Dimerization of the δ Opioid Receptor: IMPLICATION FOR A ROLE IN RECEPTOR INTERNALIZATION

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Dimerization of G-protein-coupled receptors has been increasingly noted in the regulation of their biological activity. However, its involvement in agonist-induced receptor internalization is not well understood. In this study, we examined the ability of mouse δ-opioid receptors to dimerize and the role of receptor dimerization in agonist-induced internalization. Using differentially (Flag and c-Myc) epitope-tagged receptors we show that δ-opioid receptors exist as dimers. The level of dimerization is agonist dependent. Increasing concentrations of agonists reduce the levels of dimer with a corresponding increase in the levels of monomer. Interestingly, morphine does not affect the levels of either form. It has been shown that morphine, unlike other opioid agonists, does not induce receptor internalization. This suggests a relationship between the ability of agonists to reduce the levels of dimer and to induce receptor internalization. The time course of the agonist-induced decrease of δ-opioid receptor dimers is shorter than the time course of internalization, suggesting that monomerization precedes the agonist-induced internalization of the receptor. Furthermore, we found that a mutant δ-opioid receptor, with a 15-residue C-terminal deletion, does not exhibit dimerization. This mutant receptor has been shown to lack the ability to undergo agonist-induced internalization. These results suggest that the interconversion between the dimeric and monomeric forms plays a role in opioid receptor internalization.

Short-term treatment with opioid ligands causes rapid loss of receptors from the surface of the cell as a result of the receptor endocytosis (5–7). Both of these effects require the intact C-terminal tail of the receptor (4, 7). Although deletion of the C-terminal tail substantially reduces the extent of both down-regulation and rapid internalization, point mutations within this region reduce the extent of internalization without affecting down-regulation, suggesting that these two responses are differentially regulated (7). Different opioid ligands induce different effects on rapid internalization of the opioid receptors. Morphine, unlike most of the opioid agonists, does not induce rapid internalization of the opioid receptors (5, 8). An exact mechanism of the opioid receptor internalization is not known, although it has been suggested that the rapid endocytosis of the receptors is mediated through the classic endocytic pathway (5, 7). Possible events that would precede the receptor internalization, such as the interaction of the receptor with an adapter protein or another receptor, have not yet been determined.

A number of pharmacological studies suggest the existence of opioid receptor dimers. Dimeric analogs of morphine and enkephalin exhibit higher affinity for δ- and µ-opioid receptors in membranes (9). They also have severalfold greater potency than their monomeric forms in the guinea pig ileum assay, suggesting the involvement of opioid receptor dimerization in their function. Apart from homodimerization, several pharmacological studies have suggested the existence of heterodimers between opioid receptor subtypes (10–12). Since µ-receptor ligands inhibit the binding of δ-ligands in both a competitive and noncompetitive manner, Rothman et al. (11, 12) have divided δ-receptors into two subtypes, those that are associated with µ-receptors and those that are not, further supporting the notion of opioid receptor dimerization.

The dimerization of growth factor receptors has been an extensively studied phenomenon (for review, see Ref. 13). Exposure to ligand induces the dimerization of receptors, leading to their autophosphorylation, a step that is necessary for the following intracellular signaling. The dimerization of GPCRs and the role of dimerization in the function of these receptors are not well understood. Most of the evidence suggesting the existence of GPCRs as dimers is from pharmacological studies (14–16). Studies with several GPCRs show that the co-expression of two mutant receptors, which individually do not bind or transduce signals, results in receptors that bind and transduce signals (14–16). These data imply that a functional complementation is achieved by intermolecular interaction between receptor molecules. By cross-linking and immunoprecipitation, Hebert et al. (17) have shown that the β2-adrenergic receptor can form homodimers and that a peptide derived from the transmembrane domain VI of the receptor inhibits homodimerization (17). The functional importance of dimerization is supported by the observation that this peptide also inhibits β2-adrenergic agonist-promoted stimulation of adenyl cyclase.

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activity in membranes (17). However, the possible role of dimerization in receptor internalization was not examined in these studies.

To date, there is no direct evidence for the existence of opioid receptor dimers or oligomers. In this study, we used cross-linking and immunoprecipitation of the tagged mouse δ-opioid receptor (tagged with either Flag or c-Myc epitope) to examine whether these receptors interact to form dimers. We also examined whether opioid peptides and alkaloid opiate agonists and antagonists affected the levels of receptor dimer. Furthermore, we determined the time course of the agonist-induced changes in the levels of dimer and compared it to the time course of the agonist-induced receptor internalization. Finally, we used a truncated mutant of the δ-opioid receptor to examine the role of the C-terminal tail in dimer formation. We found that δ-opioid receptors exist as dimers. The level of dimerization is agonist dependent, and an intact C-terminal tail of the δ-opioid receptor is required for receptor dimerization.

**Experimental Procedures**

**Generation of Cell Lines Expressing Wild Type and Mutant δ-Opioid Receptor**—The Flag epitope-tagged δOR was generated as described previously (4, 7). c-Myc epitope-tagged δ-opioid receptor (DOR) was generated by an overlapping extension polymerase chain reaction, using Flag epitope-tagged DOR cDNA as the template. The sequence encoding for the Flag epitope was replaced with the sequence encoding the c-Myc epitope (EQKLDSEEDLLR). The deletion mutant ΔC15 was generated using the polymerase chain reaction to amplify a region from Thr211 to Val350 of Flag epitope-tagged δ-opioid receptor that was subcloned into the pCDNA3 expression vector. Unique restriction enzyme sites were used to replace a corresponding region of the wild type receptor with the polymerase chain reaction amplification product. CHO cells stably expressing Flag epitope-tagged wild type or ΔC15 deletion mutant receptors were generated, and their binding affinities, coupling to adenyl cyclase, internalization, and down-regulation properties were characterized as described previously (4, 7). For the experiments with immunoprecipitation and Western blotting, CHO or COS cells were transfected with 20 μg of c-Myc and/or Flag-tagged DOR using the CaCl2-phosphate method (18). Cross-linking and immunodepletion were performed 48 h later as described below.

**Cross-linking**—Cells expressing either wild type or mutant δ-opioid receptor were incubated either with different doses of ligands for 10 or 30 min (see Fig. 2) or with the same dose of the ligand for different time periods (see Fig. 4) in F12 medium at 37 °C. The following ligands were used: [D-Ala2,D-Leu5]enkephalin (DADLE); [D-Ser2]Leu-enkephalin-Thr

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The rest of the text follows, discussing experimental procedures and results in detail.
resulted in the generation of a 40-kDa form and an 80-kDa form (Fig. 1A, +, +), suggesting that the interacting protein is another receptor and that the δ-opioid receptors exist as dimers. To examine whether the δ-opioid receptor dimerization was influenced by receptor expression level, cross-linking experiments were performed using CHO cells with 10-fold difference in expression levels. The ratio of the dimeric form to the monomeric form in cells with a lower expression level is similar to the ratio of these two forms in cells expressing high levels of receptor (Fig. 1B, MED and HI, respectively). These results indicate that in unstimulated cells, δ-opioid receptors exist both in monomeric and dimeric forms and that the ratio of these forms is independent of the level of receptor expression.

To directly examine the presence of δ-opioid receptor dimers, we used co-immunoprecipitation and Western blotting of differentially epitope-tagged δ-opioid receptors. Specifically, a δ-opioid receptor tagged with the c-Myc epitope (MDOR) was co-expressed with the Flag epitope-tagged receptor (FDOR) in CHO or COS cells. The receptors from the cells expressing both FDOR and MDOR were immunoprecipitated using anti-c-Myc antibody, subjected to SDS-PAGE, and immunoblotted with anti-Flag antibody. As seen in Fig. 1C, anti-Flag antibody detected dimeric forms only in cells expressing both MDOR and FDOR and not in cells expressing only FDOR or only MDOR. The dimeric forms were also detected in CHO cells expressing both MDOR and FDOR (not shown). A band of 65–70 kDa and a faint band of 100 kDa was present in COS cells expressing both FDOR and MDOR (Fig. 1C). The 65–70-kDa protein represents the receptor monomer form. The nature of the 100-kDa protein is not known and needs further investigation. It is possible that this band represents the receptor monomer complexed with a G-protein; the size of this band is consistent with such a notion. A nonspecific band of 57 kDa is detected in all three lanes; this represents cross-reactivity of the secondary antibody with IgG molecules in the immunoprecipitation mixture. The fact that MDOR and FDOR were co-immunoprecipitated as part of a dimer complex implies that the δ-opioid receptors exist as homodimers.

To examine whether the agonist modulates the levels of the dimeric form of δ-opioid receptor, cells were treated with various doses of DADLE for 10 min at 37 °C prior to cross-linking at 4 °C. The stimulation of cells with increasing doses of DADLE decreased the levels of δ-opioid receptor dimer and correspondingly increased the levels of monomer (Fig. 2A). This resulted in an agonist-induced decrease in the ratio of the dimeric form to the monomeric form. Maximal decrease of dimer to monomer ratio was caused by 1 μM DADLE, whereas 10 nM DADLE induced 50% reduction (Fig. 2B). Cells treated with increasing doses of DADLE in the presence of the antagonist naloxone did not show a change in the dimer to monomer ratio (not shown). Treatment of cells with DSLET, DPDEP, or etorphine substantially reduced the ratio of the dimeric form to the monomeric form, whereas DAMGO, a μ-opioid receptor selective agonist, did not change the level of either δ-opioid receptor form (Fig. 3). Treatment of the cells with naloxone alone did not affect the dimer to monomer ratio (Fig. 3). Taken together, these results suggest that the amount of δ-opioid receptors that exist in dimeric form can be modulated by either a peptide agonist or an alkaloid agonist and that this effect is reversible by the antagonist. Morphine, another opioid agonist, can bind to the δ-opioid receptor and induce its functional coupling to adenylyl cyclase (22, 23). Unlike other agonists, 10 μM morphine did not affect the levels of δ-opioid receptor dimer (Fig. 3). Morphine, at doses as high as 100 μM, is not able to induce rapid internalization of either δ- or μ-opioid receptor (5). This suggests that the ability of an agonist to induce interconversion of dimers to monomers is correlated with its ability to induce receptor internalization.

The time course of change in the levels of dimeric form upon treatment with 100 nM DADLE showed a time-dependent decrease in the levels of this form and a corresponding increase in the levels of the monomeric form (Fig. 4A). This decrease is rapid; with a 3-min treatment with DADLE, an approximately 50% decrease in the levels of dimer was seen (Fig. 4B). Longer exposure induced a further decrease in the dimer to monomer ratio. Cells treated for different time periods with 1 μM naloxone showed no change in the levels of the dimeric form (Fig. 4B).

Previously, it has been shown that the wild type δ-opioid receptor expressed in CHO and other cells shows rapid agonist-induced internalization (5, 7). The t½ of receptor internalization is dependent on the level of its expression. Cells with a low or medium level of the receptor expression exhibit agonist-induced internalization with a t½ of approximately 6–10 min, and cells with a high level of the receptor expression exhibit internalization with a t½ of approximately 30 min (Fig. 5). This is in contrast to the t½ of 3 min for the reduction of the dimer to monomer ratio (Fig. 5). These results show that treatment

FIG. 1. Detection of δ-opioid receptor dimers by immunoblotting and co-immunoprecipitation. A, the CHO cells expressing Flag epitope-tagged δ-opioid receptor were incubated in the absence (−) or presence (+) of 5 mM DSP as described under “Experimental Procedures.” 20 μg of the total cell proteins were resolved by 8% nonreducing SDS-PAGE, and the δ-opioid receptor was detected by Western blotting using M1 anti-Flag antibody. The shift in the size of two molecular forms due to deglycosylation by PNGaseF is shown in the right lane. Arrow points to the high molecular mass form and arrowhead to the low molecular mass form of δ-opioid receptor after deglycosylation. B, CHO-δ-opioid receptor clones with the 2,000,000 receptor/cell (HI) or 200,000 receptors/cell (MED) were incubated in the absence (−) or presence (+) of 5 mM DSP to examine the effect of the level of receptor expression on dimer formation. The positions and molecular masses (in kDa) of prestained standards (Sigma) are indicated on the left. C, COS cells transiently expressing FDOR alone, MDOR alone, or FDOR + MDOR were subjected to co-immunoprecipitation using A14 anti-c-Myc antibody as described under “Experimental Procedures.” Immunoprecipitates were resolved by 8% reducing SDS-PAGE, and co-immunoprecipitated FDOR was detected using M1 anti-Flag antibody. Co-immunoprecipitation of the FDOR can be seen only when FDOR and MDOR are co-expressed (right lane). Arrow points to the dimeric form and arrowhead to the monomeric form of the receptor. The blot is representative of four independent experiments; there was less than 10% variation between experiments.
with DADLE induces a decrease in the levels of dimeric form faster than it induces receptor internalization, suggesting that the monomerization of the receptor precedes its internalization.

We have previously reported that a 15-residue C-terminal deletion of the δ-opioid receptor substantially reduces the extent of agonist-induced internalization of the receptor (7). To further delineate a relationship between the levels of receptor dimerization and agonist-induced internalization, we examined the forms of receptor in CHO cells expressing this ΔC15 mutant. The treatment of cells with DSP did not result in a change in the levels of the dimeric form (Fig. 6). Furthermore, treatment with DADLE in the absence or in the presence of naloxone had no effect on the level of either of two forms (Fig. 6). The ratio of the dimeric form to the monomeric form in the ΔC15 mutant receptor-expressing cells treated with DSP was substantially lower than the ratio in the wild type receptor-expressing cells, and it remained same even when the mutant cells were treated with 1 μM DADLE. Taken together, these results suggest that the deletion of the 15 C-terminal amino acids prevents δ-opioid receptor dimer formation. The primary structure of the receptor shows no lysine in this region, excluding the possibility that lack of dimerization is due to deletion of the residue involved in cross-linking by DSP. The fact that the ΔC15 mutant exhibits a loss of agonist-induced changes in the levels of dimeric or monomeric form and a substantial reduction in agonist-induced internalization suggests that these two phenomena require structurally close molecular determinants of the receptor and that interconversion between the two receptor forms may have an important role in internalization. Furthermore, this result suggests that the existence of the receptor in the monomeric form is not sufficient for internalization.

**DISCUSSION**

In this study we found that in unstimulated cells, the δ-opioid receptors exist as dimers. The existence of GPCRs in dimeric form is suggested by many pharmacological studies. For example, co-expression of truncated β2ARs that individually do not transmit a signal resulted in formation of functional receptors (14). Similarly, co-expression of two different binding-defective point mutants of the angiotensin receptors restored binding activity (15). The existence of GPCR dimers is also shown by the use of two chimeric receptor molecules in which the C-terminal receptor domains were exchanged between the α1c-adrenergic receptor and the m3-muscarinic receptor; transfection of either of these receptors did not result in any detectable binding sites, whereas their co-expression resulted in the generation of a significant number of specific binding sites (16). The existence of high molecular mass forms of adrenergic, dopamine, and many other GPCRs in unstimulated cells has been also directly shown (17, 24–27). Unlike growth factor receptors (13), GPCRs exist in a dimeric form even in the absence of agonist treatment (as shown by this study and Refs. 24–27). This suggests that although dimerization may be important for the GPCR function, the exact role of dimers may be different in these two receptor families.

We found agonist-induced decrease in the level of δ-opioid receptor dimers. This effect was achieved with both peptide and alkaloid opioid agonists and it is antagonist reversible. Hebert *et al.* (17) have shown that agonist stimulation can stabilize the dimeric state of adrenergic receptor. The differences between these two findings could be due to the differences between these two receptors in the regulation of dimeric forms. Alternatively, these differences could be due to the different experimental systems used; whereas our studies were performed in the whole cell system, where cell signaling and other events that follow agonist binding are maintained, the studies with adrenergic receptor were performed with membranes (17).

Morphine does not induce changes in the levels of the dimeric form of the δ-opioid receptor or its internalization even when used in concentrations that are 100-fold higher than its *K*~D~ and EC~50~ values (5, 23). This finding shows a relationship between ability of an agonist to induce disappearance of dimers and to
induce receptor internalization. The time course of reduction in the level of dimers with a concomitant increase in monomers (t₁/₂ = 3 min) is shorter than the time course of internalization (t₁/₂ = 30 min), suggesting that the monomerization precedes internalization (Fig. 5). Furthermore, treatments that inhibit receptor internalization, such as potassium depletion or sucrose pretreatment (7), had no effect on agonist-induced reduction in the level of dimers, supporting the notion that monomerization precedes internalization. It is possible that binding of an agonist induces monomerization of the receptor, allowing interaction with adapter proteins involved in endocytosis or other proteins involved in signaling. The interconversion between dimeric and monomeric forms is agonist selective, and agonists (like morphine) that do not have the ability to induce this interconversion may also lack the ability to induce internalization.

The conformation of GPCRs has been shown to be important for their dimerization. Whereas transmembrane regions of adrenergic and dopamine receptors regulate their dimerization (17, 24), dimerization of the chimeric m3 muscarinic receptor depends on the length of the third intracellular loop (28). We found that the deletion of the 15 C-terminal residues of the δ-opioid receptor prevents its dimerization. A possible explanation for the inability of this mutant to dimerize is that it forms high molecular mass complexes (Fig. 6) by interacting differently with itself or with other proteins, due to the conformational change induced by the lack of a portion of the C-tail. Such a form of the receptor would be able to bind ligands and efficiently transmit the signal but would not be able to internalize because of its inability to bind to proteins involved in endocytosis. We found that this mutant receptor has the ability to efficiently couple to adenylyl cyclase and that it forms clusters on the cell surface in response to agonist treatment (7). A correlation between the lack of internalization and the increase in receptor clusters on the surface of the cell has been suggested by Hazum et al. (29–31). In these studies, it was shown

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2 S. Cvejic and L. A. Devi, unpublished observation.

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FIG. 4. Time course of agonist-induced changes in the level of δ-opioid receptor dimers. CHO cells expressing δ-opioid receptor were exposed to 100 nM DADLE (A), 100 nM DADLE (B), or 1 μM naltrexone (B) for the indicated time. Following treatment, cells were chilled, treated with the 5 mM DSP, and lysed. The lysates were subjected to SDS-PAGE under nonreducing conditions and to Western blotting as described under “Experimental Procedures.” The positions and molecular masses (in kDa) of prestained standards (Sigma) are indicated on the left. The blots from multiple experiments were densitized as described under “Experimental Procedures,” and the ratio of the dimer to the monomer was determined. The data represent mean ± S.E. from three to seven independent experiments.

FIG. 5. Comparison of the time course of agonist-induced changes in the dimer to monomer ratio with the time course of agonist-induced receptor internalization. The rate of change in the dimer to monomer ratio induced by 100 nM DADLE is compared with the rate of DADLE-induced δ-opioid receptor internalization, shown as a loss of surface fluorescence. The data for time course of internalization were obtained by flow cytometry as described previously (7).
that treatment of neuroblastoma cells with morphine or naloxone (ligands that cannot induce internalization) induces accumulation of clusters of opioid receptors, as detected by binding of rhodamine-enkephalin.

Although interconversion between dimeric and monomeric forms precedes internalization, the existence of the receptor in monomeric form appears not to be sufficient for its internalization. The C-terminal deletion mutant of δ-opioid receptor that exists primarily in the monomeric form is unable to undergo rapid agonist-mediated internalization, suggesting that additional steps are required for receptor internalization. These steps could be regulated by the signals determined by the secondary structure/conformation of the receptor that is involved in the interconversion between the dimeric and monomeric forms.

The finding that opioid receptors may exist in dimeric form opens additional ways of understanding the function of this receptor. δ-Opioid receptors have been classified into subgroups on the basis of their involvement in the modulation of μ-mediated antinociception (32, 33). On this basis, it has been suggested that some δ-opioid receptors form a functional complex with the μ-receptor, whereas others do not (for review, see Ref. 34). Chronic morphine treatment selectively up-regulates a subpopulation of δ-opioid receptors that is a part of the μ-δ complex (11, 12). The existence of a μ-δ receptor complex is also supported by ligand binding studies using membranes and sections of rat brain; the results show that the irreversible μ-antagonist, β-fumitrexamine, selectively alkylates the opiate receptor complex, changing the binding of either μ or δ agonists for the receptors that are part of the μ-δ complex (35, 36). Understanding possible differences in the affinity and efficacy of various agonists and antagonists for the monomeric and dimeric forms of the receptors and their differential ability to transmit signals can help explain the differences in the physiological responses they induce. The possibility of different opioid receptor subtypes forming heterodimers can help us to understand the phenomena in regions of the brain where two different subtypes of the receptors are co-expressed.

In conclusion, dimerization of GPCR is emerging as an important mechanism for the regulation of these receptors. Understanding the dimerization of opioid receptors can help us to understand the role of this form of interaction in the function of opioid receptors and of the family of GPCRs in general.

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