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Opioid Receptor Pharmacological Chaperones Act by Binding and Stabilizing Newly Synthesized Receptors in the Endoplasmic Reticulum*

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Accumulating evidence has indicated that membrane-permeable G protein-coupled receptor ligands can enhance cell surface targeting of their cognate wild-type and mutant receptors. This pharmacological chaperoning was thought to result from ligand-mediated stabilization of immature receptors in the endoplasmic reticulum (ER). In the present study, we directly tested this hypothesis using wild-type and mutant forms of the human δ-opioid receptor as models. ER-localized receptors were isolated by expressing the receptors in HEK293 cells under tightly controlled tetracycline induction and blocking their ER export with brefeldin A. The ER-retained δ-opioid receptor precursors were able to bind [^{3}H]diprenorphine with high affinity, and treatment of cells with an opioid antagonist naltrexone led to a 2-fold increase in the number of binding sites. After removing the transport block, the antagonist-mediated increase in the number of receptors was detectable at the cell surface by flow cytometry and cell surface biotinylation assay. Importantly, opioid ligands, both antagonists and agonists, were found to stabilize the ER-retained receptor precursors in an *in vitro* heat inactivation assay and the treatment enhanced dissociation of receptor precursors from the molecular chaperone calnexin. Thus, we conclude that pharmacological chaperones facilitate plasma membrane targeting of δ-opioid receptors by binding and stabilizing receptor precursors, thereby promoting their release from the stringent ER quality control.

Endoplasmic reticulum (ER)[2] quality control, involving molecular chaperones and folding factors, scrutinizes newly synthesized proteins and allows only correctly folded and assembled ones to proceed through the secretory pathway (1). Proteins that do not fulfill the criteria of the quality control are targeted for retrotranslocation and degradation in the cytosol by the ER-associated degradation pathway (2). Since ER quality control relies on conformational rather than functional criteria, even minor changes in the primary structure of a protein can lead to intracellular retention, thus preventing the affected protein from reaching its correct location in the cell. Thus, even salvageable proteins that might be functionally active can be incorrectly directed for degradation. Such an etiology is the underlying cause for a growing number of congenital and acquired conformational diseases, including those that affect G protein-coupled receptors (GPCRs), cell surface seven-transmembrane domain proteins that mediate extracellular messages into intracellular responses. Examples include nephrogenic diabetes insipidus, retinitis pigmentosa, and familial obesity that are caused by mutant forms of the V2 vasopressin receptor, rhodopsin, and melanocortin receptor 4, respectively (3).

Since many of the disease-causing proteins are not inherently nonfunctional, attempts to correct their folding and trafficking have attracted considerable attention. Several different ways to alleviate their incorrect cellular localization have been proposed, including genetic means (4–7) and approaches involving nonspecific stabilizing reagents, chemical chaperones (8–11). Recently, a more elaborate strategy has been formulated, based on pharmacological chaperones, ligands, or substrates that can be used to specifically target misfolded/mistargeted proteins. The first GPCR that was targeted with membrane-permeable ligands was the V_2 vasopressin receptor (12). So far, a total of 12 of 20 tested receptor mutants that cause X-linked nephrogenic diabetes insipidus have been rescued by small lipophilic receptor-specific antagonists to the cell surface, where most of them are functional and able to induce cAMP production in response to vasopressin stimulation (12–15). Subsequently, a similar strategy has been applied for mutant forms of the gonadotropin-releasing hormone receptor, rhodopsin, and melanin-concentrating hormone receptor 1 (16–21). Furthermore, since receptor-specific ligands have also been found to be effective in enhancing cell surface targeting of a few wild-type GPCRs (16, 21–26), pharmacological chaperones may have significance not only in rescuing mutant GPCRs but also in regulating wild-type receptor levels.

It has been hypothesized that pharmacological chaperones enhance cell surface targeting of GPCRs by binding and

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[2] The abbreviations used are: ER, endoplasmic reticulum; DPDPE, cyclic[D-Pen^2,D-Pen^5]enkephalin; GPCR, G protein-coupled receptor; BFA, brefeldin A; BSA, bovine serum albumin; CHX, cycloheximide; DDM, *n*-dodecyl-β-D-maltoside; DMEM, Dulbecco's modified Eagle's medium; Endo H, endo-β-*N*-acetylglucosaminidase H; hδOR, human δ-opioid receptor; HEK, human embryonic kidney; HRP, horseradish peroxidase; NEM, *N*-ethylmaleimide; NHS, *N*-hydroxysuccinimide; NOX, naloxone; NTB, naltriben; NTX, naltrexone; PHE, phentolamine; PBS, phosphate-buffered saline; SNC-80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*,-diethylbenzamidine.

stabilizing immature receptors in the ER (8, 27). However, direct evidence supporting this notion has so far been lacking, and further experiments are needed to directly establish their mode of action. In the present study, we have used the human δ-opioid receptor (hδOR) as a model to directly test this hypothesis. In our previous studies, we have demonstrated that only about 40% of newly synthesized hδORs are converted to mature form (28), and membrane-permeable opioid receptor antagonists and agonists are able to facilitate their maturation (22). In the present study, ER-retained hδOR precursors were found to bind [^{3}H]diprenorphine with high affinity, showing that immature GPCRs, which contain their ligand-binding domain within the seven-transmembrane domain, gain binding ability early in their biosynthetic pathway before being exported from the ER. Moreover, both opioid antagonists and agonists were found to stabilize membrane-bound receptor precursors in an *in vitro* heat inactivation assay. Finally, since the ligand treatment was found to enhance dissociation of hδOR precursors from calnexin, we were able to conclude that the opioid receptor pharmacological chaperones enhance cell surface targeting of newly synthesized receptors by direct ligand-mediated stabilization and a consequent release from ER quality control.

EXPERIMENTAL PROCEDURES

Materials—[15,16-^{3}H]Diprenorphine (50.0–54.9 Ci/mmol) and Easytag Express ^{35}S-protein labeling mix (1175 Ci/mmol) were obtained from PerkinElmer Life Sciences, and endo-β-*N*-acetylglucosaminidase H (Endo H) was from Roche Applied Science. Brefeldin A (BFA), *n*-dodecyl-β-D-maltoside (DDM), and digitonin were from Calbiochem or Alexis. Opioid ligands naloxone (NOX), naltrexone (NTX), naltriben (NTB), (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*,-diethylbenzamidin) (SNC-80), cyclic [D-Pen^2,D-Pen^5]enkephalin (DPDPE), and the α-adrenergic ligand phentolamine (PHE) were from Tocris or Sigma. Cycloheximide (CHX), poly-L-lysine, anti-FLAG M2 monoclonal antibody, anti-FLAG M2 antibody affinity resin, and FLAG peptide were products of Sigma. EZ-linked sulfo-*N*-hydroxysuccinimide (NHS)-biotin and horseradish peroxidase (HRP)-conjugated streptavidin were from Pierce. Flp-In-293 cells and Flp-In T-Rex core kit were purchased from Invitrogen and cell culture reagents were from BioWhittaker, Invitrogen, or Sigma. Anti-calreticulin (SPA-600) and anti-calnexin (SPA-860) polyclonal antibodies were obtained from Stressgen, and anti-c-Myc (9E10) monoclonal antibody was produced by the core facility at the Department of Biochemistry (University of Montreal, Canada) as ascites fluid. Concanavalin A Alexa Fluor 594-conjugate, Alexa Fluor 488 goat anti-mouse IgG, and Alexa Fluor goat 568 anti-rabbit IgG were from Molecular Probes, Inc. (Eugene, OR). Phycoerythrin-conjugated rat anti-mouse IgG_1 and 7-amino-actinomycin D were from BD Biosciences. All other reagents were of analytical grade and purchased from various commercial suppliers.

DNA Constructs—DNA construct encoding the hδOR with a cleavable influenza hemagglutinin signal peptide (KTIIALSYIFCLVFA), N-terminal Myc tag (EQKLISEEDL), and C-terminal FLAG tag (DYKDDDDK) was created. Briefly, cDNA for the hδOR (GenBank™ accession number U10504) (28) (a generous gift from P. Walker, AstraZeneca R&D Montreal (Montreal, Canada)) was amplified by polymerase chain reaction using oligonucleotides 5′-CGCGCTAGCATGGAACCGGCCCCCTCC-3′ and 5′-GCCCCTAGGGGCGGCAGCGCCACCGCC-3′, digested with NheI/BlnI, ligated into the modified pcDNA5/FRT/TO vector (Invitrogen) (called pFT-SMMF), and transformed into *Escherichia coli* JM109. The pFT-SMMF vector was created as described previously (29). The hδOR(D95A) mutant was created by introducing a single point mutation with QuikChange mutagenesis kit (Stratagene), according to the manufacturer's instructions.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (w/w) fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (complete DMEM). A stable human embryonic kidney $HEK293_i$ cell line expressing Tet repressor was established as described elsewhere (30). Stable cell lines with inducible hδOR expression were established by co-transfecting receptor constructs and pOG44 plasmid (Invitrogen) into $HEK293_i$ cells with Lipofectamin 2000 transfection reagent (Invitrogen) under BlasticidinS (4 μg/ml; Invivogen) and hygromycin (400 μg/ml; Invivogen) selection. They were isolated, expanded, and tested for zeozin sensitivity, β-galactosidase activity, and tetracycline-inducible expression of the hδOR by radioligand binding and Western blot analyses. The selected clones that were used for experiments showed very low basal but highly inducible hδOR expression (see Fig. 1), were sensitive to zeozin and lacked β-galactosidase activity. For experiments, 2 or 5×10^6 cells were plated onto 25- or 75-cm^2 culture flasks, respectively, and cultured for 3 days in complete DMEM. Receptor expression was induced by adding tetracycline (0.02–1 μg/ml; Invitrogen) into the culture medium for different periods of time as indicated in the figure legends. BFA (5 μg/ml) and opioid and α-adrenergic ligands (10 μM) were added 60 min and CHX (20 μg/ml) 7 h after tetracycline, and cells were cultured further as indicated. Before CHX addition, cells were washed twice with complete DMEM. NG-108-15 cells were cultured in DMEM supplemented with 10% (w/w) fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and HAT supplement (Invitrogen; 100 μM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) and cultured in the absence or presence of NTX for 24 h before harvesting. Cells were harvested in phosphate-buffered saline (PBS), quick frozen in liquid nitrogen, and stored at −70 °C.

Metabolic Labeling with [^{35}S]Methionine/Cysteine—For pulse-chase labeling, cells were first treated with 0.5 μg/ml tetracycline (60 min) and then incubated in methionine and cysteine-free DMEM (60 min) before labeling in fresh medium containing 100 μCi/ml [^{35}S]methionine/cysteine (40 min). After washing twice with the chase medium (DMEM supplemented with 5 mM methionine), cells were chased for different time periods as specified in Fig. 9. BFA (5 μg/ml) was included in the culture medium during the depletion, labeling, and chase, and NTB (10 μM) was included during the chase. Cells were then incubated on ice

in PBS containing 20 mM *N*-ethylmaleimide (NEM) for 10 min and harvested as described above.

Preparation and Solubilization of Membranes and Whole Cell Extracts—Cells were homogenized for radioligand binding assays in buffer A (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine) and for immunoprecipitation in buffer B (25 mM Tris-HCl, pH 7.4, 20 mM NEM, 2 mM EDTA, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-phenanthroline, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine) with a Polytron homogenizer (Ultra-Turrax T-25; Ika) using three 5-s bursts at 19,000 rpm. Homogenates were centrifuged at 1,000 × *g* for 5 min, and pellets were rehomogenized and centrifuged. The combined supernatants were centrifuged at 45,000 × *g* for 20 min. The final pellets containing crude membrane fractions were washed twice and used immediately or stored at −70 °C. Protein concentration was determined using the Bio-Rad DC protein assay kit with bovine serum albumin (BSA) as a standard.

Membranes that were subjected for immunoprecipitation were solubilized in buffer C (0.5% (w/v) DDM in buffer B containing 140 mM NaCl and without NEM) by mixing the suspension on a magnetic stirrer for 60 min at 4 °C. Solubilized receptors were collected by centrifugation at 100,000 × *g* for 60 min. Total cellular lysates were prepared by mixing thawed cells for 30 min at 4 °C in buffer C (Fig. 9) or buffer D (buffer A containing 0.5% (w/v) digitonin and 10 mM $CaCl_2$ without NEM and EDTA) (Fig. 8). Insoluble material was removed by centrifugation at 16,000 × *g* for 30 min.

Radioligand Binding Assays—Saturation and competition binding assays were carried out using 5–20 μg (HEK293 cells) or 30 μg (NG-108-15 cells) of membrane protein in a final volume of 300 μl of buffer A, containing 0.1% (w/v) BSA. One-point binding assays were performed using 5–60 μg of membrane protein. For saturation binding experiments, triplicate samples were incubated with an increasing concentration of [^{3}H]diprenorphine, the final concentration ranging from 0.04 to 14 nM. For one-point binding assays, each triplicate contained 7–12 nM [^{3}H]diprenorphine, and for competition experiments, each contained 2 nM [^{3}H]diprenorphine and either NTX, SNC-80, or DPDPE at final concentrations ranging from 0.1 pM to 100 μM. Nonspecific binding was determined using 10 μM unlabeled NTX. After incubation at 22 °C for 60 min, membranes were harvested with cold 25 mM Tris-HCl, pH 7.4, by rapid filtration over glass fiber filters (Filtermat B; PerkinElmer) presoaked in 0.1% (v/v) polyethyleneimine using a Brandel MWR-96T harvester. MeltiLex B/HS scintillator (PerkinElmer Life Sciences) was melted onto the dried filters using MeltiLex Heat-Sealer (PerkinElmer Life Sciences). Finally, radioactivity was measured with a Wallac MicroBeta TriLux scintillation counter (PerkinElmer Life Sciences).

Heat Inactivation Assay—Cellular membranes were prepared in buffer E (buffer B without NEM) as described above. Suspensions (400/800 μg of protein) were incubated in an Eppendorf thermomixer (650 rpm) with or without opioid or α-adrenergic ligands (1 μM) at 37 °C for different periods of time, as indicated in the legend to Fig. 7. After terminating the incubations by placing the samples on ice, membranes were collected by centrifugation at 20,000 × *g* for 20 min. The residual ligands were removed by washing twice with buffer E, and membranes were resuspended in 1 ml of buffer A. The one-point binding assay was performed using 100 μl of suspension and a saturating concentration of [^{3}H]diprenorphine as described above.

Immunoprecipitation—Solubilized membranes or total cellular lysates were supplemented with 0.1% (w/v) BSA (160 μg of protein) and subjected to one-step or two-step immunoprecipitation using anti-FLAG M2 antibody-agarose as described (28, 31). For SDS-PAGE, 100–150 μl of eluates was concentrated down to 25 μl by membrane filtration over Microcon-30 concentrators (Millipore), and 25 μl of SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromphenol blue) was added. Alternatively, aliquots of lysates were subjected directly to SDS-PAGE after the addition of sample buffer.

Deglycosylation of Immunoprecipitated Receptors—Immunoprecipitated receptors were eluted from anti-FLAG M2 antibody affinity resin with 1% (w/v) SDS, 50 mM sodium phosphate, pH 5.5, by incubating samples for 15 min at 22 °C and for 5 min at 95 °C. Eluates were diluted 7.5-fold with buffer F (0.5% (w/v) DDM, 50 mM sodium phosphate, pH 5.5, 50 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-phenantroline, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine), and Endo H was added to a final concentration of 50 milliunits/ml. Samples were incubated at 30 °C for 16 h, and the reaction was terminated by adding SDS-sample buffer (60 μl).

Cell Surface Biotinylation—Cell surface biotinylation was performed as described elsewhere (28), and receptors were purified by immunoprecipitation as depicted above.

SDS-PAGE, Western Blotting, and Fluorography—Samples were reduced by heating at 95 °C for 2 min in the presence of 50 mM dithiothreitol, and SDS-PAGE was run on a Bio-Rad Mini-PROTEAN 3 cell apparatus (10% SDS-polyacrylamide gels) using reagents from Bio-Rad or from Amresco (Fig. 9). Bio-Rad's broad range molecular weight standards were used as markers and stained with Ponceau S (Sigma) after blotting. Proteins were electroblotted onto Immobilon P membrane (Millipore) using the Bio-Rad Mini Trans-Blot cell apparatus at 50 mA for 16 h. Blots were probed with anti-FLAG M2 antibody (0.5 μg/ml) or anti-calnexin antibody (1:10,000), followed by HRP-conjugated anti-mouse (1:15,000; Caltaq) or anti-rabbit (1:15,000; Jackson Immunochemicals) antibodies and enhanced ECL Western blotting detection reagents from GE Healthcare. Gels containing radiolabeled samples were treated for fluorography as described previously (29). Films were scanned with an Agfa Duoscan HiD laser scanner, and data were quantified and analyzed as described (29).

Immunofluorescence Microscopy—Cells (0.5 × 10^6) were plated onto poly-L-lysine-coated 12-mm coverslips, cultured for 3 days, and treated as described in the legend to Fig. 3. Cell surface receptors were labeled under nonpermeabilizing conditions by adding anti-c-Myc antibody (1:1,000) into the medium for 30 min, and after washing twice with PBS, cell

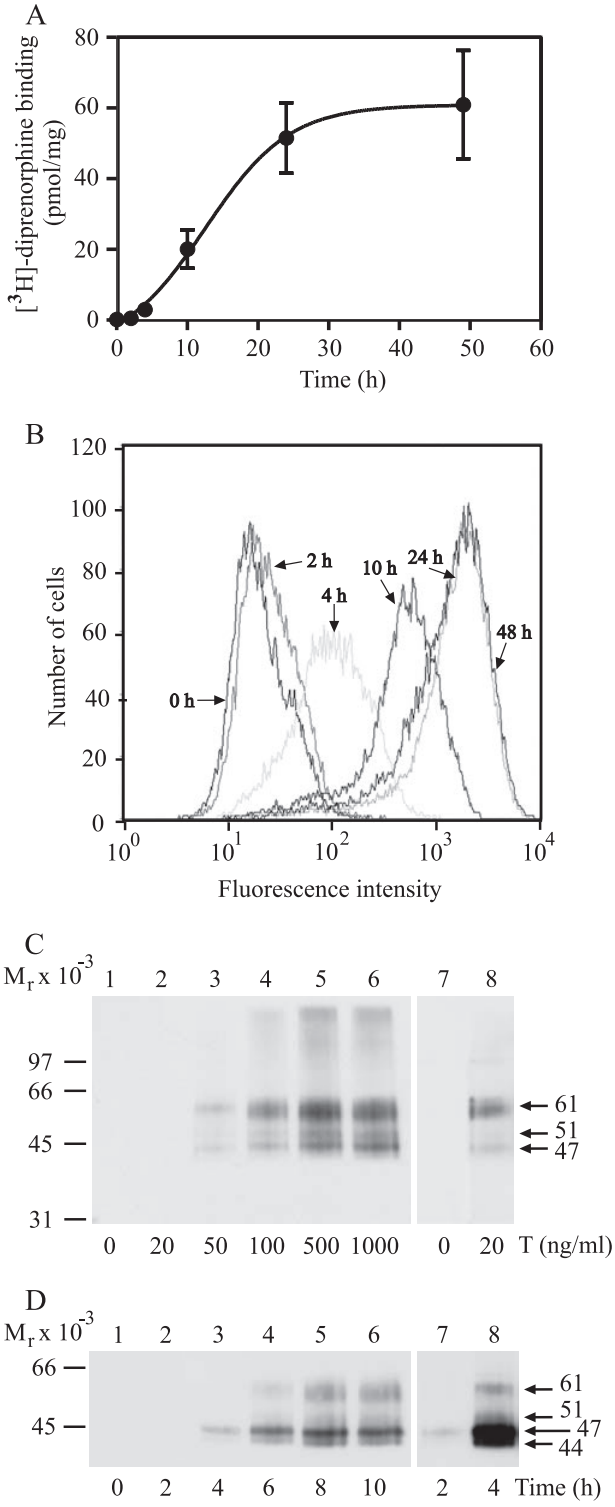

FIGURE 1. **Tetracycline-inducible expression of hδORs in HEK293$_i$-Myc-hδOR-FLAG cells.** *A*, analysis of [^{3}H]diprenorphine binding sites. Cells were incubated at 37 °C with tetracycline (0.5 μg/ml) for 0, 2, 4, 10, 24, or 48 h. Total cellular membranes were prepared and subjected to a one-point binding assay using [^{3}H]diprenorphine. The values given are the means ± S.E. of three independent experiments performed in triplicate. *B*, analysis of cell surface receptors by flow cytometry. Cells were treated as described above, labeled with anti-c-Myc antibody, and analyzed by flow cytometry. *C* and *D*, analysis of receptors by Western blotting. Cells were incubated at 37 °C with an increasing concentration of tetracycline for 24 h (*C*) or 0.5 μg/ml tetracycline for 0, 2, 4, 6, 8, or 10 h (*D*). Cellular membranes were solubilized in DDM, and immunoprecipitated receptors were subjected to SDS-PAGE and Western blotting using anti-FLAG M2 antibody. *Lanes 7* and *8* in *C* and *D* represent a longer exposure of *lanes 1* and *2* and *lanes 2* and *3*, respectively. Molecular weight markers are indicated on the *left*, and detected receptor forms are shown on the *right*. The M_r 44,000 and 47,000 bands represent receptor precursors, and the M_r 51,000 and M_r 61,000 ones represent mature receptors. The M_r 47,000 and 61,000 receptor forms contain two *N*-linked glycans, whereas the two other contain only one *N*-glycan.[3] *T*, tetracycline.

surface proteins were labeled with concanavalin A Alexa Fluor 594-conjugate in PBS (50 μg/ml) for 5 min. Cells were then washed and fixed with 2% (w/w) paraformaldehyde, PBS for 20 min and permeabilized with 0.1% (v/v) Triton X-100, 0.5% (w/v) BSA in PBS for 45 min. Finally, cells were washed with PBS and treated with Alexa Fluor 488 goat anti-mouse (1:200) antibody in the permeabilization buffer for 30 min. To label intracellular proteins, cells were fixed and permeabilized before the first antibody incubation (anti-FLAG M2 antibody (10 μg/ml) or anti-calreticulin antibody (1:200) in permeabilization buffer, 30 min), washed, and incubated with Alexa Fluor 488 goat anti-mouse (1:200) or Alexa Fluor goat 568 anti-rabbit (1:250) antibodies for 30 min in the permeabilization buffer. All incubations were performed at 22 °C. After final washes with PBS, coverslips were mounted on glass slides with Immumount (ThermoShandon) for confocal microscopy. Immunofluorescence staining was viewed with Zeiss LSM510 confocal microscope using Zeiss Plan-Apo 100 × 1.4 numerical aperture oil immersion objective under 488- and 647-nm wavelength excitation.

Flow Cytometry—Detection of plasma membrane receptors by flow cytometry was performed as described earlier (32).

Data Analysis—Data were analyzed using GraphPad Prism 4.01 software. Saturation and competition curves were fitted to one- or two- binding site models using least-squares nonlinear regression analyses. The heat inactivation data were fitted to the one-phase exponential decay model. For statistical *t* tests, the limit of significance was set at $p < 0.05$, and data are presented as mean ± S.E.

RESULTS

Inducible hδOR Expression in HEK293$_i$-Myc-hδOR-FLAG Cells—To test the hypothesis that opioid receptor pharmacological chaperones are able to bind and stabilize newly synthesized receptors, immature ER-localized receptors needed to be separated from mature cell surface ones. For this purpose, we prepared a HEK293 cell line expressing the hδOR under tetracycline induction that allowed precise control of receptor expression. A *C*-terminal FLAG epitope and an N-terminal Myc epitope were added to enable receptor purification and detection of receptor expression at the cell surface, respectively. In addition, the receptor construct was modified to contain a cleavable hemagglutinin signal sequence (33) at the N-terminal end of the Myc epitope to avoid possible ER translocation difficulties of nascent receptor molecules due to the added epitope.

To induce receptor expression, HEK293$_i$-Myc-hδOR-FLAG cells were treated with 0.5 μg/ml tetracycline for increasing periods of time, and the number of binding sites was assessed by subjecting crude membrane particles for a [^{3}H]diprenorphine binding assay. As seen in Fig. 1*A*, the number of binding sites increased in time, and the half-time for maximal binding

(60.9 ± 7.6 pmol/mg of membrane protein (mean ± S.E., n = 3)) was obtained at 13.1 ± 2.7 h. The appearance of receptors at the cell surface was then investigated by flow cytometry after labeling live cells with anti-c-Myc antibody and phycoerythrin-conjugated secondary antibody. In accordance with the binding data, the fluorescence intensity increased during induction time and reached its maximum at 24 h after initiating the induction (Fig. 1*B*).

To analyze the different receptor forms that were expressed in the HEK293$_i$-Myc-hδOR-FLAG cells, cells were treated with increasing concentrations of tetracycline for 24 h, and receptors were solubilized from the crude membrane fraction, immunoprecipitated, and subjected to SDS-PAGE and Western blotting. As seen in Fig. 1*C*, anti-FLAG M2 antibody recognized two major bands, a broad heterogeneous band of M_r 61,000 and a sharper one of M_r 47,000 from the induced cells (*lanes 2–6* and *8*), whereas no bands were apparent in cells not treated with tetracycline (*lanes 1* and *7*). The two specific bands represent mature and precursor forms of the receptor (28), based on their differential sensitivity to Endo H (see Fig. 2*B*, *lanes 1* and *2*). Occasionally, two other bands of M_r 51,000 and 44,000 were detected (see also Fig. 1*D*, *lanes 3–6* and *8*). They represent mature and immature receptor forms, respectively, carrying only one of the two *N*-linked glycans that are normally attached to the receptor.[3] The expression level of the four receptor species was dependent on the tetracycline concentration used, but, importantly, the relative amount of immature to mature receptor species remained the same irrespective of the expression level. This supports the notion that inefficient conversion of receptor precursors to mature receptors is an intrinsic property of the hδOR (28).

To find out if the relative amount of receptor precursors could be increased, the time course of appearance of different receptor forms was assessed after treating the cells with tetracycline for increasing periods of time. The immature receptor species were first detected by Western blotting at 2 h (Fig. 1*D*, *lanes 2* and *7*), and 4 h later they had reached the maximum level compared with the mature receptor forms (*lane 4*). Thereafter, their relative amount began to decrease, and mature receptors started to accumulate (Fig. 1*D*, *lanes 4–6*). After 24 h, no further changes were observed in the relative proportion of the two receptor species (Fig. 1*C*, *lane 5*). Thus, although most of the receptors existed as immature receptors following a short term induction of receptor expression, they could not be completely separated from the mature ones by shortening the induction time.

BFA Retains hδOR Precursors in the ER—To completely separate the immature receptors for further analyses, attempts were made to separate ER and plasma membrane fractions by sucrose density gradient centrifugation from cells induced with 0.5 μg/ml tetracycline for 4 h. This approach was, however, unsuccessful because of significant plasma membrane contamination in the ER fraction. The receptor precursors accumulated in the same fraction as the ER marker calnexin, but this preparation also contained detectable amounts of mature receptors (data not shown). Thus, we assessed if BFA could be used to increase the relative amount of immature receptors. This drug inhibits forward membrane vesicle traffic, leading to accumulation of newly synthesized proteins in the ER (34, 35). Receptor expression in the HEK293$_i$-Myc-hδOR-FLAG cells was induced for 7 h in the presence of BFA (5 μg/ml), and cell surface proteins were labeled with membrane-impermeable sulfo-NHS-biotin, allowing detection of cell surface receptors by HRP-conjugated streptavidin. As seen in Fig. 2*A*, only one receptor species of M_r 48,000 was immunoprecipitated from BFA-treated cells in contrast to four receptor species purified from nontreated cells (compare *lanes 1* and *2*). Streptavidin detected the mature M_r 61,000 and 51,000 receptor forms in the nontreated cells (Fig. 2*A*, *lane 3*), as expected, but no receptor species were apparent in the BFA-treated ones (Fig. 2*A*, *lane 4*). This suggests that the M_r 48,000 receptor form is located intracellularly in a similar manner as the M_r 44,000 and 47,000 receptor precursors.

The receptor form expressed in BFA-treated cells had a slightly slower mobility than receptor precursors in nontreated cells. To test the possibility that this dissimilarity in electrophoretic mobility might result from differences in *N*-glycan processing, immunoprecipitated receptors were subjected to Endo H digestion before SDS-PAGE and Western blotting. As expected, the M_r 47,000 and 44,000 receptor precursors were completely sensitive to Endo H, and their apparent molecular weight decreased to about M_r 42,000 (Fig. 2*B*, *lane 2*). In contrast, the M_r 48,000 receptor species was only partially sensitive to Endo H treatment (Fig. 2*B*, *lane 4*). This suggests that *N*-linked glycans of the hδOR precursors are trimmed to some extent in the BFA-treated cells, most likely by Golgi mannosidases and glycosyltransferases that are known to be retrotranslocated to the ER upon BFA treatment (34, 35).

The ability of BFA to block the export of hδORs from the ER was tested further by performing quantitative analysis of cell surface receptor expression by flow cytometry. A specific fluorescence signal was detected in cells not treated with BFA (Fig. 2*C*, *top*), but only a weak signal barely above the background was detected in BFA-treated cells (Fig. 2*C*, *bottom*). Immunofluorescence microscopy was then used to determine the cellular location of hδORs in more detail. For this purpose, HEK293$_i$-Myc-hδOR-FLAG cells were cultured on coverslips, receptor expression was induced with tetracycline, and BFA was added into the culture medium as described above. In non-permeabilized control cells that were not treated with BFA, clear cell surface labeling of hδORs was detected with anti-c-Myc antibody (Fig. 3*A*), and the signal co-localized with the cell surface marker concanavalin A (Fig. 3*B*), as seen in the merged image (Fig. 3*C*). In contrast, no cell surface receptors were detected in BFA-treated cells (Fig. 3, *D–F*), in agreement with the data obtained by cell surface biotinylation and flow cytometry. Intracellular localization of the hδORs was then studied by labeling receptors with anti-FLAG M2 antibody in permeabilized cells (Fig. 3, *G–L*). In control cells, receptors were found both at the cell surface (Fig. 3*G*) and intracellularly in a disperse perinuclear compartment co-localizing with the ER marker calreticulin (Fig. 3*H*), as shown in the merged image (Fig. 3*I*). As expected, no cell surface receptors were detected in BFA-

[3] P. M. H. Markkanen and U. E. Petäjä-Repo, manuscript in preparation.

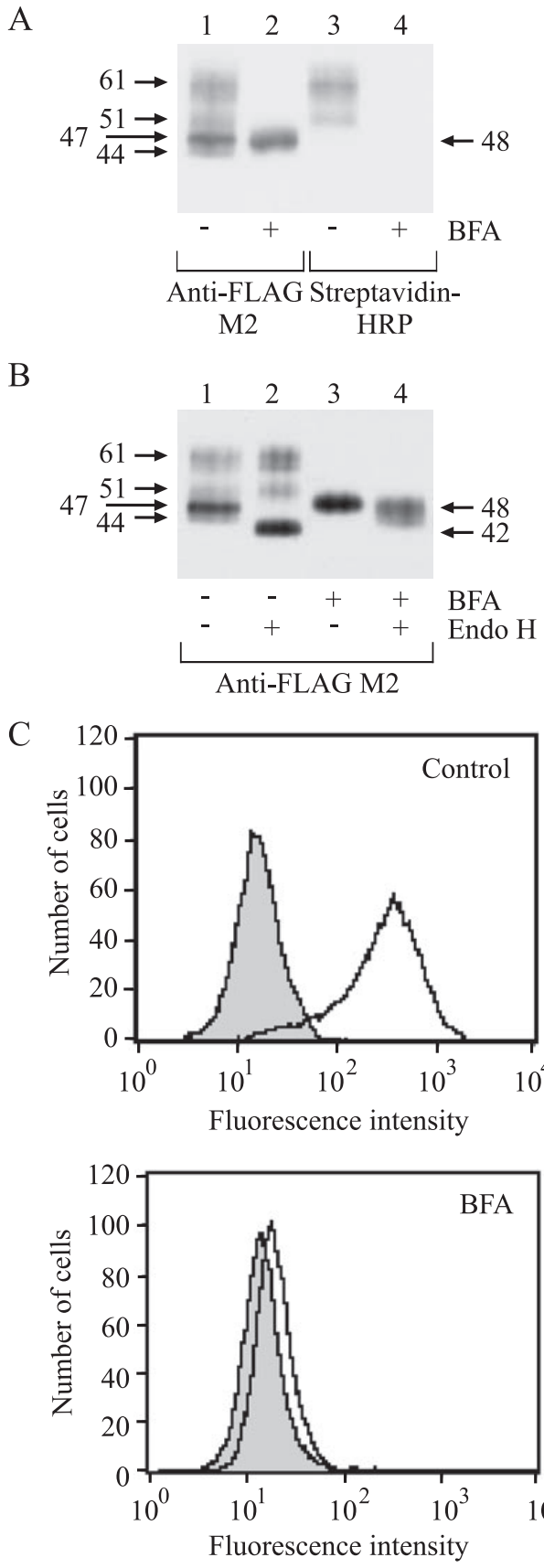

FIGURE 2. **HδORs in BFA-treated cells do not reach the cell surface and contain unprocessed *N*-linked glycans.** Expression of hδORs in HEK293$_i$-Myc-hδOR-FLAG cells was induced with tetracycline (0.5 μg/ml). BFA (5 μg/ml) or ethanol vehicle was added after 60 min, and cells were incubated at 37 °C for 6 h. *A*, analysis of plasma membrane receptors by cell surface biotinylation. Cell surface proteins were labeled with sulfo-NHS-biotin (0.5 mg/ml) on ice for 30 min. Total cellular membranes were isolated and extracted in DDM, and receptors were purified by immunoprecipitation. Aliquots of the eluates were analyzed by Western blotting using anti-FLAG M2 antibody (*lanes 1* and *2*) or HRP-conjugated streptavidin (*lanes 3* and *4*). *B*, deglycosylation of purified receptors. Receptors were isolated by immunoprecipitation and incubated with 50 milliunits/ml Endo H for 16 h (*lanes 2* and *4*). Samples were analyzed by Western blotting using anti-FLAG M2 antibody. The untreated samples (*lanes 1* and *3*) contained buffer only. *C*, analysis of plasma membrane receptors by flow cytometry. Cell surface receptors in control (*top*) and BFA-treated (*bottom*) cells were labeled with anti-c-Myc antibody and analyzed by flow cytometry. The *shaded curves* represent the background signal obtained in the absence of the primary antibody.

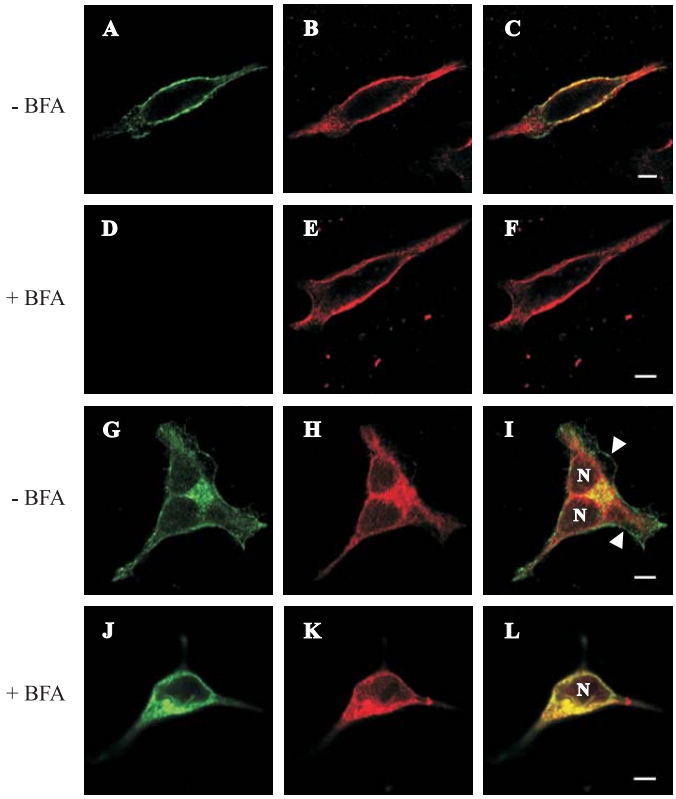

FIGURE 3. **HδORs in BFA-treated cells are retained in the ER.** HEK293$_i$-Myc-hδOR-FLAG cells were cultured on coverslips, and receptor expression was induced with tetracycline (0.5 μg/ml) in the absence (*A–C* and *G–I*) or presence (*D–F* and *J–L*) of BFA as described in the legend for Fig. 2. *A–F*, cell surface proteins were labeled with Alexa Fluor 594-conjugated concanavalin A under nonpermeabilizing conditions, and plasma membrane hδORs were labeled in parallel with anti-c-Myc antibody. The secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG was added after fixation and permeabilization. *G–L*, cells were fixed and permeabilized, and hδORs and calreticulin were labeled with anti-FLAG M2 and anti-calreticulin antibodies, respectively. Secondary antibodies were Alexa Fluor 488- and Alexa Fluor 568-conjugated goat anti-mouse and anti-rabbit IgG, respectively. The *arrowheads* in *I* indicate cell surface receptors that were absent in BFA-treated cells (*L*). *N*, nucleus; *bar*, 5 μm.

treated cells, and the intracellular receptors co-localized with calreticulin (Fig. 3, *J–L*).

ER-retained hδORs Bind Ligand with High Affinity—The above data indicate that newly synthesized hδORs in BFA-treated HEK293$_i$-Myc-hδOR-FLAG cells were completely retained in the ER, providing a convenient model to examine the mechanism of action of opioid receptor pharmacological chaperones. First, we set out to determine whether the ER-retained hδORs are capable of ligand binding by subjecting cel-

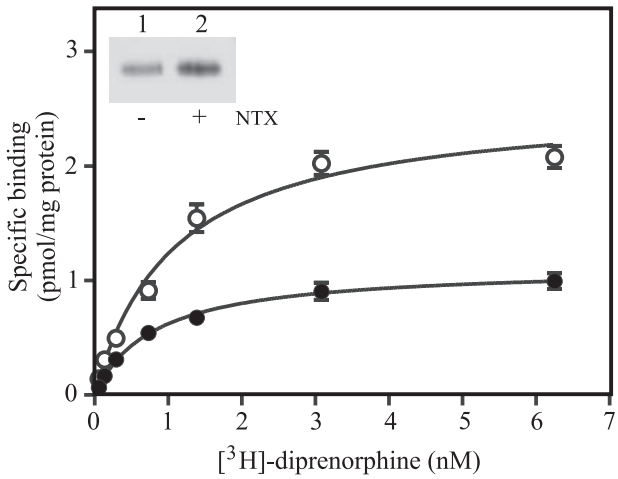

FIGURE 4. **ER-retained hδORs bind ligand with high affinity, and their number is increased by NTX treatment.** Expression of hδORs in HEK293$_i$-Myc-hδOR-FLAG cells was induced with tetracycline (0.5 μg/ml). BFA (5 μg/ml) was added after 60 min, and cells were incubated in the absence (●) or presence (○) of NTX (10 μM) at 37 °C for 6 h. Cellular membranes were prepared, and samples were incubated with an increasing concentration of [^{3}H]diprenorphine in the absence or presence of 10 μM NTX, the difference being defined as specific binding. Data points represent the mean ± S.E. of triplicate determinations that are representative of four independent experiments. *Inset*, analysis of purified receptors from control (*lane 1*) and NTX-treated (*lane 2*) cells by Western blotting. Receptors were isolated by immunoprecipitation and analyzed by Western blotting using anti-FLAG M2 antibody.

lular membranes from BFA-treated cells to saturation binding assays. As seen in Fig. 4, the receptor precursors were able to bind opioid antagonist [^{3}H]diprenorphine with high affinity with an apparent K_d of 0.9 ± 0.1 nM (n = 4). To find out if there were differences in the ligand binding ability of immature and mature hδORs, saturation binding assays were performed also using membranes that contained mature cell surface receptors. These samples were prepared from cells that were not treated with BFA but were incubated after the 7-h tetracycline induction in the presence of 20 μg/ml CHX for 12 h. This treatment was necessary to deplete the ER from receptor precursors and to allow accumulation of mature receptors at the cell surface, as was confirmed by Western blot analysis of immunoprecipitated receptors. Interestingly, the affinity of [^{3}H]diprenorphine for the mature hδORs was significantly higher than that obtained for the ER-retained receptors (0.5 ± 0.08 nM, $p < 0.005$). The two membrane preparations were then subjected to displacement binding assays to find out if the ER- and plasma membrane-localized receptors differ in their ability to bind agonists. Similar to what was observed for the antagonist, the affinity for the two tested agonists SNC-80 and DPDPE was slightly lower for the ER-retained receptors, and only one high affinity binding site was detected for the two membrane preparations (data not shown).

Opioid Ligands Increase the Number of hδOR Precursors in the ER and Enhance Their Transport to the Cell Surface—To test the ability of opioid antagonists to increase the number of ligand binding competent receptors in the ER, membranes from BFA- and NTX-treated HEK293$_i$-Myc-hδOR-FLAG cells were subjected to saturation binding assays. The binding capacity of receptors in the BFA-treated cells increased 2.2-fold when NTX was added into the culture medium (B_{max} = 3.38 ± 0.70 and 1.55 ± 0.30 pmol/mg of membrane protein for cells treated

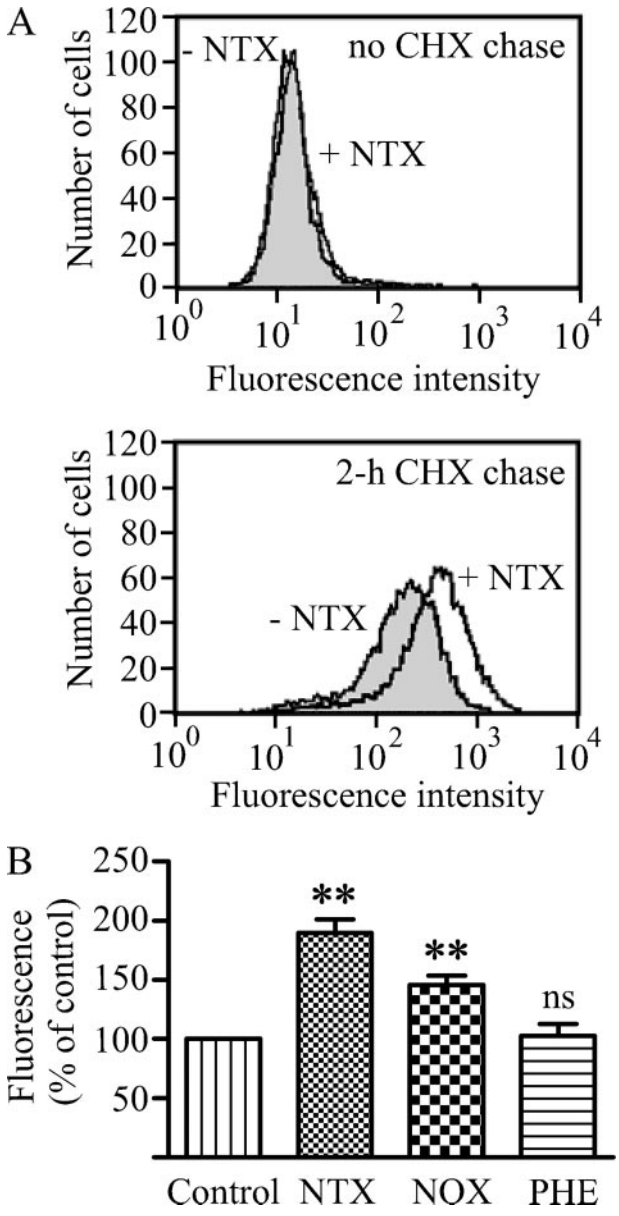

FIGURE 5. **Opioid ligands enhance transport of ER-retained hδORs to the plasma membrane.** Analysis of plasma membrane receptors by flow cytometry. *A*, expression of hδORs was induced with tetracycline (0.5 μg/ml) in stably transfected HEK293$_i$-Myc-hδOR-FLAG cells. BFA (5 μg/ml) was added after 60 min, and cells were incubated at 37 °C for 6 h in the presence or absence of NTX (10 μM) as indicated. Cells were then harvested (*A*, *top*) or washed and incubated for 2 h in the presence of CHX (20 μg/ml) (*A*, *bottom*). Cell surface receptors were labeled with anti-c-Myc antibody and analyzed by flow cytometry. A 1.9 ± 0.2-fold (mean ± S.E., n = 5) increase in the fluorescence intensity was detected in the NTX-treated cells. *B*, HEK293$_i$-Myc-hδOR-FLAG cells were treated as described above using NTX, NOX, or PHE, and cell surface receptors were labeled with anti-c-Myc antibody and analyzed by flow cytometry. The values (mean ± S.E., n = 4–5) indicate the relative mean cell fluorescence compared with values in control cells that were set to 100%. **, $p < 0.001$; *ns*, not significant.

or not with NTX, respectively, $p < 0.005$; n = 4) (Fig. 4). A similar 1.5 ± 0.1-fold increase in the number of receptors was also detected following immunoprecipitation and analysis by Western blotting (Fig. 4, *inset*). In contrast, no receptors were detected at the cell surface by flow cytometry (Fig. 5*A*, *top*), indicating that the NTX-mediated increase in receptor number occurred intracellularly.

Since the BFA-mediated block in protein transport is known to be reversible (34, 35), we tested whether ER-retained receptors could reach the cell surface after removing the drug from the culture medium. In order to prevent new receptor synthesis, CHX (20 μg/ml) was added, and cells were incubated further for 2 h. The appearance of receptors at the cell surface was then assessed by flow cytometry. As seen in Fig. 5*A* (*bottom*), specific fluorescence signal was detected, and, importantly, treatment of cells with NTX caused a 1.9-fold increase in the cell surface fluorescence. A significant increase in the cell surface fluorescence was also detected when cells were treated with another opioid antagonist, NOX, but not with the α-adrenergic receptor antagonist PHE (Fig. 5*B*), establishing the specificity of the antagonist effect. The relatively low increase in the number of receptors following opioid antagonist treatment is in line with our previous study, in which NTX was found to enhance hδOR maturation by 2-fold, increasing the efficiency of maturation from about 40% to nearly 100% (22).

The appearance of ER-retained receptors at the plasma membrane was also followed by a cell surface biotinylation approach. Receptor expression was induced in HEK293$_i$-Myc-hδOR-FLAG cells in the presence of BFA as described above, and cells were incubated for 2 h in a CHX-containing medium in the absence or continued presence of BFA. Cell surface proteins were then labeled with sulfo-NHS-biotin, and immunoprecipitated receptors were analyzed by Western blotting using either anti-FLAG M2 antibody or HRP-conjugated streptavidin. As expected, streptavidin was not able to detect any receptor species when they were purified from cells in which the transport block was not removed during CHX chase (Fig. 6*A*, *lane 3*), although the M_r 48,000 receptor was clearly detected by anti-FLAG M2 antibody (*lane 7*). In contrast, a broad heterogeneous M_r 48,000–52,000 receptor species was detected by both streptavidin and anti-FLAG M2 antibody when the chase was performed in the absence of BFA, (Fig. 6*A*, *lanes 1* and *5*, respectively), and an ~2-fold increase in the intensity of this receptor species was detected when cells were treated simultaneously with NTX (*lanes 2* and *6*). The heterogeneity in the molecular weight of receptors that reached the cell surface suggests that the receptor glycans were not processed in a normal manner following removal of the transport block. Since the hδORs are transported through the Golgi in only about 10 min (28), it is understandable that they were transported to the cell surface before processing enzymes had reached their correct locations in the Golgi. However, Endo H digestion revealed that the receptors became less sensitive to the enzyme treatment during the 2-h CHX chase (Fig. 6*B*, compare *lanes 2* and *6*), suggesting that the glycans were processed to some extent. Interestingly, the antagonist treatment appeared to increase the resistance to Endo H. This appeared to take place whether receptors were retained in the ER or were allowed to reach the cell surface (Fig. 6*B*, compare *lanes 2* and *4* and *lanes 6* and *8*).

Opioid Ligands Stabilize ER-retained hδOR Precursors—To test the hypothesis that binding of opioid ligands leads to stabilization of newly synthesized receptors, membrane-bound hδOR precursors were subjected to an *in vitro* heat inactivation assay, an assay that has been widely used to establish structural instability of constitutively active mutant and wild-type GPCRs (36–43). Cellular membranes were prepared from HEK293$_i$-Myc-hδOR-FLAG cells that were induced to express the receptor in the presence of BFA. They were then incubated at 37 °C with or without 1 μM NTX for 0–180 min, and the remaining [^{3}H]diprenorphine binding activity was assessed after removing the antagonist by washing. The binding affinities of the receptor preparations that were treated with NTX were unaltered, confirming that the antagonist was removed before the binding assay. The binding of membrane-bound receptor precursors decreased in time, with half-maximal inactivation occurring within 14.2 ± 1.8 min (Fig. 7*A*), assuming an exponential decay. In contrast, the inactivation slowed down significantly when membranes were incubated in the presence of NTX (half-time for inactivation 32.9 ± 2.9 min, $p < 0.0001$), without any detectable difference in the receptor protein level (Fig. 7*A*, *inset*). These results are in line with the hypothesis that ligand binding can stabilize ER-localized receptors. In cells, this stabilization is likely to spare the receptors from premature degradation in proteasomes (31).

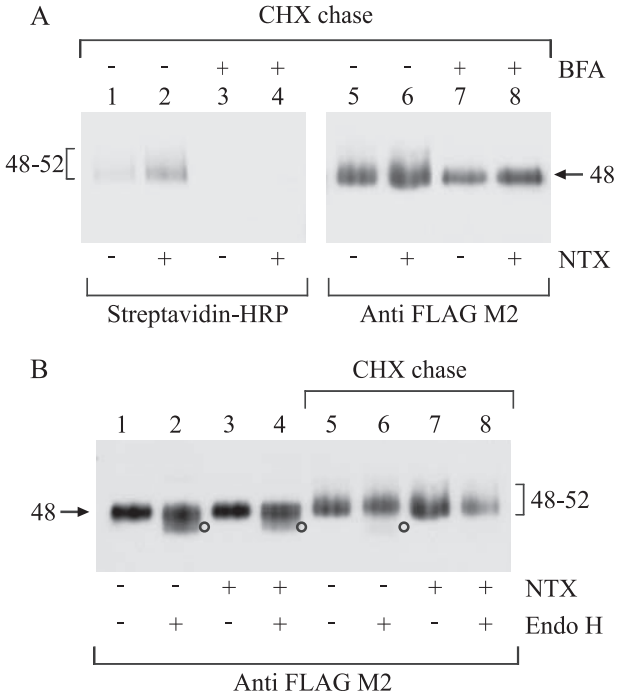

FIGURE 6. **Opioid ligands enhance transport of ER-retained hδORs to the plasma membrane.** Analysis of plasma membrane receptors by cell surface biotinylation. *A*, expression of hδORs was induced with tetracycline (0.5 μg/ml) in stably transfected HEK293$_i$-Myc-hδOR-FLAG cells. BFA (5 μg/ml) was added after 60 min, and cells were incubated at 37 °C for 6 h in the presence or absence of NTX (10 μM). Cells were then washed and incubated for 2 h in a medium containing CHX (20 μg/ml) in the absence or continued presence of BFA (5 μg/ml) as indicated. Cell surface proteins were biotinylated before harvesting, and receptors were purified by immunoprecipitation. Samples were analyzed by Western blotting using anti-FLAG M2 antibody (*lanes 5–8*) or HRP-conjugated streptavidin (*lanes 1–4*). *B*, expression of hδORs was induced in the presence of BFA and NTX as described above and either harvested immediately (*lanes 1–4*) or incubated for 2 h in a medium containing CHX (20 μg/ml) (*lanes 5–8*). Immunoprecipitated receptors were subjected to Endo H digestion (50 milliunits/ml; 16 h) and analyzed by Western blotting using anti-FLAG M2 antibody. *Open circles*, Endo H-sensitive receptors.

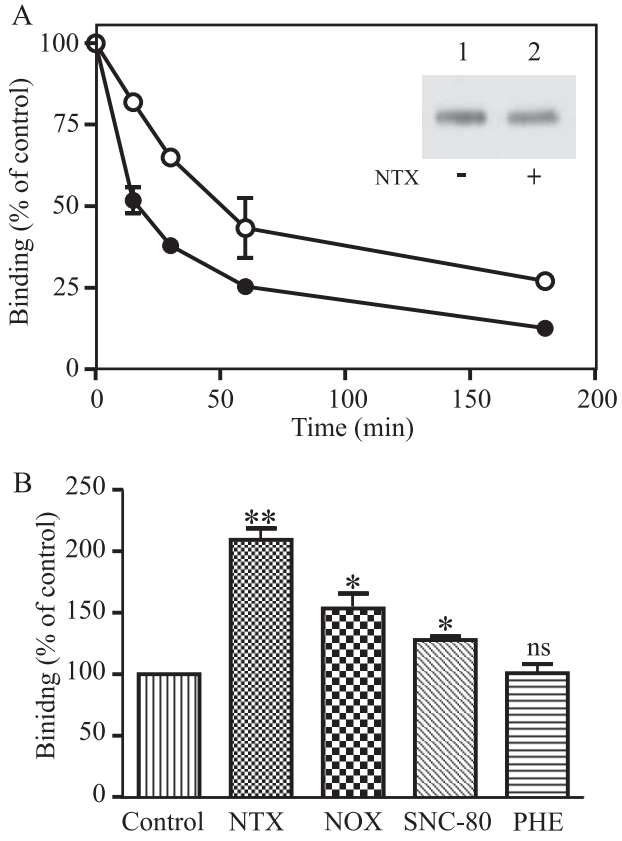

FIGURE 7. **Binding of opioid ligands to ER-retained hδORs increases their stability.** Receptor expression in HEK293$_i$-Myc-hδOR-FLAG cells was induced with tetracycline (0.5 μg/ml), and cells were treated with BFA as described in the legend for Fig. 2. *A*, cellular membranes were prepared and incubated with (○) or without (●) NTX (1 μM) at 37 °C for 0, 15, 30, 60, or 180 min. After removing residual NTX, samples were subjected to a one-point binding assay using [^{3}H]diprenorphine. Data are given as remaining binding activity in percentage of control binding at $t = 0$ (mean ± S.E., $n = 4$). *Inset*, Western blot analysis of immunoprecipitated receptors from membranes incubated for 180 min in the absence (*lane 1*) or presence (*lane 2*) of NTX. *B*, membranes were incubated at 37 °C for 30 min without or with 1 μM NTX, NOX, SNC-80, or PHE, and samples were subjected to a one-point binding assay as described above. The values (mean ± S.E., $n = 4$) indicate the relative specific binding compared with values in control cells that were set to 100%. **, $p < 0.001$; *, $p < 0.05$; *ns*, not significant.

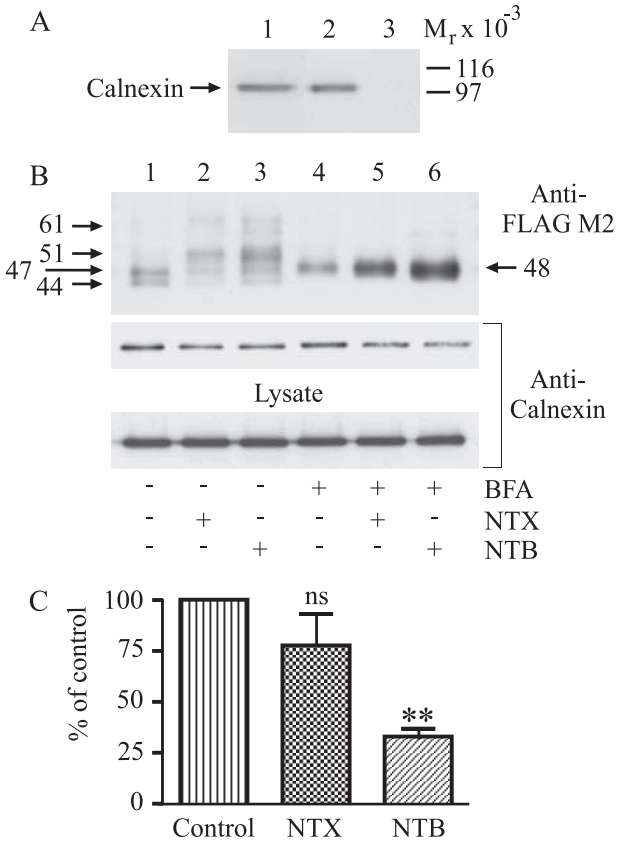

FIGURE 8. **Binding of opioid antagonists to hδOR precursors enhances their dissociation from calnexin.** *A*, expression of hδORs in HEK293$_i$-Myc-hδOR-FLAG cells was induced with tetracycline (0.5 μg/ml) for 7 h. The total cellular lysate was divided into two aliquots and subjected to immunoprecipitation with immobilized anti-FLAG M2 antibody (*lane 2*) or mouse IgG (*lane 3*), and immunoprecipitates were analyzed by Western blotting using anti-calnexin antibody. An aliquot of the lysate was loaded on *lane 1*. *B* and *C*, HEK293$_i$-Myc-hδOR(D95A)-FLAG cells were induced to express the hδOR(D95A) mutant with tetracycline (0.5 μg/ml). BFA (5 μg/ml) or ethanol vehicle was added after 60 min, and cells were incubated in the absence or presence of NTX (10 μM) or NTB (10 μM) at 37 °C for 3 h. Cellular membranes were solubilized and subjected to immunoprecipitation with anti-FLAG M2 antibody, and aliquots of the eluates were analyzed by Western blotting with either anti-FLAG M2 (*first panel*) or anti-calnexin antibodies (*second panel*). An aliquot of the solubilized membranes was probed with anti-calnexin antibody, indicating that the amount of calnexin in each sample was equal (*third panel*). The data were quantified by densitometric analysis, and the calnexin/receptor precursor ratio was calculated for the BFA-treated samples (*C*). The calnexin/precursor ratio in control cells not treated with antagonists was set to 100%. Values given are the mean ± S.E. of three independent experiments. **, $p < 0.001$; *ns*, not significant. For unknown reasons, one of the two consensus sequences for *N*-glycosylation for the hδOR(D95A) is used very inefficiently, and the receptor is mainly expressed in precursor and mature forms that contain only one *N*-glycan.

To test whether other opioid ligands in addition to NTX could stabilize hδOR precursors, membranes from BFA-treated cells were incubated at 37 °C for 30 min without or with 1 μM NTX, NOX, SNC-80, or PHE. All of the opioid ligands, whether antagonists (NTX or NOX) or agonists (SNC-80), increased the amount of remaining [^{3}H]diprenorphine binding by 2.1 ± 0.09-, 1.5 ± 0.12-, and 1.3 ± 0.29-fold, respectively ($n = 3$) (Fig. 7*B*). In contrast, PHE had no effect, confirming the specificity of the opioid ligand-mediated effect.

Binding of Opioid Ligands to hδOR Precursors Leads to Dissociation from Calnexin and Enhances Processing of Receptor Oligosaccharides—It has been demonstrated previously that a mutant form of the V2 vasopressin receptor that can be rescued by pharmacological chaperones is normally retained in the ER by calnexin (44). Since this ER chaperone is an important component of the glycoprotein quality control (1), we wondered whether the opioid ligand-mediated stabilization of hδOR precursors might lead to their dissociation from calnexin. To find out if the receptor interacts with calnexin, HEK293$_i$-Myc-hδOR-FLAG cells were induced for 7 h, and total cellular lysates were subjected to immunoprecipitation and Western blotting. As seen in Fig. 8*A*, anti-calnexin antibody was able to detect calnexin from anti-FLAG M2 antibody immunoprecipitates but not from those obtained with immobilized mouse IgG (compare *lanes 2* and *3*), indicating that calnexin indeed interacts with the receptor. The effect of opioid ligands on the calnexin interaction was then studied by including antagonists in the culture medium during the induction. No reproducible effect on the amount of co-precipitated calnexin could be detected for the wild-type hδOR, which most likely resulted from the fact that only part of the newly synthesized hδORs is

retained in the ER (28, 31) and can be rescued by pharmacological chaperones (22). We therefore tested whether the effect could be detected for a mutant form of the hδOR that has more difficulties in exiting the ER. Mutation of the conserved aspartate to alanine (D95A) in the second transmembrane helix of the hδOR has been shown previously to reduce receptor maturation efficiency (22). When HEK293$_i$ cells expressing the hδOR(D95A) were treated with either NTX or another opioid antagonist NTB, the enhancement in receptor maturation was clearly evident (Fig. 8*B*, compare *lanes 1–3* in the *first panel*). In nontreated cells that were induced to express the receptor, hardly any mature receptors were detected (*lane 1*), but in antagonist-treated cells, the amount of mature receptors was substantially increased, and that of precursors decreased (*lanes 2* and *3*). The antagonists also decreased the amount of calnexin that co-immunoprecipitated with anti-FLAG M2 antibody (Fig. 8*B*, compare *lanes 1–3* in the *second panel*). This decrease was not entirely due to reduction in the total amount of receptor precursors, since the same decrease was detected even in BFA-treated samples (Fig. 8*B*, compare *lanes 4–6* in the *second panel*), in which the amount of ER-localized M_r 48,000 receptor was increased (Fig. 8*B*, compare *lanes 4–6* in the *first panel*). The antagonist-mediated decrease in the amount of co-immunoprecipitated calnexin was apparent for both antagonists used but reached a significant level only for NTB (Fig. 8*C*).

The finding that opioid antagonists enhanced dissociation of hδORs from calnexin is in line with the observation that NTX treatment increased receptor Endo H resistance in cells that were treated with BFA (see Fig. 6*B*). This implies that the receptor *N*-glycans had become more accessible to glycosidases and glycosyltransferases that were retrotranslocated to the ER. To investigate this possibility in more detail and to follow the fate of receptor precursors in BFA-treated cells, metabolic pulse-chase labeling experiments were performed. HEK293$_i$-Myc-hδOR(D95A)-FLAG cells were induced to express the receptor for 60 min, labeled with [^{35}S]methionine/cysteine in the presence of BFA for 40 min, and chased for various periods of time in the continued presence of BFA and in the absence or presence of NTB. At each time point, cells were harvested, and solubilized receptors were subjected to immunoprecipitation with anti-FLAG M2 antibody and analyzed by SDS-PAGE and fluorography. As seen in Fig. 9*A*, the antagonist treatment decreased the receptor turnover (compare *lanes 2–6* with *lanes 7–11*), indicating that the newly synthesized receptors were spared from degradation. During the chase, the electrophoretic mobility of the receptors decreased in time, and they became gradually Endo H-resistant (Fig. 9*B*), confirming the data obtained by Western blotting (see Figs. 2*B* and 6*B*). Furthermore, the conversion of receptor precursors to the slower migrating forms was enhanced in NTB-treated cells. This was most clearly seen in cells chased for 40 or 60 min (Fig. 9*B*, compare *lanes 3* and *4* with *lanes 8* and *9*). Thus, these results confirm the calnexin co-immunoprecipitation results and indicate that the opioid antagonist-mediated stabilization of hδOR precursors results in dissociation from ER glycoprotein quality control.

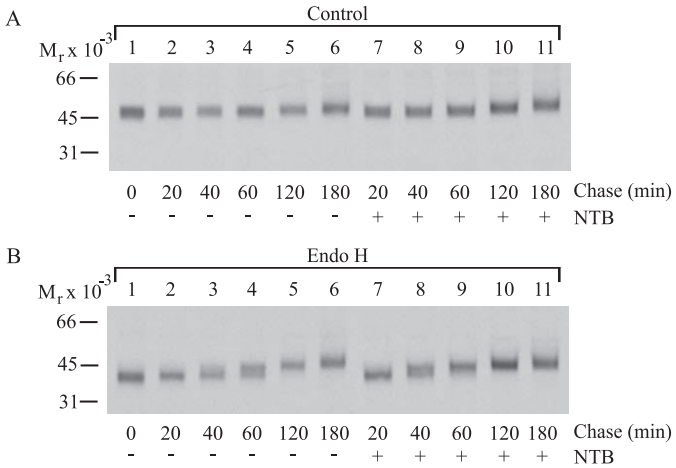

FIGURE 9. **Binding of NTB to ER-retained hδORs decreases their turnover and enhances processing of oligosaccharides.** HEK293$_i$-Myc-hδOR(D95A)-FLAG cells were induced to express the hδOR(D95A) mutant with tetracycline (0.5 μg/ml) for 60 min, pulse-labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 40 min in the presence of BFA (5 μg/ml), and chased for different periods of time in the continued presence of BFA and in the presence or absence of NTB (10 μM) as indicated. Receptors were purified by two-step immunoprecipitation with anti-FLAG M2 antibody from solubilized membranes and treated (*B*) or not (*A*) with 50 milliunits/ml Endo H for 16 h. Samples were analyzed by SDS-PAGE and fluorography.

NTX Treatment Increases the Number of Endogenously Expressed Mouse δORs in NG-108-15 Cells—To investigate whether opioid ligands could act as pharmacological chaperones for endogenously expressed δORs, NG-108-15 cells expressing the mouse δOR were treated or not with NTX for 24 h. Cellular membranes were then prepared and subjected to saturation binding assays. The binding affinity of [^{3}H]diprenorphine for membrane-bound receptors remained unaltered (K_d of 4.2 ± 0.3 and 4.6 ± 0.6 nM for control and NTX-treated cells, respectively, $n = 5$), but the 24-h NTX treatment increased the number of receptors by 1.4 ± 0.08-fold. Thus, opioid ligands may regulate receptor expression levels by the pharmacological chaperone-mediated manner also in cells expressing δORs at an endogenous level.

DISCUSSION

In the past few years, accumulating evidence has indicated that GPCR ligands can act as pharmacological chaperones and enhance cell surface targeting of wild-type and mutant receptors (12–26, 45, 46). Although a wealth of indirect evidence has suggested that they act on newly synthesized receptors before they have been inserted into the plasma membrane, their precise mechanism of action has remained elusive. The present study provides the first direct evidence that this enhancement in cell surface targeting results from binding of membrane-permeable ligands to newly synthesized receptors in the ER. Furthermore, binding of opioid ligands was found to lead to conformational stabilization and dissociation of ER-retained hδORs from calnexin, thus supporting a notion that stabilization redirects a subpopulation of newly synthesized receptors away from the degradation pathway and allows them to exit the ER and reach the cell surface. Understanding the mechanism of action of opioid receptor pharmacological chaperones may be important not only with respect to opioid receptors but poten-

tially for other GPCRs as well, both wild-type and mutants forms. In addition, mechanisms that can be identified in heterologous expression systems are likely to be involved in the regulation of receptor levels in cells that endogenously express the receptor, as was suggested by the finding that NTX treatment was found to increase the number of mouse δORs in NG-108-15 cells.

Ligand binding ability of ER-localized hδORs was demonstrated by saturation binding assays. Separation of ER and plasma membranes by sucrose density gradient centrifugation was unsuccessful, but using an inducible expression system and the protein transport blocker BFA, we were able to have a preparation devoid of interfering mature cell surface receptors. This was verified by cell surface biotinylation assay, immunofluorescence, and flow cytometry. To our knowledge, only one other family A GPCR that binds extracellular ligands and contains its binding site within the seven-transmembrane bundle has so far been demonstrated to gain binding activity before the receptors reach the cell surface. Hurt *et al.* (47) showed that murine α_{1C}-adrenergic receptors are able to bind the radioligand RX820021 with high affinity in an enriched ER membrane fraction. In line with this observation, the human β_2-adrenergic receptor was found to gain ligand binding ability in an *in vitro* translation system (48), and recently, GPR30 was shown to be able to bind estrogen intracellularly (49). In comparison, a number of studies have reported that GPCRs that interact with their cognate ligands via their extracellular domain are able to gain ligand binding ability before they reach the plasma membrane. For example, a few intracellularly retained mutant forms of the luteinizing hormone receptor have been shown to bind ligand, but only after cells have been solubilized (50). In addition, the precursor form of this receptor can be purified by ligand affinity chromatography (29). However, these family A GPCRs are exceptional, because their large extracellular ligand binding domain is likely to fold independently of the transmembrane domain.

Although ER-retained hδORs were able to bind [^{3}H]diprenorphine with high affinity, the affinity was still significantly lower than that of mature cell surface receptors. The same applied also for two opioid agonists, DPDPE and SNC-80. The reason for this difference is not known but may be related to different properties of the lipid bilayer in the two cellular locations. It can be argued that this could either directly or indirectly have an effect on receptor conformation and thus binding ability. Whereas the plasma membrane is enriched in sphingolipids and cholesterol, the ER membrane contains almost exclusively glycerol phospholipids. In addition, the relative proportion of glycerol phospholipids and aminophospholipids differs in the two membrane bilayers (51). Membrane lipids are known to affect the function of integral membrane proteins in several ways (52). For example, cholesterol has been found to modulate the function of GPCRs either indirectly by changing the membrane properties or directly by interacting with the receptor (52). Nevertheless, cholesterol is unlikely to be the cause for the different binding affinities observed for the mature and immature hδORs, because we were unable to detect any changes in the binding affinity for either antagonists or agonists following cholesterol depletion of membranes with methyl-β-cyclodextrine.[4]

The opioid antagonist-mediated stabilization of hδOR precursors was demonstrated in an *in vitro* heat inactivation assay. Incubation of membranes at 37 °C led to a time-dependent loss in the number of [^{3}H]diprenorphine binding sites that was slowed down significantly in the presence of NTX and other opioid ligands. A similar antagonist-mediated stabilization has been demonstrated previously for a number of constitutively active mutant GPCRs, in which stabilizing intramolecular constraints that keep the receptor in an inactive conformation are disrupted (38–43). Thus, it is likely that binding of opioid ligands to newly synthesized hδORs stabilizes the native state of the receptor by introducing additional constraints within the seven-transmembrane helix bundle. This may then promote more stable packing of the transmembrane α-helices. This is analogous to other small molecular ligands that have been shown to increase protein thermostability and reduce flexibility (53–55). In addition, this notion is supported by the observation that several mutant GPCRs that have been rescued by pharmacological chaperones carry substitutions in amino acids that are known to be involved in intramolecular interactions (12, 14, 21, 23). Interestingly, all membrane-permeable opioid ligands that have been tested are found to enhance cell surface expression of opioid receptors (22, 25, 45), suggesting that both the inactive receptor conformation, stabilized by antagonists or inverse agonists, and the active conformation(s), stabilized by agonists, are compatible with ER export.

Binding of antagonists was also found to lead to dissociation of precursors of the hδOR(D95A) mutant from calnexin, similarly to what has been reported recently for mutant forms of the melanin-concentrating hormone receptor 1 and V3 vasopressin receptor (21, 46). Binding of substrates to calnexin or its soluble homolog, calreticulin, is mediated by monoglucosylated *N*-glycans (1). The glucoses are removed by ER glucosidase II, but if the protein has not reached its stable correct fold, it is reglucosylated by the folding sensor, UDP-glucose:glycoprotein glucosyltransferase, allowing the protein to bind again to calnexin or calreticulin (1). This so-called calnexin cycle thus retains incompletely folded glycoproteins in the ER. The finding that NTB significantly decreased the number of hδOR(D95A) precursors that were bound to calnexin indicates that the ligand binding stabilizes the receptor molecules in such a way that they are no longer recognized by the folding sensor and are therefore able to exit the calnexin cycle. This notion was also supported by the finding that opioid antagonists were found to enhance trimming of the receptor *N*-glycans by retrotranslocated Golgi mannosidases and glycosyltransferases in BFA-treated cells. It can thus be argued that since the ligand-stabilized receptor precursors are no longer recognized as misfolded, they are redirected away from the disposal pathway and are spared from degradation.

The ability of opioid ligands to dissociate hδOR precursors from calnexin and enhance their processing and maturation suggests that the receptors are inappropriately retained in the

[4] T. T. Leskelä and U. E. Petäjä-Repo, unpublished data.

ER and are targeted to degradation prematurely. The inherent conformational flexibility that is needed for the functional activity of GPCRs may thus make these proteins very susceptible for inappropriate ER retention and in some cases may result in slow kinetics of folding and maturation. This may also apply to a few other wild-type GPCRs, like the rat luteinizing hormone and mouse V2 vasopressin receptors and the human D4 dopamine, GnRH, and Pael receptors that have been shown to mature inefficiently (16, 23, 24, 29, 56). The slow folding kinetics is probably not entirely disadvantageous, however, and may even allow cells to regulate the level of mature receptors at the plasma membrane. For example, final maturation of luteinizing hormone receptors appears to be developmentally regulated in gonads, since the developing organs express only immature receptors that are likely to be nonfunctional (57). Assuming that the inefficient maturation of GPCRs is a more common phenomenon than presently anticipated, it can be hypothesized that pharmacological chaperoning may take part in regulating the number of cell surface wild-type receptor levels, especially taking into account the vast number of membrane-permeable GPCR ligands that are in clinical use. For mutant disease-causing GPCRs, pharmacological chaperoning has already been shown to have pharmacological potential, since V2 vasopressin receptor antagonists were recently found to relieve symptoms of patients suffering from nephrogenic diabetes insipidus caused by ER-retained mutant receptors (58).

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Opioid Receptor Pharmacological Chaperones Act by Binding and Stabilizing Newly Synthesized Receptors in the Endoplasmic Reticulum

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