Differential Sorting of Human δ-Opioid Receptors after Internalization by Peptide and Alkaloid Agonists*

Received for publication, January 6, 2003, and in revised form, March 31, 2003
Published, JBC Papers in Press, April 2, 2003, DOI 10.1074/jbc.M30084200

Nicolás Marie‡, Isabelle Lecoq, Philippe Jauzac, and Stéphane Allouche§

From the Laboratoire de Pharmacologie Moléculaire de la Tolérance aux Opiacés, Université de Caen, Centre Hospitalier et Universitaire de Caen, 14033 Caen Cedex, France

Desensitization and internalization of G protein-coupled receptors observed after agonist activation are considered two important regulatory processes of receptor transduction. Endogenous human δ-opioid receptors (hDOR) are differentially regulated in terms of desensitization by peptide ([D-Pen²,⁵]enkephalin (DPDPE) and Deltorphin I) and alkaloid (etorphine) agonists in the neuroblastoma cell line SK-N-BE (Allouche, S., Roussel, M., Marie, N., and Jauzac, P. (1999) Eur. J. Pharmacol. 371, 235–240). In the present study, we examined the role of hDOR internalization and down-regulation in this differential desensitization. Sustained activation by peptides for 30 min caused a marked decrease of both [³H]diprenorphine binding sites and hDOR immunoreactivity, observed in a Western blot, whereas a moderate reduction by receptor was observed after a 30- and 60-min etorphine exposure in binding experiments without opioid receptor degradation. Using fluorescence microscopy, we visualized hDOR internalization promoted by different agonists in SK-N-BE cells expressing FLAG-tagged hDOR. Agonist withdrawal results in a greater recycling process correlated with a stronger hDOR resensitization after etorphine treatment compared with DPDPE or Deltorphin I, as shown in binding, immunocytochemical, and functional experiments. This suggests a distinct sorting of opioid receptors after their internalization. We demonstrated a lysosomal hDOR targeting upon peptides by using chloroquine in binding, Western blot, and immunocytochemical experiments and by colocalization of this receptor with a late endosome marker. In contrast, when the recycling endosome blocker monensin was used, acceleration of desensitization associated with a strong intracellular immunostaining was observed upon etorphine treatment. The possibility of separate endocytic pathways responsible for the differential sorting of hDOR upon peptide and alkaloid ligand exposure was ruled out by binding and immunocytochemical experiments using sucrose hypertonie solution. First, these results showed complex relationships between hDOR internalization/down-regulation and desensitization. Second, we demonstrated for the first time that the same receptor could undergo a distinct sorting after internalization by peptide and alkaloid agonists.

Opioid receptors are the targets of endogenous and exogenous opioid peptides and alkaloid agonists that bind these receptors with high affinity. The opioid receptor family is composed of three types, namely μ, δ, and κ, whose activation produces many effects including analgesia (1). In vitro, the agonist-activated opioid receptors regulate, via heterotrimeric G proteins, downstream effectors such as adenylate cyclase, Ca²⁺ and K⁺ channels, phospholipase C (2), and [Ca²⁺]³ (3). Upon prolonged activation (from hours to days), opioid receptors undergo down-regulation, a complex mechanism resulting from both internalization and degradation of receptors in lysosomes (4, 5). In contrast, short time activation (from minutes to hours) promotes receptor translocation from plasma membrane into endosomes (or sequestration) that allows their redistribution back to the cell surface (6, 7). Beside the variation of opioid receptor number upon agonist treatment, a decrease of signal transduction from these receptors, a process known as desensitization, is frequently observed. However, the role of sequestration in desensitization is obviously ambiguous since, depending on the model under study, internalization can either contribute to desensitization by reducing functional receptors at the cellular membrane (8) or conversely, can reduce desensitization by promoting receptor recycling to cell surface in an active state (6, 7). The most representative example of this complex relationship between desensitization and internalization is illustrated by the difference in desensitization kinetics of the two μ-opioid receptor isoforms (MOR1 and MOR1B).¹

The relative resistance in desensitization of MOR1B is related to its rapid internalization, compared with MOR1, which allows its resensitization (6). Conversely for the protease-activated receptor-1, internalization is directly responsible for the desensitization induced by thrombin (9). Once internalized, receptors can be either targeted to lysosomes for their degradation or to endosomes for their recycling as demonstrated for the mouse δ-opioid receptors (mDOR) and for the β₂-adrenergic receptors, respectively (10). While the C-terminal tail of GPCR seems to be a major determinant in the sorting process, the molecular mechanisms governing the targeting of sequestrated receptors to endosomes or lysosomes need to be clarified.

Most of the studies carried out on opioid receptor desensitization and internalization have been realized on transfected non-neuronal cells overexpressing opioid receptors, which is known to directly affect the rate of both sequestration and desensitization (11). The SK-N-BE cells represent a more suitable model to study these phenomena for several reasons. (i)

These authors contributed equally to this work.

1 The abbreviations used are: MOR, μ-opioid receptors; mDOR, mouse δ-opioid receptors; DPDPE, [D-Pen²,⁵]enkephalin; Deltorphin I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂; GPCR, G protein-coupled receptor; hDOR, human δ-opioid receptors; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ANOVA, analysis of variance; LBPA, lysobisphosphatidic acid.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from the Ministère de l’Éducation nationale, de la recherche et de la technologie and from the Fondation pour la recherche médicale.

§ To whom correspondence should be addressed: Laboratoire de Biochimie A, avenue Côte de Nacre, CHU de Caen, 14033 Caen cedex, France. Tel.: 33-231064568; Fax: 33-231064985; E-mail: allouche-s@chu-caen.fr.

This paper is available on line at http://www.jbc.org

22795
This cell line presents the neuronal phenotype. (ii) It is a human cell line, and (iii) it expresses only endogenously δ-opioid receptors at physiological levels (12, 13). Upon chronic activation, hDOR undergo a rapid desensitization that was observed on the inhibition of adenylyl cyclase (14, 7). Recently, we reported a differential regulation of those receptors by peptide and alkaloid agonists, which was characterized by a more robust desensitization in the presence of DPDPE and Deltorphin I than in the presence of etorphine (15) and by a differential G protein activation (16).

To address the possibility that this differential desensitization could be related to differences in opioid receptor trafficking, we performed a detailed comparison of hDOR internalization, down-regulation, recycling, desensitization, and resensitization produced by peptide (DPDPE and Deltorphin I) and alkaloid (etorphine) agonists. Visualization of δ receptor internalization was realized using immunofluorescence experiments within the SK-N-BE cells expressing the FLAG-tagged hDOR. Opioid receptor down-regulation and recycling were performed in SK-N-BE cells with binding studies to quantify the number of opioid binding sites. Desensitization and resensitization were determined by functional assays on the inhibition of cAMP accumulation.

In this work, we demonstrated a complex relationship between hDOR internalization/down-regulation and desensitization. We found that the profound desensitization of hDOR promoted by DPDPE and Deltorphin I was correlated with a marked down-regulation of the receptor. While etorphine promoted a strong hDOR desensitization and internalization after a 60-min period, we found no evidence for down-regulation. Exploring the postendocytic fate of hDOR demonstrated that the differential targeting, either to lysosomes for peptide agonists or to recycling endosomes for etorphine, would account for the different rate of desensitization rather than down-regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—SK-N-BE cells, endogenously expressing hDOR, were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Invitrogen), 1% antibiotic-antimycotic mixture (Sigma), and 2 mm l-glutamine at 37 °C in a water-saturated atmosphere containing 5% CO2.

To visualize hDOR internalization by fluorescence and confocal microscopy, SK-N-BE cells were transfected by electroporation with pcDNA3 alone or containing the CDNA encoding for the FLAG-tagged hDOR (pcDNA3-hDOR). Stably transfected cells were obtained after selection with 0.5 mg/ml geneticin (Sigma), and the whole pool of resistant cells was used without clonal selection.

Binding Experiments—Radioisotopic binding studies were performed on attached cells as described previously (4). Briefly, SK-N-BE cells were seeded in 24-well plates at a density of 75,000–100,000 cells/well. Monolayers of cell were treated for various times (30 or 60 min) in the presence of peptide or alkaloid agonists at 37 °C in DMEM/20 mm Hepes buffer. When inhibitors of either internalization (0.5 mM sucrose) or lysosomal degradation (200 μM chloroquine) were used, cells were preincubated with the inhibitor for 30 or 5 min, respectively. In recycling assays, the medium was removed, and SK-N-BE cells were left in DMEM/20 mm Hepes buffer for 30 min. Before binding studies, cells were rinsed with DMEM/20 mm Hepes buffer followed by phosphate-buffered saline (PBS)/0.2% (v/v) bovine serum albumin (BSA) and incubated for 30 min at 37 °C with appropriate concentrations of [3H]hDOR (PerkinElmer Life Sciences) (0.05–5 nM) in the presence of 10% FBS (nonspecific binding) or in the absence (total binding) of 20 μM levorphanol (Sigma), in a final volume of 300 μl of 50 mm Tris-HCl, 1% (v/v) BSA, pH 7.4. The efficiency of washing procedure was checked using control and agonist-pretreated cells. Under these conditions it is worthy to note that no modification of [3H]hDOR binding was detected, indicating that the agonist was completely removed (data not shown). Cells were harvested in 200 μl of 1 M NaOH and placed into vials in the presence of 3 ml of a scintillation mixture (PicoFlour-40, Packard). Vials were counted for radioactivity in a scintillation counter (Packard). Each determination was carried out in triplicate. All experiments were repeated at least three times with similar results. Scatchard analysis was performed using LIGAND software (17) to calculate Kd and Bmax values.

Determination of cAMP Accumulation—Inhibition of adenylyl cyclase was determined by measuring [3H]cAMP accumulation. SK-N-BE cells were seeded in 24-well plates at a density of 100,000–150,000 cells/well in the presence of 0.6 μCi/well [3H]adenine (PerkinElmer Life Sciences) for 12–15 h. Then, cells were challenged with or without different agonists, in the presence of 1 μM isobutylmethylxanthine (IBMX) and 40 μM forskolin for 5 min at 37 °C. When using inhibitors, SK-N-BE cells were pretreated for 5 min (200 μM chloroquine) or 30 min (0.5 μM sucrose and 50 μM monensin). The reaction was stopped by removing the medium and by addition of 5% (v/v) trichloroacetic acid. [3H]cAMP was separated by chromatography on acid alumina columns, and radioactivity was determined using liquid scintillation counting.

Inhibition of adenylate cyclase was determined using Ligand software (17) to calculate Kd.

Images were collected with a Nikon confocal microscope (Eclipse TE-2000, lens ×60) and processed with Adobe Photoshop software. After overlaying green and red labeling, we extracted only the overlapping signal that was converted into grayscale. The merged pictures were collected.
Differential hDOR Targeting by Peptide and Alkaloid Agonists

RESULTS

Down-regulation of δ-Opioid Receptors Induced by Peptide and Alkaloid Agonists—Previously, we reported in the SK-N-BE cell line that peptide agonists, DPDPE and Deltorphin I, promoted a faster desensitization of hDOR after a 30-min pretreatment when compared with an alkaloid agonist, etorphine (15). To determine the reduction of [3H]diprenorphine binding sites, the same conditions as in Ref. 15 were used corresponding to the concentration of the agonist producing the maximal and an identical inhibition of cAMP accumulation (10 nM Deltorphin I, 100 nM DPDPE, and etorphine). In naive cells, determination of Bmax values by Scatchard analysis revealed a number of hDOR ranging between 100 and 150 fmol/mg of protein and was considered as 100%. While etorphine pretreatment produced a reduction of opioid binding sites by ~30% after a 30-min period, a significantly greater decrease of ~50% was observed with peptides (50 ± 4.9% for DPDPE and 50.5 ± 6.6% for Deltorphin I versus 29.7 ± 0.8% for etorphine, ANOVA, Bonferroni-Dunn test, p < 0.05) (Fig. 1A). When etorphine pretreatment was prolonged up to 60 min, no further significant difference in opioid binding site decrease was noted with SK-N-BE cells pretreated with etorphine for 30 min (Fig. 1A). It is worthy to note that no significant modification of Kd values was observed after agonist pretreatment (data not shown).

Western blot analysis was performed to correlate the reduction of [3H]diprenorphine binding sites with the hDOR immunoreactivity in naive and agonist-treated SK-N-BE cells (Fig. 1B). The polyclonal mDOR antibody detected a major band of ~50 kDa in the whole SK-N-BE cell extracts whereas in human fibroblast, no labeling could be observed (data not shown) demonstrating the specificity of the mDOR antibody. A decrease of hDOR immunoreactivity was evident in cells treated with peptides (Dp, Del) for 30 min compared with naive cells (cont) while no significant modification was detected after etorphine pretreatment (Fig. 1B).

Internalization of δ-Opioid Receptors Induced by Peptide and Alkaloid Agonists—Localization of hDOR in SK-N-BE cells was determined by immunocytochemical experiments in naive and agonist-treated cells using the polyclonal mDOR antibody. Under these conditions, a weak but specific immunolabeling was observed as a sharp staining at the plasma membrane of naive cells. However, upon agonist treatment we could only detect a loss of the staining without clearly visualizing opioid receptor internalization. To overcome this problem, immunofluorescence experiments on FLAG-tagged hDOR cells were conducted. Binding experiments revealed about 15–20-fold more opioid receptors in FLAG-tagged hDOR cells compared with SK-N-BE cells (Table I) and pcDNA3-transfected cells (data not shown). Overexpression of FLAG-tagged hDOR does modify neither the affinity of δ-opioid receptors for [3H]diprenorphine nor the ability of the different agonists to promote opioid receptor desensitization (Table I).

While in non-transfected (data not shown) or pcDNA3-transfected SK-N-BE cells, no staining was detected (Fig. 2, pcDNA3), a strong immunofluorescent labeling could be visualized in FLAG-tagged hDOR-transfected cells (Fig. 2, Cont). Using confocal microscopy and sequential sections of cells, we confirmed that the immunostaining was confined to the plasma membrane.

After 30 min of DPDPE and Deltorphin I exposure, an obvious loss of hDOR immunoreactivity from plasma membrane associated with a concomitant appearance of cytoplasmic punctiform staining was observed (Fig. 2, Dp 30 and Del 30). As shown in Fig. 2 (Eto 60), when FLAG-tagged hDOR-transfected cells were incubated in the presence of the alkaloid agonist for 30 data not shown) or 60 min, a strong vesicular labeling was observed with a marked decrease of the plasma membrane staining. Analysis using confocal microscopy confirmed the cytosolic localization of the internalized opioid receptor.

Recycling and Resensitization of hDOR—To investigate the recycling process of hDOR in an active state, SK-N-BE and FLAG-tagged hDOR-transfected cells were either treated for 30 min with DPDPE and Deltorphin I or 60 min with etorphine to obtain a strong internalization and almost a similar desensitization (14, 15). Then, cells were placed in an agonist-free me-

**Fig. 1.** Down-regulation of hDOR by etorphine, DPDPE, and Deltorphin I. A, SK-N-BE cells were pretreated for 30 or 60 min in the presence of 100 nM etorphine, DPDPE, or 10 nM Deltorphin I (filled bars). For recycling experiments, the medium was removed, and cells were left in agonist-free medium for 30 min (hatched bars). The total number of opioid receptors was determined using [3H]diprenorphine. hDOR down-regulation is expressed as a loss of opioid binding sites compared with naive SK-N-BE cells. Data are means ± S.E. of n experiments. ANOVA followed by the Bonferroni-Dunn test or Student’s t test as appropriate was used to determine the statistical significance (Statview, Abacus).

Statistical Analysis—All results are expressed as the mean ± S.E. of n experiments. ANOVA followed by the Bonferroni-Dunn test or Student’s t test as appropriate was used to determine the statistical significance (Statview, Abacus).
Differential hDOR Targeting by Peptide and Alkaloid Agonists

Comparison of binding parameters and desensitization between SK-N-BE cells and FLAG-tagged hDOR transfected SK-N-BE cells

Binding parameters were determined as described under “Experimental Procedures” using \(^{3}H\)lindrenorphine. The opioid agonist-induced inhibition of cAMP accumulation was determined both in naive and agonist-pretreated cells to calculate the percentage of desensitization. Data are means ± S.E. of 3-5 different experiments performed in triplicate. Statistical analysis revealed no significant difference between the two cell lines.

|                          | SK-N-BE   | hDOR/FLAG |
|--------------------------|-----------|-----------|
| **Binding parameters**   |           |           |
| \( K_d \) (nM)           | 0.70 ± 0.15 | 1.26 ± 0.29 |
| \( B_{max} \) (fmol/mg of protein) | 134 ± 9 | 2652 ± 169 |
| **Desensitization (%)**  |           |           |
| Etorphine 100 nM, 60 min | 83.4 ± 5.7 | 82.1 ± 5.8 |
| DPDPE 100 nM, 30 min     | 82.3 ± 5.6 | 88.8 ± 8.9 |
| Deltorphin I 10 nM, 30 min | 86.1 ± 5.6 | 96.8 ± 1.5 |

![Fig. 2. Internalization and recycling of FLAG-tagged hDOR.](image)

Fig. 2. Internalization and recycling of FLAG-tagged hDOR. SK-N-BE cells were transfected either with pcDNA3-hDOR or the empty vector (pcDNA3). Using the anti-FLAG M2 antibody, we visualized the opioid receptor distribution in naive (Cont) and agonist-pretreated cells (Eto 60, etorphine 60 min; Dp 30, DPDPE 30 min; Del 30, Deltorphin I 30 min). In recycling experiments, cells were left in agonist-free medium for 30 min, and opioid receptor localization was determined using fluorescence microscopy (Eto 60 + 30; Dp 30 + 30; Del 30 + 30).

Functional experiments were further conducted on the inhibition of adenylate cyclase to ensure that the recovery of opioid binding sites was associated with a hDOR resensitization. When activated by the alkaloid and peptide agonists, hDOR inhibited cAMP accumulation by 50 and 40%, respectively. To ensure comparison between agonists, the inhibition produced by each molecule was referred as 100% in naive cells. SK-N-BE cells were first incubated for 30 or 60 min in the presence of peptides or etorphine, respectively, to promote a similar desensitization of about 80–90% (Fig. 3). hDOR were allowed to resensitize in an agonist-free medium for various times (15 and 30 min), and then the inhibition produced by each agonist was measured. Fig. 3 shows that etorphine-pretreated cells displayed a faster resensitization than peptide-treated cells. However, this difference of resensitization between etorphine and peptides reached a statistical significance only after a 30-min period in agonist-free medium.

**Lysosomal Degradation after Peptide-induced hDOR Sequestration**—After internalization, GPCR are either recycled to plasma membrane, trapped in endosomal compartments or targeted to lysosomes. The latter contains proteases that can be inhibited by chemicals such as chloroquine by elevating the luminal pH. Data presented in the Figs. 1–3 strongly suggested that peptide-induced hDOR internalization would be followed by their degradation since, unlike etorphine, no complete recovery of opioid receptors was observed when cells were left in agonist-free medium for 30 min. To assess this hypothesis, binding, immunofluorescence, and functional studies were carried out using 200 μM chloroquine, a concentration known to inhibit mDOR degradation in lysosomes (10). SK-N-BE cells were pretreated with chloroquine for 5 min and, to induce down-regulation and desensitization, agonists were then added in the same conditions as described above. As shown in Fig. 4A, chloroquine pretreatment did not affect the loss of opioid receptors induced by etorphine. In contrast, we could observe a significant decrease in the ability of peptide agonists (from 50 to 95%) to induce down-regulation of hDOR but with a more pronounced effect for Deltorphin I than DPDPE. Immunoblot experiments were conducted in the presence or in the absence of the lysosomal proteases inhibitor and showed no difference between naive cells indicating that chloroquine was devoid of any effect on basal level of hDOR. Data presented in Fig. 4B indicated that peptide-induced decrease of hDOR immunoreactivity, observed in Fig. 1B, was totally blocked by chloroquine pretreatment.

Effects of chloroquine were further explored in internalization experiments. SK-N-BE cells stably expressing FLAG-tagged hDOR were treated or not for 30 min with DPDPE or Deltorphin I in the presence or in the absence of chloroquine. All these results are depicted in Fig. 4C and suggest both an enhancement of the intracellular vesicle staining and the number of those vesicles compared with chloroquine-untreated transfected cells (Fig. 4C, see arrows).

To corroborate all the data obtained with chloroquine that strongly argued for an opioid receptor degradation in lysosomes after peptide pretreatment, we investigated a putative localization of the receptor with lysobisphosphatidic acid. This phospholipid is found in late endosomes and thus considered as a marker of the degradative pathway (18). After ensuring that secondary antibodies did not cross-react with the inappropriate primary antibody, we were unable to observe any colocalization of the FLAG-tagged hDOR with LBPA in naive cells (Fig. 5,
In peptide-treated cells, we clearly observed a colocalization of internalized opioid receptor with the late endosomes marker (Fig. 5, Dp 30 and Del 30, arrows). Conversely, despite a massive opioid receptor internalization promoted by etorphine, colocalization of hDOR with LBPA is lacking (Fig. 5, Eto 60).

In order to explore the functional consequences of chloroquine effects, desensitization, and resensitization experiments were conducted. In naive cells, no marked modification of the inhibitory action of agonists was observed (data not shown). After normalization of data, no substantial effect of chloroquine was detected either on desensitization or resensitization, whatever the agonist used (Fig. 6, A–C).

**Evidence for hDOR Recycling upon Etorphine Exposure**

Data presented in Figs. 1 and 2 strongly suggest that upon etorphine activation, hDOR would be recycled after its endocytosis. To test this hypothesis, we examined opioid receptor desensitization, internalization and recycling in the presence or in the absence of a recycling blocker, monensin, after etorphine treatment. To avoid a maximum rate of desensitization, SK-N-BE cells were exposed for 30 min with 100 nM etorphine. In the absence of monensin, etorphine desensitized hDOR by 30% whereas a 30-min pretreatment with 50 μM monensin dramatically increased receptor desensitization by 60% (Fig. 7A). Monensin experiments were also conducted with peptide agonists. However, this recycling inhibitor dramatically re-
A pretreated cells). While all opioid agonists were able to promote naive cells (100% for control cant effect of sucrose pretreatment on the basal hDOR level in A was observed when SK-N-BE cells were pretreated with hyper-
agonists. Sucrose pretreatment totally prevents FLAG-tagged 

Fig. 8

Dp 30

Del 30

Identification of hDOR Internalization Pathways—GPCR sequestration could be mediated either by the clathrin-coated pits pathway or by an alternative endocytic pathway involving caveolin (19). The differential sorting of opioid receptor could result from distinct endocytic pathways. To answer to this question, sucrose hypertonic solution was used to block the clathrin-dependent internalization. SK-N-BE cells were pretreated for 30 min with or without 0.5M sucrose, and the internalization was measured. After normalization of values with monensin-pretreated cells, we were unable to detect any action of monensin on peptide-induced desensitization (data not shown).

In immunofluorescence studies, monensin pretreatment appeared to enhance both the size and the staining of cytoplasmic vesicles after etorphine exposure (Fig. 7B). When etorphine was removed and cells were left in agonist-free medium supplemented with monensin for 30 min, we noticed that FLAG-tagged hDOR failed to recycle back to the plasma membrane (Fig. 7B).

Relationship between hDOR Internalization and Desensitization—As demonstrated for some GPCR, internalization is a prerequisite for their desensitization by reducing active receptors at the plasma membrane (8). Functional studies were conducted to establish the relationships between sequestration and desensitization in our cellular model. SK-N-BE cells were pretreated with or without 0.5 m sucrose for 30 min, then opioid receptor desensitization was induced by etorphine (100 nM, 60 min), DPDPE (100 nM, 30 min), or Deltorphin I (10 nM, 30 min). In sucrose-pretreated cells, we observed a significant reduction of hDOR desensitization by 40% only in the presence of etorphine (ANOVA, Bonferroni-Dunn test, p < 0.05) (Fig. 9). Concerning peptide agonists, we were unable to see any significant effect of hypertonic sucrose solution on opioid receptor desensitization after 30 min (Fig. 9).

FIG. 5. Peptides induced a targeting of internalized FLAG-tagged hDOR in late endosomes. SK-N-BE cells stably expressing FLAG-tagged-hDOR were pretreated or not (Cont) with opioid agonists (Eto 60, Dp 30, and Del 30). Visualization of the opioid receptor (green labeling) and the late endosomes marker LBPA (red labeling) was realized as described under "Experimental Procedures." The colocalized pixels were determined and are shown in the merged pictures. Arrows indicate colocalization of FLAG-tagged hDOR with LBPA. These images are representative of many cells examined in different separate experiments.

Relationship between hDOR Internalization and Desensitization—As demonstrated for some GPCR, internalization is a prerequisite for their desensitization by reducing active receptors at the plasma membrane (8). Functional studies were conducted to establish the relationships between sequestration and desensitization in our cellular model. SK-N-BE cells were pretreated with or without 0.5 m sucrose for 30 min, then opioid receptor desensitization was induced by etorphine (100 nM, 60 min), DPDPE (100 nM, 30 min), or Deltorphin I (10 nM, 30 min). In sucrose-pretreated cells, we observed a significant reduction of hDOR desensitization by 40% only in the presence of etorphine (ANOVA, Bonferroni-Dunn test, p < 0.05) (Fig. 9). Concerning peptide agonists, we were unable to see any significant effect of hypertonic sucrose solution on opioid receptor desensitization after 30 min (Fig. 9).

DISCUSSION

Molecular mechanisms of opioid receptor desensitization and internalization have been extensively studied but the role of internalization and down-regulation in the desensitization processes is still a matter of debate. While in COS-7 (20) and in CHO cells (21), chronic activation of opioid receptors leads to desensitization without down-regulation, Joseph and Bidlack (22) observed a down-regulation of κ-opioid receptor without desensitization following U50,488 exposure. Conversely, in other studies, a close relationship between these events was observed (23, 6). In the present work, we attempted to delineate the role of hDOR internalization/down-regulation in the differential desensitization upon peptide and alkaloid agonist exposure that we previously reported (15).

To answer to this question, immunocytochemical studies were conducted to visualize hDOR internalization, and the quantification of down-regulation was achieved by binding experiments. Using SK-N-BE cells transfected with FLAG-tagged hDOR, to increase the opioid receptor fluorescence staining, we observed a massive and similar internalization after peptide agonists but also after etorphine pretreatment suggesting that the differential desensitization of opioid receptor is not directly linked to its endocytosis and that other mechanisms could occur after receptor sequestration. However, after 30-min exposure, we showed a significant difference in the loss of opioid binding sites between DPDPE and Deltorphin I, on one hand, and etorphine, on the other hand. These observations are correlated with the marked reduction of hDOR immunoreactivity detected on immunoblots experiments in peptide-pretreated cells only demonstrating a degradation of receptors. Comparison between down-regulation data and desensitization experiments suggests that the important decrease of active opioid receptors at the plasma membrane would participate in the strong desensitization produced by DPDPE and Deltorphin I. A similar observation was reported by Prather et al. (24), who observed that hDOR desensitization was associated with their down-regulation following DPDPE exposure in SH-SY5Y cells.

So, we explored the relationship between the disappearance of opioid receptors from the cell surface and the desensitization process using hypertonic sucrose solution to prevent hDOR internalization via the clathrin-coated pits pathway. If the inhibitory response of opioid agonists was directly linked to the level of opioid receptors at the cell surface, we expected to observe a decrease of desensitization in sucrose-pretreated cells. Despite of an effective blockade of hDOR internalization and down-regulation by sucrose, we were unable to observe any effect of this treatment on peptide-induced desensitization. However, a partial reduction by 50% of etorphine-induced desensitization was measured when opioid receptors endocytosis was impaired. These functional data using sucrose demonstrate that internalization is partially responsible for etor-
Phine-induced desensitization by reducing the number of active receptors at the cell surface as demonstrated for the [D-ala², N-MePhe⁴, Gly-ol⁵]enkephalin (25). The sucrose-insensitive desensitization would be probably due to opioid receptor uncoupling from G proteins as demonstrated for the majority of GPCRs (26). On the other hand, peptide-induced desensitization of hDOR does not require receptor endocytosis as shown by sucrose experiments. So, we can assume that desensitization would be produced either by receptor uncoupling or down-regulation at the plasma membrane as demonstrated for the β₂-adrenergic receptor (27). In the case of a cell surface degradation of opioid receptors, sucrose pre-treatment would be unable to affect down-regulation. However, as we observed an effective blockade of down-regulation by sucrose, we can reasonably suppose that receptor uncoupling is the main process involved in peptide-induced desensitization. These data revealed an underestimated complexity between receptor endocytosis, down-regulation, and signaling upon peptide and alkaloid agonist treatment. So we can assume that the difference in hDOR down-regulation is not per se responsible for the differential desensitization produced by chemically different agonists but probably involves other molecular mechanisms and consequently suggests an unexpected behavior of hDOR trafficking depending on the agonist.

Next, we explored the mechanisms underlying the quantitative difference in down-regulation observed between etorphine and peptides. First, we hypothesized that this differential hDOR down-regulation could result from receptor internalization by different endocytic pathways. GPCR internalization, including opioid receptors, is mainly achieved via the clathrin-
dependent pathway (4, 6, 28) but the role of caveolae has been also demonstrated for B2 bradykinin, angiotensin II type 1, and endothelin type A receptors (29–31). When examining the endocytic pathway using hypertonic sucrose solution as a classical inhibitor of clathrin-dependent internalization, we showed that whatever the agonist used, hDOR were internalized into clathrin-coated pits. These data can rule out our first hypothesis. Second, we can assume that peptides and etorphine would promote a differential sorting of hDOR after its internalization. In the presence of DPDPE and Deltorphin I, opioid receptors would be preferentially targeted to lysosomes to be degraded and consequently down-regulated while with the alkaloid agonist, these receptors would be trapped into endosomes and then recycled to the cell surface. This hypothesis is based on the studies of von Zastrow’s group (10) who showed that two different GPCRs, the β2-adrenergic receptor and the mDOR, can undergo differential sorting responsible for a down-regulation in the case of opioid receptor. In Western blot experiments, we were unable to observe a decrease of hDOR immunoreactivity upon etorphine treatment suggesting that the opioid receptors were not degraded. Binding and immunocytochemical experiments showed that hDOR could recycle to plasma membrane and adenyl cyclase assays revealed that these receptors were in an active state. In contrast, when SK-N-BE cells were challenged with peptide agonists, we showed a marked hDOR degradation on immunoblot, a poor recycling process correlated with a weak resensitization. All these data strongly argue for hDOR targeting either to lysosomes following peptide exposure or to recycling endosomes after etorphine treatment.

This differential targeting was evidenced using a recycling inhibitor, monensin and a lysosomal proteases blocker, chloroquine. Indeed, monensin both potentiated desensitization produced by etorphine and almost prevented hDOR recycling demonstrating that opioid receptors were mainly sequestrated into

---

**Fig. 8.** Blockade of agonist-induced hDOR down-regulation and internalization by hypertonic sucrose solution. A, SK-N-BE cells were pretreated with DMEM (filled bars) or with DMEM/0.5 M sucrose (hatched bars) for 30 min. Then, cells were exposed or not to different agonists. The total number of opioid receptors was determined using [3H]diprenorphine. Receptor down-regulation is expressed as a loss of opioid binding sites compared with naive SK-N-BE cells. Data are means ± S.E. of three different experiments performed in triplicate. *, significantly different from sucrose-untreated cells; p < 0.01, Student’s t test. B, SK-N-BE cells stably expressing FLAG-tagged-hDOR were pretreated or not with sucrose 0.5 M. Then, cells were exposed or not to different agonists as described above. Localization of opioid receptor was visualized by fluorescence microscopy using anti-FLAG M2 antibody. Cont, naive cells; Eto 60, etorphine 60 min; Dp 30, DPDPE 30 min; Del 30, Deltorphin I 30 min.

**Fig. 9.** Effects of hypertonic sucrose solution on hDOR desensitization. SK-N-BE cells were pretreated with DMEM or with DMEM/0.5 M sucrose for 30 min. Then, cells were pretreated (hatched bars) or not (filled bars) with etorphine (60 min), DPDPE (30 min), or Deltorphin I (30 min), and the cAMP accumulation was determined as described under “Experimental Procedures.” Data are means ± S.E. of 3–8 independent experiments performed in triplicate. *, significantly different from sucrose non-pretreated cells; p < 0.05 ANOVA, Bonferroni-Dunn test.
Differential hDOR Targeting by Peptide and Alkaloid Agonists

22803

reconciling endosomes. In this case, hDOR internalization would allow dephosphorylation of inactive receptors into endosomes and their redistribution to the plasma membrane as we previously demonstrated (4). When regarding peptide agonists, we showed that chloroquine effectively blocked hDOR degradation as observed on Western blot and in binding experiments. These data strongly suggest that DPDPE and Deltorphin I promote opioid receptor degradation in lysosomal compartments following their internalization. However, it is noteworthy that chloroquine blocked only partially DPDPE-induced down-regulation, suggesting an alternative mechanism that could involve the ubiquitin-proteasome pathway as demonstrated for the murine µ- and δ-opioid receptors expressed in HEK293 cells (32). So, the concept of a different hDOR regulation by peptide and alkaloid agonists seems to be attractive but regulatory mechanisms of these receptors are probably more subtle. The colocalization of internalized receptor with LBPA, described by Kobayashi et al. (18) as a late endosome marker, and thus a marker of the degradative pathway, supported the lysosomal targeting of opioid receptor upon peptide exposure. All those data clearly demonstrate that DPDPE and Deltorphin I promote hDOR degradation in lysosomes. A similar lysosomal degradation process was described for the mouse δ-opioid receptor expressed in Neuro2A cells after [d-Ala², D-Leu⁶] enkephalin (DADLE) treatment but with longer time exposure (24 h) (5). Unexpectedly, we observed the lack of any effect by chloroquine on both desensitization and resensitization despite an effective blockade of hDOR degradation. These data suggest that once trapped into lysosomes, the receptor is unable to recycle in an active state assuming that the targeting of opioid receptor into lysosomal compartments is quite irreversible.

The time of agonist pretreatment is another essential parameter for the opioid receptor fate following internalization. Indeed, our data showed that hDOR is differentially regulated by peptides and etorphine after short time exposure (30–60 min) but when the time treatment is prolonged until 4 h, the opioid receptor behavior upon etorphine exposure is similar to those observed after DPDPE and Deltorphin I. Under these conditions, resensitization and recycling processes are no longer noted indicating that hDOR are down-regulated (4). These data suggest that despite early distinct molecular processes, largely dependent on chemically distinct agonists, the opioid receptors would converge toward down-regulation independent of the chemical nature of the agonist after long term agonist exposure.

Molecular mechanisms underlying the differential sorting of internalized GPCR either to recycling endosomes or lysosomes are still poorly understood but recent data pointed out the role of accessory proteins such as arrestins and the recently cloned GASP (GPCR-associated sorting protein) (33). By switching the C-terminal tail of β2-adrenergic and vasopressin V2 receptors, Oakley et al. (34) showed that the presence of serine/threonine residue clusters of the vasopressin V2 receptor allowed strong interactions with β-arrestins, and consequently, the co-internalization of receptors with arrestins would avoid a rapid recycling by trapping those complexes in intracellular compartments. In our cellular model, we observed that different kinases are involved in peptide and alkaloid agonist-induced hDOR desensitization. Accordingly, the different phosphorylation state of hDOR promoted by peptides and etorphine would modulate its affinity for β-arrestins. Upon peptide activation, the phosphorylated hDOR would form high affinity complexes with β-arrestins, which could be retained into endosomes and turned toward lysosomes. Conversely for the alkaloid agonist, the lack of co-internalization of hDOR with β-arrestins would mainly direct the receptor to the recycling pathway. Interestingly, Whistler et al. (33) discovered a new factor that acts as a post-endocytic sorting protein. Indeed, when this protein interacts with the C-terminal region of mDOR, it follows its degradation in lysosomes while in the case of the µ-opioid receptor, which weakly binds GASP, a major recycling process is observed after agonist treatment. The role of this protein should be explored in our cellular model.

In conclusion, we demonstrated that hDOR internalization and its sorting either to endosomes or lysosomes are important processes that participate in the regulation of cell responsiveness to opioid agonists. For the alkaloid agonist, hDOR internalization has a dual role: by reducing active receptors, internalization initiates desensitization, and by allowing its dephosphorylation and recycling, it reduces the rate of desensitization. While hDOR internalization is not the primary event responsible for peptide-induced desensitization, it potentiates this process by promoting its degradation into lysosomes. Our data demonstrate for the first time that the same receptor could be differentially targeted to endosomes or lysosomes by chemically distinct opioid agonists. The poor propensity of a given agonist to induce tolerance would be linked to its ability to promote opioid receptor internalization and recycling as it was proposed by Finn and Whistler (35). Indeed, they showed that the “cellular tolerance” to morphine was higher in cells expressing a chimeric µ receptor targeted to lysosomes (D MOR) compared with those expressing R MOR, a mutant that recycled following its activation. In this study, we came to the same conclusions as Finn and Whistler (35), showing that opioid receptor desensitization was linked to receptor degradation.

Acknowledgments—We thank Drs. Brigitte Sola (Université de Caen, France) and Florence Noble (Université de Paris V, France) for critical reading of the manuscript. We also thank Prof. M. Bouvier (Université de Montréal, Canada) for generously providing pDNAS-FLAG-tagged hDOR, Dr. T. Kobayashi (Supra-Biomicolecular Research Group, RIKEN, Hirosawa, Japan) for the generous gift of lysobiosphophatic acid antibody, and Prof. A. Buissin (CNRS UMR 6551, Caen, France) for technical assistance on confocal microscopy.

REFERENCES
1. Pasternak, G. W. (1993) Clinical Pharmacol. Ther. 63, 1–18
2. Law, P. Y., Wong, Y. H., and Lo, H. H. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 389–430
3. Allouche, J., Polastron, J., and Jauzac, P. (1996) J. Neurochem. 66, 2461–2470
4. Hasbi, A., Allouche, S., Michelet, F., Stanislas, L., Massotte, D., Landemore, G., Polastron, J., and Jauzac, P. (2000) J. Pharmacol. Exp. Ther. 293, 257–264
5. Ko, J. L., Arvidsson, U., Williams, F. G., Law, P. Y., Elde, R., and Loh, H. H. (1999) Mol. Brain Res. 69, 171–185
6. Koch, T., Schulz, S., Schröder H., Wolf, R., Rauf, E., and Holt, V. (1998) J. Biol. Chem. 273, 13652–13657
7. Hasbi, A., Polastron, J., Allouche, S., Stanislas, L., Massotte, D., and Jauzac, P. (1998) J. Neurochem. 70, 2129–2138
8. Sibley, D. R., and Leffkowitz, R. J. (1985) Nature 317, 124–129
9. Trejo, J., Hammes, S. R., and Coughlin, S. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 13098–13072
10. Tsao, P. I., and von Zastrow, M. (2000) J. Biol. Chem., 275, 11130–11140
11. Ichiyama, T., Maehara, N., Ishii, T., Nakamura, Y., Nakamura, Y., and Kuriyama, H. (1999) J. Biol. Chem. 274, 13525–13534
12. Polastron, J., Mur, M., Maazarguil, H., Puget, A., Meunier, J. C., and Jauzac, P. (1994) J. Neurochem. 62, 898–906
13. Allouche, S., Hasbi, A., Ferey, F., Sola, B., Jauzac, P., and Polastron, J. (2000) Biochim. Biophys. Acta, 915–925
14. Namir, N., Polastron, J., Allouche, S., Hasbi, A., and Jauzac, P. (1997) J. Neurochem. 68, 1764–1772
15. Allouche, S., Roussel, M., Marie, N., and Jauzac, P. (1999) Eur. J. Pharmacol. 371, 235–240
16. Allouche, S., Polastron, J., Hasbi, A., Homburger, V., and Jauzac, P. (1999) Biochem. J. 342, 71–78
17. McPherson, G. A. (1985) J. Pharmacol. Methods, 14, 213–228
18. Kobayashi, T., Stang, E., Fang, Y., de Moorhouse, P., Parton, R. G., and Grunenberg, J. (1998) Nature 392, 193–197
19. Ferguson, S. G. S. (2001) Pharmacol. Rev. 53, 1–4
20. Raynor, K., Kong, H., Hines, J., Kong, G., Benezre, A., Yasuda, K., Bell, G. I., and Reisine, T. (1994) J. Pharmacol. Exp. Ther. 270, 1381–1386
21. Zhu, J., Luo, L., Yao, C., Ashby, B., and Liu-Chen, L. Y. (1998) J. Pharmacol. Exp. Ther. 285, 28–36

S. Allouche, N. Marie, A. Hasbi, and P. Jauzac, manuscript in preparation.
22804

Differential hDOR Targeting by Peptide and Alkaloid Agonists

22. Joseph, D. B and Bidlack, J. M. (1995) *J. Pharmacol. Exp. Ther.* **272**, 970–976
23. Bot G., Blake A. D., Li S., and Reisine, T. (1997) *Mol. Pharmacol.* **52**, 272–281
24. Prather, P. L., Tsai, A. W., and Law, P. Y. (1994) *J. Pharmacol. Exp. Ther.* **270**, 177–184
25. Pak., Y., Kouvelas, A., Scheideler, M. A., Rasmussen, J., O'Dowd, B. F., and George, S. R. (1996) *Mol. Pharmacol.* **50**, 1214–1222
26. Bunemann, M. and Hosey, M. M. (1999) *J. Physiol.* **517**, 5–23
27. Jockers, R., Angers, S., Da Silva, A., Benarech, P., Struberg, A. D., Bounier, M., and Marullo, S. (1999) *J. Biol. Chem.* **274**, 28900–28908
28. Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L., and Liu-Chen, L. Y. (1999) *J. Biol. Chem.* **274**, 12087–12094
29. De Weerd, W. F., and Leeb-Lundberg, L. M. (1997) *J. Biol. Chem.* **272**, 17858–17866
30. Ishizaka, N., Griendling, K. K., Lassegue, B., and Alexander, R. W. (1998) *Hypertension* **32**, 459–466
31. Okamoto, Y., Ninomiya, H., Miwa, S., and Masaki, T. (2000) *J. Biol. Chem.* **275**, 6439–6446
32. Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001) *J. Biol. Chem.* **276**, 12345–12355
33. Whistler, J. L., Enquist, J., Marley, A., Feng, J., Gladher, F., Tsuruda, P., Murray, S. R., and von Zastrow, M. (2002) *Science* **5581**, 615–620
34. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *J. Biol. Chem.* **274**, 32248–32257
35. Finn, A. K., and Whistler, J. L. (2001) *Neuron* **325**, 829–839
Differential Sorting of Human δ-Opioid Receptors after Internalization by Peptide and Alkaloid Agonists
Nicolas Marie, Isabelle Lecoq, Philippe Jauzac and Stéphane Allouche

J. Biol. Chem. 2003, 278:22795-22804.
doi: 10.1074/jbc.M300084200 originally published online April 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300084200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 19 of which can be accessed free at http://www.jbc.org/content/278/25/22795.full.html#ref-list-1