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Standard Opioid Agonists Activate Heteromeric Opioid Receptors: Evidence for Morphine and [D-Ala^2-MePhe^4-Glyol^5]Enkephalin as Selective $\mu-\delta$ Agonists

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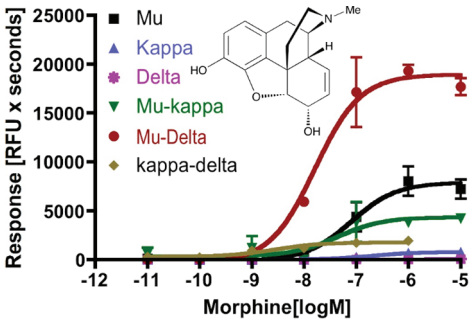
Abstract



Research in the opioid field has relied heavily on the use of standard agonist ligands such as morphine, [D-Ala^2-MePhe^4-Glyol^5]enkephalin (DAMGO), U69593, bremazocine, [D-Pen^{2}D-Pen^5]enkephalin (DPDPE), and deltorphin-II as tools for investigating the three major types of opioid receptors, MOP (μ), KOP (κ), and DOP (δ), that mediate antinociception. The functional selectivity of these ligands has been based on the assumption that opioid receptors exist as homomers. As numerous studies in cultured cells have suggested that opioid receptors can associate both as homomers and heteromers, we have investigated the selectivity of these standard ligands using intracellular calcium release and [^{35}S]GTPγS assays in HEK-293 cells that contain singly and coexpressed opioid receptors. The present study reveals that morphine and DAMGO, traditionally classified as μ selective agonists, selectively activate $\mu-\delta$ heteromeric opioid receptors with greater efficacy than homomeric opioid receptors. Moreover, standard ligands that have been widely employed as κ- and δ-selective agonists display little or no differences in the activation of homomeric and heteromeric opioid receptors. The far-reaching implications of these results are discussed.

Keywords: Opioid, receptors, heterodimers, heteromers, morphine, standard opioids

Opioid receptors are members of the class A subfamily of G protein-coupled receptors (GPCRs). The most prominent physiologic and pharmacologic function mediated by opioid receptors is the modulation of nociceptive pathways in the brain and spinal cord to modulate pain. The three types of opioid receptors (IUPHAR Web site: http://www.iuphar-db.org/PRODDATABASE/FamilyIntroductionForward?familyId=50), MOP (μ), KOP (κ), and DOP (δ), involved in such modulation have been cloned and expressed in cultured cells (*1*−*4*). Since the concept of multiple opioid receptors was first proposed (*5*) and subsequently established (*6*, *7*), the selectivity of new opioid ligands for these receptor types has been evaluated mainly with standard agonist ligands whose selectivity was based primarily on their binding affinities. Thus, morphine (*8*, *9*) and D-Ala^2-MePhe^4-Glyol^5 enkephalin (DAMGO; *10*, *11*)) have been described and used widely as selective ligands for μ receptors, [D-Pen^{2}D-Pen^{5}enkephalin] (DPDPE; *12*) and deltorphin-II (*13*, *14*) for δ receptors, and U69593 (*15*, *16*) and bremazocine (*16*) for κ receptors.

Over the past decade, it has been demonstrated that numerous GPCRs associate to form oligomerized homo- and heteromeric structures in cultured cells (*17*, *18*). This property is particularly prominent among opioid receptors with greater than nine heteromeric opioid receptors having been reported (*17*, *19*−*29*). Moreover, it has been demonstrated that opioid receptors and other GPCRs form heteromers in the endoplasmic reticulum prior to being transported to the cell surface (*30*−*32*) where they are constitutively expressed. Also μ and δ opioid receptors have been shown to colocalize in the frontal cortex and striatum of the rat brain (*32*). Taken together, these reports point to the likelihood that GPCR heteromers exist *in vivo*. Indeed, the recent development of a number of ligands that apparently selectively antagonize or activate opioid receptor heteromers in mice has suggested their presence in the CNS (*33*−*35*). Additionally, it has been very recently

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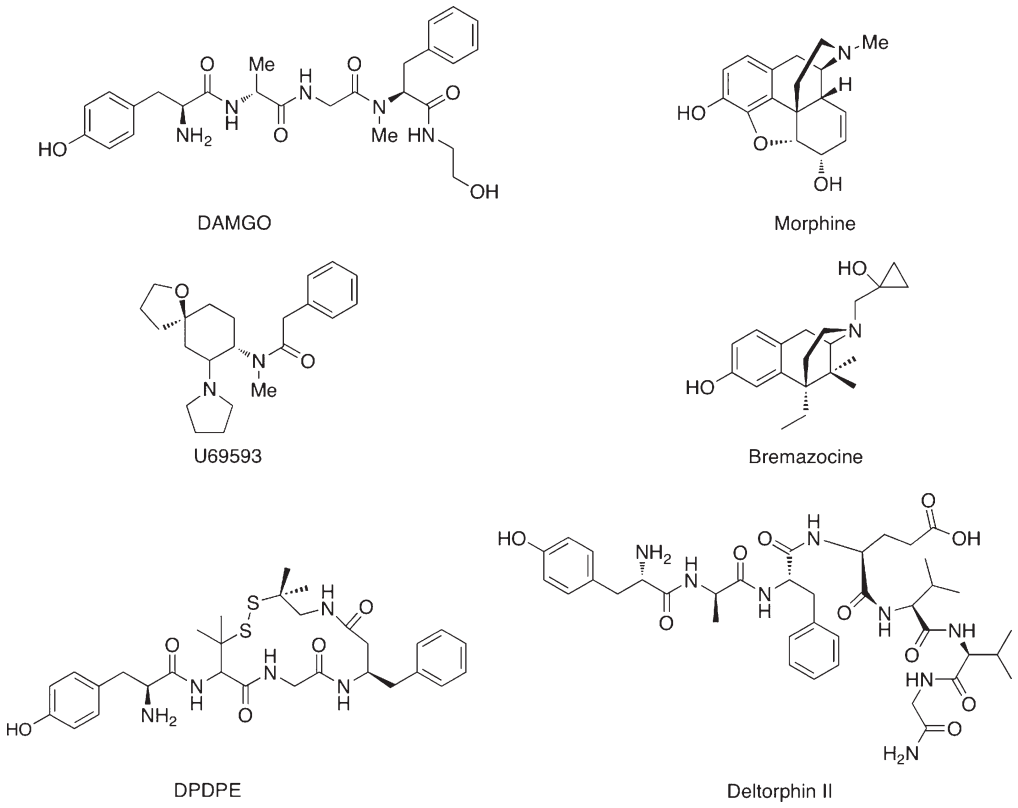

Figure 1. Chemical structures for opioid standard ligands tested in the study.

reported that the clinically employed mixed agonist−antagonist analgesics selectively activate heteromers in cultured cells (*36*).

To date, no studies on the selectivity of standard opioid ligands in activating both homomers and heteromers have been reported. The existence of opioid receptor heteromers in cultured cells may have significant ramifications concerning the interpretation of *in vivo* data, particularly if standard agonists (Figure 1) that are widely employed as tools in opioid research were to activate them more efficiently than homomers. In order to investigate whether this is a possibility, we have evaluated standard μ-, κ-, and δ-opioid agonist ligands using HEK-293 cells that contain singly or doubly expressed opioid receptors. Here we demonstrate that although the functional selectivity of these standard ligands is in harmony with their reported binding selectivity to homomeric opioid receptors, they often exhibit greater or equivalent activity at opioid receptor heteromers. In this regard and most significantly, the standard agonists, morphine and DAMGO, selectively activate $\mu-\delta$ heteromers rather than μ receptors as traditionally viewed. These studies call into question the conclusions derived from a multitude of published *in vivo* pharmacologic studies whose data was interpreted based on the assumption that these standard ligands selectively activate μ opioid receptor homomers.

Results and Discussion

Researchers in the pharmacologic sciences have traditionally depended on selective ligands as tools to establish the identity of receptor targets. In this regard, over the years, the field of opioid research has seen the development of an array of ligands that target opioid receptors. After the existence of multiple opioid receptors was suggested (*5*) and later established (*6*, *7*), standard ligands were developed that were apparently selective for each of the opioid receptor types, and these became cornerstones in the field (*8*−*16*, *37*).

An additional layer of complexity has more recently been added with the numerous reports that GPCRs, including opioid receptors, can associate to form higher order structures that include heteromers in cultured cells (*17*, *19*−*29*). The existence of heteromers raises cogent questions concerning receptor recognition, activation, and signal transduction. Since opioid receptor heteromers represent potential targets that have not been considered previously, we investigated frequently employed standard opioid ligands using the intracellular calcium release and [^{35}S]GTPγS assays on HEK-293 cells stably expressing homomeric and heteromeric opioid receptors.

MOP Receptor Selective Agonists

The HEK-293 cell lines stably coexpressing hemagglutinin (HA)- or FLAG-tagged opioid receptors,

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which were characterized previously by coimmunoprecipitation and shown to contain heteromeric receptors (*34*, *38*), were employed in the present study. To determine the relative densities of the receptors in these dual transfected cells, two-color immunoflourescence was used. High-resolution confocal images obtained for HEK-293 cells stably coexpressing HA-δ and FLAG-μ opioid receptors show that both receptors were colocalized on the plasma membrane and similarly expressed (Figure 2).

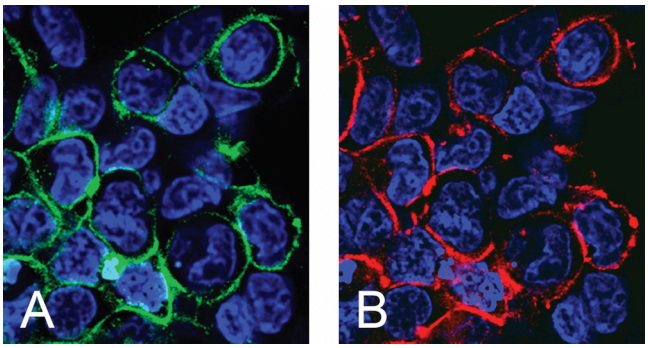

Figure 2. High-magnification confocal images of double-labeling immunofluorescence for HA-δ and FLAG-μ opioid receptors. HEK-293 cells stably expressing both HA-δ and FLAG-μ opioid receptors are shown labeled for μ (A, green fluorescence) and for δ (B, red fluorescence). DAPI (blue fluorescence) has been used to stain the nuclei.

The functional selectivity of morphine and DAMGO, which are considered to be μ-selective agonists (*8*−*11*), was evaluated using the intracellular calcium release method with minor modifications (*34*). A transiently transfected chimeric G protein, Δ6-$G_{qi4\text{-}myr}$ (*39*) whose activation is coupled to calcium release, was employed. Morphine was observed to have the highest potency and efficacy (curve height) in cells coexpressing μ and δ opioid receptors, relative to cells containing other singly or coexpressed receptors of equal density (Figure 3a). Since the coexpressed cells are known (*19*−*21*, *34*, *38*) to contain opioid receptor heteromers, the selective activation of μ−δ receptors may be due to conformational differences when compared with homomeric μ receptors or other opioid heteromers. The potency of morphine was at least 5-fold greater at μ−δ heteromers (16.4 nM) than at homomeric μ-opioid receptors (89.4 nM) or μ−κ-opioid receptor heteromers (141.6 nM). There was little observable activation at the κ, δ, or κ−δ heteromeric opioid receptors (Figure 3a).

DAMGO (Figure 3c) was 7-fold more potent at μ−δ opioid receptor heteromers (EC_{50} = 1.5 nM) relative to

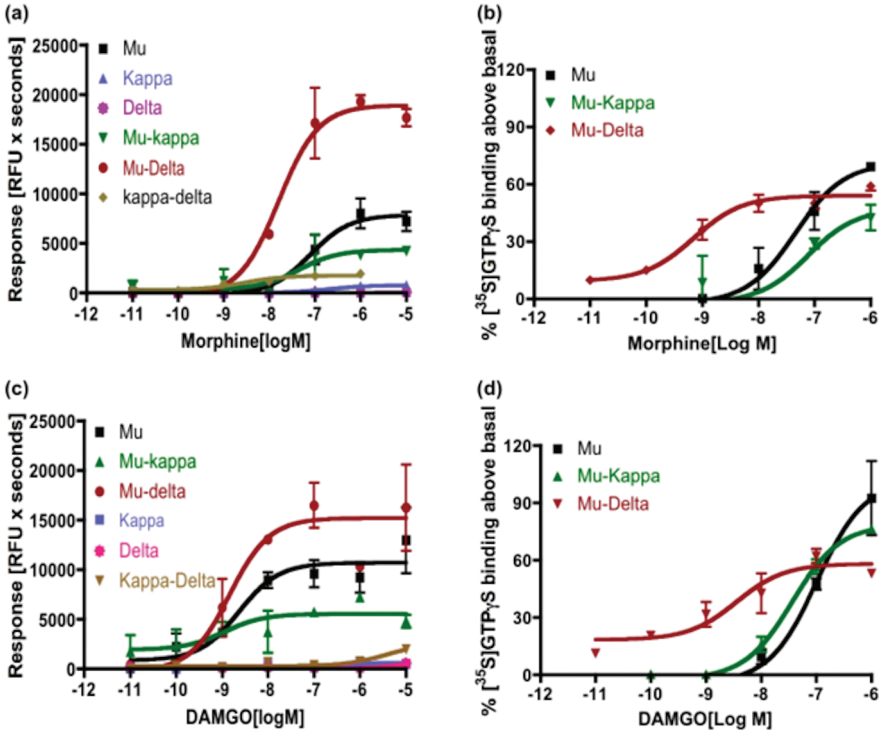

Figure 3. Morphine and DAMGO are highly selective agonists for μ−δ opioid receptor heteromers. Intracellular Ca^{2+} ion release mediated by increasing concentrations of the agonists (a) morphine and (c) DAMGO was measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. Response was measured as relative fluorescence units (RFU), and time was measured in seconds. The *Y*-axis plots the area under the curve (AUC) values ± SEM (n = 12−16), which are represented as RFU × seconds. Concentration−response curves for stimulation of [^{35}S]GTPγS by (b) morphine and (d) DAMGO, as measured in HEK-293 cell membranes expressing various opioid receptors.

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μ opioid receptors (EC_{50} = 10.6 nM). Interestingly, unlike morphine, DAMGO possessed similar efficacy at μ, $\mu-\delta$, and $\mu-\kappa$ receptors. Again, there was little observable activation at the κ, δ, or $\kappa-\delta$ heteromeric opioid receptors. The difference between DAMGO and morphine could be related to their divergent structures.

We also employed the [^{35}S]GTPγS assay in order to evaluate receptor activation at the Gα-protein level as opposed to a downstream signal transduction effect. Here also we observed that morphine and DAMGO both more potently activated $\mu-\delta$ opioid receptor heteromers (Figure 3b,d). It is noteworthy that the [^{35}S]GTPγS and intracellular calcium assays differed in the observed output though giving similar results. The [^{35}S]GTPγS assay also revealed the greater selectivity of both morphine and DAMGO for activation of $\mu-\delta$ heteromers, as manifested by the nearly 100-fold lower EC_{50} when compared with the μ opioid receptors (leftward shift of the concentration−response curve, Figure 3b,d), while the calcium assay was characterized by an increased curve height or efficacy (higher AUC_{peak} values, Figure 3a,c).

Though widely employed as an analgesic, the well-known side effects of morphine, such as tolerance and physical dependence, limit its chronic use. Some insight into the origin of these side effects was obtained when it was reported that coadministration of morphine with δ-opioid antagonists abolished the development of tolerance and dependence in mice, with a concomitant increase in agonist potency (*40*). These results are consistent with the absence of morphine-induced tolerance in mice lacking δ-opioid receptors (*41*) and absence of both tolerance and dependence in δ receptor antisense experiments (*42*, *43*). Additionally, it is particularly noteworthy that bivalent ligands containing both μ agonist and δ antagonist pharmacophores produce potent antinociception without tolerance or dependence in mice, only when the linker length between the pharmacophores is long enough to presumably permit bridging of receptors, suggesting that μ and δ receptors are in close proximity, perhaps as heterodimers (*35*, *44*). A possible mechanism for this effect is that the δ antagonist pharmacophore effectively blocks the development of tolerance and dependence by allosterically altering the signaling pathway normally induced by the activation of the μ recognition site of the $\mu-\delta$ heteromer. The alteration in the signaling pathway could occur at the G protein level, in view of the report that G_z rather than G_i or G_o is activated by interaction of morphine with $\mu-\delta$ heteromers in cultured cells (*32*).

The observation that both morphine and DAMGO selectively activate $\mu-\delta$ opioid receptor heteromers in the calcium release and [^{35}S]GTPγS assays (Figure 3) has important implications. When taken together with the literature suggesting interaction between μ and δ receptors (*35*, *40*−*43*, *45*), the activation selectivity data for morphine suggests a major role for $\mu-\delta$ heteromers in mediating morphine analgesia and the development of tolerance and dependence. Similarly, the central and peripheral tolerance and dependence by DAMGO (*46*−*48*) further implicates the role of $\mu-\delta$ receptors in these side effects.

KOP Receptor Agonists

There have been suggestions of multiple subtypes for κ receptors based mainly on differences between the *in vivo* pharmacology (*49*−*51*) of the benzeneactamides (eg, U50488, U69593) and bremazocine. On this basis, the benzeneacetamides and bremazocine have been referred to as κ_1 and κ_2 opioid receptor agonists, respectively. However, since only a single κ receptor has been cloned (*3*) and in view of evidence for of κ receptor heteromers in cultured cells, it appears that the receptor "subtype" may actually represent a phenotypic κ receptor in the form of a heteromer (*19*, *27*, *33*, *51*).

Based on the above considerations, we have investigated two κ-opioid receptor ligands, U69593 and bremazocine, at various opioid receptors in HEK-293 cells. Though U69593 has long been considered a highly selective κ-opioid agonist, the activation at $\kappa-\delta$ opioid receptors (EC_{50} = 2.9 nM, AUC_{peak} = 11803 RFU × s) was at least 80% more efficacious and 11-fold more potent than at κ-opioid receptors alone (EC_{50} = 33.7 nM, AUC_{peak} = 6704 RFU × s; Figure 4a). There was no significant activation observed at μ, δ, and $\mu-\delta$ receptors.

On the other hand, bremazocine produced comparable activity at all the receptors tested except for μ opioid receptors, where it was inactive (Figure 4b). Interestingly, the activity of bremazocine at $\kappa-\delta$ receptors (AUC_{peak} = 5916 RFU × s) resembled that of a partial agonist with AUC_{peak} values around half of that of U69593. Bremazocine also activated κ, δ, and $\mu-\delta$ receptors to the levels of a partial agonist thus showing no functional selectivity to κ opioid receptors. In this regard, bremazocine has been recently suggested to be a partial agonist at κ opioid receptors using a similar calcium release method (*52*). It has been previously shown that 68% and 27% of [^{3}H]bremazocine binding was lost in μ and δ knockout mice, respectively, while 15% of binding was retained in μ/δ dual knockout mice (*53*). The higher μ and δ binding data could be explained by our finding that bremazocine has the highest functional activity at $\mu-\kappa$ and $\kappa-\delta$ receptors (Figure 4b), while also activating δ and $\mu-\delta$ opioid receptors. When taken together with the reported *in vivo* binding (*53*), the results underscore the nonselective nature of bremazocine, making the interpretation of its *in vivo* pharmacology problematic. Thus, it appears likely that the difference in the *in vivo* pharmacological profiles observed between U69593 and bremazocine

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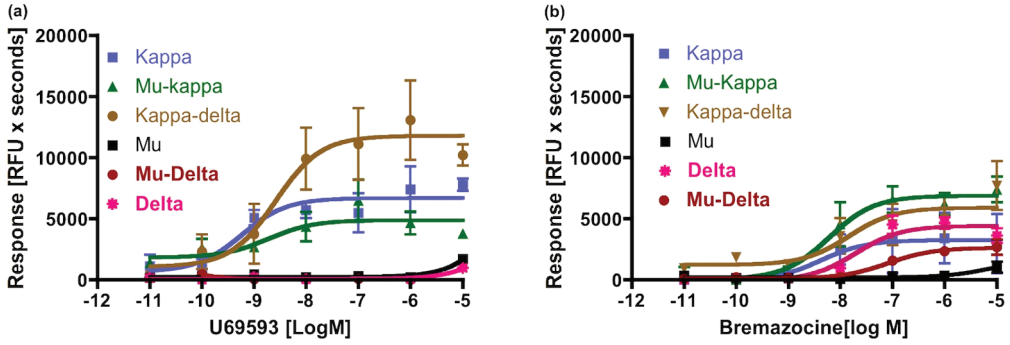

Figure 4. U69593 and bremazocine are both nonselective for κ-opioid receptor-containing homomers and heteromers. Intracellular Ca^{2+} ion release was mediated by increasing concentrations of the agonists (a) U69593 and (b) bremazocine in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. Response was measured as relative fluorescence units (RFU), and time was measured in seconds. The *Y*-axis plots the AUC values ± SEM (n = 12−16), which are represented as RFU × seconds.

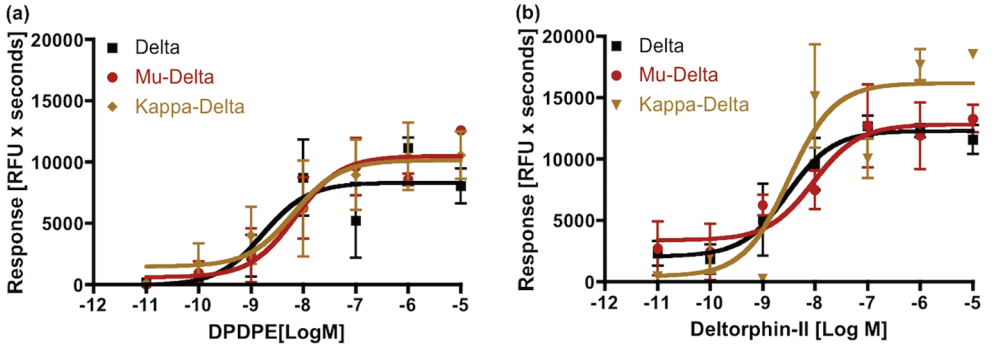

Figure 5. DPDPE and deltorphin-II are nonselective agonists for δ-opioid containing homomers and heteromers. Intracellular Ca^{2+} ion release mediated by increasing concentrations of the agonist, (a) DPDPE and (b) deltorphin-II, was measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. The *Y*-axis plots the AUC values ± SEM (n = 12−16), which are represented as RFU × seconds.

may in part be due to κ-opioid receptors that are heteromerized with μ, δ, or a nonopioid GPCR.

DOP Receptor Agonists

The two standard agonist ligands that are most frequently employed as research tools for investigating δ-opioid receptors are the peptides DPDPE (*12*) and deltorphin-II (*13*, *14*). They have been classified as δ_1 and δ_2 agonists, respectively. They are selectively antagonized *in vivo* by the δ antagonists, BNTX (δ_1) (*54*) and naltriben, also called NTB (δ_2) (*55*). The *in vivo* selective antagonism by BNTX and NTB has led to the proposal for δ opioid receptor subtypes (*54*−*57*). However, to date, genotypic δ_1 and δ_2 receptors have not been reported. In view of reports for the heterodimerization of δ receptors expressed in cultured cells (*19*−*21*, *27*, *34*, *38*), it seems plausible that the δ_1 and δ_2 subtypes actually represent phenotypic receptors that display different sensitivity to the selective antagonists (*33*, *51*).

In order to address the aforementioned questions concerning the agonist selectivity of DPDPE and deltorphin-II, we have evaluated their ability to activate homomeric and heteromeric δ receptors. The calcium release data for DPDPE reveal little, if any, difference in the activation of homomeric and heteromeric δ-opioid receptors (Figure 5a). DPDPE potently elicited a calcium response in all the cell lines (Figure 5a). DPDPE was completely nonselective, as the response was equi-efficacious and equipotent in δ, μ−δ, and κ−δ cell lines.

Similarly, deltorphin-II also activated δ, μ−δ ,and κ−δ opioid receptors to the same degree (Figure 5b). However, when compared with the other standard ligands, deltorphin-II was most active at the κ/δ opioid receptor heteromers. The apparent absence of selectivity in the activation by deltorphin-II suggests that it may produce antinociception via multiple phenotypic δ-containing opioid receptors. Moreover, the possibility that such phenotypic receptors may show tissue-specific expression increases the variability of ligand selectivity and potency *in vivo* and brings into focus the importance of route of administration (*33*, *34*, *51*). If this is the case, the antagonist selectivity for δ_1 and δ_2 phenotypes observed for BNTX and naltriben may represent their ability to target different populations of δ receptor heteromers. In this connection, it has been reported that antagonist selectivity ratios (eg, δ_1/δ_2) for BNTX and

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naltriben (NTB) differ as a function of the route of administration (*51*).

In the [^{35}S]GTPγS assay, however, DPDPE showed 60-fold lower EC_{50} at $\kappa-\delta$ receptors when compared with κ receptors. Because this is the only instance where there is a quantitative difference in the observations between the intracellular calcium release and [^{35}S]GTPγS assays, it may be related to the fact that the calcium release method employs a chimeric G protein whereas the [^{35}S]GTPγS assay measures [^{35}S]GTPγS binding to endogenous G proteins. Also, since the calcium release assay is performed in whole cells, it measures a response that is downstream to receptor activation, while in the [^{35}S]GTPγS assay the change in binding affinity is directly coupled to receptor activation via the endogenous G protein. However, although the assay outputs are different, the general qualitative similarity in results between the two assays are reassuring. If the higher selectivity of DPDPE for $\kappa-\delta$ opioid receptors observed in the [^{35}S]GTPγS experiments reflects its selectivity *in vivo*, the behavioral differences between DPDPE and deltorphin-II may indeed be due to their actions on different phenotypic receptors.

Conclusions

In order to assess the possible role of heteromers in mediating antinociception, the present study has evaluated the agonist selectivity of μ, κ, and δ standard opioid ligands that are currently widely employed in opioid research. The results reveal that all of the ligands exhibit no agonist selectivity for their presumed target homomeric opioid receptors. In fact, morphine and DAMGO selectively activate $\mu-\delta$ heteromers, which is consistent with a variety of studies that implicate the involvement of μ- and δ-opioid receptors in analgesia, tolerance, and dependence (*35*, *40*−*44*). Given the multiplicity of heteromeric μ receptors reported in cultured cells, perhaps many of the side effects associated with opioids can be traced to multiple opioid receptor heteromers. Opioid behavioral effects have been studied using standard ligands whose selectivity was based on binding studies and on the assumption that such tools identify the response of only homomeric receptors (*58*). It is therefore imperative to confirm the functional selectivity of standard ligands on both homomeric and heteromeric GPCRs in order to confidently use them as tools for pharmacological studies. Moreover, in view of the results obtained for the standard opioid agonist ligands in the present study, the anatomical distribution of colocalized opioid receptors in the CNS should be investigated as a prelude to evaluating the possible involvement of heteromers. Finally, designing ligands that selectively target specific heteromers could represent a general approach to developing analgesics with minimal side effects.

Methods

Materials

Morphine, bremazocine, U69593, deltorphin-II in salt form, and the peptide ligands DAMGO and DPDPE were provided as gifts from the National Institute on Drug Abuse.

Cells

Human embryonic kidney cells (HEK-293) stably expressing single opioid receptors (human μ and κ and mouse δ) were generated. HEK-293 cells coexpressing human opioid receptors in pairs were procured from Dr. Jennifer Whistler (University of California, San Fransisco). Their construction and characterization has been described previously (*34*). Briefly, HEK-293 cells were cotransfected with HA-δ and FLAG-μ ($\mu-\delta$), HA-δ and FLAG-κ ($\kappa-\delta$), or HA-μ and FLAG-κ ($\mu-\kappa$) to make the different stable coexpression cell lines. The stably expressing cells were checked for the expression of $\mu-\delta$ and $\kappa-\delta$ opioid receptor heteromers using coimmunoprecipitation (*34*, *38*). All the single and dual stable transfected cell lines were grown at 37 °C and 10% CO_2 in Dulbelcco's modified medium (GIBCO) supplemented with 10% FBS and 1% penicillin−streptomycin antibiotic solution. G418 alone was used for selecting cells expressing opioid receptors singly, while both G418 and Zeocin were used to select for dual coexpression cell lines.

Immunocytochemistry

Two-color immunofluorescence was employed to analyze coexpression of μ- and δ-opioid receptors. Briefly, HEK-293 cells stably coexpressing HA-δ and FLAG-μ opioid receptors were incubated in the presence of goat anti-HA (Abcam, cat. no. ab9134) antibody mixed with rabbit anti-FLAG (Abcam, cat. no. 1162) antibody for 60 min at 4 °C (final working dilution for both antibodies was 1:100). Cells were then rinsed with PBS and fixed with 2% formaldehyde for 10 min at room temperature. Then cells were washed (3 × 15 min) with 50 mM PBS (pH 7.2) and incubated 1 h at room temperature with the mixture of fluorescent secondary antibodies: anti-goat NL-493 (cat. no. NL003, R&D Systems, Inc.) and anti-rabbit NL-557 (cat. no. NL004; R&D Systems, Inc.) both diluted 1:200. After that cells were washed in PBS (3 × 15 min), counterstained with DAPI and mounted under coverslips with antifade mounting media iBright Plus (cat. no. SF40000-10; Neuromics, Inc.). Images of labeled cells were collected using Olympus FluoView1000 confocal microscope.

Intracellular Ca^{2+} Release Assay

HEK-293 cells stably expressing opioid receptors obtained from Dr. Jennifer Whistler (*34*) were transiently transfected with a chimeric G_α-protein (*39*), $\Delta6$-$G_{qi4\text{-}myr}$ at a concentration of 200 ng/20 000 cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA). The DNA for the chimeric G-protein was the only DNA that was transiently transfected. Cells were grown to a confluency of approximately 2 million cells in a petri dish. The cells were then counted, and DNA for the chimeric G-protein was added to a ratio of 200 ng/20 000 cells. Lipofectamine 2000 at a ratio of 1:2 w/v (DNA/Lipofectamine) was used for the transfection. The cells were then seeded 24 h later into half area black 96 well plates (Corning) at 20 000 cells per well. The FLIPR calcium kit (Molecular Devices) was used for the assay. Cells were incubated with a Ca^{2+} ion chelating dye from the kit, 48 h

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after transfection, and incubated for an hour. The plates were then assayed in a Flexstation-III apparatus (Molecular Devices) using a range of concentrations of the opioid ligand. The response was measured as relative fluorescence units (RFUs), and the time of the response was measured in seconds. A response window of 33 s after ligand addition was used to measure the response before calcium ion reuptake mechanisms caused a drop in fluorescence. Area under the curve (RFU × seconds) was computed for each concentration, which was then plotted as a concentration−response curve using nonlinear regression. To incorporate well−well variability, four well replications were performed for each concentration of the ligand. Importantly, each ligand was tested in at least three independent replications, where each replicate experiment consists of cells transiently transfected with the chimeric G-protein on a separate day thus ensuring true biological replication. The representative curves, EC_{50} and AUC_{peak} values are all thus calculated from the four internal/dependent and three independent replications. Thus any variability due to transfection is contained in the error bars and has been accounted for.

[^{35}S]GTPγS Assay

The [^{35}S]GTPγS assay as described previously (*59*) was used to measure the level of activation of endogenous $G_{i/o}$ proteins due to receptor activation. Briefly, with varying concentrations of ligand, HEK-293 cell membranes expressing opioid receptors and [^{35}S]GTPγS (Perkin-Elmer) were incubated together in a 96 well plate in membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 0.5% BSA) for an hour at 37 °C. The incubated mixtures were then filtered onto a filter plate (Multiscreen HTS, Millipore) and counted for [^{35}S]. At least three replications were performed for each treatment.

Supporting Information Available

Table of EC_{50} and AUC_{peak} data for the intracellular calcium release experiments and table of EC_{50} and E_{max} data for the [^{35}S]GTPγS experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations

GPCR, G-protein coupled receptor; DAMGO, [D-Ala^2, MePhe^4, Glyol^5]-enkephalin; DPDPE, [D-Pen^{2}D-Pen^5]-enkephalin; RFU, relative fluorescence units; HEK-293 cells, human embryonic kidney cells; NTB, naltriben; BNTX, 7-benzylidenenaltrexone; FBS, fetal bovine serum; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin.

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