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Distinct Subcellular Localization for Constitutive and Agonist-modulated Palmitoylation of the Human δ Opioid Receptor*

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Protein palmitoylation is a reversible lipid modification that plays important roles for many proteins involved in signal transduction, but relatively little is known about the regulation of this modification and the cellular location where it occurs. We demonstrate that the human δ opioid receptor is palmitoylated at two distinct cellular locations in human embryonic kidney 293 cells and undergoes dynamic regulation at one of these sites. Although palmitoylation could be readily observed for the mature receptor (M_r 55,000), [^{3}H]palmitate incorporation into the receptor precursor (M_r 45,000) could be detected only following transport blockade with brefeldin A, nocodazole, and monensin, indicating that the modification occurs initially during or shortly after export from the endoplasmic reticulum. Blocking of palmitoylation with 2-bromopalmitate inhibited receptor cell surface expression, indicating that it is needed for efficient intracellular transport. However, cell surface biotinylation experiments showed that receptors can also be palmitoylated once they have reached the plasma membrane. At this location, palmitoylation is regulated in a receptor activation-dependent manner, as was indicated by the opioid agonist-promoted increase in the turnover of receptor-bound palmitate. This agonist-mediated effect did not require receptor-G protein coupling and occurred at the cell surface without the need for internalization or recycling. The activation-dependent modulation of receptor palmitoylation may thus contribute to the regulation of receptor function at the plasma membrane.

Palmitoylation, a post-translational lipid modification, plays an important role in the structure and function of a variety of proteins involved in cell signaling (1, 2). Among them several G protein-coupled receptors (GPCRs),[3] mainly belonging to family A, have been shown to be palmitoylated on one or more cysteine residues located in the proximal region of their C-terminal tail (2). The attached palmitate has therefore been proposed to promote the formation of a fourth cytoplasmic loop through its anchoring in the membrane (3). Consequently, palmitoylation may have a profound effect on the local conformation of this domain and possibly controls interaction of GPCRs with specific regulatory proteins. It has been shown to display diverse roles in GPCR function, including G protein coupling, desensitization, trafficking, and targeting of the receptors (2).

Unlike other lipid modifications such as myristoylation and prenylation, palmitoylation is a highly dynamic modification. The 16-carbon saturated palmitate that is attached to the specific cysteine residues via an acyl-thioester bond has been demonstrated to turn over rapidly (4–8) and palmitoylation/depalmitoylation cycles may therefore regulate protein function. It has been shown that the palmitoylation/depalmitoylation cycle is activation-dependent for several G protein α-subunits (4, 9–11), endothelial nitric-oxide synthase (12), and several GPCRs (the β_2-adrenergic (5, 13), D_1 dopamine (14), α_{2A}-adrenergic (15), m_2-muscarinic (16), serotonin 5-HT4a (17, 18), and V_{1a} vasopressin receptors (19)).

The biochemical processes that regulate protein palmitoylation have remained poorly understood, and so far only a few enzymes involved in the formation or hydrolysis of palmitoyl-protein thioesters have been characterized (2, 20). In addition, the subcellular site for palmitoylation of membrane proteins has remained elusive. For example, viral glycoproteins appear to be palmitoylated in the pre-/early Golgi compartment (21–24), whereas attachment of fatty acids to cellular integral membrane proteins has been suggested to occur either in the endoplasmic reticulum (ER) (25) or at the plasma membrane (26, 27).

The human δ opioid receptor (hδOR) is one of the three opioid receptor subtypes (μ, δ, κ) that are targets for opiate drugs and endogenous opioid neuropeptides. It contains several cysteine residues in the proximal part of its C-terminal end (28, 29) and thus may be palmitoylated. Because biosynthesis, maturation, and trafficking of the δORs have been characterized in detail (30, 31), we selected this receptor as a study model to examine the subcellular location and dynamic regulation of GPCR palmitoylation. We have demonstrated that hδOR palmitoylation can occur at two distinct cellular locations. The newly synthesized receptors are first palmitoylated early in their biosynthetic pathway before they are transported to the cell surface, but palmitoylation/depalmitoylation cycles can also occur on the mature receptor at the plasma membrane. We also found that such constitutive receptor-bound palmitate turnover is increased upon agonist-promoted receptor activation. This regulation occurred locally at the plasma membrane and did not require G protein coupling, internalization, or recycling of the receptor.

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[3] The abbreviations used are: GPCR, G protein-coupled receptor; BFA, brefeldin A; CHX, cycloheximide; 2-Br, 2-bromopalmitate; DMEM, Dulbecco's modified Eagle's medium; DPDPE, cyclic[D-Pen^2,D-Pen^5]enkephalin; DDM, *n*-dodecyl-β-D-maltoside; ER, endoplasmic reticulum; FBS, fetal bovine serum; hδOR, human δ opioid receptor; HEK293, human embryonic kidney 293; HRP, horseradish peroxidase; LE, leucine-enkephalin; MON, monensin; NHS, *N*-hydroxysuccinimide; NOC, nocodazole; PBS, phosphate-buffered saline; PTX, pertussis toxin.

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EXPERIMENTAL PROCEDURES

Materials—[^{3}H-9,10]Palmitate (60 Ci/mmol) and [9-^{3}H]bremazocine (26.6 Ci/mmol) were purchased from PerkinElmer Life Sciences and brefeldin A (BFA), monensin (MON), and *n*-dodecyl-β-D-maltoside (DDM) from Calbiochem or Alexis. The anti-FLAG M2 antibody, FLAG-peptide, isoproterenol, leucine-enkephalin (LE), cyclic[D-Pen^2,D-Pen^5]enkephalin (DPDPE), pertussis toxin (PTX), nocodazole (NOC), and cycloheximide (CHX) were products of Sigma. 2-Bromopalmitate (2-Br) was obtained from Fluka, and sulfo-*N*-hydroxysuccinimide (NHS)-biotin, sulfo-NHS-*S-S*-biotin, immobilized streptavidin, and horseradish peroxidase (HRP)-conjugated streptavidin were obtained from Pierce. Phycoerythrin-conjugated rat anti-mouse IgG$_1$ and 7-amino-actinomycin D were from BD Biosciences. The mouse anti-c-Myc antibody (9E10) was produced by the core facility at the Department of Biochemistry, University of Montréal, as ascites fluid. Cell culture reagents were either from Invitrogen, Wisent, or Sigma. All the other reagents were of analytical grade and were obtained from various commercial suppliers.

DNA Constructs and Cell Culture—The hδOR (Gene accession number P41443) in pcDNA3 (Invitrogen) was tagged at the C-terminal end with a *Flag* epitope (DYKDDDDK) and expressed in stably transfected human embryonic kidney (HEK) 293S cells (HEK293S-hδOR-*Flag* cells; 10.5 pmol/mg membrane protein, as determined by saturation binding using [^{3}H]bremazocine) as described previously (30). A DNA construct encoding the hδOR with a cleavable influenza hemagglutinin signal peptide (KTIIALSYIFCLVFA), N-terminal *Myc* epitope (EQKLISEEDL) and C-terminal *Flag* epitope was created, and a stable cell line with inducible expression was established as described elsewhere (32, 33).[4] Receptor expression in the HEK293$_i$-*Myc*-hδOR-*Flag* cells was induced by adding tetracycline (0.5 μg/ml; Invitrogen) into the culture medium.

Labeling with [^{3}H[Palmitate—For [^{3}H]palmitate labeling, cells were first preincubated in Dulbecco's modified Eagle's medium (DMEM) without fetal bovine serum (FBS) for 60 min at 37 °C, and labeling was performed in fresh serum-free medium containing 500 μCi/ml [^{3}H]palmitate for the times indicated in the figures and text. For pulse-chase experiments, the medium was removed, and cells were washed and incubated in the chase medium (DMEM supplemented with FBS and 100 μM palmitate) for different periods of time as specified in Figs. 2, 3, and 5 and in the text. For the HEK293$_i$-*Myc*-hδOR-*Flag* cells, the chase medium was supplemented with CHX (20 μg/ml). The receptor ligands were added at the beginning of the labeling or chase, as indicated. When the labeling was performed in the presence of BFA (5 μg/ml), NOC (20 μg/ml), MON (10 μM), PTX (100 ng/ml), or 2-Br (100 μM), the reagents were added 15 min (NOC), 30 min (BFA, MON), or 16 h (PTX, 2-Br) before the labeling. For HEK293$_i$-*Myc*-hδOR-*Flag* cells, BFA was added 6 h before labeling, and no preincubation in a serum-free medium was performed. To block receptor internalization, cells were detached and incubated in DMEM, 20 mM Hepes, pH 7.5, 5 mM EDTA in the absence or presence of sucrose (0.35 M) for 30 min at 37 °C before adding [^{3}H]palmitate.

Isolation of Cell Surface Receptors—Cells were biotinylated using sulfo-NHS-biotin, and cell surface receptors were isolated by a two-step purification using immobilized streptavidin and anti-FLAG M2 antibody as described (30). Alternatively, immunoprecipitated receptors were subjected to Western blot analysis using HRP-conjugated streptavidin.

Inhibition of Internalization and Detection of Internalized Receptors—The assay used to detect internalized receptors was modified from Cao *et al.* (34). Briefly, cells were first washed with warm Dulbecco's PBS, cooled on ice, and incubated with gentle agitation at 4 °C for 30 min in the same buffer containing 0.3 mg/ml sulfo-NHS-*S-S*-biotin. Excess biotin was quenched by adding Tris-HCl, pH 7.4, to a final concentration of 50 mM, after which the incubation was continued for 10 min at 4 °C. Thereafter, cells were detached and incubated in warm DMEM, 20 mM Hepes, pH 7.5, 5 mM EDTA in the presence or absence of sucrose (0.35 M) for 30 min at 37 °C. Internalization was induced by adding LE (10 μM) and incubating cells for 30 min at 37 °C. After cooling on ice, the medium was removed by centrifugation (500 × *g*, 10 min), and cells were washed with 20 mM Hepes, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10% (v/v) FBS. Biotin that remained attached to the cell surface was removed by incubating cells on ice for 10 min in 50 glutathione, 20 mM Hepes, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10% (v/v) FBS. Finally, to quench the residual glutathione, cells were incubated on ice for 10 min in 20 mM *N*-ethylmaleimide, 1% (w/v) bovine serum albumin, 5 mM EDTA in PBS, pH 7.4. The efficiency of biotin removal was confirmed by verifying that no biotin labeling could be detected in the absence of agonist-induced internalization following glutathione treatment.

Immunoprecipitation—Crude cellular membranes were prepared as described (30) and solubilized in 0.5% (w/v) DDM, 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, 20 mM *N*-ethylmaleimide, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM 1,10-phenantroline for 60 min at 4 °C. After centrifugation at 100,000 × *g* for 60 min, the *Flag*-tagged receptor was immunoprecipitated from the supernatant fraction by two-step immunoprecipitation using immobilized anti-FLAG M2 antibody (35).

Hydroxylamine Treatment—For hydroxylamine treatment, receptors were eluted from immobilized anti-FLAG M2 antibody using a solution composed of 1% (w/v) SDS and 20 mM *N*-ethylmaleimide. Samples were then divided into two equal aliquots and treated with either 1 M hydroxylamine (pH 7.2) or 1 M Tris-HCl (pH 7.2) at 20 °C for 2 h. The reaction was stopped by adding equal volume 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).

SDS-PAGE and Western Blotting—For SDS-PAGE (10% separating gels), samples were denatured by heating at 95 °C for 2 min under non-denaturing conditions. For detection of radioactivity, gels were treated with Enhance (PerkinElmer Life Sciences) according to the manufacturer's instructions, dried, and exposed at −80 °C for 20–120 days using the Biomax MS film and intensifying screens (Kodak). For Western blotting, proteins resolved in SDS-PAGE were transferred electrophoretically onto Immobilon P membrane (Millipore), and bound proteins were probed using anti-FLAG M2 antibody as described (30, 35) or HRP-conjugated streptavidin (1:40,000). The relative intensities of the bands on autoradiograms and Western blots were analyzed by densitometric scanning with Agfa Arcus II or Agfa Duoscan HiD laser scanners and quantitation using Scion Image 4.02 software, subtracting the local background from each lane.

Flow Cytometry—Cells were cooled on ice and detached from cell culture flasks with PBS. To label plasma membrane receptors, 1 × 10^6 cells/sample were incubated for 30 min on ice with monoclonal anti-c-Myc antibody (1:1,000) in PBS, 1% (v/v) FBS. After the residual unbound primary antibody was removed, cells were incubated with 0.5 μg/ml phycoerythrin-conjugated rat anti-mouse IgG$_1$ in PBS, 1% (v/v) FBS for 30 min. After washing, dead cells were labeled with 7-amino-actinomycin D (0.25 μg/1 × 10^6 cells) for 10 min. All incubations were performed

[4] T. T. Leskelä, P. M. H. Markkanen, E. M. Pietilä, J. T. Tuusa, and U. E. Petäjä-Repo, manuscript in preparation.

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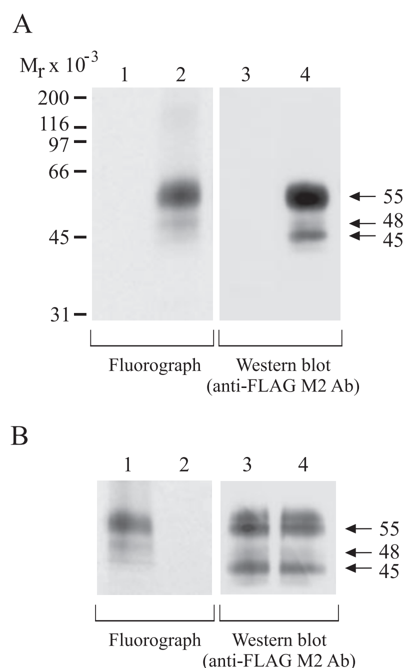

FIGURE 1. **hδOR is palmitoylated via thioester bonds.** *A*, nontransfected HEK293S cells (*lanes 1* and *3*) or HEK293S-hδOR-*Flag* cells stably expressing the *Flag* epitope-tagged hδOR (*lanes 2* and *4*) were labeled with 500 μCi/ml [^{3}H]palmitate for 60 min at 37 °C. Cellular membranes were isolated and solubilized in DDM, and receptors were immunoprecipitated using immobilized anti-FLAG M2 antibody. Samples were analyzed by SDS-PAGE and fluorography (*lanes 1* and *2*) or Western blotting (*lanes 3* and *4*) using anti-FLAG M2 antibody. Molecular weight markers are indicated on the *left*. *Arrows* indicate the molecular weights of the mature (M_r 48,000 and 55,000) and precursor (M_r 45,000) forms of the receptor. *B*, HEK293S-hδOR-*Flag* cells were labeled with [^{3}H]palmitate for 60 min at 37 °C, and immunoprecipitated receptors were incubated in the presence of 1 M Tris-HCl (pH 7.2) (*lanes 1* and *3*) or 1 M hydroxylamine (pH 7.2) (*lanes 2* and *4*) at 20 °C for 2 h. Samples were analyzed by SDS-PAGE and fluorography (*lanes 1* and *2*) or Western blotting (*lanes 3* and *4*). The data shown are representative of three independent experiments.

on ice. Ten thousand cells of each sample were analyzed on a BD Biosciences FACSCalibur™ flow cytometer. Cells were excited with a 488-nm argon ion laser, and phycoerythrin and 7-amino-actinomycin D emission was detected with a 585 ± 21-nm band-pass filter and a 670-nm long-pass filter, respectively. Data analysis to quantitate changes in mean surface receptor fluorescence values were performed using the CellQuest ™Pro 4.02 software (BD Biosciences). Mean fluorescence of live cells minus mean fluorescence of cells stained only with phycoerythrin-conjugated secondary antibody was used for calculations.

Data Analysis—Data were analyzed using GraphPad Prism 4.02 software. For statistical *t* tests, the limit of significance was set at $p < 0.05$, and data are presented as the mean ± S.E.

RESULTS

hδOR Is Palmitoylated via Thioester Bonds—To determine whether the hδOR is palmitoylated, we assessed whether it incorporates [^{3}H]palmitate during metabolic labeling. HEK293S cells expressing the C-terminally *Flag*-tagged receptor (HEK293S-hδOR-*Flag* cells) were incubated for 60 min with [^{3}H]palmitate, and the receptor was immunoprecipitated using immobilized anti-FLAG M2 antibody and subjected to SDS-PAGE analysis and fluorography. As shown in Fig. 1*A*, [^{3}H]palmitate was incorporated into one major protein of M_r 55,000 that comigrated with the mature receptor on Western blots (compare *lanes 2* and *4*) (30), whereas no labeled proteins were detected in nontransfected cells (*lanes 1* and *3*). This clearly demonstrates that, as is the case for many family A GPCRs, the hδOR is modified by addition of palmitate. No distinct labeled band comigrating with the M_r 45,000 receptor precursor (30) was detected, but occasionally an ill-defined [^{3}H]palmitate-labeled band migrating faster than the M_r 55,000 mature receptor (~M_r 48,000) was observed (Fig. 1*A*, *lane 2*). This species represents a partially glycosylated mature receptor form that contains only one *N*-linked glycan.[5]

Covalent attachment of palmitate into proteins occurs primarily through a thioester linkage to cysteine residues; this linkage is chemically labile and can be cleaved by weak nucleophiles such as hydroxylamine at neutral pH. To assess the nature of the acyl linkage on the hδOR, the immunoprecipitated [^{3}H]palmitate-labeled receptor was treated with either 1 M hydroxylamine (pH 7.2) or 1 M Tris-HCl (pH 7.2) and analyzed by SDS-PAGE, fluorography, and Western blotting. Treatment with hydroxylamine led to a loss of the ^{3}H signal on the fluorograms, whereas the protein remained intact (Fig. 1*B*, *lanes 2* and *4*, respectively), demonstrating that the acyl moiety on the receptor was attached via a thioester bond.

hδOR Palmitoylation Is a Dynamic Process, Involving Newly Synthesized Receptors in Transit to the Plasma Membrane as well as Mature Receptors at the Cell Surface—The major hδOR species that was found to incorporate [^{3}H]palmitate during the 60-min labeling period was the mature fully glycosylated form of M_r 55,000 (Fig. 1*A*, *lane 2*), and this was observed even when the labeling was shortened to 10 min (see Fig. 4*B*). Given that the ER residency time of newly synthesized hδORs is relatively long (half-time of maturation ~ 120 min) (30), the fast labeling kinetics of the mature receptor suggests that palmitoylation is a late post-translational event and might even take place once the protein has been inserted into the plasma membrane. To investigate further in which cellular subcompartment palmitoylation takes place, we first assessed which hδOR species could incorporate [^{3}H]palmitate in cells treated with BFA. This drug interferes with protein transport to the cell surface and leads to the accumulation of newly synthesized proteins in the ER (36). When BFA was added to the labeling medium 30 min before [^{3}H]palmitate addition and maintained during the 30-min labeling period, label was incorporated into two major proteins of M_r 45,000 and 55,000 (Fig. 2*A*, *lane 2*), the label of which was removable by hydroxylamine treatment (compare *lanes 1* and *2* in Fig. 2*B*). The smaller receptor species represents newly synthesized receptor precursors that accumulate in the ER in BFA-treated cells (30, 37),[4] suggesting that these receptors were palmitoylated early in the biosynthetic pathway as their transport to the cell surface was blocked. However, because the M_r 45,000 receptor species was not labeled in nontreated cells (Fig. 2*A*, *lane 1*), it is unlikely that palmitoylation occurs to any considerable extent in the ER. To further investigate this early site of palmitate attachment to the hδOR, the cells were treated with NOC, a microtubule-depolymerizing agent that inhibits microtubule-mediated vesicular transport and leads to accumulation of secretory proteins in pre-Golgi intermediates that fail to translocate to the Golgi (38). As seen in Fig. 2*A*, *lane 3*, the M_r 45,000 receptor precursor incorporated [^{3}H]palmitate in the NOC-treated cells. Thus, these results suggest that palmitate is attached to the newly synthesized hδORs shortly after their export from the ER, most likely at the ER exit sites or the ER-Golgi intermediate compartment.

The observation that the M_r 55,000 mature receptor was labeled in BFA- and NOC-treated cells (Fig. 2*A*, *lanes 2* and *3*, respectively) indicates that palmitoylation can also occur after the receptor has been fully processed in the Golgi. This is suggested by the observation that a 30-min pretreatment with BFA is sufficient to completely block the transport of newly synthesized receptors to the cell surface (37).[4] To

[5] P. M. H. Markkanen, J. T. Tuusa, and U. E. Petäjä-Repo, manuscript in preparation.

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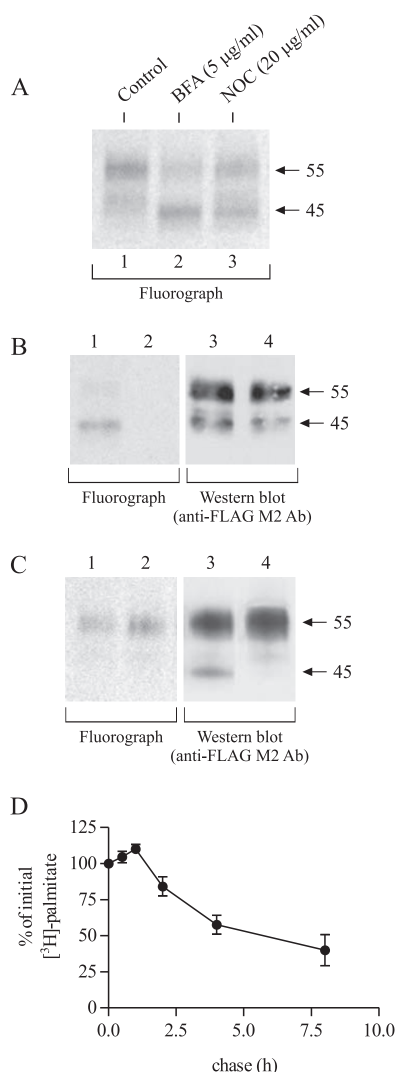

FIGURE 2. **hδOR is palmitoylated at two cellular locations.** *A*, HEK293S-hδOR-*Flag* cells were labeled with [^{3}H]palmitate for 30 min at 37 °C in the absence (*lane 1*) or presence of BFA (5 μg/ml) (*lane 2*) or NOC (20 μg/ml) (*lane 3*). BFA and NOC were added to the medium at 30 and 15 min, respectively, before labeling. Receptors were immunoprecipitated using immobilized anti-FLAG M2 antibody and analyzed by SDS-PAGE and fluorography. The fluorograph shown is representative of three to six independent experiments. *B*, HEK293S-hδOR-*Flag* cells were labeled with [^{3}H]palmitate in the presence of BFA as described in *A*. Immunoprecipitated receptors were incubated in the presence of 1 M Tris-HCl (pH 7.2) (*lanes 1* and *3*) or 1 M hydroxylamine (pH 7.2) (*lanes 2* and *4*) at 20 °C for 2 h. Samples were analyzed by SDS-PAGE and fluorography (*lanes 1* and *2*) or Western blotting (*lanes 3* and *4*). The data shown are representative of two independent experiments. *C*, HEK293S-hδOR-*Flag* cells were biotinylated with sulfo-NHS-biotin (0.5 mg/ml) at 4 °C for 30 min and then labeled with [^{3}H]palmitate for 30 min at 37 °C. Cellular membranes were solubilized, and receptors from 25% of the extract were immunoprecipitated using immobilized anti-FLAG M2 antibody to isolate the total pool of labeled receptors (*lanes 1* and *3*). The rest of the extract was used to isolate the cell surface receptors by sequential precipitations using first immobilized streptavidin and then anti-FLAG M2 antibody (*lanes 2* and *4*). Purified receptors were analyzed by SDS-PAGE and fluorography (*lanes 1* and *2*) or Western blotting (*lanes 3* and *4*). The data shown are representative of four independent experiments. *D*, HEK293S-hδOR-*Flag* cells were labeled for 120 min with [^{3}H]palmitate at 37 °C and chased with DMEM supplemented with 10% (v/v) FBS and 100 μM palmitate for the indicated times. Cell surface proteins were then biotinylated with sulfo-NHS-biotin, and cell surface receptors were purified by sequential precipitations using immobilized streptavidin and anti-FLAG M2 antibody. The purified receptors were analyzed by SDS-PAGE and fluorography. The intensity of the mature M_r 55,000 receptor was quantified by densitometric scanning of the fluorograms, and values were normalized to the intensity at 0 h of chase. The graph describes the time course of disappearance of the M_r 55,000 receptor. The values given are means ± S.E. of three independent experiments.

determine whether this second event of palmitoylation occurs before exit from the Golgi/*trans*-Golgi network or at the plasma membrane, the HEK293S-hδOR-*Flag* cells were biotinylated with a membrane-impermeable reagent, sulfo-NHS-biotin, prior to [^{3}H]palmitate labeling. This allowed us to isolate receptors that were labeled at the plasma membrane by sequential purification using immobilized streptavidin and anti-FLAG M2 antibody (30). When the purified receptors were analyzed by Western blotting, only the mature M_r 55,000 and M_r 48,000 receptors were detected (Fig. 2*C*, *lane 4*), and as seen on *lane 2*, these receptors were labeled with [^{3}H]palmitate. Thus, the hδOR palmitoylation can take place after the protein has been transported to the plasma membrane and does not only occur early in the biosynthetic pathway.

The most likely explanation for the finding that palmitate addition to the hδOR can take place at two cellular locations is the reversible nature of the thioester bond, which can result in dynamic palmitoylation/depalmitoylation of the receptor. To find out whether the fast turnover of receptor-bound palmitate could contribute to the efficient incorporation of [^{3}H]palmitate into the mature receptors at the cell surface, we performed pulse-chase labeling experiments. The HEK293S-hδOR-*Flag* cells were first labeled for 120 min and then incubated in the presence of an excess of unlabeled palmitate for varying lengths of time. At each time point cell surface proteins were biotinylated, and cell surface receptors were isolated. As can been seen in Fig. 2*D*, the labeling intensity of the mature cell surface receptors increased at the earliest chase times studied. This is possibly due to the presence of significant amounts of tracer in the palmitoylation donor pool (39) and/or to receptors that were labeled early after biosynthesis and transported to the cell surface during the chase. After 60 min, however, the labeling declined rapidly with an apparent half-time of 110 ± 15 min ($n = 3$). This is clearly shorter than the half-life of the cell surface receptor protein (15–20 h) as determined previously in [^{35}S]methionine/cysteine pulse-chase experiments (30), indicating that palmitate that is bound to the mature cell surface receptor turns over more rapidly than the protein itself.

Palmitoylation Is Needed for Efficient Transport of Newly Synthesized Receptors to the Cell Surface—The results presented above suggest that the hδOR can be palmitoylated at two cellular locations, early in the biosynthetic pathway as well as at the cell surface. To investigate palmitoylation of the newly synthesized receptors in more detail, we created a stably transfected HEK293 cell line (HEK293$_i$-*Myc*-hδOR-*Flag*) that expresses the *Myc* and *Flag* epitope-tagged hδOR under tetracycline induction.[4] Cells were treated with tetracycline for 7 h in the presence of BFA, and cell surface proteins were biotinylated with sulfo-NHS-biotin followed by immunoprecipitation of the receptor with anti-FLAG M2 antibody. Only the precursor form of the receptor was detected on Western blots with anti-FLAG M2 antibody (Fig. 3*B*, *lane 1*), but no cell surface receptors could be detected with HRP-conjugated streptavidin (Fig. 3*C*, *lane 1*), confirming the effective block of receptor cell surface trafficking with BFA. These intracellular receptors were able to incorporate [^{3}H]palmitate (Fig. 3*A*, *lane 1*), confirming the notion that the newly synthesized receptors are initially palmitoylated before they are transported to the cell surface. When BFA was removed and cells were incubated for 2 h in the presence of CHX, a heterogeneous band was detected both with anti-FLAG M2 antibody and HRP-conjugated streptavidin (*lane 3* in Fig. 3, *B* and *C*, respectively), indicating that the intracellular receptors were able to reach the cell surface following removal of the transport block. The heterogeneity of the receptor band is most likely due to inefficient processing of the *N*-linked glycans by glycosyltransferases that need to be translocated from the ER to their correct locations in the Golgi after BFA removal. As seen on Fig. 3*A*, *lane 3*, the 2-h chase incubation reduced the intensity of the [^{3}H]palmitate-labeled receptors by about 45% (compare *lanes 1* and *3*). This suggests that the turnover of the ER-retained precursor-bound palmitate is

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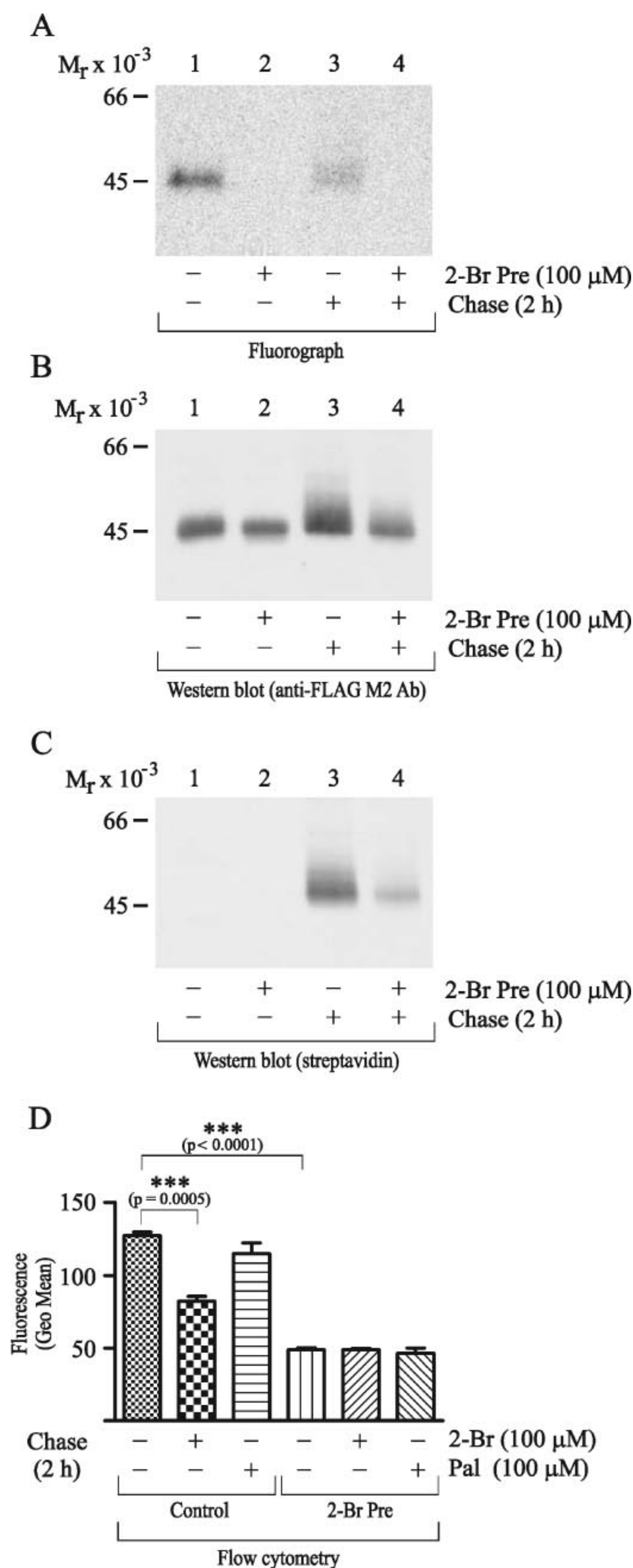

FIGURE 3. **Addition of palmitate to newly synthesized hδORs is needed for their efficient transport to the cell surface.** *A–C*, HEK293$_i$-*Myc*-hδOR-*Flag* cells expressing the *Myc* and *Flag* epitope-tagged hδOR in a tetracycline-inducible manner were pretreated or not with 2-Br (*2-Br Pre*, 100 μM) for 16 h as indicated. Receptor expression was induced by adding tetracycline (0.5 μg/ml) to the culture medium, and transport of newly synthesized receptors to the cell surface was inhibited with BFA (5 μg/ml). Cells were incubated for 7 h in the continued absence or presence of 2-Br and then were labeled with [^{3}H]palmitate for 60 min at 37 °C (*A*). Alternatively, cells were treated as described in *A* without adding [^{3}H]palmitate, and cell surface proteins were labeled with sulfo-NHS-biotin (0.5 mg/ml) at 4 °C for 30 min (*B* and *C*). Cells were then harvested immediately (*lanes 1* and *2*) or after a 2-h chase in the absence of BFA, tetracycline, and 2-Br but in the presence of CHX (20 μg/ml) and palmitate (100 μM) (*lanes 3* and *4*). Total cellular membranes were isolated and extracted in DDM, and receptors were isolated by immunoprecipitation. Aliquots of the eluates were analyzed by SDS-PAGE and fluorography (*A*) or by Western blotting using anti-FLAG M2 antibody (*B*) or HRP-conjugated streptavidin (*C*). The results shown are representative of two to three independent experiments. *D*, HEK293$_i$-*Myc*-hδOR-*Flag* cells were pretreated (*2-Br Pre*) or not (*Control*) with 2-Br, and receptor expression was induced with tetracycline, as described in *A*, in the presence of BFA. A 2-h CHX chase was then performed in the absence of BFA and tetracycline and in the presence or absence of 2-Br (100 μM) or palmitate (*Pal*, 100 μM) as indicated. Cell surface receptors were labeled with monoclonal anti-c-Myc antibody and analyzed by flow cytometry. The values given are means ± S.E. of three independent experiments. The data were analyzed using an unpaired *t* test.

approximately the same as that of the cell surface receptors (compare with Fig. 2*D*). Furthermore, because the transit time of receptors through the Golgi is relatively fast, ~10 min, (30), these data indicate that a majority of the receptors that are inserted into the plasma membrane still contain palmitate that has been incorporated into the receptor during their transport thought the secretory pathway.

To investigate the potential role of palmitoylation for transport of the newly synthesized receptors to the cell surface, the HEK293$_i$-*Myc*-hδOR-*Flag* cells were treated with 2-Br for 16 h before inducing receptor expression. As seen on Fig. 3*A*, *lane 2*, this treatment was able to completely block incorporation of [^{3}H]palmitate into the newly synthesized receptors without any major effect on the protein content (Fig. 3*B*, *lane 2*), consistent with findings obtained previously for other proteins (8, 25, 40, 41). The nonpalmitoylated receptors were able to reach the cell surface, albeit at a reduced level (Fig. 3*C*, compare *lanes 4* and *3*). This suggests that palmitoylation greatly facilitates cell surface expression of the newly synthesized receptors but is not absolutely required. To study the significance of palmitate incorporation more quantitatively, we assessed the appearance of newly synthesized receptors at the cell surface by flow cytometry. As seen in Fig. 3*D*, blocking of palmitoylation with 2-Br decreased cell surface expression of the receptors by 61.4 ± 0.4% (compare the first and fourth bar), supporting the notion that palmitoylation has a substantial facilitating effect. This was also demonstrated by the fact that there was a significant decrease in the number of cell surface receptors (35.6 ± 2.0%) even when 2-Br was added to the culture medium only during the 2-h chase incubation (compare the first and second bar in Fig. 3*D*). In contrast, adding an excess of palmitate into the culture medium during the chase had no effect (compare the first and third bar in Fig. 3*D*).

hδOR Palmitoylation Is Modulated by Opioid Agonists—The fact that palmitoylation of the hδOR appears to be a reversible and a dynamic modification that can also occur at the cell surface prompted us to investigate whether it could be modulated by opioid agonists. HEK293S-hδOR-*Flag* cells were labeled for 30 min with [^{3}H]palmitate in the absence or presence of 10 μM LE, and the incorporated radiolabel was assessed by immunoprecipitation followed by SDS-PAGE and fluorography. As shown in Fig. 4*A*, LE elicited a clear increase in incorporation of [^{3}H]palmitate into the mature M_r 55,000 receptor (compare *lanes 1* and *2*, *left panel*) (179 ± 14% of the control value, $n = 9$), whereas there was no change in the amount of receptor protein (compare *lanes 1* and *2*, *right panel*). This LE-induced increase in [^{3}H]palmitate incorporation into the receptor was rapid, being clearly apparent as early as 10 min following receptor stimulation, the earliest labeling time point studied (Fig. 4*B*). [^{3}H]Palmitate incorporation into the M_r 55,000 mature receptor was also increased in the presence of another peptidic opioid agonist, DPDPE, but not in the presence of a β_2-adrenergic

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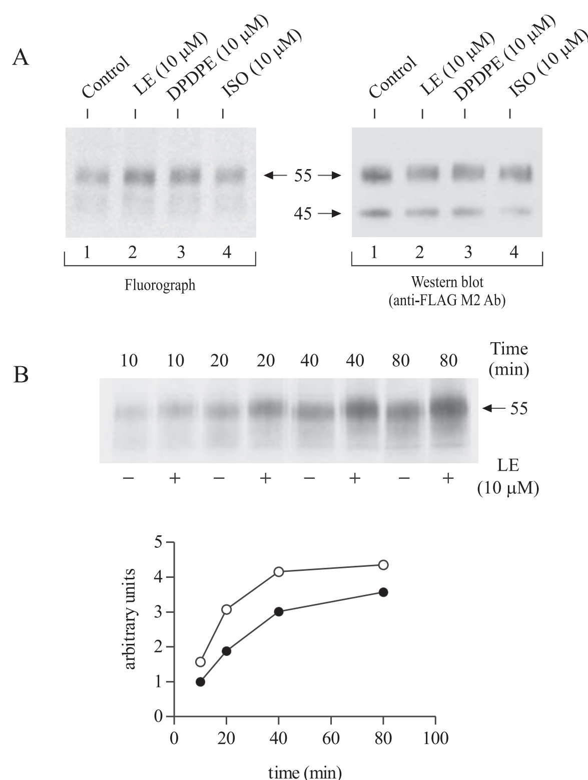

FIGURE 4. **Opioid agonists increase incorporation of [^{3}H]palmitate into the hδOR.** *A*, HEK293S-hδOR-*Flag* cells were labeled with [^{3}H]palmitate for 30 min at 37 °C in the absence or presence of 10 μM LE, DPDPE, or isoproterenol (*ISO*) as indicated. Cellular membranes were solubilized, and immunoprecipitated receptors were analyzed by SDS-PAGE and fluorography (*left panel*) or Western blotting (*right panel*). *B*, HEK293S-hδOR-*Flag* cells were labeled with [^{3}H]palmitate for 10, 20, 40, or 80 min at 37 °C in the absence or presence of 10 μM LE; cellular membranes were solubilized, and immunoprecipitated receptors were analyzed by SDS-PAGE and fluorography. The graph describes the time-dependent incorporation of [^{3}H]palmitate into the mature M_r 55,000 receptor in non-treated (●) and LE-treated (○) cells. Intensities of the mature receptor species were obtained by densitometric scanning of the fluorograms, and the values were normalized to the intensity of that species in nontreated cells labeled for 10 min. The results shown are representative of three to nine independent experiments for *A* and four for *B*.

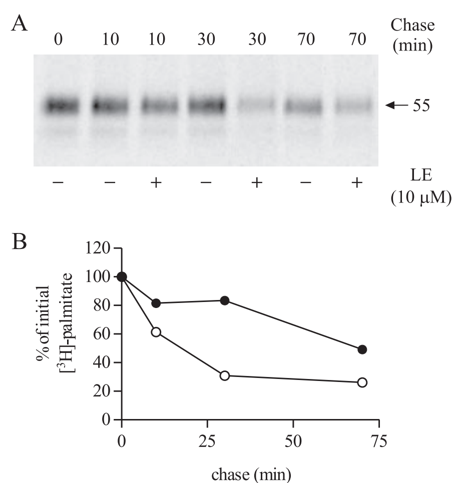

FIGURE 5. **Agonist treatment increases the turnover of hδOR-bound [^{3}H]palmitate.** HEK293S-hδOR-*Flag* cells were labeled for 120 min at 37 °C with [^{3}H]palmitate and chased with DMEM supplemented with 10% (v/v) FBS and 100 μM palmitate in the absence or presence of LE (10 μM) for the indicated times. LE was added following a 60-min preincubation to avoid the initial increase in receptor labeling seen in Fig. 2*D*. Cellular membranes were solubilized, and immunoprecipitated receptors were analyzed by SDS-PAGE and fluorography (*A*). The graph (*B*) describes the time course of disappearance of the M_r 55,000 receptor species in nontreated (●) and LE-treated (○) cells. Intensities of this labeled receptor species were obtained by densitometric scanning of the fluorograms, and values were normalized to the intensity at 0 h of chase. The results shown are representative of three independent experiments.

receptor agonist, isoproterenol (Fig. 4*A*, *left panel*, *lanes 3* and *4*, respectively), establishing the specificity of the agonist-promoted incorporation of the radiolabel. Taken together, these findings suggest that opioid agonists are able to modulate receptor palmitoylation, possibly by increasing the turnover rate of hδOR-bound palmitate (exchange of nonlabeled receptor-bound palmitate for labeled palmitate).

To test this idea more directly, pulse-chase studies were performed. The HEK293S-hδOR-*Flag* cells were labeled with [^{3}H]palmitate for 120 min followed by incubation with 100 μM unlabeled palmitate in the presence or absence of 10 μM LE for varying lengths of time. The agonist was added following a 60-min preincubation to avoid the initial increase in receptor labeling (see Fig. 2*D*). As shown in Fig. 5, LE promoted a greater decrease in receptor-bound [^{3}H]palmitate at all time points tested without any change in the amount of receptor protein (data not shown). The maximal effect of the agonist treatment was observed following 30 min of chase, the remaining receptor-bound [^{3}H]palmitate in LE-treated cells being only 56 ± 11% (n = 5) of that in the control cells. Because the potential confounding effects of recycling of the palmitate donor pool (39) were not taken into account in these studies, one cannot determine the absolute turnover rates. Nevertheless, the data clearly demonstrate that agonist stimulation increases palmitate turnover on the receptor. Combined with the observation that an opioid but not an adrenergic agonist increased incorporation of [^{3}H]palmitate during pulse labeling of the hδOR (Fig. 4*A*), these results suggest that the state of palmitoylation is dynamically regulated in a receptor-activation dependent manner. We therefore investigated whether the agonist-modulated receptor palmitoylation requires coupling of the receptor to G proteins and modulation of its downstream effectors. For that purpose, we tested the effect of PTX on the LE-induced increase in incorporation of [^{3}H]palmitate into the receptor. Treatment with PTX, which efficiently uncoupled the hδOR from G_i proteins as confirmed by its ability to block the LE-induced inhibition of cyclic AMP accumulation (data not shown), was without effect on the LE-promoted increase in [^{3}H]palmitate incorporation into the mature M_r 55,000 receptor (Fig. 6, compare *lanes 1* and 3 with *lanes 2* and *4*). This indicates that agonist-modulated palmitoylation of the hδOR requires receptor activation but is independent of G protein coupling.

Agonist-induced Increase in the Turnover Rate of hδOR-bound Palmitate Occurs at the Cell Surface and Does Not Require Internalization—Because LE is a peptidic agonist that cannot cross the plasma membrane (37), it is expected that the increase in the turnover rate of receptor-bound palmitate occurs at the cell surface. To verify this hypothesis, we first assessed the effect of LE treatment on [^{3}H]palmitate incorporation into the hδOR in HEK293S-hδOR-*Flag* cells treated with BFA. As seen in Fig. 7*A*, LE was able to enhance labeling of the mature M_r 55,000 receptor, whereas it did not increase that of the intracellularly retained M_r 45,000 receptor precursor (compare *lanes 3* and *4*). Similar results were also obtained using MON (Fig. 7*A*, *lanes 5* and *6*), a drug that interferes with protein transport, leading to accumulation of newly synthesized proteins in the medial Golgi (42). In another set of experiments, the cells were labeled with [^{3}H]palmitate in the presence or absence of the agonist after biotinylating the cell surface receptors with sulfo-NHS-biotin. As seen in Fig. 7*B*, the agonist-induced increase in palmitate incorporation was apparent in the cell surface pool of receptors (compare *lanes 3* and *4*).

The data presented above indicate that dynamic regulation of receptor palmitoylation can occur at the cell surface. However, because binding of opioid agonists to their cognate receptors leads to rapid internalization (31), enhancement in the palmitate turnover might require internalization of the receptors followed by their recycling to the cell

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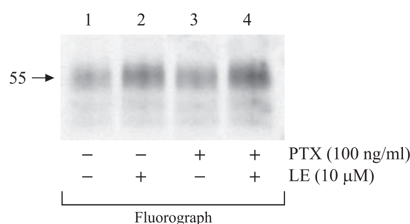

FIGURE 6. **Agonist-mediated enhancement in [³H]palmitate labeling of hδOR is not dependent on G protein coupling.** HEK293S-hδOR-*Flag* cells were pretreated without (*lanes 1* and *2*) or with (*lanes 3* and *4*) PTX (100 ng/ml) for 16 h and labeled with [³H]palmitate for 30 min at 37 °C in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of LE (10 μM). Cellular membranes were solubilized, and immunoprecipitated receptors were analyzed by SDS-PAGE and fluorography. The fluorogaph shown is representative of two independent experiments.

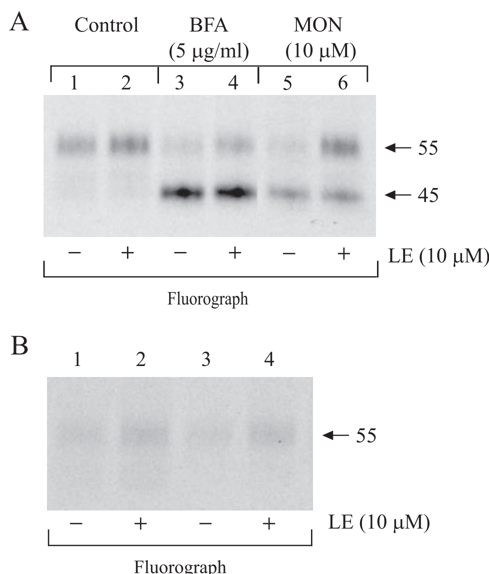

FIGURE 7. **Agonist treatment increases incorporation of [³H]palmitate into hδOR at the cell surface.** *A*, HEK293S-hδOR-*Flag* cells were labeled with [³H]palmitate for 30 min at 37 °C in the absence (*lanes 1*, *3*, and *5*) or presence (*lanes 2*, *4*, and *6*) of LE (10 μM). BFA (5 μg/ml) or MON (10 μM) was added to the medium 30 min before the labeling as indicated. Cellular membranes were solubilized, and receptors were immunoprecipitated and analyzed by SDS-PAGE and fluorography. *B*, HEK293S-hδOR-*Flag* cells were biotinylated with sulfo-NHS-biotin (0.5 mg/ml) for 30 min at 4 °C and then labeled with [³H]palmitate for 30 min at 37 °C in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of LE (10 μM). Cellular membranes were solubilized, and the total pool (*lanes 1* and *2*) and cell surface pool (*lanes 3* and *4*) of receptors were purified as described in the legend for Fig. 2. Samples were analyzed by SDS-PAGE and fluorography. The data shown are representative of three to six independent experiments for *A* and four for *B*.

surface. To test this possibility, we assessed whether inhibition of receptor internalization by sucrose treatment could blunt the agonist-enhanced incorporation of [³H]palmitate. To confirm that the sucrose treatment inhibited internalization of the hδOR in the HEK293S-hδOR-*Flag* cells, we used a biochemical assay to measure internalization (34). For this purpose, cells were biotinylated using a cell-impermeable reagent, sulfo-NHS-*S-S*-biotin, and following a 30-min pretreatment with or without sucrose, LE was added and the cells were incubated for another 30 min. The remaining biotin at the cell surface was then removed by a cell-impermeable reducing agent, glutathione, allowing detection of the internalized receptors by their resistance to cleavage. Analysis of the immunoprecipitated receptors on Western blot using HRP-conjugated streptavidin revealed that agonist treatment induced internalization of the receptor (Fig. 8*A*, compare *lanes 3* and *4*), which was efficiently inhibited with sucrose (Fig. 8*A*, compare *lanes 6* and *4*) (70.7 ± 4.7%, $n = 3$). For unknown reasons, sucrose treatment led to a decrease in [³H]palmitate incorporation into the receptor both at basal and agonist-stimulated conditions. However, the agonist treatment still

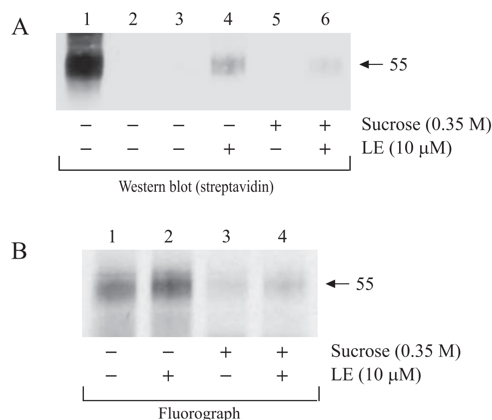

FIGURE 8. **Agonist-mediated enhancement in [³H]palmitate labeling of hδOR does not require receptor internalization.** *A*, HEK293S-hδOR-*Flag* cells were biotinylated with sulfo-NHS-SS-biotin (0.3 mg/ml) at 4 °C for 30 min and incubated at 37 °C in the absence or presence of LE (10 μM) and/or sucrose (0.35 M) for 30 min as indicated. Sucrose was added to the medium 30 min prior to the opioid agonist. Cell surface-bound biotin was then cleaved with membrane-impermeable glutathione as described under "Experimental Procedures." Cellular membranes were solubilized, and receptors were immunoprecipitated with immobilized anti-FLAG M2 antibody and analyzed by SDS-PAGE and Western blotting using HRP-conjugated streptavidin. The cleavage buffer contained no glutathione for the sample on *lane 1*, and no internalization was induced for the sample on *lane 2*. *Lanes 1* and *2* thus represent the total pool of biotinylated receptors and the quantitative loss of biotinylated receptors following cleavage, respectively. *B*, HEK293S-hδOR-*Flag* cells were labeled with [³H]palmitate for 30 min at 37 °C in the absence or presence of LE (10 μM) and/or sucrose (0.35 M), as indicated. Sucrose was added to the medium 30 min before the labeling. Cellular membranes were solubilized, and receptors were immunoprecipitated and analyzed by SDS-PAGE and fluorography. The data shown are representative of three independent experiments.

promoted a 1.6-fold increase in receptor [³H]palmitate incorporation (Fig. 8*B*, compare *lanes 3* and *4*) that was comparable with the 1.4-fold increase observed in the sucrose-nontreated cells (Fig. 8*B*, compare *lanes 1* and *2*). This indicates that the agonist-induced enhancement in the turnover of receptor-bound palmitate is not dependent on internalization of the receptors. Similarly, recycling of the internalized receptors to the cell surface appears not to be necessary for the agonist-mediated effect on the hδOR palmitoylation. This was shown by the inability of MON, a drug that inhibits recycling of cell surface receptors (42), to block the agonist-enhanced incorporation of [³H]palmitate into the M_r 55,000 receptor (see Fig. 7*A*, *lanes 5* and *6*). Taken together, these data support the notion that agonist-induced palmitoylation/depalmitoylation cycle of the hδOR occurs at the plasma membrane and does not require internalization or cycling of the receptors between the plasma membrane and endocytic vesicles.

DISCUSSION

The results of the present study indicate that the hδOR is modified by post-translational addition of palmitate, thus joining the growing number of membrane proteins that have been shown to be palmitoylated. We demonstrate that the newly synthesized hδORs are palmitoylated constitutively while in transit to the cell surface and that the modification is needed for their efficient transport. However, because of the dynamic nature of the modification, the receptor-bound palmitate turns over rapidly, and binding of an agonist to the receptor increases the turnover rate even further in a receptor activation-dependent manner at the cell surface. This agonist-mediated effect on receptor palmitoylation did not require receptor-G protein coupling, internalization, or recycling of the receptor, thus suggesting that it is a consequence of conformational changes promoted by agonist binding. Such plasma membrane-restricted regulation of receptor palmitoylation provides new insights into the potential role of this modification and of the enzymatic machinery involved.

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Determining the cellular location in which palmitoylation of membrane proteins takes place has proven to be a difficult task, and several cellular locations for this modification have been proposed. For example, palmitoylation of the immunoglobulin E receptor (27), transferrin receptor (26), and caveolin-1 (43) is suggested to occur at the plasma membrane, whereas several other membrane proteins appear to be modified prior to the final processing of their *N*- and *O*-linked oligosaccharides in the Golgi (21, 22, 24). In addition, α-bungarotoxin receptors were found to be palmitoylated in the ER during their assembly (25). These apparently contradictory results may result from the fact that proteins may move rapidly from one cellular site to another, which makes it difficult to pinpoint the cellular location in which addition of palmitate into the protein initially occurs. Another possibility is that membrane proteins may be palmitoylated in more than one location within the cell because of the reversibility of the modification. Our results support the latter possibility and demonstrate directly that the hδOR is palmitoylated constitutively at two cellular locations.

The newly synthesized hδORs were initially palmitoylated within the secretory pathway before they were inserted into the plasma membrane. This was demonstrated by the ability of the M_r 45,000 receptor precursor to incorporate [^{3}H]palmitate in BFA-, NOC-, and MON-treated cells in which protein transport to the cell surface was blocked. However, in nontreated cells that expressed the receptor constitutively, only the mature M_r 55,000, and 48,000 receptor forms were labeled, indicating that normally palmitoylated precursor forms do not accumulate. This suggests that the initial hδOR palmitoylation is a late post-translational modification, occurring either concomitantly or just before the *N*- and *O*-linked oligosaccharides are being processed in the Golgi. The most likely sites are the ER exit sites or the ER-Golgi intermediate compartment. Our interpretations are in agreement with those made previously for viral glycoproteins, which are palmitoylated either in the ER-Golgi intermediate compartment or in the *cis*-Golgi (22–24). Similarly, rhodopsin has been proposed to be palmitoylated early in the secretory pathway (44).

Efficient incorporation of [^{3}H]palmitate into the ER-retained receptor precursors in BFA-treated but not in nontreated cells could be due to retrotranslocation of Golgi-resident protein palmitoyltransferases to the ER. This possibility is, however, unlikely, because the M_r 45,000 receptor precursors were also palmitoylated in NOC-treated cells, a treatment that does not promote rapid retrotranslocation of Golgi-resident enzymes (38). An alternative possibility is that the receptor precursors may be palmitoylated in BFA-treated cells because of their recycling between the ER and the ER exit sites, a pathway that apparently remains functional following BFA treatment (45). In any case, hδORs that are unable to leave the ER and accumulate in this compartment before being degraded (35) do not appear to undergo palmitoylation. Indeed, receptor precursors failed to incorporate [^{3}H]palmitate to any detectable level in nontreated cells despite the fact that a substantial proportion of the receptors exist in the precursor form at steady state. This notion is consistent with previous findings showing that ER-retained mutants of the luteinizing hormone receptor and rhodopsin are not palmitoylated (46, 47). Interestingly, permanently misfolded vesicular stomatitis virus G proteins have been shown to be unable to enter the ER exit sites (48), which may be the case for the misfolded/incompletely folded GPCRs as well. This might explain the difference in palmitoylation observed for the ER-retained misfolded/incompletely folded receptors and wild type folding-competent receptors in BFA-treated cells.

The addition of palmitate to the newly synthesized hδORs prior to their plasma membrane insertion suggests that the modification may have a role in the transport process. This was supported by the fact that blocking the palmitoylation with 2-Br reduced the number of receptors that reached the cell surface. This observation is in line with previously published studies (25, 41, 49–51). For example, blocking palmitoylation with 2-Br or mutation of the three C-terminal palmitoylated cysteines of the CCR5 receptor was found to lead to intracellular accumulation of the protein (41, 51).

The occurrence of palmitoylation at later steps in the hδOR life cycle was first suggested by the fast labeling kinetics of the mature, fully processed receptors (M_r 48,000 and M_r 55,000), which were the only [^{3}H]palmitate-labeled receptor species observed even during very short labeling periods in cells expressing the receptor constitutively. Indeed, these data indicated that [^{3}H]palmitate could still be incorporated into the receptor following the final processing of the carbohydrates, pointing to the *trans*-Golgi network and/or the plasma membrane as potential sites for active palmitoylation. The finding that the mature receptors were able to incorporate [^{3}H]palmitate even following treatment with the protein transport blockers BFA, NOC, and MON is also consistent with late palmitate incorporation. However, it made the *trans*-Golgi network an unlikely location for the observed palmitoylation reaction because the transit time through the Golgi has been shown to be very short (~10 min) (30). Thus all of the receptors should have exited the Golgi before the labeling was initiated because the blocks were initiated 15 min (NOC) or 30 min (BFA and MON) before adding [^{3}H]palmitate. Because the transport of newly synthesized receptors was blocked before the labeling, it follows that the M_r 55,000 and, to a lesser extent, the M_r 48,000 receptor species were palmitoylated after they reached the plasma membrane. This interpretation was confirmed in experiments in which plasma membrane receptors were biotinylated prior to metabolic labeling. Detection of streptavidin-purified receptors that had incorporated [^{3}H]palmitate proved that palmitoylation of the hδOR is not entirely dependent on newly synthesized receptors and can occur once the receptors have reached the cell surface. This is in agreement with previous studies showing that red blood cells and platelets support protein palmitoylation in the absence of the Golgi or ER (52, 53), as do isolated nerve growth cones (54) and retinal outer segments (55). This is also consistent with the finding that the hδOR-bound palmitate turns over faster than the protein itself, indicating that the mature receptor is subject to a dynamic constitutive palmitoylation/depalmitoylation cycle at the plasma membrane.

The notion that dynamic palmitoylation events occur at the cell surface was further supported by the ability of the membrane-impermeable opioid agonists LE and DPDPE to increase the turnover of the receptor-bound palmitate in pulse and pulse-chase labeling experiments. Interestingly, the LE-promoted increase in [^{3}H]palmitate incorporation into the receptor was observed even when the receptor-G protein coupling was impaired by PTX, ruling out the contribution of classical G protein-mediated signaling pathways in the control of the palmitoylation/depalmitoylation cycle. Although one cannot exclude the potential role of accessory proteins that could be recruited to the receptor in a G protein-independent manner, the simplest hypothesis is to propose that the conformational changes promoted by the agonist increase the accessibility of the palmitoylated residues to palmitoyl-protein thioesterases and/or transferases.

Activation-dependent increase in the turnover of receptor-bound palmitate has been demonstrated previously for the β_2-adrenergic (5), α_{2A}-adrenergic (15), and serotonin 5-HT4a receptors (17, 18). Similarly, the α-subunits of the G_s and G_i proteins have been found to undergo activation-dependent cycles of palmitoylation/depalmitoylation (4, 9–11). This activation-dependent modulation of palmitoylation of the

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two key components in the GPCR signal transduction pathway argues that regulation of the palmitoylation state of these proteins has an important functional role. Among the roles that have been suggested (2) and could be controlled by palmitoylation/depalmitoylation cycles at the plasma membrane, modulation of receptor-G protein coupling efficacy as well as receptor desensitization and internalization come to mind. However, the activation-dependent palmitoylation/depalmitoylation cycle of the hδOR did not require receptor internalization, because inhibiting endocytosis with sucrose did not prevent the LE-mediated increase in [^{3}H]palmitate incorporation. Similarly, receptor recycling from the endosomes to the plasma membrane was not found to be required for efficient agonist-promoted turnover of the receptor-bound palmitate, as the LE-mediated increase in [^{3}H]palmitate incorporation was not affected by MON. These results are in contrast to the regulation of another post-translational modification of GPCRs, namely phosphorylation. Indeed, at least for the β_2-adrenergic receptor, dephosphorylation has been shown to occur in the endosomes before the receptor is recycled back to the plasma membrane (56). This apparent difference in the cellular location of regulated palmitoylation and phosphorylation cycles of GPCRs may reflect differences in the subcellular distribution of the participating enzymes.

Membrane-bound protein palmitoyltransferase activities have been identified in the mammalian ER, Golgi, and plasma membrane fractions (21, 57, 58), but the instability of the enzymatic activity has hampered purification and molecular identification of the enzymes. Recently, however, a family of proteins containing a conserved DHHC cysteine-rich domain has emerged as potential enzymes capable of catalyzing the transfer of palmitate onto a variety of substrates (59–65). Interestingly, one of these proteins found in yeast, Akr1p, was found to have a role in pheromone signaling and to interact with the C-terminal tail of the **a**-factor receptor (Ste3p) and G protein β-subunit (Ste4p) (66–68). However, it is not known whether Akr1p is able to function as a palmitoyl transferase for the **a**-factor receptor. Similarly, none of the DHHC-containing proteins has been found to catalyze palmitoylation of mammalian GPCRs to date.

In contrast to potential protein palmitoyltransferases, the molecular characterization of palmitoyl-protein thioesterases has been less successful. Only one putative mammalian cytosolic protein thioesterase, the acyl-protein thioesterase 1, has been purified and cloned (69). This enzyme was found to catalyze depalmitoylation of the G protein α-subunits, p21^{ras}, and endothelial nitric-oxide synthase (69–71). However, no additional substrates have been identified since these original reports. The identity of the enzyme responsible for agonist-mediated hδOR depalmitoylation thus remains an open question.

Taken together, the results of the present study indicate that palmitoylation of the hδOR is a highly dynamic and reversible modification, occurring constitutively while receptors are transported to the cell surface, and that addition of palmitate into the newly synthesized receptors appears important for the efficient intracellular transport of these proteins. Furthermore, because hδOR palmitoylation also appears to be regulated at the plasma membrane in an activation-dependent manner, it is likely to have an important role in agonist-induced signal transduction.

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