Heterodimerization of μ and δ 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 μ opioid receptors, a number of studies have shown that δ 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 μ and δ receptors could account for the cross-modulation previously observed between these two receptors. We find that co-expression of μ and δ receptors in heterologous cells followed by selective immunoprecipitation results in the isolation of μ–δ heterodimers. Treatment of these cells with extremely low doses of certain δ-selective ligands results in a significant increase in the binding of a μ receptor agonist. Similarly, treatment with μ-selective ligands results in a significant increase in the binding of a δ receptor agonist. This robust increase is also seen in SKNSH cells that endogenously express both μ and δ receptors. Furthermore, we find that a δ receptor antagonist enhances both the potency and efficacy of the μ receptor signaling; likewise a μ antagonist enhances the potency and efficacy of the δ receptor signaling. A combination of agonists (μ and δ receptor selective) also synergistically binds and potentiates signaling by activating the μ–δ 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; DPDPE; TIPPΨ; G-protein-coupled receptor; oligomerization; MAP kinase

Opioid receptors can regulate several biological effects, including analgesia, miosis, bradycardia, general sedation, feeding, and hypothermia (Herz, 1993). Morphine, a prototype opioid agonist, binds to μ and δ opioid receptors and inhibits neurotransmitter release (MacDonald and Nelson, 1978; Yaksh, 1993). Studies with transgenic animals lacking μ receptors show that morphine functions primarily via μ receptors (Matthes et al., 1996). Interestingly, δ ligand-mediated analgesia is altered in these animals, suggesting an interaction between the two receptors (Sora et al., 1997).

A number of pharmacological studies have suggested that μ and δ receptors interact and influence each other's properties (Traynor and Elliot, 1993). For example, mice treated with δ antagonists exhibit diminished development of morphine tolerance and dependence (Abdelhamid et al., 1991; Zhu et al., 1999). Selective reduction of δ receptors by antisense oligonucleotides attenuates the development of morphine dependence (Sanchez-Blazquez et al., 1997). In δ receptor knockout animals, the extent of dependence attributable to morphine administration is also selectively altered (Zhu et al., 1999). Ligand binding studies show that μ-selective ligands inhibit the binding of δ-selective ligands in both competitive and noncompetitive manners (Rothman and Westfall, 1981; Rothman et al., 1983). Findings such as these led to the proposal that δ receptors exist as two subtypes: those that are associated with μ receptors and those that are not. However, the biochemical basis for this association was not explored.

Early studies using radioligand binding and electrophysiology suggested that both μ and δ receptors colocalize to cells in the dorsal root ganglia (Fields et al., 1980; Egan and North, 1981; Zieglgansberger et al., 1982). Immunohistochemical studies of the opioid receptor distribution in the CNS have shown that μ and δ receptors colocalize to the same axonal terminals of the superficial dorsal horn (Arvidsson et al., 1995). Ultrastructural analysis of the dorsal horn neurons also revealed colocalization of δ receptors with μ receptors in the plasmallema (Cheng et al., 1997). In addition, several neuroblastomas have been shown to co-express these two opioid receptors (Yu et al., 1986; Kazmi and Mishra, 1987; Baumhaker et al., 1993; Palazzi et al., 1996). Taken together, these studies provide evidence for colocalization of μ and δ receptors and suggest that the existence of δ and μ receptor complexes is physically possible.

Receptor dimerization is a potential mechanism for modulation of their function (Salahpour et al., 2000). Early studies

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showing that dimeric analogs of oxymorphone and enkephalin exhibit higher affinity and potency than their monomeric forms suggested that μ 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 (Miotto 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). Naltriben (NTB), benzylidenenaltrexone (BNTX), and SNC 80 were from Tocris Cookson. D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) and [D-Pen^2, Pen^5] enkephalin (DPDPE) were from Peninsula Inc. ^{3}H-DAMGO and ^{3}H-deltorphin II were from NEN (DuPont). Anti-*myc*, anti-Flag, and anti-tubulin antibodies were from Sigma. Monoclonal antibody against pMAPK (E10) was from Cell Signaling Technologies (New England Biolabs). Tyr-Ticψ(CH_2NH)-Phe-Phe (TIPPψ) 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 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; Trapaidze 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 $MgCl_2$, and 1 mM $CaCl_2$ 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-*myc* 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 as described using M1, monoclonal anti-Flag antibody.

Whole-cell binding assays. The binding assay was performed essentially as described (Gomes et al., 2000). Briefly, cells were incubated with indicated concentrations of ^{3}H-DAMGO or ^{3}H-Deltorphin II in 50 mM Tris-Cl buffer, pH 7.4, for 2 hr at 37°C in the absence or presence of various ligands (at 10 nM). Under these conditions the level of agonist-mediated receptor internalization is insignificant (I. Gomes and L. A. Devi, unpublished observations). Cells were washed three times with cold buffer, and the radioactivity was determined after solubilization as described (Gomes et al., 2000). Concentrations of ^{3}H-DAMGO or ^{3}H-Deltorphin II were from 0.1 to 10 nM for saturation analysis, and ^{3}H-DAMGO was 3 nM for the determination of EC_{50} values. Nonspecific binding was determined with 100 nM DAMGO, Deltorphin II, or Diprenorphine.

Functional assays. The opioid-induced increase in MAP kinase phosphorylation in SKNSH cells or CHO cells co-expressing μ and δ receptors was essentially as described previously (Jordan et al., 2000; Trapaidze et al., 2000). Briefly, cells were treated for 5 min at 37°C with indicated concentrations of either DAMGO ± 10 nM TIPPΨ or Deltorphin II ± 10 nM CTOP. The level of phosphorylated MAPK (p44/42 MAPK; Erk1/2) was determined by Western blotting using anti-phospho-MAP kinase antibody and the levels of tubulin using anti-tubulin antibody.

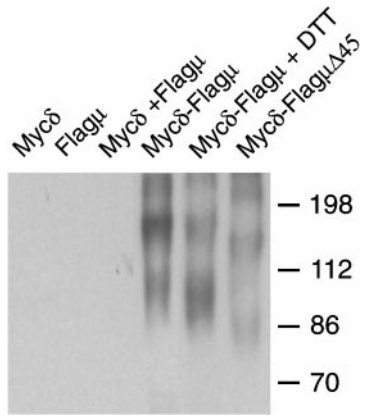

Figure 1. μ and δ receptors interact with each other to form heterodimers. Immunoprecipitation of cell lysates from HEK-293 cells individually expressing either *myc*-δ (*Myc*δ) or Flag-μ (*Flag*μ) receptors, mixed cells individually expressing *myc*-δ or Flag-μ receptors (*Myc*δ + *Flag*μ), or cells co-expressing *myc*-δ and Flag-μ (*Myc*δ–*Flag*μ) was performed using anti-*myc* antibodies. Western blotting of these immunocomplexes using anti-Flag antibodies shows a ~150 kDa protein representing the 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 (*Myc*δ–*Flag*μ + *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 (*Myc*δ–*Flag*$\mu\Delta$*42*).

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 (TIPPΨ) to modulate the binding of a μ agonist, ^{3}H-DAMGO. We find a substantial increase (~100%) in the number of μ binding sites in the presence of TIPPΨ only in cells express-

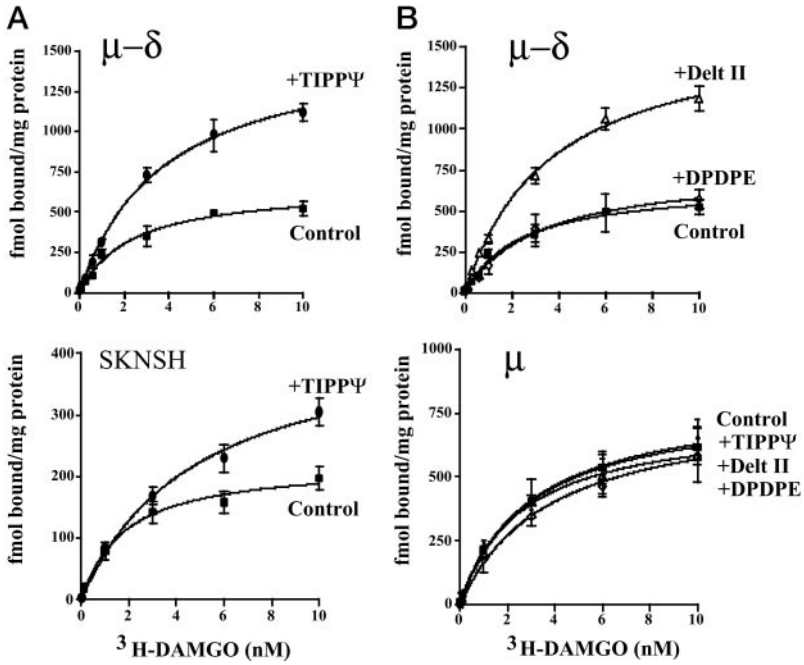

Figure 2. *A*, Binding of μ agonist to CHO cells stably co-expressing μ and δ receptors (μ–δ) or neuroblastoma cells expressing endogenous μ and δ receptors (*SKNSH*) in the absence or presence of δ antagonist. Cells were incubated with indicated concentrations of ^{3}H-DAMGO in the absence (*Control*)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

Table 1. Ligand binding properties

	K_d (nM)			B_{max} (fmol/mg protein)		
	μ–δ	SKNSH	μ	μ–δ	SKNSH	μ
^{3}H-DAMGO	2.3 ± 0.4	1.8 ± 0.4	2.8 ± 0.1	660 ± 41	222 ± 14	786 ± 14
+ TIPP	3.8 ± 0.3	4.8 ± 0.9*	3.0 ± 0.7	1572 ± 55**	440 ± 37**	816 ± 70
+ Delt II	3.7 ± 0.3	ND	2.6 ± 0.2	1644 ± 61**	ND	735 ± 17
	μ–δ	SKNSH	δ	μ–δ	SKNSH	δ
^{3}H-Delt II	1.1 ± 0.3	3.8 ± 2.2	0.7 ± 0.2	61.8 ± 4.1	94 ± 23	41.8 ± 2.6
+ CTOP	0.7 ± 0.2	ND	0.9 ± 0.2	91.9 ± 4.8**	ND	42.2 ± 2.5
+ DAMGO	ND	6.1 ± 2.9	0.9 ± 0.3	ND	191 ± 4.5**	44.5 ± 3.8

ND, Not done. Data represent mean ± SEM. Statistically significant differences (Dunnett's test) from tritiated agonists alone are indicated. *$p < 0.05$, **$p < 0.01$ (n = 3–7).

ing both μ and δ receptors (Fig. 2*A*, Table 1). This effect is also seen in SKNSH cells, a neuroblastoma cell line that expresses endogenous μ and δ receptors (Fig. 2*A*, Table 1). An increase (~60%) in ligand binding is also observed when the binding assay is performed at 4°C for 5 hr (Gomes and Devi, unpublished observations), suggesting that one of the mechanisms for the observed drug effect could be the modulation of the μ binding site by direct interaction with δ receptors. Taken together, these results support the notion that TIPPΨ is able to synergize with ^{3}H-DAMGO to reveal a hidden population of receptors in heterologous cells expressing μ and δ receptors as well as in neuroblastoma cells endogenously expressing these two receptors.

We next examined whether two agonists are able to synergize in cells expressing μ–δ heterodimers and whether this would lead to an increase in μ binding sites. We find that treatment with Deltorphin II, but not DPDPE, leads to a significant increase in μ agonist binding; this increase is not seen in cells expressing only μ receptors (Fig. 2*B*, Table 1). Taken together, these results show that both the δ-selective antagonist and the agonist can synergize with the μ receptor agonist and uncover a population of receptors with unique binding properties. This property of the μ–δ heterodimer is distinct from that of the κ–δ heterodimer because in the latter case the δ antagonist was not able to synergize with the κ agonist; only two agonists or two antagonists were able to show synergistic effects (Jordan and Devi, 1999).

μ ligands can uncover a population of δ receptors

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

μ–δ heterodimers have unique ligand binding properties

To further characterize the binding properties of the μ–δ heterodimers, we took advantage of the availability of various δ

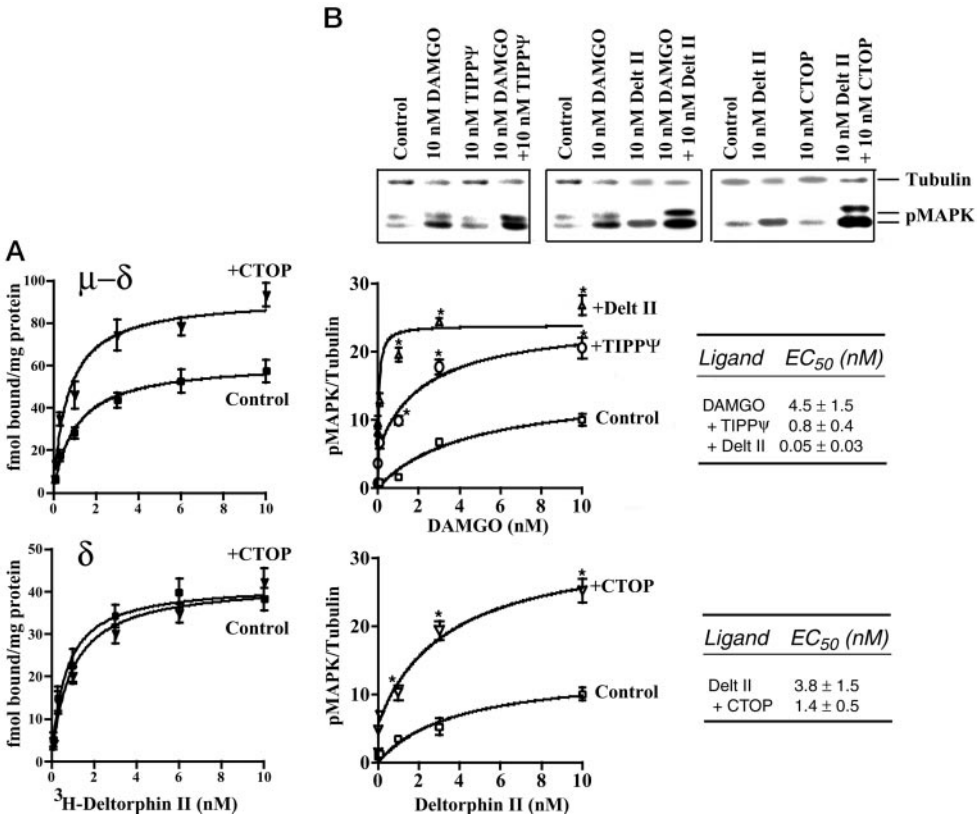

Figure 3. *A*, Binding of a δ agonist, ^{3}H-Deltorphin II, to CHO cells stably co-expressing μ and δ receptors (μ–δ) or δ receptor alone (δ) in the absence or presence of μ antagonist, CTOP. Cells were incubated with indicated concentrations of ^{3}H-Deltorphin II in the absence (*Control*) or presence of 10 nM CTOP (*+CTOP*), and the extent of ^{3}H-Deltorphin II binding to cells was measured as described. The population of δ receptors is increased in the presence of CTOP; this increase is not seen in cells expressing only δ receptors (δ). Data representing mean ± SEM (n = 3) are shown. *B*, Functional properties of μ–δ heterodimer. SKNSH cells endogenously expressing μ and δ receptors were treated with indicated concentrations of DAMGO in the absence or presence of 10 nM TIPPΨ or 10 nM Deltorphin II. Alternatively, cells were treated with indicated concentrations of Deltorphin II in the absence or presence of 10 nM CTOP. The extent of MAP kinase phosphorylation was determined using Western blot analysis as described. The extent of phospho-MAP kinase (*pMAPK*) in cells treated with individual or a combination of ligands at a fixed concentration is shown in the *top panel*. *pMAPK/Tubulin* refers to the ratio of phospho-MAPK levels to the tubulin levels; the level in untreated cells is taken as zero. Data represent mean ± SEM (n = 4). Statistically significant differences (Dunnett's test) from the control values are indicated by $^*p = <0.005$. EC_{50} = dose that gives 50% of maximum response.

receptor-selective ligands. Among the ligands tested, treatment with a relatively low dose (picomole) of TIPPΨ or Deltorphin II results in a substantial increase (approximately twofold) in ^{3}H-DAMGO binding (data not shown). At a higher dose (nanomole), BNTX is also able to increase ^{3}H-DAMGO binding albeit to a lower extent (~1.3-fold). Interestingly, neither DPDPE nor a structurally unrelated agonist, SNC 80, is effective even at relatively high doses (1 μM). These results suggest that the synergistic binding exhibits ligand selectivity and that this cooperative binding requires very low doses of a subset of selective ligands.

μ–δ heterodimers represent functional receptors

We also examined whether the heterodimer represents a functional receptor and whether the synergistic binding leads to a potentiation of effector function. The activation of opioid receptors results in an increase in the level of phosphorylated MAP kinase (Trapaidze et al., 2000). We examined whether the δ antagonist (TIPPΨ) or agonist (Deltorphin II) could potentiate the μ agonist (DAMGO)-induced phosphorylation of p-42/44 MAP kinases (Erk1/2). Treatment with TIPPΨ or Deltorphin II leads to a significant increase in the potency (~6- or 90-fold, respectively) and efficacy (approximately twofold) of MAP kinase phosphorylation by DAMGO (Fig. 3*B*). In a reciprocal experiment, we examined whether the μ antagonist (CTOP) could potentiate the δ agonist (Deltorphin II)-induced signaling. As shown in Figure 3*B*, treatment with CTOP significantly increases the potency and efficacy of MAP kinase phosphorylation by Deltorphin II (Fig. 3*B*). These results imply that μ–δ heterodimers represent functional receptors and that the selective ligands are able to enhance signaling by opioid agonists.

DISCUSSION

μ receptors associate with δ receptors to form μ–δ heterodimers

In this paper we provide biochemical, pharmacological, and functional evidence for dimerization between μ and δ 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 δ and μ receptors (or individually expressing these receptors) to demonstrate direct physical and functional interactions between these two classic opioid receptors.

Does the μ–δ heterodimer represent a δ 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 δ-selective ligands enhanced μ receptor-mediated analgesia; these ligands were thus proposed to bind to δ receptors complexed with μ receptors (Traynor and Elliot, 1993). Two independent studies demonstrated that the μ–δ-complexed receptors were sensitive to characteristic δ2 selective ligands, suggesting that they represented the δ2 receptor subtype (Porreca et al., 1992; Xu et al., 1993). Interestingly, recent work with antisense oligonucleotides to δ receptors and δ 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 neither 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.

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