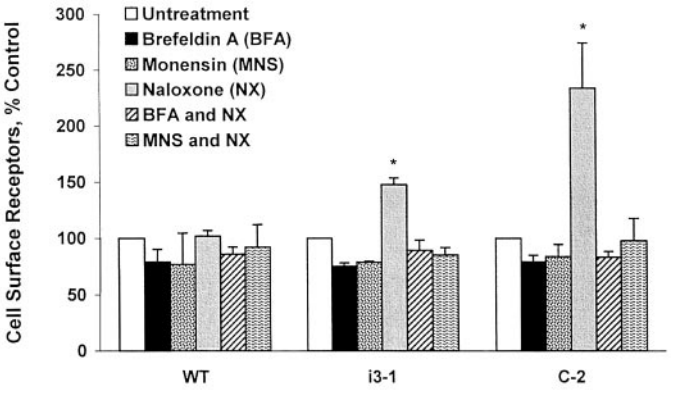

Fig. 8. Brefeldin A and monensin block naloxone-enhanced expression of mutated receptors. Cell surface receptor numbers on HEK293 cells were determined by FACS analysis. The bars represent the mean and error bars represent the S.E.M. of two separated experiments in triplicate. *, $p < 0.05$ significantly different from untreated receptors.

are retained in the ER. In addition, the study of mutation of the rLHR (Rozzell et al., 1995) and the human vasoactive intestinal peptide 1 receptor (Couvineau et al., 1996) demonstrated that the isolated ER-retaining immature mutants of rLHR and vasoactive intestinal peptide receptors remained in the high affinity binding state for their cognate ligands. The wild-type human δ-opioid receptors detected in ER maintained high-affinity binding to the ligands as illustrated by the abilities of hydrophobic ligands to increase the cell surface expression of the receptors (Petaja-Repo et al., 2002). Hence, it is possible that the i3-1 and C-2 mutant retained in the ER remained in the immature high-mannose glycosylated form and the high-affinity binding to opioid ligand. This was reflected by the ability of the mutant receptors to retain their stereo-selectivity for the opioid ligand, because (+)-naloxone and *d*-methadone proved to be inactive in the rescuing these mutants (Fig. 3, Table 1).

Hence a model is proposed in which naloxone, by binding to the immature i3-1 and C-2 mutants would result in a conformational change, allowing maturation and transport to the cell surface. This model implies that structure or conformation, rather than the function of the deleted sequences, governs ability of receptors to undergo one or more stages of maturation, such as export from ER, complete maturation in the Golgi, or targeting to plasma membrane. This model is in contrast with reported studies with the V2 vasopressin receptor in which a specific sequence of the receptor, a conserved glutamate/dileucine motif, was required to exit the ER (Schulein et al., 1998; Krause et al., 2000). This motif of the vasopressin receptor may serve as the targeting signal for exiting from ER to Golgi apparatus (Bradbury et al., 1997). Alternatively, this motif may be required for transport-competent folding of the receptor (Rozell et al., 1998). The fusion protein consisting of the carboxyl tail and the fragment of the amino terminus of the first cytoplasmic loop of V2 vasopressin receptor was detected on the cell surface. Similar fusion proteins with the mutations in the glutamate/dileucine motif were also detected on the cell surface (Rozell et al., 1998). In contrast, the full-length receptors with similar mutations in the glutamate/dileucine motif were retained in the ER (Rozell et al., 1998). Consequently, we concluded that the glutamate/dileucine motif is required for transport-competent folding of the receptor rather than as a transport signal. Use of computer modeling suggested that this motif contributed to the formation of a U-like loop within the carboxyl tail and the interaction of this U-like loop with the first intracellular loop (Rozell et al., 1998). It is unlikely, however, that either of the sequences (^{258}RLSKV^{262} or ^{344}KRCFR^{388}) that were deleted from the i3-1 or C-2 mutants, respectively, was required for the transport-folding state of the receptor. If these two motifs were required for transport-folding state of the μ-opioid receptor, naloxone, etorphine, or other hydrophobic ligands should not have been able to rescue the trafficking of the i3-1 and C-2 mutants that lacked ^{258}RLSKV^{262} or ^{344}KRCFR^{388} motifs, respectively. Furthermore, mutation of the Asp^{340} of the μ-opioid receptor to Ala, the putative site of chaperonin 7 subunit interaction, resulted in the retention of this mutant receptor in the ER (Smirnov et al., 2002). Prolonged treatment with naloxone did not result in the up-regulation of the D340A mutant receptor. Thus, it is likely that the deletion of the i3-1 and C-2 sequences within the μ-opioid receptor resulted in the exposure of sequences rec-

ognized by ER chaperones, which carry the retaining signal. Reversal of interaction with a retaining protein by a naloxone-induced conformational change would allow release of the nascent receptor to the Golgi apparatus and would be targeted to the plasma membrane.

Several studies provide data consistent with this hypothesis. As shown in studies of rLHR and rFSHR, by using coimmunoprecipitation (Rozell et al., 1998), the immature forms of both receptors are associated with calnexin, which is an ER chaperone. The association of chaperone with the immature protein has also been detected in many other glycoproteins, such as transferrin receptor (Williams and Enns, 1993), P-glycoprotein (Loo and Clarke, 1994), cystic fibrosis transmembrane conductance regulator (Yang et al., 1993; Pind et al., 1994), α1-antitrypsin (Le et al., 1994; Wu et al., 1994), and melanocyte-specific enzyme tyrosinase (Halaban et al., 2000). Some mutations of these proteins result the retention of the mutants in the ER. These ER-retaining mutants prolonged the time associated with one or two of these chaperones: immunoglobulin heavy chain binding protein (Williams and Enns, 1993), calnexin (Loo and Clarke, 1994; Pind et al., 1994; Rozell et al., 1998), calriticulin (Halaban et al., 2000), or heat shock protein (Yang et al., 1993; Halaban et al., 2000). In contrast, the wild-type proteins were able to dissociate from the chaperone before their transport to the Golgi apparatus (Yang et al., 1993) and to be targeted to the plasma membrane (Loo and Clarke, 1994; Pind et al., 1994). Although a coimmunoprecipitation study was not carried out in the current studies, the colocalization of the mutant receptors and calnexin in the untreated HEK293 cells (Fig. 6) suggested such a scenario. Recently, similar to our results of i3-1 and C-2 treatment with antagonist, cell-permeable antagonist SR121453A rescued cell surface expression of the ER-retaining V_2 vasopressin mutant receptor with the deletion of three amino acids in the first intracellular loop (Morello et al., 2000).

The fact that mutant V2 vasopressin receptors and the i3-1 and C-2 μ-opioid receptor mutants behaved similarly suggests the existence of a common transport pathway among GPCRs. A possible common property could be the existence of hydrophobic motifs responsible for prolonged interaction with chaperones. In the wild-type receptor, these hydrophobic motifs are probably masked, allowing the receptor to pass the "quality control" or the conformation-dependent molecular sorting process in ER. In contrast, it is possible that these hydrophobic domains remain exposed in the newly synthesized mutant receptors. The binding of ligand to the mutant receptor may result in a conformational change that masks these hydrophobic sequences. A similar mechanism may explain how hydrophobic ligands facilitate maturation and ER export of the wild-type δ-opioid receptor (Petaja-Repo et al., 2002). Only a fraction of newly synthesized δ-opioid receptors leave the ER and reach the cell surface (Petaja-Repo et al., 2000). The binding of hydrophobic ligands may release a portion of those δ-opioid receptors remaining in the ER, explaining the ability of membrane-permeable ligands to increase the number of wild-type receptors on the cell surface. The identities of these sequences, and the chaperones that are involved in the trafficking of the μ-opioid receptor, remain to be elucidated.

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References

Bradbury FA, Kawate N, Foster CM, and Menon KM (1997) Post-translational processing in the Golgi plays a critical role in the trafficking of the luteinizing hormone/human chorionic gonadotropin receptor to the cell surface. *J Biol Chem* **272:**5921–5926.

Chen CA and Okayama H (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6:**632–638.

Cheung AH, Huang RR, and Strader CD (1992) Involvement of specific hydrophobic, but not hydrophilic, amino acids in the third intracellular loop of the beta-adrenergic receptor in the activation of Gs. *Mol Pharmacol* **41:**1061–1065.

Chicchi GG, Graziano MP, Koch G, Hey P, Sullivan K, Vicario PP, and Cascieri MA (1997) Alterations in receptor activation and divalent cation activation of agonist binding by deletion of intracellular domains of the glucagon receptor. *J Biol Chem* **272:**7765–7769.

Couvineau A, Fabre C, Gaudin P, Maoret JJ, and Laburthe M (1996) Mutagenesis of N-glycosylation sites in the human vasoactive intestinal peptide 1 receptor. Evidence that asparagine 58 or 69 is crucial for correct delivery of the receptor to plasma membrane. *Biochemistry* **35:**1745–1752.

Georgoussi Z, Merkouris M, Mullaney I, Megaritis G, Carr C, Zioudrou C, and Milligan G (1997) Selective interactions of mu-opioid receptors with pertussis toxin-sensitive G proteins: involvement of the third intracellular loop and the c-terminal tail in coupling. *Biochim Biophys Acta* **1359:**263–274.

Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H, and Kobilka BK (1997) Structural Instability of a constitutively active g protein-coupled receptor. Agonist-independent activation due to conformational flexibility. *J Biol Chem* **272:**2587–2590.

Halaban R, Svedine S, Cheng E, Smicun Y, Aron R, and Hebert DN (2000) Endoplasmic reticulum retention is a common defect associated with tyrosinase-negative albinism *Proc Natl Acad Sci U S A* **97:**5889–5894.

Heinflink M, Nussenzveig DR, Grimberg H, Lupu-Meiri M, Oron Y, and Gershengorn MC (1995) A constitutively active mutant thyrotropin-releasing hormone receptor is chronically down-regulated in pituitary cells: evidence using chlordiazepoxide as a negative antagonist. *Mol Endocrino* **9:**1455–1460.

Huang P, Li J, Chen C, Visiers I, Weinstein H, and Liu-Chen LY (2001) Functional role of a conserved motif in TM6 of the rat mu opioid receptor: constitutively active and inactive receptors result from substitutions of Thr6.34(279) with Lys and Asp. *Biochemistry* **40:**13501–13509.

Krause G, Hermosilla R, Oksche A, Rutz C, Rosenthal W, and Schulein R (2000) Molecular and conformational features of a transport-relevant domain in the C-terminal tail of the vasopressin V2 receptor. *Mol Pharmacol* **57:**232–242.

Law PY, Wong YH, and Loh HH (1999) Mutational analysis of the structure and function of opioid receptors. *Biopolymers* **51:**440–455.

Law PY, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL, and Loh HH (2000) Receptor density and recycling affect the rate of agonist-induced desensitization of μ-opioid receptor. *Mol Pharmacol* **58:**388–398.

Le A, Steiner JL, Ferrell GA, Shaker JC, and Sifers RN (1994) Association between calnexin and a secretion-incompetent variant of human α1-antitrypsin. *J Biol Chem* **269:**7514–7519.

Li J, Huang P, Chen C, de Riel JK, Weinstein H, and Liu-Chen LY (2001) Constitutive activation of the mu opioid receptor by mutation of D3.49(164), but not D3.32(147): D3.49(164) is critical for stabilization of the inactive form of the receptor and for its expression. *Biochemistry* **40:**12039–12050.

Loo TW and Clarke DM (1994) Prolonged association of temperature-sensitive mutants of human P-glycoprotein with calnexin during biogenesis. *J Biol Chem* **269:**28683–28689.

MacEwan DJ and Milligan G (1996) Inverse agonist-induced up-regulation of the human β2-adrenoceptor in transfected neuroblastoma X glioma hybrid cells. *Mol Pharmacol* **50:**1479–1486.

McLean AJ, Bevan N, Rees S, and Milligan G (1999) Visualizing differences in ligand regulation of wild-type and constitutively active mutant β2-adrenoceptor-green fluorescent protein fusion proteins. *Mol Pharmacol* **56:**1182–1191.

Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, 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.

Oksche A, Dehe M, Schulein R, Wiesner B, and Rosenthal W (1998) Folding and cell surface expression of the vasopressin V2 receptor: requirement of the intracellular C terminus. *FEBS Lett* **424:**57–62.

Pei G, Samama P, Lohse M, Wang M, Codina J, and Lefkowitz RJ (1994) A Constitutively active mutant β2-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc Natl Acad Sci U S A* **91:**2699–2702.

Petaja-Repo UE, Hogue M, Laperriere A, Walker P and Bouvier (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human ∂ opioid receptor. *J Biol Chem* **275:**13727–13736.

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 delta opioid receptor maturation. *EMBO (Eur Mol Biol Organ) J* **21:**1628–1637.

Pind S, Riordan JR, and Williams DB (1994) Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **269:**12784–12788.

Ray K, Fan GF, Goldsmith PK, and Spiegel AM (1997) The carboxyl terminus of the human calcium receptor. Requirements for cell-surface expression and signal transduction. *J Biol Chem* **272:**31355–31361.

Rozzell TG, Wang H, Liu X, and Segaloff DL (1995) Intracellular retention of mutant gonadotropin receptors results in loss of hormone binding activity of the follitropin receptor but not of the lutropin/choriogonadotropin receptor. *Mol Endocrino* **9:**1727–1736.

Rozell TG, Davis DP, Chai Y, and Segaloff DL (1998) Association of gonadotropin receptor precursors with the protein folding chaperone calnexin. *Endocrinology* **139:**1588–1593.
Samama P, Bond RA, Rockman HA, Milano CA, and Lefkowitz RJ (1997) Ligand-induced overexpression of a constitutively active β2-adrenergic receptor: Pharmacological creation of a phenotype in transgenic mice. *Proc Natl Acad Sci USA* **94:**137–141.
Schulein R, Hermosilla R, Oksche A, Dehe M, Wiesner B, Krause G, and Rosenthal W (1998) A dileucine sequence and an upstream glutamate residue in the intracellular carboxyl terminus of the vasopressin V2 receptor are essential for cell surface transport in COS M6 cells. *Mol Pharmacol* **54:**525–535.
Smirnov D, Usachev YM, Law PY, and Loh HH (2002) Role of the delta opioid receptor carboxyl tail in receptor translocation. Program No. 515.1. 2002 Abstract Viewer/Itinerary Planner. Society for Neuroscience, Washington DC. Online.
Stevens PA, Bevan N, Rees S, and Milligan G (2000) Resolution of inverse agonist-induced up-regulation from constitutive activity of mutants of the α1b-adrenoceptor. *Mol Pharmacol* **58:**438–448.
Unson CG, Cypess AM, Kim HN, Goldsmith PK, Carruthers CJ, Merrifield RB, and Sakmar TP (1995) Characterization of deletion and truncation mutants of the rat glucagon receptor. *J Biol Chem* **270:**27720–27727.
Wess J (1998) Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol Ther* **80:**231–264.
Williams AM and Enns CA (1993) A region of the C-terminal portion of the human transferrin receptor contains an asparagine-linked glycosylation site critical for receptor structure and function *J Biol Chem* **268:**12780–12786.
Wonerow P, Schoneberg T, Schultz G, Gudermann T, and Paschke R (1998) Deletions in the third intracellular loop of the thyrotropin receptor. A new mechanism for constitutive activation. *J Biol Chem* **273:**7900–7905.
Wu Y, Whitman I, Molmenti E, Moore K, Hippenmeyer P, and Perlmutter DH (1994) A lag in intracellular degradation of mutant α1-antitrypsin correlates with the liver disease phenotype in homozygous PiZZ α1-antitrypsin deficiency. *Proc Natl Acad Sci USA* **91:**9014–9018.
Yang Y, Janich S, Cohn JA, and Wilson JM (1993) The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. *Proc Natl Acad Sci USA* **90:**9480–9484.

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