Heterodimerization of $\mu$ and $\delta$ Opioid Receptors: A Role in Opiate Synergy


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Opiate analgesics are widely used in the treatment of severe pain. Because of their importance in therapy, different strategies have been considered for making opiates more effective while curbing their liability to be abused. Although most opiates exert their analgesic effects primarily via $\mu$ opioid receptors, a number of studies have shown that $\delta$ receptor-selective drugs can enhance their potency. The molecular basis for these findings has not been elucidated previously. In the present study, we examined whether heterodimerization of $\mu$ and $\delta$ receptors could account for the cross-modulation previously observed between these two receptors. We find that co-expression of $\mu$ and $\delta$ receptors in heterologous cells followed by selective immunoprecipitation results in the isolation of $\mu$-$\delta$ heterodimers. Treatment of these cells with extremely low doses of certain $\delta$-selective ligands results in a significant increase in the binding of a $\mu$ receptor agonist. Similarly, treatment with $\mu$-selective ligands results in a significant increase in the binding of a $\delta$ receptor agonist. This robust increase is also seen in SKNSH cells that endogenously express both $\mu$ and $\delta$ receptors. Furthermore, we find that a $\delta$ receptor antagonist enhances both the potency and efficacy of the $\mu$ receptor signaling; likewise a $\mu$ antagonist enhances the potency and efficacy of the $\delta$ receptor signaling. A combination of agonists ($\mu$ and $\delta$ receptor selective) also synergistically binds and potentiates signaling by activating the $\mu$-$\delta$ heterodimer. Taken together, these studies show that heterodimers exhibit distinct ligand binding and signaling characteristics. These findings have important clinical ramifications and may provide new foundations for more effective therapies.

Key words: receptor subtypes; agonist; antagonist; enkephalin; Deltorphin II; DAMGO; DPDP; TIPP$\Psi$; G-protein-coupled receptor; oligomerization; MAP kinase

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showing that dimeric analogs of oxyphorphone and enkephalin exhibit higher affinity and potency than their monomeric forms suggested that δ receptors could function as dimers (Hazum et al., 1982). We have previously shown that δ receptors exist as homodimers and undergo agonist-mediated monomerization (Cvejic and Devi, 1997). Furthermore, δ receptors heterodimerize with κ receptors, and heterodimerization affects their ligand binding and signaling properties (Jordan and Devi, 1999). Because δ receptors show significant sequence homology with μ receptors at the amino acid level (Miotti et al., 1995), we examined whether δ receptors physically associate with μ receptors and whether this interaction alters their properties. Here we show that μ and δ receptors associate to form a ~150 kDa heterodimer, and these heterodimers exhibit distinct ligand binding and signaling properties. Therefore, μ-δ heterodimerization represents a novel mechanism that could modulate the function of these receptors.

MATERIALS AND METHODS

Materials. Tyr-d-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO), Tyr-d-Ala-Phe-Glu-Val-Val-Gly (Deltorphin II), and diprenorphine were from RBI (Natick, MA) and Sigma (St. Louis, MO). Naftiben (NTB), benzylidene-dinitrate (BNTX), and SNC 80 were from Tocris Cookson. D-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP) and [D-Pen2, Pen5] enkephalin (DPDPE) were from Peninsula Inc. [3H]DAMGO and [3H]Deltorphin II were from NEN (DuPont). Anti-myct, anti-Flag, and anti-tubulin antibodies were from Sigma. Monoclonal antibody against pMAPK (E10) was from Cell Signaling Technologies (New England Biolabs). Tyr-Tic(4CH2NH)-Phe-Phe (TIPPP) was a gift from Dr. Peter Schiller (Institut de Reserches Cliniques de Montreal, Canada).

Cell culture and transfection. Human embryonic kidney (HEK)-293 cells expressing wild-type mouse myc-δ receptors alone or wild-type mouse Flag-μ receptors alone, or co-expressing wild-type myc-δ receptors with wild-type Flag-μ receptors or wild-type myc-δ with C-terminally truncated Flag-μ receptors were generated as described previously (Jordan et al., 2000; Trapadizde et al., 2000). Chinese hamster ovary (CHO) cells stably expressing wild-type Flag-μ and wild type myc-δ receptors were generated using Lipofectamine reagent (Life Technologies) as described (Jordan and Devi, 1999). SKNSH cells that express endogenous μ and δ receptors were grown in DMEM containing 10% FBS.

Coimmunoprecipitation and Western Blotting. Immunoprecipitation and Western blotting analysis of receptors expressed in HEK-293 cells were essentially as described previously (Jordan and Devi, 1999). Briefly, cells were lysed for 1 hr in buffer G (1% Triton X-100, 10% glycerol, 300 mM NaCl, 1.5 mM MgCl2, and 1 mM CaCl2 and 50 mM Tris-Cl, pH 7.4) containing 10–100 mM iodoacetamide and a protease inhibitor mixture (Jordan and Devi, 1999). For immunoprecipitation, 100–200 μg of protein was incubated with 1–2 μg of polyclonal anti-μ or anti-δ antibody overnight at 4°C. Immunocomplexes were isolated by incubation with 10% v/v protein A-Sepharose for 2–3 hr. The beads were washed three times with buffer G, resolved on a nonreducing 8% SDS-PAGE, and subjected to Western blotting using anti-Flag antibodies. Western blotting of these immunocomplexes with anti-Flag antibodies shows a ~150 kDa band representing μ-δ heterodimer only in cells co-expressing both myc-δ and Flag-μ receptors. Pretreatment of cells co-expressing μ and δ receptors with 1 mM DTT (Myct-Flagμ + DTT) results in the destabilization of dimers. μ-δ heterodimers are also seen in cells co-expressing wild-type δ receptors and C-terminally truncated μ receptors (Myct-FlagμΔ2).

RESULTS

μ and δ receptors associate to form detergent-stable heterodimers

A number of pharmacological studies have provided indirect evidence for the interaction between μ and δ receptors (Traynor and Elliot, 1993). We directly examined the association between these two classic receptors by co-expressing myc-tagged δ receptors with Flag-tagged μ receptors. Myc-tagged δ receptors in cell lysates were immunoprecipitated with polyclonal anti-myc antibodies and the Flag-tagged μ receptors in the immunoprecipitate were visualized with monoclonal anti-Flag antibody (Cvejic and Devi, 1997). We find that μ receptors interact with δ receptors to form a ~150 kDa heterodimer only in cells co-expressing both receptors (Fig. 1). We also see the presence of higher molecular weight forms representing oligomers only in cells co-expressing both receptors. Pretreatment of cells with a reducing agent (1 mM DTT) results in the destabilization of dimers (Fig. 1). These heterodimers are not induced during solubilization/immunoprecipitation conditions because they are not seen in immunoprecipitates from a mixture of cells individually expressing μ and δ receptors (Fig. 1). Interestingly, when a mutant μ receptor lacking C-terminal 42 amino acids is co-expressed with wild-type δ receptors, a band representing μ-δ heterodimer is seen; the decrease in the size of the band is consistent with the size of the truncated μ receptor (Fig. 1), suggesting that the C terminus of μ receptors does not play an important role in the heterodimerization of these two receptors.

Δ ligands are able to uncover a population of μ receptors

To explore whether heterodimerization affects the ligand binding properties of the receptors, we examined the ability of a δ antagonist (TIPPP) to modulate the binding of a μ agonist, [3H]DAMGO. We find a substantial increase (~100%) in the number of μ binding sites in the presence of TIPPP only in cells express-
that both the \( \mu \) and \( \delta \) receptors (\( \mu-\delta \)) or neuroblastoma cells expressing endogenous \( \mu \) and \( \delta \) receptors (SKNSH) in the absence or presence of \( \delta \) antagonist. Cells were incubated with indicated concentrations of \( ^{3}H\)-DAMGO in the absence (Control) or presence of 10 nM TIPP\(^\Psi\) (+TIPP\(^\Psi\)) as described above. In both cell types, the population of \( \mu \) receptors increased in the presence of TIPP\(^\Psi\); this increase is not seen in cells expressing only \( \mu \) receptors \((B, \mu)\). Data represent mean \( \pm \) SEM from seven independent experiments performed in triplicate. Similar results were obtained in additional clones expressing a lower number of \( \mu \) and \( \delta \) receptors. \( B, \mu \) agonist binding in the presence of \( \delta \) antagonists, Deltorphin II, and DPDPE. CHO cells stably expressing \( \mu-\delta \) heterodimers \((\mu-\delta)\) or \( \mu \) receptors alone \((\mu)\) were incubated with indicated concentrations of \( ^{3}H\)-DAMGO in the absence (Control) or presence of 10 nM Deltorphin II (+Delt II) or 10 nM DPDPE (+DPDPE) as described. The population of \( \mu \) receptors is increased in the presence of Deltorphin II but not DPDPE; this increase is not seen in cells expressing only \( \mu \) receptors \((\mu)\). Data represent mean \( \pm \) SEM from three to five independent experiments performed in triplicate.

### Table 1. Ligand binding properties

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( K_d ) (nM)</th>
<th>( B_{max} ) (fmol/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>( \mu-\delta )</td>
<td>SKNSH</td>
</tr>
<tr>
<td>( ^{3}H)-DAMGO</td>
<td>2.3 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>+ TIPP(^\Psi)</td>
<td>3.8 ± 0.3</td>
<td>4.8 ± 0.9*</td>
</tr>
<tr>
<td>+ Delt II</td>
<td>3.7 ± 0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( \mu-\delta )</th>
<th>SKNSH</th>
<th>( \delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ^{3}H)-Delt II</td>
<td>1.1 ± 0.3</td>
<td>3.8 ± 2.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>+ CTOP</td>
<td>0.7 ± 0.2</td>
<td>ND</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>+ DAMGO</td>
<td>ND</td>
<td>6.1 ± 2.9</td>
<td>0.9 ± 0.3</td>
</tr>
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ND, Not done. Data represent mean \( \pm \) SEM. Statistically significant differences (Dunnett’s test) from tritiated agonists alone are indicated. *\( p < 0.05 \), **\( p < 0.01 \) (\( n = 3-7 \)).

**Figure 2.** A, Binding of \( \mu \) agonist to CHO cells stably co-expressing \( \mu \) and \( \delta \) receptors \((\mu-\delta)\) or neuroblastoma cells expressing endogenous \( \mu \) and \( \delta \) receptors (SKNSH) in the absence or presence of \( \delta \) antagonist. Cells were incubated with indicated concentrations of \( ^{3}H\)-DAMGO in the absence (Control) or presence of 10 nM TIPP\(^\Psi\) (+TIPP\(^\Psi\)) as described above. In both cell types, the population of \( \mu \) receptors increased in the presence of TIPP\(^\Psi\); this increase is not seen in cells expressing only \( \mu \) receptors \((B, \mu)\). Data represent mean \( \pm \) SEM from seven independent experiments performed in triplicate. Similar results were obtained in additional clones expressing a lower number of \( \mu \) and \( \delta \) receptors. \( B, \mu \) agonist binding in the presence of \( \delta \) antagonists, Deltorphin II, and DPDPE. CHO cells stably expressing \( \mu-\delta \) heterodimers \((\mu-\delta)\) or \( \mu \) receptors alone \((\mu)\) were incubated with indicated concentrations of \( ^{3}H\)-DAMGO in the absence (Control) or presence of 10 nM Deltorphin II (+Delt II) or 10 nM DPDPE (+DPDPE) as described. The population of \( \mu \) receptors is increased in the presence of Deltorphin II but not DPDPE; this increase is not seen in cells expressing only \( \mu \) receptors \((\mu)\). Data represent mean \( \pm \) SEM from three to five independent experiments performed in triplicate.

**Figure 2.** A, Binding of \( \mu \) agonist to CHO cells stably co-expressing \( \mu \) and \( \delta \) receptors \((\mu-\delta)\) or neuroblastoma cells expressing endogenous \( \mu \) and \( \delta \) receptors (SKNSH) in the absence or presence of \( \delta \) antagonist. Cells were incubated with indicated concentrations of \( ^{3}H\)-DAMGO in the absence (Control) or presence of 10 nM TIPP\(^\Psi\) (+TIPP\(^\Psi\)) as described above. In both cell types, the population of \( \mu \) receptors increased in the presence of TIPP\(^\Psi\); this increase is not seen in cells expressing only \( \mu \) receptors \((B, \mu)\). Data represent mean \( \pm \) SEM from seven independent experiments performed in triplicate. Similar results were obtained in additional clones expressing a lower number of \( \mu \) and \( \delta \) receptors. \( B, \mu \) agonist binding in the presence of \( \delta \) antagonists, Deltorphin II, and DPDPE. CHO cells stably expressing \( \mu-\delta \) heterodimers \((\mu-\delta)\) or \( \mu \) receptors alone \((\mu)\) were incubated with indicated concentrations of \( ^{3}H\)-DAMGO in the absence (Control) or presence of 10 nM Deltorphin II (+Delt II) or 10 nM DPDPE (+DPDPE) as described. The population of \( \mu \) receptors is increased in the presence of Deltorphin II but not DPDPE; this increase is not seen in cells expressing only \( \mu \) receptors \((\mu)\). Data represent mean \( \pm \) SEM from three to five independent experiments performed in triplicate.

**μ ligands can uncover a population of \( \delta \) receptors**

To examine whether \( \mu \) receptor-selective ligands are able to modulate the binding of a \( \delta \) agonist, we examined the effect of a \( \mu \)-selective antagonist (CTOP) on the binding of a \( \delta \)-selective agonist, \( ^{3}H\)-Deltorphin II. We find that there is a significant increase in the number of \( \delta \) binding sites in the presence of CTOP only in cells expressing \( \mu-\delta \) heterodimers and not in cells expressing only \( \delta \) receptors (Fig. 3, Table 1). Furthermore, a \( \mu \)-selective agonist, DAMGO, is also able to increase the number of \( \delta \) binding sites as seen in SKNSH cells (Table 1). These results suggest that \( \mu \)-selective ligands are able to uncover a hidden population of \( \delta \) receptors in cells expressing \( \mu \) and \( \delta \) receptors.

**μ–δ heterodimers have unique ligand binding properties**

To further characterize the binding properties of the \( \mu-\delta \) heterodimers, we took advantage of the availability of various \( \delta \)
and the extent of $^3$H-Deltorphin II binding

As shown in Figure 3B, treatment with CTOP significantly increases the potency and efficacy of MAP kinase phosphorylation by Deltorphin II (Fig. 3B). These results imply that $\mu$-$\delta$ heterodimers represent functional receptors and that the selective ligands are able to enhance signaling by opioid agonists.

**DISCUSSION**

**$\mu$ receptors associate with $\delta$ receptors to form $\mu$-$\delta$ heterodimers**

In this paper we provide biochemical, pharmacological, and functional evidence for dimerization between $\mu$ and $\delta$ opioid receptors. Previous studies using moderately selective ligands provided indirect evidence for the interaction between these two receptors (Traynor and Elliot, 1993). This led to alternate explanations of the findings because these ligands could interact with both receptors. In the present study, we used highly selective ligands and heterologous cells expressing both $\delta$ and $\mu$ receptors (or individually expressing these receptors) to demonstrate direct physical and functional interactions between these two classic opioid receptors.

**Does the $\mu$-$\delta$ heterodimer represent a $\delta$ receptor subtype?**

The complexities of opioid receptor pharmacology have often been attributed to two different phenomena: receptor–receptor interactions and opioid receptor subtypes. A number of investigators have proposed that receptor–receptor interactions could form the basis for some of the opioid receptor subtypes (Porreca et al., 1992; Xu et al., 1993; Jordan and Devi, 1999). It was shown that a subset of $\delta$-selective ligands enhanced $\mu$ receptor-mediated analgesia; these ligands were thus proposed to bind to $\delta$ receptors complexed with $\mu$ receptors (Traynor and Elliot, 1993). Two independent studies demonstrated that the $\mu$-$\delta$-complexed receptors were sensitive to characteristic $\delta_2$ selective ligands, suggesting that they represented the $\delta_2$ receptor subtype (Porreca et al., 1992; Xu et al., 1993). Interestingly, recent work with antisense oligonucleotides to $\delta$ receptors and $\delta$ receptor knockout mice...
showed a blockade of analgesia induced by a δ2-selective ligand (Deltorphin II) but not that induced by a δ1-selective ligand, DPDPE (Bilsky et al., 1996; Zhu et al., 1999). Remarkably, no DPDPE or Deltorphin II binding could be observed in either case. These results would then suggest that the cloned δ receptor may represent the δ2 subtype and not the previously thought δ1 subtype. However, when expressed in heterologous cells, the cloned δ receptor is able to bind both δ1- and δ2-selective ligands with high affinity. It is therefore unclear as to what represents a δ2 (and a δ1) binding site. We have shown previously that heterodimers of δ and κ receptors reveal ligand binding sites that are virtually identical to previously described κ2 subtypes (Jordan and Devi, 1999). It is therefore likely that the heterodimers described here represent a novel δ receptor subtype. Consistent with this notion, we find that the μ–δ heterodimer is able to bind some δ1 (BNTX)– and δ2 (Deltorphin II)-selective ligands and not others (DPDPE, NTB).

Direct interactions and G-proteins in receptor dimerization

One interesting observation is that only a subset of selective ligands including antagonists are able to enhance μ agonist binding. This effect is also seen (to a lesser extent) when the binding assay is performed at 4°C. These findings strongly point to a phenomenon occurring at the level of ligand binding. Because most, if not all, cellular processes arrest at low temperatures and given the ability of antagonists to synergize with agonists, it is apparent that not all downstream effects nor receptor activation are required for this phenomenon to occur. The simplest explanation is that heterodimerization alters the binding pocket of both receptors and that the binding of one ligand can “restore” the binding site of the other.

The possibility that G-proteins may be involved in the interactions cannot be ignored. It is possible that G-protein switching between receptors may cause these alterations in affinity and the number of binding sites. In such a model, the binding of receptor selective ligands (agonists and antagonists) could cause the selective uncoupling of G-proteins that, if in limiting conditions, may significantly affect the binding of ligands to a nearby receptor that uses a similar G-protein. It is therefore possible that G-proteins could play a role not only in determining ligand selectivities but also in mediating the synergistic effects on downstream signaling observed as a potentiation of function.

In summary, heterodimerization between μ and δ represents a novel mechanism that could modulate receptor function and provides a new strategy for the development of novel therapies.

REFERENCES


