Internalization and Recycling of δ-Opioid Receptor Are Dependent on a Phosphorylation-Dephosphorylation Mechanism

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Accepted for publication January 11, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

Internalization, recycling, and resensitization of the human δ-opioid receptor (hDOR) were studied in the neuroblastoma cell line SK-N-BE, endogenously expressing this receptor. Conventional and confocal fluorescence microscopy observations, corroborated by Scatchard analysis, indicated that after a 100 nM Eto treatment, 60 to 70% of hDOR were rapidly internalized ($t_{1/2} < 15$ min). This agonist-triggered internalization was reversible for a treatment not exceeding 1 h and became irreversible for prolonged treatment (4 h), leading probably to the degradation and/or down-regulation of the receptor. The rapid internalization of hDOR was totally blocked in the presence of heparin, known as an inhibitor of G protein-coupled receptor kinases (Benovic et al., 1989), a result indicating that phosphorylation of the internalized hDOR by okadaic acid totally suppressed its recycling to the plasma membrane and its subsequent resensitization. These results indicate that regulatory events leading to desensitization, internalization, and recycling in a functional state of hDOR involve phosphorylation by a G protein-coupled receptor kinase, internalization via clathrin-coated vesicles, and dephosphorylation by acid phosphatases.

The opioid receptors are well characterized by pharmacological (Lord et al., 1977; Chang and Cuétricasas, 1979; Kosterlitz et al., 1981; Meunier et al., 1983; Loh and Smith, 1990) and molecular studies (Evans et al., 1992; Kieffer et al., 1992; Yasuda et al., 1993; Wang et al., 1993). They belong to the G protein-coupled receptor (GPCR) superfamily and their three major types, namely, µ, δ, and κ (for review, see Fowler and Fraser, 1994) are coupled with many effectors such as adenyl cyclase (Kosiki et al., 1982), Ca$^{2+}$, and K$^+$ channels (Seward et al., 1989, William and North, 1984), phosphatidylinositol pathway (Jin et al., 1994), and are able to mobilize the Ca$^{2+}$ intracellular stores (Jin et al., 1994, Allouche et al., 1996). A repeated or sustained treatment by opioid agonists elicits their desensitization and reduces their ability to modulate the second messengers, as described for cAMP accumulation (Law et al., 1983), Ca$^{2+}$ voltage-dependent channel (Nomura et al., 1994), and K$^+$ channels (Mestek et al., 1995).

The mechanisms of the desensitization of other GPCRs have been extensively studied, such as for the β2-adrenergic receptors (Hausdorff et al., 1990; von Zastrow and Kobilka, 1992; Moore et al., 1995), the α2-adrenergic receptors (Kurose and Lefkowitz, 1994), the A1 adenosine receptors (Ciruela et al., 1997), or the m2-muscarinic receptors (Richardson et al., 1999; Pals-Rylaasm et al., 1997; Schlador and Nathanson, 1997). Based on extensive observations, the desensitization of such heptahelical receptors seems to involve at least two mechanisms: 1) the phosphorylation of the receptor itself, a covalent modification that initiates the uncoupling between the receptor and its associated G protein(s) blocking thus the transduction pathway; and 2) the reduction of the receptor number at the cell surface, either by sequestration (internalization), which involves the receptor endocytosis, or by down-regulation which includes the receptor sequestration and degradation.

Mechanisms of the opioid receptor desensitization also were extensively studied and, in human embryonic kidney 293 cells transfected with the δ-opioid receptor (DOR) cDNA,
Pei et al. (1995) have shown that desensitization was dependent on the activity of G protein-coupled receptor kinase (GRK) 2, a member of the GRK family. Identical observations for the μ-opioid receptor (MOR) transfected in Chinese hamster ovary cells were reported by Yu et al. (1997) and for the κ-opioid receptor in guinea pig hippocampal slices (Appleyard et al., 1997).

In our laboratory, we have demonstrated that the human δ-opioid receptor (hDOR) desensitized rapidly in human neuroblastoma SK-N-BE cells (Namir et al., 1997; Hasbi et al., 1998; Allouche et al., 1999), a cell line endogenously expressing a relatively large amount (200–300 fmol/mg protein) of the hDOR (Polastron et al., 1994). Such a model seems more suitable than transfected cells to study either the function or the transduction and the desensitization mechanisms of the hDOR because it permits to avoid many artifacts due to the overexpression of the receptor. In this model, we have recently shown that phosphorylation by a GRK was involved in its rapid agonist-induced desensitization (Hasbi et al., 1998). Furthermore, the receptor desensitization was reversible and the blockade of protein phosphatases I and IIa by okadaic acid (OA) abolished this reversibility, suggesting that phosphorylation and desensitization were tightly associated and that dephosphorylation of the receptor was required for its functional recovery (i.e., resensitization) as it was studied for β-adrenergic receptor (Ferguson et al., 1997; Ferguson and Caron, 1998).

Recently, it was reported that the agonist-induced reduction of the opioid receptors at cell surface was due to their internalization (Arden et al., 1995; Chakrabarti et al., 1997; Gaudriault et al., 1997; Segredo et al., 1997), but, and in fact some indirect data, as will be discussed, the putative relationships between the agonist-induced phosphorylation of the opioid receptor and its sequestration have not been clearly established. Furthermore, the function of this agonist-induced internalization remains unclear.

In this study, we investigated the links between GRK-dependent phosphorylation of the hDOR and its sequestration and between its endosomal dephosphorylation and its recycling. We show that desensitization occurs even in conditions impairing internalization. Furthermore, the sequestration of hDOR, in clathrin-coated pits, is dependent on phosphorylation by a GRK, whereas the receptor recycling in a functional state requires the integrity of the phosphatases I and/or IIa activities.

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**Fig. 1.** Internalization of hDOR visualized by confocal fluorescence microscopy. SK-N-BE cells were treated as described in Materials and Methods and observed with a fluorescence microscope at ×63 lens. A, control cells; B, cells pretreated with 100 nM Eto for 30 min.
Fig. 2. Cross sections of internalized hDOR visualized by confocal microscopy. A, untreated SK-N-BE cells; B, cells were treated for 30 min with 100 nM Eto before permeabilization and immunostaining with anti-hDOR antibody. Cross sections of the cells were performed following the horizontal and vertical axis (indicated by arrows) to show the precise localization of the immunostaining in both experimental conditions; A1 and B1: cross sections following horizontal axis of naive and 100 nM Eto treated cells, respectively; A2 and B2: cross sections following the vertical axis of naive and 100 nM Eto treated cells, respectively.
Materials and Methods

Cell Culture. Human neuroblastoma SK-N-BE cells (Biedler and Spengler, 1976; Biedler et al., 1978) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% antibiotic-antimycotic solution (penicillin, 1000 U/ml; streptomycin, 10 mg/ml; amphotericin B, 25 μg/ml; Sigma Chemical Co., St. Louis, MO). The cells were allowed to grow at 37°C in a humidified atmosphere of 5% CO₂, 95% air. At 90% confluence, they were harvested in buffer D (137 mM NaCl, 3 mM KCl, 6 mM NaHPO₄, 15 mM KH₂PO₄, and 0.5 mM EDTA; pH 7.5) and dissociated with a Pasteur pipette.

Production of Anti-hDOR-NH₂ Terminus Antibody. Polyclonal anti-hDOR antibody was raised by two of the present authors (L.S. and D.M.) and used for δ-opioid receptor characterization (Hasbi et al., 1998). Briefly, amino acids 1 to 52 of the hDOR were cloned in pGE2X2TK (Pharmacia, Piscataway, NJ) as a COOH-terminal fusion to glutathione-S-transferase. The sequence encoding the N terminus of hDOR was amplified by polymerase chain reaction (PCR) using primers described, before [3H]diprenorphine binding: in cells not washed with buffer D (137 mM NaCl, 3 mM KCl, 6 mM NaHPO₄, 15 mM KH₂PO₄, and 0.5 mM EDTA; pH 7.5) and dissociated with a Pasteur pipette.

Radioligand-Binding Studies. Radioligand-binding studies were performed on attached cells. SK-N-BE cells were seeded in 24-well plates at a density of 150,000 cells/well and allowed to grow for 48 h. Plates were rinsed twice (PBS/0.2% BSA), and the cells were incubated for 30 min at 37°C, with appropriate concentrations of [3H]diprenorphine (0.05 to 5 nM) in the presence (unspecific binding) or in the absence (total binding) of 10 μM leverehanol, in a 0.5-ml final volume. The cells were washed (twice with PBS/0.2% BSA and once with PBS), then harvested in 250 μl of NaOH (0.01 N), and placed into vials in the presence of 3 ml of a scintillation cocktail (PicoFluor-40; Packard, Meriden, CT). The 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.

For sequestration studies, the cells were treated with 100 nM etorphine (Eto) for 15 min, 30 min, and 1, 2, and 4 h; rinsed twice with cold PBS/0.2% BSA; and subsequently used for [3H]diprenorphine binding as described. Control experiments were performed to test the ability of washing procedure with PBS/0.2% BSA to eliminate residual Eto and show that diprenorphine binding in resensitized cells was the same as in naive cells. In other experiments, cells were incubated for 10 min (to avoid internalization) in the presence of 10 nM Eto and washed twice in the conditions previously described, before [3H]diprenorphine binding: in cells not washed with PBS, the binding of the titrated antagonist was not detectable, whereas in washed cells it was of the same order of magnitude as in naive cells.

For resensitization/recycling experiments, the cells were desensitized as described above, extensively washed with PBS/0.2% BSA, allowed to resensitize for 1 h in fresh culture medium and in the absence or in the presence of 10 nM OA, and then used for [3H]diprenorphine binding. The Kᵦ and B₂max were calculated with Radlig software.

Immunocytochemistry. Cells were grown on glass coverslips in 6-well plates until 20 to 40% confluence. They were wshed twice with cold PBS/0.2% BSA and treated with 100 nM Eto for various times (15 and 30 min, and 1, 2, and 4 h). Then they were washed three times with PBS/0.2% BSA and fixed with freshly prepared 4% paraformaldehyde. To block the remaining reactive aldehyde groups, the cells were washed with 0.02 M glycine. The permeabilization of cells was carried out in the presence of 0.1% saponin in PBS for 5 min. After nonspecific sites blockade (1 h in presence of PBS/0.2% BSA), the fixed cells were washed with PBS and incubated with a polyclonal primary antibody raised against the N terminus 52 amino acid of hDOR and diluted at 1:200 in PBS. The secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit IgG; Sigma Chemical Co.), diluted at 1:200 in PBS, was applied. The incubation was carried out in darkness for 2 h at room temperature. After washing, the cells were incubated for 4% paraformaldehyde. For confocal microscopy experiments, the functional integrity of the hDOR was controlled by measuring its ability to inhibit the cAMP accumulation elicited by 40 μM forskolin with the method described by Alvarez and Daniels (1992), slightly modified in our laboratory (Hasbi et al., 1998).

Results

Sequestration of hDOR. We visualized the redistribution of the δ-opioid receptors, before and after 100 nM Eto treatment, with the anti-hDOR as primary antibody for immunofluorescence detection. In untreated SK-N-BE cells (control), a bright staining restricted to the cell surface was observed, suggesting that most of the receptors were localized at the plasma membrane (Fig. 1A). The 100 nM Eto treatment induced a rapid modification of the hDOR localization, characterized by a dramatic reduction of the cell...
surface staining and the advent of an important cytoplasmic labeling, detectable after only 15 min of treatment, suggesting an internalization of hDOR. This effect reached a maximum after 30 min of exposure to the agonist (Fig. 1B). This agonist-induced sequestration was antagonized by 1 μM naloxone, demonstrating that it was an opioid agonist-specific effect (data not shown).

To eliminate the eventuality of misinterpretation due to the depth of field and to show the precise localization of the immunostaining of hDOR, cross sections of the cells were performed by confocal microscopy (Fig. 2), following a horizontal (arrow 1) and a vertical (arrow 2) axis. Thus, in naive cells (Fig. 2A), cross sections (A1-A2) demonstrate that hDOR are localized at plasma membrane. After 100 nM Eto-treatment (Fig. 2B), hDOR is internalized as shown by cross sections (B1-B2).

The morphological observations of this sequestration closely paralleled with binding data because Scatchard analysis showed that the [3H]diprenorphine $B_{\text{max}}$ decreased to a maximum of 30 to 40% of control values after 30 min of exposure to Eto and that no further decrease occurred after 4 h of agonist treatment (Fig. 3). According to these results, the following experiments were carried out at indicative times, 30 min (for monitoring rapid sequestration) and 4 h (for studying long-term sequestration).

Effect of Sucrose and Concanavalin A on hDOR Sequestration. Hypertonic sucrose and concanavalin A are known to inhibit the sequestration of some GPCRs involving a clathrin-coated vesicles mechanism (for review, Ferguson et al., 1997). The results summarized in Fig. 4 show that, in the presence of 0.5 M sucrose, the rapid sequestration of the δ-opioid receptors was abolished (Fig. 4A–C) because, after a

Fig. 4. Effects of hypertonic sucrose on hDOR internalization. hDOR was visualized by fluorescence microscopy in control cells (A) and cells treated for 30 min with 100 nM Eto, in the absence (B) or in the presence (C) of 0.5 M sucrose. Scatchard plots (D) were performed with untreated cells in the absence (●) or in the presence (○) of 0.5 M sucrose and with cells treated for 30 min with 100 nM Eto in the absence (▲) or in the presence (△) of 0.5 M sucrose. Data are representative of three identical experiments, each determination being realized in triplicate.
Effects of Sucrose on hDOR Desensitization and Resensitization. As shown on Fig. 6, hypertonic sucrose (0.5 M) was devoid of any significant effect on desensitization of the hDOR. Thus, the loss of inhibition of \(^{3}H\)cAMP accumulation, elicited by treatment with 100 nM Eto for 30 min, was in the same order of magnitude either in the absence or in the presence of 0.5 M sucrose (≈50%). When the cells were allowed to resensitize by a further incubation in fresh medium for 1 h in the absence of Eto, a striking difference was observed: cells treated in the absence of sucrose resensitized almost completely, whereas resensitization was impaired in the presence of hypertonic sucrose, suggesting that sequestration was a key-step for resensitization but not for desensitization.

Sequestration of hDOR and Phosphorylation. In this series of experiments, the agonist pretreatment was carried out in the presence of low \(M_{f}\) (≈6000) heparin. Heparin was allowed to enter the cells by preincubating the cultures overnight in the presence of 1 \(\mu\)M heparin together with 5 \(\mu\)g/ml lipofectin before agonist exposure. Heparin by itself, in the presence of lipofectin, did not affect the binding of \(^{3}H\)diprenorphine (data not shown) and, as shown in Fig. 7, the inhibition of GRK by heparin in these conditions (Fig. 7D) blocked totally the \(\delta\)-opioid receptor internalization (Fig. 7, B and C) in the SK-N-BE cells as visualized by confocal fluorescence microscopy (Fig. 7, A–D).

Resensitization/Recycling of hDOR. After exposure to 100 nM Eto for 30 min, the agonist was removed and cells were further incubated in fresh culture medium for 1 h at 37°C to allow recycling of the receptor to the cell surface and resensitization. In these conditions, the sequestration observed after 30 min of agonist treatment (Fig. 8B) appeared to be reversible and, as shown in Fig. 8C, the inhibition of GRK by heparin in these conditions (Fig. 7D) blocked totally the \(\delta\)-opioid receptor internalization (Fig. 7, B and C) in the SK-N-BE cells as visualized by confocal fluorescence microscopy (Fig. 7, A–D).

To investigate a putative relationship between the phosphorylation of the \(\delta\)-opioid receptor and its recycling to plasma membrane, the resensitization/recycling experiments were carried out in the presence or in the absence of 10 nM OA, known as a protein phosphatases I and IIA inhibitor (Cohen et al., 1990). Figure 8, D and E show that, for short period of agonist treatment (30 min), the recycling of the hDOR was abolished in the presence of the phosphatase inhibitor (Table 1), the receptors remaining internalized. This result demonstrates that the phosphatase activity is required for the receptor recycling at the cell surface. As shown in Table 1, OA by itself had no significant effect on \(^{3}H\)diprenorphine binding to the hDOR.

In contrast, when Eto treatment was sustained during 4 h the receptors remained internalized, even in the absence of OA during resensitization (Fig. 9; Table 1), the sequestration becoming thus irreversible after long-term exposure to the agonist. As shown in Table 2, recycling of hDOR to the plasma membrane was accompanied by restoration of its function: its ability to inhibit the forskolin-stimulated cAMP production.
accumulation; the values becoming again close to those observed in naive cells.

Discussion

In this study, we examined the internalization of the hDOR, naturally expressed in neuroblastoma SK-N-BE cells (Polastron et al., 1994), and we also investigated the recycling and resensitization of the receptor. We particularly focused our study on possible relationships between the phosphorylation/dephosphorylation state of the receptor and its desensitization, its internalization, and its recycling/resensitization.

Our results show clearly that a 100 nM Eto pretreatment leads to a rapid ($t_{1/2} = 10$–15 minutes) and specific internalization of hDOR, as visualized by confocal immunocytochemistry and corroborated by a drastic decrease of $\text{[3H]}\text{diprenorphine-specific binding}$. In treated cells, receptors seem to lose their uniform distribution at the cell surface. Such clusters also were observed with transfected opioid receptors (Gaudriault et al., 1997) and are usually related to receptor oligomerization during the desensitization step. This agonist-induced internalization was blocked by naloxone and was inhibited either by hypertonic sucrose or by concanavalin A pretreatment. Such treatments are currently used to demonstrate the clathrin-dependent internalization of many other receptors belonging to the GPCR family (for review, see Ferguson et al., 1997; Koenig and Edwardson, 1997; Krupnick and Benovic, 1998). These findings are in good agreement with recent studies, carried out in transfected systems, indicating that $\delta$-opioid receptors (Trapaidze et al., 1996; Gaudriault et al., 1997), $\mu$-opioid receptors (Arden et al., 1995; Sternini et al., 1996; Gaudriault et al., 1997; Keith et al., 1998), or $\kappa$-opioid receptors (Zhu et al., 1998) underwent a rapid and agonist-promoted internalization. In most cases, the agonist-induced internalization of opioid receptors occurred rapidly ($t_{1/2} < 15$ minutes) via clathrin-coated vesicles because they were blocked either by hypertonic sucrose treatment (Trapaidze et al., 1996; Segredo et al., 1997), phenylarsine oxide treatment, or low temperature (0°C) (Gaudriault et al., 1997). Nevertheless, morphine was demonstrated to be unable to trigger either $\mu$- or $\delta$-opioid receptor sequestration (Arden et al., 1995; Keith et al., 1998; Zhang et al., 1999). This apparent discrepancy could be because morphine is unable to maintain the receptor in a conformation allowing its internalization. A constitutive sequestration, in the absence of agonists, was even observed with $\mu$-opioid receptor mutated at a Ser/Thr-rich domain of the C terminus of MOR (Trunc354), which could be a negative regulator of its internalization (Segredo et al., 1997).

Whereas the agonist-promoted receptor phosphorylation is now clearly established as a mechanism triggering the receptor desensitization (Arden et al., 1995; Pei et al., 1995; Hasbi et al., 1998), a possible role of this phosphorylation as a preliminary step preceding receptor internalization remains more controversial. Directed mutagenesis studies indicated that the phosphorylation of $\mu$-opioid receptor expressed in Neuro 2a cells (Burd et al., 1998) or of $\delta$-opioid receptor expressed in human embryonic kidney 293 cells (Murray et al., 1998) is not required in their agonist-induced internalization. In contrast, other studies have demonstrated that the truncation of the carboxyl-terminus tail of DOR, and point-mutation within this cytoplasmic tail, which contains putative phosphorylation sites, reduced the rate of internalization and/or down-regulation of the receptor (Trapaidze et al. 1996; Afify et al., 1998), suggesting that phosphorylation of one or several of these amino acids was a crucial step in the agonist-promoted internalization of opioid receptors. Another argument for the involvement of the phosphorylation in the internalization process comes from the difference observed between MOR1 and MOR1b. These receptor subtypes, which

Fig. 7. Effects of heparin on hDOR internalization. hDOR were visualized by confocal fluorescence microscopy in naive SK-N-BE cells (A), and in cells pretreated for 30 min (B) or 4 h (C) with 100 nM Eto. Cells (D) were pretreated for 30 min with 100 nM Eto after an overnight preincubation with 1 $\mu$M, low $M_r$ ($\sim$6000) heparin in the presence of 5 $\mu$g/ml lipofectin.
differ in their carboxyl-terminus tail lengths, display different kinetics of desensitization, internalization, and recycling (Zimprich et al., 1995; Koch et al., 1998). These differences have been related to the absence of a putative site of phosphorylation in the carboxyl-terminus tail of MOR1b. In agreement with this suggestion, we report herein that the phosphorylation of hDOR is required, not only for receptor desensitization as previously demonstrated (Hasbi et al., 1998) but also for its internalization because heparin blocks the receptor sequestration. Heparin is known as an inhibitor of GRK family (Benovic et al., 1989) and more precisely as an inhibitor of GRK2 whose catalytic domain contains a binding site for heparin (Palczewski, 1997). Preliminary data indicate that GRK2 is the unique member of GRK family expressed by the SK-N-BE cell line and that it is rapidly translocated from cytosol to plasma membrane after agonist exposure (A.H., S.A., F.S., L.S., D.M., G.L., J.P., and P.J., unpublished data). These results strongly suggest that GRK2 is the key kinase involved in the desensitization/internalization process of the human δ-opioid receptor in this cell line. This hypothesis, under investigation in our laboratory with negative dominant mutants of GRK2, is in good agreement with the recent observations of Zhang et al. (1999) in transfected cells.

For agonist exposure not exceeding 1 h, the internalization of hDOR is clearly reversible after agonist removal. Thus,

**Fig. 8.** Recycling of hDOR after 30 min of 100 nM Eto pretreatment. hDOR was visualized by fluorescence microscopy in naive SK-N-BE cells (A), in cells pretreated for 30 min with 100 nM Eto (B), and in cells allowed to resensitize for 1 h in fresh medium in the absence (C) or in the presence (D) of 10 nM OA. Scatchard plots (E) were performed with naive cells (○), with cells pretreated for 30 min with 100 nM Eto (△), and in cells allowed to resensitize in the absence (▲) or in the presence (●) of 10 nM OA.

**TABLE 1**

Parameters of [3H]diprenorphine binding to the δ-opioid receptor on attached SK-N-BE cells

<table>
<thead>
<tr>
<th></th>
<th>$K_d$</th>
<th>$B_{max}$</th>
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<tbody>
<tr>
<td></td>
<td>nM</td>
<td>fmol/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>1.35 ± 0.14</td>
<td>160 ± 30</td>
</tr>
<tr>
<td>Eto 30 min</td>
<td>1.18 ± 0.15</td>
<td>64.5 ± 10*</td>
</tr>
<tr>
<td>Eto 4 h</td>
<td>0.81 ± 0.25</td>
<td>48 ± 8.5*</td>
</tr>
<tr>
<td>Control + OA</td>
<td>1.41 ± 0.12</td>
<td>170 ± 25</td>
</tr>
<tr>
<td>Eto 30 min Res</td>
<td>1.22 ± 0.12</td>
<td>125 ± 17</td>
</tr>
<tr>
<td>Eto 30 min Res + OA</td>
<td>0.96 ± 0.34</td>
<td>75.5 ± 12*</td>
</tr>
<tr>
<td>Eto 4 h Res</td>
<td>1.14 ± 0.31</td>
<td>79 ± 31*</td>
</tr>
<tr>
<td>Eto 4 h Res + OA</td>
<td>0.98 ± 0.18</td>
<td>62 ± 7*</td>
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* $P < .05$, significantly different from control values (t test).
receptors are able to return to the plasma membrane (i.e., recycling) in a functional state (i.e., resensitization), as shown by immunocytochemistry, binding studies, and cAMP accumulation measurement. These recycling and resensitization processes are dependent on acid phosphatase activity because they are blocked by OA, known as an acid phosphatase inhibitor (Cohen et al., 1990). Okadaic acid, by itself, has no effect either on the agonist-receptor binding or on the cell viability examined by functional studies (cAMP accumulation) and cytomorphological observations (normal shapes and trypan-blue exclusion).

Furthermore, hypertonic sucrose treatment, blocking hDOR internalization without affecting the receptor desensitization, blocks receptor resensitization. These results suggest that the internalization is not responsible per se of receptor desensitization, but could be viewed as an important step carrying the receptor in an intracellular compartment containing the appropriate phosphatases and allowing it to return to the plasma membrane in a dephosphorylated and a functional state.

Finally, when Eto treatment was prolonged for 4 h, the agonist removal was no longer able to promote hDOR recycling and resensitization, suggesting that a supplementary process, such as receptor degradation, could occur. Such a long-time regulation was proposed to explain the β2-adrenergic and m2-muscarinic receptor down-regulation (Gagnon et al., 1998; Tsuga et al., 1998). This hypothesis is under investigation in our laboratory and could involve more complex regulatory pathways such as increasing activity of adenylyl cyclase and PKA or MAPK (A.H., S.A., F.S., L.S., D.M., G.L., J.P., and P.J., unpublished data).

Collectively, our results indicate that agonist-promoted and GRK-mediated phosphorylation of hDOR leads not only to the receptor desensitization (Hasbi et al., 1998) but also to its internalization (this study). They have been obtained with
δ-opioid receptor endogenously expressed by a human cell line and could be representative of a “physiological” regulation. In transfected systems, contradictory data could be the consequence of artificial modifications of the stoichiometrical ratio between the different components of the regulatory pathways or a consequence of differences in the rate of expression of proteins, such as GRK or arrestins, depending on the nature of the host cell. In SK-N-BE cells, the agonist-promoted internalization of hDOR occurs in two separate steps. First, hDOR internalizes in early endosomes via a clathrin-mediated mechanism. Its dephosphorylation by acid phosphatases allows its recycling to the plasma membrane and its resensitization (i.e., functional state recovering). This internalization, by itself, is not responsible for receptor desensitization but, in contrast, seems to be a key step leading to hDOR resensitization. Under prolonged exposure to the agonist, the internalization becomes an irreversible process because neither the recycling nor the resensitization of hDOR occurred. Probably, for longer duration of agonist treatment, the hDOR is targeted to another intracellular compartment and/or down-regulated.

References


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