A Role for the Distal Carboxyl Tails in Generating the Novel Pharmacology and G Protein Activation Profile of μ and δ Opioid Receptor Hetero-oligomers*

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Opioid receptor pharmacology in vivo has predicted a greater number of receptor subtypes than explained by the profiles of the three cloned opioid receptors, and the functional dependence of the receptors on each other shown in gene-deleted animal models remains unexplained. One mechanism for such findings is the generation of novel signaling complexes by receptor hetero-oligomerization, which we previously showed results in significantly different pharmacology for μ and δ receptor hetero-oligomers compared with the individual receptors. In the present study, we show that deltorphin-II is a fully functional agonist of the μ-δ heteromer, which induced desensitization and inhibited adenyl cyclase through a pertussis toxin-insensitive G protein. Activation of the μ-δ receptor heteromer resulted in preferential activation of Goi, illustrated by incorporation of GTPγS, whereas activation of the individually expressed μ and δ receptors preferentially activated Gox. The unique pharmacology of the μ-δ heteromer was dependent on the reciprocal involvement of the distal carboxyl tails of both receptors, so that truncation of the distal μ receptor carboxyl tail modified the δ-selective ligand-binding pocket, and truncation of the δ receptor distal carboxyl tail modified the μ-selective binding pocket. The distal carboxyl tails of both receptors also had a significant role in receptor interaction, as evidenced by the reduced ability to co-immunoprecipitate when the carboxyl tails were truncated. The interaction between μ and δ receptors occurred constitutively when the receptors were co-expressed, but did not occur when receptor expression was temporally separated, indicating that the hetero-oligomers were generated by a co-translational mechanism.

The endogenous opioid systems mediate important physiological functions related to pain perception, locomotion, motivation, reward, autonomic function, immune modulation, and hormone secretion. Opioid receptors, mediating the actions of the opioid peptides, belong to the family of G protein-coupled receptors (GPCRs) and have distinct pharmacological profiles with discrete but overlapping distributions in brain (reviewed Refs. 1 and 2). The pharmacology of the opioid receptors obtained in brain and other tissues has consistently predicted a greater number of receptor subtypes such as μ1, μ2, and δ1, δ2, which are not explained by the individual pharmacological profiles obtained for the three cloned opioid receptors, μ, δ, and κ, when expressed individually in heterologous systems. One possible mechanism for these findings, gaining increasing credence, has been that of direct receptor-receptor interactions creating novel signaling units and generating distinct post-receptor functional effects.

The ability of GPCRs to form homo-oligomers and hetero-oligomers has been described by us and others (reviewed in Refs. 3–5). We have provided evidence for the direct interaction of μ and δ opioid receptors to form hetero-oligomers, with generation of novel pharmacology and G protein coupling properties, distinct from that demonstrated when each receptor was expressed individually (6). Novel functional properties generated by the formation of hetero-oligomeric opioid receptor complexes have been reported for μ and δ opioid receptors (6, 7) and δ and κ opioid receptors (8). Receptor homo-oligomerization appears to be a universal occurrence for GPCRs, with functional roles emerging in cellular processes such as receptor trafficking after synthesis in the endoplasmic reticulum (9). The functional roles for hetero-oligomerization of GPCRs appear varied, ranging from chaperone-like aiding of cell surface localization to novel pharmacology and signal transduction properties, depending on the interacting receptors (4).

μ and δ opioid receptors share 65% overall amino acid homology and even greater homology in the transmembrane domains (1). We have previously shown that the receptors expressed individually formed robust SDS-resistant homodimers and higher order homo-oligomers, whereas under identical conditions co-expressed μ and δ receptors did not form SDS-resistant heterodimers (6), suggesting a different or weaker interaction between them. The co-expressed receptors were shown to have significant alterations of their individual pharmacology and signal transduction properties, with the emergence of unique properties (6, 7) and additive signaling functions (10). In the present study, we sought to further characterize the differences in the pharmacology and signaling mechanisms arising from the formation of the μ-δ hetero-oligomeric complex and to identify a specific agonist for the heteromer. We also sought to define the structural basis for the receptor-receptor interaction, which has not been characterized. We demonstrated that deltorphin-II was an agonist of the μ-δ heteromer, capable of inducing desensitization and selectively activating the pertussis toxin-insensitive Go protein Gz, whereas agonist activation of the individual μ and δ receptors activated the pertussis toxin-sensitive Gz. We also determined that the distal carboxyl tails of both receptors participated in the structural basis for the generation of the unique pharmacology of the μ and δ heteromer and had a significant role in the interaction between the receptors.
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Pertussis Toxin Treatment—Cells co-expressing μ and δ receptors were incubated with pertussis toxin (1 μg/ml) or vehicle for 24 h and then membranes were harvested.

Adenylyl Cy clase Assay—Adenylyl cyclase assays were conducted essentially as described (11). The assay mixture contained 0.02 ml of membrane suspension from cells (10–25 μg of protein), 0.1 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phosphoenolpyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase, 1 mM forskolin, and 0.13 μCi of [3H]ATP in a final volume of 0.05 ml. The mixture was incubated with 10–12 M agonist at 22 °C for 20 min, and enzyme activities were determined. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [3H]cAMP (25,000 cpm). cAMP was isolated by sequential column chromatography using Dowex cation-exchange resin and aluminum oxide. Data were expressed as picomole of cAMP/pmol of receptor and analyzed by computer-fitted nonlinear least-squares regression.

Electrophoresis and Immunoblotting—The membrane samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions using 10 or 12% precast acrylamide gels (Novex) and transferred to nitrocellulose as described previously (5). Immunoreactivity was revealed with antibodies raised against the c-Myc epitope (Santa Cruz Biotechnology) and the FLAG epitope (Sigma), horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad), and with the enhanced chemiluminescence detection kit (Amersham Biosciences).

Immunoprecipitation Studies—The P2 membrane pellet was re-suspended and stirred at 4 °C overnight with protease inhibitors as previously described (5). The homogenate was centrifuged and the solubilized fraction was washed and concentrated. The washed fraction was precleared with agarose-fixed goat anti-mouse IgG overnight, and the solubilized receptors were immunoprecipitated with the mouse monoclonal anti-Myc or anti-FLAG antibody and agarose-fixed goat anti-mouse IgG as previously described. The immunoprecipitate was washed, solubilized in SDS sample buffer, and electrophoresed by SDS-polyacrylamide gel electrophoresis.

Measurement of Ga-specific [35S]GTPγS Binding—Cells were washed with phosphate-buffered saline and lysed by Polytron disruption in ice-cold 5 mM Tris-HCl and 2 mM EDTA buffer containing 5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor as described previously (11). The lysate was subjected to centrifugation at 100,000 × g for 20 min at 4 °C to prepare the membrane fraction. Membrane protein was determined by the Bradford assay (Bio-Rad) according to the manufacturer’s instructions.

Radioligand Binding Assays—Saturation binding experiments were performed with 25 μg of P2 membrane protein incubated with increasing concentrations of [125I]naloxone or [3H]diprenorphine to determine receptor densities (Bmax) and ligand affinities (KD) as described previously (12). Each drug concentration was examined in duplicate or triplicate and incubated for 2 h at 22 °C in a total volume of 1 ml of binding buffer (50 mM Tris-HCl, 5 mM EDTA, 1.5 mM CaCl2, 5 mM MgCl2, 5 mM KCl, and 120 mM NaCl) with protease inhibitors. Nonspecific binding was defined as that not displaced by 10 μM naltrexone. Competition experiments were performed in triplicate with increasing concentrations of competing ligand. The concentration of radioligand used in the competition assays was approximately equivalent to its KD. Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel) using GF/C filters (Whatman). Filters were washed with 10 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and placed in glass vials with scintillation fluid (Universol) and counted for tritium. Data were analyzed by nonlinear least-squares regression using Prism (GraphPAD Software). A two-site fit was designated only when a statistically significant improvement of the fit over a one-site model was obtained by comparison of the coefficients of the goodness-of-fit by an F test.

Agonist Treatment—Cells co-expressing μ and δ receptors were incubated with agonist (10 μM) or vehicle for 1 h at 22 °C and then membranes were harvested. The membranes were washed three times by resuspension in binding buffer and centrifugation to ensure that agonist did not remain bound to the receptors. Cells were also exposed to agonist for 1–4 h, and following three washes in binding buffer were analyzed by whole cell binding.

Materials and Methods

Construction of μ and δ Opioid Receptor Expression Vectors—cDNAs encoding the rat μ and δ opioid receptors were inserted into the mammalian expression vector pcDNA3 as previously described (5). For immunoprecipitation studies, the receptors were tagged with c-Myc (EQKLISEEDL) or FLAG (DYKDDDDK) epitope tags, using the Transformam site-directed mutagenesis kit (Clontech), also as described (5). The absence of sequence errors and the correct orientation of the polymerase chain reaction products in the expression vectors were verified by sequencing on both strands.

Expression in Mammalian Cells—COS-7 monkey kidney cells and Chinese hamster ovary cells (CHO-K1; American Type Culture Collection) were maintained as monolayer cultures at 37 °C in minimal essential medium, supplemented with 10% fetal bovine serum and antibiotics, and transfected using Lipofectamine (Invitrogen). An equal amount of pcDNA3 vector was co-transfected with each receptor construct so that the total amount of DNA used was consistent with studies involving transfections with two constructs. The receptor expression levels ranged between 600 and 1000 fmol/mg of protein after 24–48 h post-transfection. For the staggered expression, the receptor cDNA, and receptor cDNA so that the total amount of DNA used was consistent with studies involving transfections with two constructs. The receptor expression levels ranged between 600 and 1000 fmol/mg of protein after 24–48 h post-transfection. For the staggered expression, the receptor cDNA, and receptor cDNA were transfected first, followed 24 h later by the receptor cDNA, and receptor cDNA, and cells harvested 24 h later. In separate experiments, the receptors were also transfected in the reverse order.

Membrane Preparation—Cells were washed extensively with phosphate-buffered saline and lysed by Polytron disruption in ice-cold 5 mM Tris-HCl and 2 mM EDTA buffer containing 5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor as described previously (11). The lysate was subjected to centrifugation at 100,000 × g to pellet unbroken cells and nuclei and to recover the supernatant, which was centrifuged at high speed (40,000 × g for 20 min at 4 °C) to prepare the membrane fraction. Membrane protein was determined by the Bradford assay (Bio-Rad) according to the manufacturer’s instructions.

Radioligand Binding Assays—Saturation binding experiments were performed with 25 μg of P2 membrane protein incubated with increasing concentrations of [125I]naloxone or [3H]diprenorphine to determine receptor densities (Bmax) and ligand affinities (KD) as described previously (12). Each drug concentration was examined in duplicate or triplicate and incubated for 2 h at 22 °C in a total volume of 1 ml of binding buffer (50 mM Tris-HCl, 5 mM EDTA, 1.5 mM CaCl2, 5 mM MgCl2, 5 mM KCl, and 120 mM NaCl) with protease inhibitors. Nonspecific binding was defined as that not displaced by 10 μM naltrexone. Competition experiments were performed in triplicate with increasing concentrations of competing ligand. The concentration of radioligand used in the competition assays was approximately equivalent to its KD. Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel) using GF/C filters (Whatman). Filters were washed with 10 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and placed in glass vials with scintillation fluid (Universol) and counted for tritium. Data were analyzed by nonlinear least-squares regression using Prism (GraphPAD Software). A two-site fit was designated only when a statistically significant improvement of the fit over a one-site model was obtained by comparison of the coefficients of the goodness-of-fit by an F test.

Agonist Treatment—Cells co-expressing μ and δ receptors were incubated with agonist (10 μM) or vehicle for 1 h at 22 °C and then membranes were harvested.
Western blots confirmed that the antibodies identified the specific Gα species in membranes and after immunoprecipitation by the epitope-tagged μ opioid receptor. Verification of the specific band was obtained by use of the antigenic peptide sequence to which the antibody was generated.

Receptor Immunocytochemistry—Cells expressing epitope-tagged myc-μ and FLAG-δ receptors prepared by co-transfecting the cDNA for the two receptors or staggering them 24 h apart were examined by a LSM 510 confocal microscope (Zeiss) after incubation with the specific c-myc and FLAG antibodies, followed by the species-specific secondary antibodies labeled with fluorescein isothiocyanate and TRITC.

RESULTS

Radioligand binding competition assays were conducted to identify high affinity agonists for the co-expressed μ and δ receptors. Membranes from cells co-expressing μ and δ receptors bound the nonselective antagonists [3H]naloxone and [3H]diprenorphine with high affinity. Competition by opioid agonists revealed highest affinity for the δ agonists deltorphin-I and DSLET compared with the δ agonist DPDPE or the μ agonist DAMGO. Therefore deltorphin-II was selected for detailed study. In the absence of drug treatment (n = 4), deltorphin-II-detected Kᵢ was 3.2 ± 0.9 nM and Kᵢ was 4.9 ± 1.0 μM with 57 ± 6% of...
receptors in the high affinity state. Pretreatment of cells with deltorphin-II induced a lowering of the affinity of the agonist-detected high affinity state, indicative of desensitization (Fig. 1A). Following deltorphin-II pretreatment for 60 min, the affinities shifted to $K_i$ values of 15.8 nM and 25.8 ± 13.3 μM with a reduction of receptors in the high affinity state to 35 ± 1%. In contrast, similar treatments with the μ-selective DAMGO or the δ-selective DPDPE did not induce any change in the affinity constants or profiles in the μ-δ cells (Fig. 1, B and C) nor did it induce any receptor down-regulation. Because deltorphin-II appeared to function as an agonist of the heteromeric μ-δ receptors, expressing both receptors were exposed to the drug for 1–4 h ($n = 3$), and receptor density was evaluated on the cell surface by whole cell binding. The receptor heteromers exhibited desensitization, with a 10-fold shift in affinity of the KH from 2 to 20 nM (Fig. 1D). With deltorphin-II treatment for 2 h, there was evidence of significant down-regulation as well, with reduction both of the total density of receptors (by 32%) and further reduction in the fraction of receptors in the high affinity state (by 70%) (Fig. 1E). When agonist treatment was extended to 4 h, there was a further reduction in the total density of receptors, but no further change in the $K_i$ and $K_R$ values. Treatment of cells expressing μ or δ receptors singly showed desensitization and down-regulation of the receptors in response to treatment with DAMGO or DPDPE. Exposure of μ receptor-expressing cells to DAMGO for 1 h resulted in an increase of $K_i$ from 5 to 42 nM and a reduction in receptor density by 38% (Fig. 1F). Similarly, treatment of δ receptor-expressing cells by DPDPE for 1 h resulted in a shift of the $K_i$ from 2 to 42 nM and a reduction in receptor density by 37% (Fig. 1G).

The nature of the deltorphin-II detected high affinity site in the heteromeric μ-δ receptor complex was further evaluated by determining its sensitivity to guanine nucleotides and pertussis toxin. Incubation of membranes co-expressing μ and δ opioid receptors with GTPγS (500 μM) resulted in a rightward shift of the deltorphin-II detected affinity states, indicating uncoupling of the complex from G protein (Fig. 2A). This was in contrast to the effects observed with DAMGO, for which there was no shift in affinity by GTPγS (Fig. 2B). We have previously shown that the G protein involved in the generation of the agonist-detected high affinity state in cells expressing the μ-δ opioid receptor complex was pertussis toxin-insensitive and different from the pertussis toxin-sensitive G proteins coupling of μ and δ opioid receptors expressed individually (6). Pertussis toxin treatment (1 μg/ml for 24 h) did not abolish the ability of deltorphin-II to inhibit forskolin-stimulated adenylyl cyclase activity but reduced the potency (Fig. 3). This is in marked contrast to the complete abolition of adenylyl cyclase activity inhibition by deltorphin-II when μ or δ opioid receptors were expressed individually and treated with pertussis toxin (Fig. 3).

The affinity profiles of several opioid agonists were tested before and after pertussis toxin (PTX) treatment, to evaluate the pharmacological rank order of these compounds at the μ-δ-PTX-insensitive G protein complex (Fig. 4). Agonist-detected high and low affinity sites were evident after PTX treatment for all agonists tested (Fig. 4, A–D). The relative rank order of agonist potencies following PTX treatment was DSLET > deltorphin-II > DAMGO > DPDPE. The affinities for deltorphin-II were $K_i$ values of 3.1 nM and 4.6 μM following vehicle treatment, and $K_i$ values of 35.5 nM and 72.7 μM following PTX treatment ($n = 3$). Shown in contrast are the effects of PTX to shift the agonist affinity of DAMGO for the μ receptor (Fig. 4E) and DPDPE for the δ receptor (Fig. 4F) to a single low affinity site. To identify the nature of the G protein involved, we examined for a PTX-insensitive G protein capable of coupling negatively to adenylyl cyclase. Western blots of HEK and COS cells identified $G_\alpha_\delta$ in the membrane fraction, although present in much lower concentrations than $G_\alpha_\mu$ (data not shown). To determine whether the concentration of $G_\alpha_\delta$ in the cells had a limiting role, $G_\alpha_\delta$ was co-expressed with μ and δ receptors. This had no effect on the affinity profile of deltorphin-II for the co-expressed μ-δ heteromeric receptors (Fig. 5A), but significantly enhanced the affinity of DAMGO for the μ-δ heteromers (Fig. 5B). In sharp contrast, co-expression of $G_\alpha_\delta$ did not affect the affinities of DAMGO for the μ receptor (Fig. 5C) or DPDPE for the δ receptor (Fig. 5D) when each was expressed individually. The relative levels of endogenous $G_\alpha_\delta$ (Fig. 5F, lanes 4–6) and levels of endogenous $G_\alpha_\mu$ (Fig. 5E, lane 7) and following its transfection are shown (Fig. 5E, lanes 4–6) as well as the ability to immunoprecipitate the G proteins using the c-myc epitope-tagged μ receptor (Fig. 5, E, lanes 1–3 and F, lanes 1–3). The specificity of the antibody-detected band was confirmed by its displacement by blocking peptide representing the antigen against which the antibody was directed, as shown for $G_\alpha_\delta$ (Fig. 5G).
To evaluate the role of the $G_\delta$ protein in agonist-mediated signal transduction via the $\mu-\delta$ heteromer, the ability of various agonists to induce GTP$^{35S}$ binding to specific G proteins was evaluated. In membranes from cells expressing $\mu$ opioid receptors, DAMGO induced a robust increase in GTP$^{35S}$ binding to $G_\delta$ within 1 min of agonist treatment, which waned by 15 min (Fig. 6A). In contrast, DAMGO had no effect on GTP$^{35S}$ binding to $G_\delta$ when $\mu$ receptors were exclusively activated (Fig. 6B). In cells expressing $\delta$ receptors individually, both deltorphin-II and DPDPE induced GTP$^{35S}$ binding to $G_\delta$ (Fig. 6C), but only very minor increases in GTP$^{35S}$ binding to $G_\delta$ (Fig. 6D) were seen. In membranes from cells co-expressing $\mu$ and $\delta$ receptors along with $G_\delta$, deltorphin-II induced a 75% increase in GTP$^{35S}$ binding to $G_\delta$ at 1 min, which persisted at 5 min of agonist activation (Fig. 7A) showing the time course of $G$ protein activation. In this preparation, DAMGO and DPDPE had much smaller effects on incorporation of GTP$^{35S}$ into $G_\delta$ (Fig. 7A) or $G_\delta$ (Fig. 7B).

We have previously shown that $\mu$ and $\delta$ receptors formed SDS-resistant homodimers and oligomers, whereas the $\mu-\delta$ hetero-oligomer was easily disrupted under similar experimental conditions (6), suggesting that a less robust interaction may mediate the formation of this complex, compared with the formation of receptor homodimers. Moreover, because agonist-induced internalization of the $\delta$ receptor was prevented by complexing with the $\mu$ opioid receptor (6) and the internalization of the $\mu$ receptor has been proposed to involve the distal carboxyl tail (13), we hypothesized that the interaction between $\mu$ and $\delta$ opioid receptors may involve the carboxyl tail of the $\delta$ receptor.

The $\mu$-agonist DAMGO had distinctly different affinities for the individually expressed and co-expressed $\mu$ and $\delta$ receptors as shown (Fig. 8A). The terminal 15 amino acid residues of the carboxyl tail of the $\delta$ opioid receptor from Val$^{357}$ to Ala$^{372}$ were deleted and this construct was termed $\delta$T1. Expression of the $\mu$ opioid receptor with the truncated $\delta$T1 receptor partially restored the profile of the $\mu$-selective agonist DAMGO, particularly detectable at the high affinity site (Fig. 8B). To determine whether a larger truncation would have additional effects, a second deletion was made following Ser$^{344}$ to generate the $\delta$T2 receptor. Co-expression of $\delta$T2 with the $\mu$ opioid receptor revealed complete
FIGURE 5. Effect of co-expression of Gz on the pharmacology of co-expressed or individually expressed μ and δ opioid receptors and ability of G proteins to immunoprecipitate (IP) with the opioid receptors. Competition of [3H]diprenorphine binding to membranes from cells transfected (open symbols) or not transfected (solid symbols) with Gz, by (A) deltorphin-II and (B) DAMGO to μ-δ heteromers, (C) DAMGO to μ receptors, and (D) DPDPE to δ receptors. The detection of Gz endogenous levels and following transfection (E) and the endogenous levels of Gz3 in COS cell membranes (F) and their ability to be immunoprecipitated by c-myc-tagged μ opioid receptor under various conditions is shown. The specificity of the Gz band detected is confirmed by its displacement by the antigenic peptide (G). IB, immunoblot.

FIGURE 6. Incorporation of GTPγS into Gα proteins by selective agonist activation of μ or δ opioid receptors. Membranes from cells expressing μ or δ receptors were treated with vehicle (C, control), DAMGO (DM), deltorphin II (DL), or DPDPE (DP) for the times specified in the presence of GTPγS, and the Gα protein was immunoprecipitated by specific antibodies. The Gα3 assayed was endogenous, whereas Gz was transfected. Data are expressed as a percentage of the basal value and are the mean ± S.E. of 3 experiments. Significant difference from control is denoted by the asterisk, *p < 0.05.
normalization of the DAMGO-detected binding to both the high affinity and low affinity sites of the μ opioid receptor (Fig. 8C). Western blot and radioligand binding analysis of these membranes established that both constructs were expressed normally in equivalent amounts. These data indicated that the deletion of the carboxyl tail residues of the δ receptor enabled restoration of μ opioid pharmacology even when the receptors were co-expressed. Thus, the co-expression of the μ receptor with the truncation mutant of the δ receptor permitted the recovery of detection by DAMGO of a typical μ opioid binding pocket. However, the pharmacology of the δT1 and δT2 receptors detected by DPDPE when expressed alone or co-expressed with the μ opioid receptor was only partially restored to approximate that of the wild type δ receptor, likely because of the truncation of the carboxyl tail residues (Fig. 9, A and B).

To determine whether the carboxyl tail of the μ opioid receptor also had any role in the altered pharmacology of the μ-δ heteromeric complex, two deletion mutants of the μ opioid receptor at positions comparable with that selected for the δ receptor were made. The first construct, missing 23 amino acids distal to Ser352 in the carboxyl tail termed μT1, and the second, truncated distal to Ser286 in the carboxyl tail termed μT2, were generated. Co-expression of these μ receptor truncation mutants with the δ opioid receptor revealed recovery of affinity with respect to the DPDPE-detected high affinity site but without full recovery of affinity of the low affinity site (Fig. 10). These results indicate that truncation of the terminal carboxyl tail portions of μ and δ opioid receptors resulted in preserved high affinity agonist-detected μ- and δ-selective pharmacology when the two receptors were co-expressed.

Although the carboxyl tails of the μ and δ opioid receptors have been shown to have a role in the novel pharmacology generated by their association, it remained to be determined whether the interaction between these receptors resided solely within the carboxyl tails. Epitope tag containing μ and δ opioid receptors could be efficiently co-immunoprecipitated with each other (Fig. 11A, lanes 2 and 3). Immunoblot analyses of the carboxyl tail-truncated μT1 (not shown) and δT1 receptors (Fig. 11D, lane 2) revealed no alteration of their ability to form homo-oligomeric assemblies, indicating that the carboxyl tails were not an important component of the homodimeric receptor-receptor interactions of μ or δ opioid receptors. Moreover, the ability of differentially epitope-tagged μ receptors to immunoprecipitate with each other was not affected by truncation of the carboxyl tail (data not shown). However, when the c-myc epitope-tagged μ opioid receptor was co-expressed with the FLAG-tagged δT1 receptors, their efficacy of co-immunoprecipitation was diminished (Fig. 11B, lane 1 and C, lane 2) compared with the full-length receptors (Fig. 11A, lanes 2 and 3). These results indicate that the carboxyl tails are likely important domains of contact within the heteromeric μ-δ receptor complex, but are not the only points of contact between μ and δ receptors.

Because we have previously shown that mixing membranes separately expressing μ and δ opioid receptors resulted in preservation of their distinct pharmacological profiles detected by the selective agonists, in contrast to the co-expression of the receptors in the same membrane (6), we determined whether the temporal dissociation of synthesis of the two receptors within the same cells could affect their heteromeric association. The staggered transfection of μ and δ opioid receptors was compared with co-transfection of these receptors. The affinity of DAMGO for the μ opioid receptor when co-expression was staggered by 24 h was in the low nanomolar range for the high affinity site, as is typical for μ opioid receptors expressed singly, with a 10-fold lower

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**Figure 7.** Incorporation of GTPγ35S into Go proteins by selective agonist activation of the μ-δ heteromeric opioid receptors. Membranes from cells co-expressing μ and δ receptors were treated with vehicle (C, control), DAMGO (DM), deltorphin II (DL), or DPDPE (DP) for the times specified in the presence of GTPγ35S, and the Go protein was immunoprecipitated by specific antibodies. The Go2, assayed was endogenous, whereas Go1, was transfected. Data are expressed as a percentage of the basal value and are the means of four to six experiments.

**Figure 8.** Effect of truncation of the distal carboxyl tail of the δ opioid receptor on the μ-selective agonist profile at co-expressed μ and δ receptors. The serially truncated receptors δT1, δT2, or the wild type δ receptor were co-expressed with the μ opioid receptor. Cell membranes were harvested and analyzed by [3H]diprenorphine radioligand competition assays. Results shown are representative of n = 3–6 experiments.
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**FIGURE 9.** Effect of truncation of the distal carboxyl tail of the \( \delta \) opioid receptor on the \( \delta \)-selective agonist profile at co-expressed \( \mu \) and \( \delta \) receptors. The serially truncated mutants \( \delta T1, \delta T2, \) or the wild type \( \delta \) receptor were co-expressed with the \( \mu \) opioid receptor. Cell membranes were harvested and analyzed by radioligand competition assays using \( [3H] \) diprenorphine. Results shown are representative of \( n = 3-4 \) experiments.

**FIGURE 10.** Effect of truncation of the distal carboxyl tail of the \( \mu \) opioid receptor on the \( \delta \)-selective agonist profile at co-expressed \( \mu \) and \( \delta \) receptors. The serially truncated mutants \( \mu T1, \mu T2, \) or the wild type \( \mu \) receptor were co-expressed with the \( \delta \) opioid receptor. Cell membranes were harvested and analyzed by radioligand competition assays using \( [3H] \) diprenorphine. Results shown are representative of \( n = 3 \) experiments.

**FIGURE 11.** Immunoblot (IB) analysis of \( \mu \) and \( \delta \) opioid receptors (A) or the truncation mutant \( \delta T1 \) expressed with the \( \mu \) opioid receptor (B and C), showing the ability of \( \delta \) and \( \delta T1 \) receptors to immunoprecipitate (IP) with the \( \mu \) opioid receptor. Cell membranes expressing (D) the \( \delta T1 \) compared with the \( \delta \) receptor expressed alone shows the difference in their sizes. \( \delta \) and \( \delta T1 \) receptors were tagged with the FLAG epitope. The c-myc-tagged \( \mu \) opioid receptor was co-expressed with FLAG-\( \delta \) or FLAG-\( \delta T1 \) and immunoprecipitated using the FLAG antibody and visualized with the c-myc antibody or vice versa.

affinity for the \( \mu-\delta \) heteromer when the receptors were co-expressed (Fig. 12A). Similarly, the high affinity site for DPDPE binding remained intact when \( \mu \) and \( \delta \) receptor co-expression was staggered, while it was lower, shifted 10-fold to the right, when the receptors were co-expressed (Fig. 12B). Identical results were obtained when the order of the transfections was reversed. The cellular distribution of \( \mu \) and \( \delta \) receptors was examined by confocal laser microscopy. Both receptors were seen to have the same distribution on the cell surface and in the cytoplasm when co-transfected (Fig. 12C) or when transfection was staggered (Fig. 12D). These results indicated that the heteromeric receptor interaction likely occurs during or soon following translation and not at the cell surface. Furthermore, this demonstrated that receptors can exist within the same cellular compartments without interacting with each other.

**DISCUSSION**

The heteromeric interaction between \( \mu \) and \( \delta \) opioid receptors generating novel pharmacological and functional properties indicates formation of a unique signaling complex, distinct from that present when the receptors are expressed individually, forming homo-oligomers. We have established that deltorphin-II is a potent agonist at the \( \mu-\delta \) complex, with detection of agonist high affinity binding sites that were sensitive to guanine nucleotide and exhibited desensitization on prolonged agonist exposure by shifting to a lower affinity state. In the \( \mu-\delta \) complex, deltorphin-II mediated these agonist effects through the involvement of a PTX-insensitive G protein, G\(_3\). The switch from G\(_{i3}\) activation by agonist interaction of \( \mu \) and \( \delta \) receptor homo-oligomers to G\(_{i}\) activation by \( \mu-\delta \) heteromer activation represents the first definitive demonstration of a radical shift in G protein specificity for a hetero-oligomeric receptor complex to one that is different from the specificity of that of its receptor constituents. We also identified that the structural basis for the \( \mu-\delta \) interaction in part, involved the distal portions of the carboxyl tails of both \( \mu \) and \( \delta \) receptors as crucial elements necessary for generating the novel pharmacological profile of the hetero-oligomer. The carboxyl tail interaction was also responsible partly for the physical association of \( \mu \) and \( \delta \) receptors but not for the \( \mu-\mu \) or \( \delta-\delta \) homo-oligomeric interactions.

Analysis of opioid receptor pharmacology has provided evidence for the existence of a hybrid and varied pharmacology in different tissues (14, 15), which even before the cloning of the opioid receptors, was proposed to be because of the formation of “complexes” between distinct opioid receptor types (16). Subsequently, we and others showed that, at least in part, the molecular basis for these observations may be
the hetero-oligomerization of $\mu$ and $\delta$ opioid receptors (6, 7). Interestingly, the earlier studies had demonstrated the greater efficacy of ligands such as deltorphin-II rather than DPDPE at this complex (14, 15), which corroborates our current findings exactly. However, the agonist affinity profile of the co-expressed $\mu$ and $\delta$ receptors probably best approximates that described for the pharmacological $\delta_2$ subtype, but exceptions to the pharmacological rank order have been demonstrated (7), which preclude the definitive conclusion that the $\mu$-$\delta$ heteromer represents the previously characterized opioid $\delta_2$ receptor subtype defined in tissues.

In our studies, deltorphin-II appears to function as a true agonist at the $\mu$-$\delta$ heteromeric complex, as evidenced by the detection of receptors in a high affinity state, their sensitivity to guanine nucleotides, and evidence of desensitization and down-regulation with prolonged exposure to deltorphin-II. The novel pharmacological profile obtained with the co-expressed $\mu$ and $\delta$ receptors indicated that the binding pockets of each receptor had been altered. In fact, both DAMGO and DPDPE, considered to be $\mu$-selective and $\delta$-selective respectively, had lower but equivalent affinities for the $\mu$-$\delta$ heteromeric receptors. The continued ability of deltorphin-II to behave as an agonist following treatment with pertussis toxin indicated that the $\mu$-$\delta$ receptors were associated with a PTX-insensitive G protein. Because the predominant interactions of $\mu$ and $\delta$ opioid receptors with G proteins involve $G_\delta$ proteins, we examined the effects of PTX to irreversibly ADP-ribosylate $G_\delta/G_\alpha$ proteins and uncouple it from the receptors, and demonstrated that adenylyl cyclase signaling via the heteromeric $\mu$-$\delta$ opioid receptors persisted even after $G_\delta/G_\alpha$ inactivation. The ability of deltorphin-II to inhibit adenylyl cyclase after PTX treatment of cells expressing $\mu$-$\delta$ receptors indicated that the second messenger coupling resulting in inhibition of adenylyl cyclase activity was mediated by a PTX-insensitive G protein, possibly $G_\delta$ (17). Even though opioid receptors have the ability to couple to multiple G proteins, including $G_\delta$ (18, 19), we have shown previously for the $\mu$ opioid receptor, that inactivation of the $G_\delta/G_\alpha$ proteins by PTX results in complete loss of the DAMGO-detected high affinity site, with 100% of receptors shifted to the agonist-detected low affinity state (13, 20). Moreover, we have shown that adenylyl cyclase inhibition mediated by $\mu$ or $\delta$ opioid receptors is completely abolished by treatment with PTX (6), indicating preferential coupling to $G_\delta$ proteins when $\mu$ or $\delta$ receptors are expressed individually. In light of these findings, we surmised that the agonist ligand affinity profile obtained following PTX treatment should reflect the affinities of these compounds for the $\mu$-$\delta$ heteromer coupled to the novel PTX-insensitive G protein. The rank order of affinities obtained after PTX treatment: DSLET > deltorphin-II > DAMGO > DPDPE is therefore unique to the $\mu$-$\delta$ heteromeric complex. The comparable rank order profile of these agonists for adenylyl cyclase inhibition following PTX treatment indicates that the $\mu$-$\delta$ complex mediated this function. Most importantly, we have provided evidence for the direct incorporation of GTP$\gamma$S into $G_\delta$ by deltorphin-II activation of the $\mu$-$\delta$ heteromer, in marked contrast to the pref-

FIGURE 12. Effect of co-expression or staggered expression of $\mu$ and $\delta$ opioid receptors. Cells were co-transfected with the cDNA constructs for $\mu$ and $\delta$ receptors, or transfected 24 h apart. Membranes were harvested and analyzed by $[^3H]$diprenorphine competition assays using DAMGO (A) and DPDPE (B). Results shown are representative of n = 3 experiments. Confocal microscopy of cells (C) co-transfected with myc-$\mu$ and FLAG-$\delta$ receptors or (D) with staggered expression of FLAG-$\delta$, then myc-$\mu$ receptors 24 h later, were fixed and evaluated by immunocytochemistry using primary antibodies against the epitopes and secondary antibodies coupled to fluorescein isothiocyanate or TRITC.
Role for Carboxyl Tails in Opioid μ-δ Receptor Heteromer

The identification of novel G protein coupling by the co-expressed μ and δ receptors and our previous demonstration of the abolition of δ opioid receptor internalization by a δ agonist when the receptor was co-expressed with μ receptors (6), led us to hypothesize that the heteromeric association of these receptors may have induced conformational differences in the intracellular domains of the μ and δ receptors. Indeed, the sum of the findings suggests that the ligand binding pocket and the conformation of the intracellular domains of the μ-δ complex differed from that in the homomeric μ-μ or δ-δ receptor complexes.

In particular, the carboxyl tails of GPCRs have been shown to have important functional roles in mediating many of the steps necessary for agonist-induced receptor internalization (21, 22). More specifically, sites are likely present as well, because co-immunoprecipitation of carboxyl tails had no role in generating the homomeric assemblies of identical manner (13). Our data show that the carboxyl tail of the δ opioid receptor internalization was somehow occluded. To test this, we selectively truncated the distal 15-amino acid segment of the δ opioid receptor carboxyl tail, and then a further stretch of 14 amino acids, to determine that removal of these residues restored the classic DAMGO-detected μ opioid receptor pharmacology in the co-expressed receptors, indicating that the carboxyl tail of the δ opioid receptor was involved in mediating the change to the DAMGO binding site of the μ opioid receptor. Because the DPDPE-detected high affinity state was not fully restored by this maneuver, comparable truncations were also made in the μ opioid receptor. When the μ receptor truncation mutants were co-expressed with the δ opioid receptor, the DPDPE-detected high affinity site remained intact, indicating that the carboxyl tail of the μ receptor had contributed to the alteration of the DPDPE binding site in the δ receptor. These data reveal that the carboxyl tails of both receptors were reciprocally involved in generating the novel pharmacology of the μ-δ heteromer.

The contribution of the distal portions of the carboxyl tails to the overall ability of the receptors to hetero-oligomerize was investigated. Each of the δ and μ truncation mutants when expressed alone formed robust SDS-resistant homodimers and higher order oligomers, in contrast to what has been reported for the δ opioid receptor truncated in the identical manner (13). Our data show that the carboxyl tails had no role in generating the homomeric assemblies of μ or δ opioid receptors. The reduced ability of the truncated δ receptor to co-immunoprecipitate with the wild type μ receptor and vice versa, indicated that the interactions involving the carboxyl tail were significant in keeping the heteromeric complex intact. However, interactions between μ and δ receptors in other sites are likely present as well, because co-immunoprecipitation was not abolished. The μ-δ interaction only occurred when the receptors were coexpressed, indicating that the formation of the hetero-oligomers likely occurred co-translationally or soon after translation. The staggered expression of the two receptors by 24 h prevented hetero-oligomerization and resulted in the pharmacological profile typical of μ and δ homo-oligomers.

The co-dependence of μ and δ opioid receptor functions, such as shown in models of analgesia (26), antinociception (14), morphine dependence, and tolerance (27–30), may provide a physiological basis for the function of receptor hetero-oligomers, demonstrating that ablating either one of the constituents of a heteromeric complex will abolish signaling via the complex. The physiologic significance of μ-δ interactions is very well illustrated in opioid receptor gene-deleted animals. In contrast to wild type animals, μ opioid receptor knock-out mice did not show place preference to deltorphin-II, considered to be a δ-selective agonist, indicating an absence of deltorphin-II rewarding effects in these mice (31). Furthermore, μ receptor-deficient mice did not develop deltorphin-II dependence, as measured by a lack of naloxone-precipitated withdrawal and an absence of protein kinase C up-regulation (31). These data provide excellent validation of our conclusions regarding the ability of deltorphin-II to function as an agonist of the μ-δ heteromer. In addition, several δ receptor-mediated functions were impaired in μ receptor-deficient mice (26, 32, 33), and a loss of morphine tolerance in mice lacking the δ opioid receptor (29) has been shown. These functional changes in selective receptor gene-deleted animals, occurring in the absence of alterations in the expression levels of the other opioid receptors (29, 34), clearly point to significant physiological functional interactions between μ and δ opioid receptors. Even at a cellular level, it has been shown that μ and δ receptors exist within the same neurons (35), and that stimulation of neurons with the μ-selective agonist morphine markedly increased recruitment of intracellular δ receptors to the cell surface (36), indicating that the cellular trafficking of the δ receptor could be influenced by activation of the μ opioid receptor selectively.

In summary, we have shown that the heteromeric association between μ and δ opioid receptors generates a distinct and novel pharmacology, which results from interactions mediated by the distal carboxyl tails of the receptors. The identification of deltorphin-II as an agonist of the heteromeric complex, and the novel association of this receptor complex with a PTX-insensitive G protein identified as Gz, indicates the formation of a signaling unit different from that present when μ or δ receptor homo-oligomers are individually expressed. The hetero-oligomerization of μ and δ opioid receptors broadens the repertoire of signal transduction mechanisms available to these receptors and offers a novel biological premise for the study and therapeutic targeting of opioid mechanisms. The hetero-oligomerization of μ and δ opioid receptors to generate a unique pharmacologic signaling unit also has significant implications for drug discovery, providing a highly novel and untested drug target, which may have major physiological relevance in processes such as analgesia, tolerance, and drug dependence.

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