Heterodimerization of \( \mu \)- and \( \delta \)-Opioid Receptors Occurs at the Cell Surface Only and Requires Receptor-G Protein Interactions*

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Homo- and heterodimerization of the opioid receptors with functional consequences were reported previously. However, the exact nature of these putative dimers has not been identified. In current studies, the nature of the heterodimers was investigated by producing the phenotypes of the 1:1 heterodimers formed between the constitutively expressed \( \mu \)-opioid receptor (MOR) and the ponasterone A-induced expression of \( \delta \)-opioid receptor (DOR) in EcR293 cells. By examining the trafficking of the cell surface-located MOR and DOR, we determined that these two receptors endocytosed independently. Using cell surface expression-deficient mutants of MOR and DOR, we observed that the corresponding wild types of these receptors could not rescue the cell surface expression of the mutants, whereas the antagonist naloxone could. Furthermore, studies with constitutive or agonist-induced receptor internalization also indicated that MOR and DOR endocytosed independently and could not “drag in” the corresponding wild types or endocytosis-deficient mutants. Additionally, the heterodimer phenotypes could be eliminated by the pretreatment of the EcR293 cells with pertussis toxin and could be modulated by the deletion of the RRTR sequence in the third intracellular loop that is involved in the receptor-G protein interaction and activation. These data suggest that MOR and DOR heterodimerize only at the cell surface and that the oligomers of opioid receptors and heterotrimeric G protein are the bases for the observed MOR-DOR heterodimer phenotypes.

The ability of G protein-coupled receptors (GPCRs) to homo- or heterodimerize has implications in the functions of the receptors. Dimerization of the receptors has been reported for the class A GPCRs such as the adenosine (1), adrenergic (2–5), angiotensin (6), dopamine (7, 8), muscarinic (9), vasopressin (2, 10), and opioid (11–15) receptors and the class C GPCRs such as the calcium-sensing (16), the metabotropic glutamate receptors (17), and the \( \gamma \)-amino-\( \beta \)-butyric acid type B (GABA\(_B\)) receptors (18–20). The homo- and heterodimerization of these receptors have been demonstrated by co-immunoprecipitation experiments (11, 21) and subsequently by the fluorescence resonance energy transfer or bioluminescence resonance energy transfer techniques (3, 12, 23). The heterodimerization of the GPCRs was shown to be selective, with formation of heterodimers with some but not all subtypes of the receptors (13, 24, 25). Most importantly, there are functional differences between the monomers and the homo- and heterodimers of the GPCRs. The classic example is the inability of individual GABA\(_A\) and GABA\(_B\) subunit to form a functional receptor (18–20). Alteration in the GPCR function or expression was also observed with the heterodimerization of 5HT1B and -1D (26), dopamine D1 and adenosine A1 (27), muscarinic M2 and M3 (28), or dopamine and somatostatin (29) receptors. Heterologimerization of the GPCRs with other receptor types, such as the ionotropic GABA\(_B\) receptor, has been observed, resulting in the alteration in the ion-gating properties of the channels (30). As for the opioid receptors, which belong to the rhodopsin subfamily of the GPCRs, homo- and heterodimerization of the receptors have been reported. Homodimerization of the \( \delta \)-opioid receptor (DOR) was reported with immunoprecipitation, and agonist-induced receptor internalization appeared to be related to the dimers formation (11). The agonist-induced change in DOR oligomerization was not observed with bioluminescence resonance energy transfer experiments (12). The \( \kappa \)-opioid receptor is reported to exist in homodimers and could heterodimerize with DOR but not with the \( \mu \)-opioid receptor (MOR) (13). The heterodimerization of DOR and \( \kappa \)-opioid receptor resulted in the decrease in the affinities of receptor-selective ligands (13). The DOR and \( \kappa \)-opioid receptor could also heterodimerize with the \( \beta_2 \)-adrenergic receptor, resulting in an alteration of \( \beta_2 \)-adrenergic receptor functionality (31). The most interesting of the heterodimers is the reported heterodimerization of the MOR and DOR receptors. Using different epitope-tagged receptors, both George et al. (15) and Gomes et al. (14) reported the ability of MOR and DOR to heterodimerize. Both groups reported a change in functionality of the receptor, but George et al. (15) reported that the heterodimer function appeared to be insensitive to pertussis toxin pretreatment, implying coupling to G proteins other than G\( \gamma \) (15).

The exact nature of the opioid receptor homo- and heterodimerization has not been defined. Two general schemes for the dimerization of GPCRs have been proposed by Reynolds and co-workers (32); 1) the 1:1 stoichiometric molecular complexes of
the receptors or contact dimers and 2) the swapping of the transmembrane domains of the GPCRs. The domain swapping was initially suggested by Gouldson et al. (5) to explain the ability of adrenergic-muscarinic receptor fragments 1–5 and 6–7 to function as autonomous folding units when co-expressed in the same cells (33, 34). Using computational three-dimensional models of the opioid receptors, Filizola and Weinstein (35) suggested the most likely interfaces between the opioid receptor homodimers are TM4-TM4, TM4-TM5, and TM5-TM5 for MOR, and TM4-TM5 for κ-opioid receptor. These hypotheses do not consider the possible roles intracellular domains could have, as in the case of GABA<sub>δ</sub> receptors heterodimerization (36, 37) and of adrenergic-muscarinic receptor heterodimerization (34). Hence, to investigate the nature of the putative MOR and DOR heterodimers, DOR expression in the EcR293 cells constitutively expressing MOR was controlled by the edcsyne-inducible mammalian expression system. The role of the intracellular domains in the formation of the MOR and DOR heterodimers, particularly those domains within the third intracellular loop that are involved in G protein coupling, was investigated. It could be demonstrated from our current studies that the observed heterodimer phenotypes are the results of opioid receptors interaction with the G proteins.

**EXPERIMENTAL PROCEDURES**

**Expression of MOR and DOR in the EcR293 Cells—**The mouse MOR tagged with the human hemagglutinin (HA) epitope (YPYDVPDYA) and mouse DOR tagged with the FLAG epitope (DYKDDDKD) were subcloned in pcDNAm and in the pLDISP1 vectors, respectively. EcR293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mg/ml Zeocin, and 10% fetal calf serum (DMEM growth medium) in a 10% CO<sub>2</sub> incubator. The cells observed from background the biotinylated. EcR293 cells were grown in 10-cm dishes and treated with or 2 μM ponasterone A (PA) for 48 h before harvesting by phosphate-buffered saline-EDTA (0.1 M NaCl, 0.01 M Na<sub>2</sub>PO<sub>4</sub>, 0.04% EDTA, pH 7.4). The cell pellets were then homogenized in a sucrose-HEPES solution (0.32M sucrose, 10 mM HEPES, pH 7.7) with a glass homogenizer. After centrifuging at 1000 × g for 10 min to remove the unbroken cell and nuclei, the membranes were isolated by centrifuging at 100,000 × g for 60 min. The final pellets were resuspended in the sucrose-HEPES solution. After the protein concentrations of the pellets were determined by the Lowry method, [H]diprenorphine (2 nM) binding in the presence or absence of 10 μM naloxone were carried out as to determine the specific binding. The percentages of MOR and DOR in the total specific binding were determined by carrying out the [H]diprenorphine binding in the presence of 1 μM CTP (selective antagonist for MOR) or 1 μM TIPPP (selective antagonist for DOR), respectively. Competition binding assays were performed by varying the concentrations of agonists and antagonists from 10<sup>−11</sup> to 10<sup>−4</sup> M. Prism 3 program (GraphPad, San Diego, CA) was used to analyze and determine the relative K<sub>i</sub> values from the competition binding experiments. All binding experiments were performed in triplicate, and 3–6 competition binding assays were carried out for individual agonist or antagonists.

**Intracellular cAMP Measurements—**The ability of opioid agonists to inhibit the forskolin-stimulated adenyl cyclase activity was determined by measuring the intracellular cAMP level in the EcR293 expressing either MOR alone or MOR and DOR together. The amplified luminescent proximity homogenous assays (AlphaScreen<sup>™</sup>) for cAMP supplied by PerkinElmer Life Sciences (Boston, MA) were used to determine the cAMP levels. Twenty-four hours before the assays EcR293 cells treated with 0 or 2 μM PA for 24 h were plated into 96-well plates. Various concentrations of opioid agonists and antagonists were diluted with Krebs-Ringer-Hepes-buffer containing 10 μM forskolin and 0.5 mM isobutylmethylxanthine. After removal of the growth medium, the 96-well plates were placed on ice, and 100 μl of the drug solution was added to the well, with 4 wells per drug concentration. After sealing the plates with HotSeal™ (Diversified Biotech, Boston, MA), the plates were incubated at 37°C for 15 min. Afterward, the plates were placed in a water bath at 85–90°C for 5 min so as to lyse the cells and to release the intracellular cAMP. After then, 12 μl of the donor solutions (500 × g for 2 min, the amount of cAMP in 4 μl of the supernatant was determined using the donor beads coated with streptavidin, acceptor beads coated with anti-cAMP antibodies, and biotinylated cAMP in the AlphaScreen<sup>™</sup> assay system. The cAMP concentrations, from 10<sup>−11</sup> to 10<sup>−4</sup> M, were used to construct the standard curve. The donor beads (final 20 μg/ml), the acceptor beads (final 15 μg/ml), and biotinylated cAMP (final 1 μM) were coated in the 1× control (HEPES, pH 7.4, and 0.3% Tween 20 (60%) and Hanks’ buffer salts saline (40%)). 4 μl of the reaction supernatant or standard cAMP solutions were pipetted into duplicate wells of a 384-well Opti-plate (PerkinElmer Life Sciences) with a Biomek 2000 (Beckman-Coulter, Fullerton, CA) in a dimly lit room at 4°C. The plate was then sealed with TopSeal<sup>™</sup> adhesive sealing films (PerkinElmer Life Sciences) and incubated for 10 min. Then 12 μl of the donor beads were pipetted into the wells, and the mixtures were incubated in the dark at 4°C for 18–24 h. After equilibrating to room temperature in the dark (4 h), the content of the cAMP in each well was determined by exciting the donor beads at 680 nm, generating a singlet O<sub>2</sub>, resulting in the fluorescence emission of the acceptor beads at 520–620 nm. The fluorescence of the wells was measured with the Fusion<sup>™</sup> (PerkinElmer Life Sciences) plate reader (PerkinElmer Life Sciences) plate reader, and the amount of cAMP in each well was extrapolated from the standard curve. The dose response curves of the opioid agonists were constructed accordingly. The IC<sub>50</sub> value and the maximal inhibition level for individual agonist were obtained from Prism 3 program analyses of 3–12 dose-response curves.

**Fluorescence Flow Cytometry—**The opioid receptors located on the plasma membrane was quantified by assaying the cell surface immunoreactivity. EcR293 cells were grown in 10-cm dishes and treated with 0 or 2 μM PA for 48 h. Then cells were treated with 1 μM etorphine for different time periods or with various concentrations of etorphine for 60 min so as to induce receptor internalization. For the experiments in which the turnover of the cell surface-labeled receptors was measured, media containing PA were removed, and the cells were washed and resuspended with fresh medium. The amplified luminescent proximity homogenous assays (AlphaScreen<sup>™</sup>) for cAMP supplied by PerkinElmer Life Sciences (Boston, MA) were used to determine the cAMP levels. The cells were then isolated by treating the cells with 90% confluency in the dishes. After washing twice with serum-free DMEM, the cells were rinsed twice with serum-free DMEM. Then the cells were incubated at 4°C for 60 min in serum-free DMEM with either anti-HA (1:500 dilution) or anti-FLAG (1:300) antibody. Afterward, the cells were washed twice with serum-free DMEM and incubated with Alexa488<sup>™</sup>-labeled goat anti-mouse IgG secondary antibody (1:400 dilution) at 4°C for an additional 1 h. Then the cells were washed and fixed with 3% formaldehyde before quantitating the receptor immunoreactivity with fluorescence flow cytometry (FACSScan, BD Biosciences). Fluorescence intensity of 10,000 cells was collected for each sample. Cell Quest software (BD Biosciences) was used to calculate the mean fluorescence intensity of the cells population. All FACS analyses were conducted three times with triplicate in each experiment.

**Surface Biotinylation—**To examine the constitutive internalization of MOR and DOR, the cell surface-labeled opioid receptors were biotinylated. EcR293 cells were grown in 10-cm dishes and treated with 0 or 2 μM PA for 48 h. Normally, biotinylation was carried out with cells reaching 90% confluency in the dishes. After washing twice with ice-cold phosphate-buffered saline, the cell surface proteins were biotinylated by incubating with 300 μg/ml sulfo-NHS-Ss-biotin in ice for 10 min. After washing with cold phosphate-buffered saline, the cells were biotinylated by incubating with 3-μg/ml biotinylated streptavidin for 15 min at 4°C. After washing with cold phosphate-buffered saline, the cells were rinsed twice with serum-free DMEM. Then the cells were incubated at 4°C for 60 min in serum-free DMEM with either anti-HA (1:500 dilution) or anti-FLAG (1:300) antibody. Afterward, the cells were washed twice with serum-free DMEM and incubated with Alexa488<sup>™</sup>-labeled goat anti-mouse IgG secondary antibody (1:400 dilution) at 4°C for an additional 1 h. Then the cells were washed and fixed with 3% formaldehyde before quantitating the receptor immunoreactivity with fluorescence flow cytometry (FACSScan, BD Biosciences). Fluorescence intensity of 10,000 cells was collected for each sample. Cell Quest software (BD Biosciences) was used to calculate the mean fluorescence intensity of the cells population. All FACS analyses were conducted three times with triplicate in each experiment.
RESULTS

The ability of MOR and DOR to heterodimerize has been reported previously (14, 15). In those studies the binding properties and functions of various ligands were examined in cells expressing one type of the receptors and compared with those in cells expressing both receptors. Because cellular background could affect the opioid responses, we decided to re-evaluate the possible interaction between MOR and DOR in the same population of cells that express one type of the receptors and, by induction, express both types of the receptors. This was accomplished by selecting EcR293 cells constitutively expressing MOR and additionally culturing these cells in the presence of PA to stimulate the expression of both MOR and DOR. As summarized in Fig. 1A, in the absence of PA, 0.46 ± 0.025 pmol/mg of MOR was expressed in the EcR293 cells, as defined by the amount of [3H]diprenorphine binding competed by 1 μM CTP, a MOR-selective antagonist. Meanwhile, 0.057 ± 0.003 pmol/mg of DOR was detected in these EcR293 cells, as defined by specific binding in the presence of 1 μM TIPP, a DOR-selective antagonist. The low level or absence of DOR expression in the absence of PA was substantiated by the absence of DOR protein in the Western analyses as shown in the left-hand panel of Fig. 1B. However, when the EcR293 was treated with various concentrations of PA for 48 h, there was a concentration-dependent increase in the MOR-specific binding, with a slight decrease in the MOR binding to a plateau level of ~0.35 pmol/mg of protein. At 2 μM PA, the amount of DOR (0.43 ± 0.039 pmol/mg of protein) statistically was not different from the MOR level (0.37 ± 0.033 pmol/mg of protein) expressed in these EcR293 cells. The presence of DOR protein was also demonstrated with the Western analyses (Fig. 1B). Interestingly, the constitutively expressed MOR could be co-immunoprecipitated with anti-FLAG M2 monoclonal antibodies only when the FLAG-DOR was induced by PA treatment and was PA concentration-dependent (Fig. 1B). Thus, in subsequent experiments EcR293 cells were treated with 2 μM PA for 48 h to achieve a 1:1 MOR:DOR ratio and apparent receptor heterodimerization.

In addition to co-immunoprecipitation, one of the evidences in support of MOR/DOR heterodimerization was the differences in the affinities of the ligands between the receptor heterodimers and homodimers or monomers (14, 15). In current studies the affinities of several MOR-selective agonists for the homodimers or monomers were compared with those for the heterodimers. To limit the differences in cellular background, EcR293 expressing only DOR was used in our comparison. As summarized in Table I, the MOR-selective agonists, endomorphin-2 and PL017, exhibited low or no affinities for the DOR. Interestingly, although endomorphin-1 and -2 have been reported previously to be highly selective agonists for MOR (39), endomorphin-1 exhibited some affinity for DOR, whereas endomorphin-2 did not. Endomorphin-2 has similar selectivity for MOR and DOR as PL017 (Table I). The affinities of these two endorphins for MOR were very similar, with the high affinity binding lower than those observed with DAMGO and morphine.

However, when the DOR level was increased to that of MOR in these EcR293 cells, a decrease in the binding affinity was observed. For DAMGO, morphine, and endomorphin-1, there were 3–10-fold decreases in the high affinity binding (Table I). There was a 3-fold decrease in PL017 binding affinity. In contrast, in the presence of DOR the high affinity binding for endomorphin-2 was increased 10-fold. Because both endomorphin-2 and PL017 did not exhibit affinity for DOR, the observed alterations in the binding affinities for MOR exhibited by these agonists could not be accounted by their affinities for DOR. Such changes in these ligand affinities for MOR could reflect the alteration in the binding domains within the receptor as a result of heterodimerization.

Apparently, the changes in the ligand affinities for MOR in the presence of DOR were limited to agonists. When the affinities of naloxone and naltrexone were determined, these two antagonists exhibited selectivity for MOR over DOR with a ratio of 1:13 to 1:15 (Table I). Interestingly, when the DOR level was increased to that of MOR in EcR293 cells, these two antagonists exhibited multiple affinity states binding. The affinities of these two antagonists for R1 and R2 paralleled the affinities for MOR and DOR, respectively. The percentage of...
receptor in RH (~40%) approached the 1:1 ratio of MOR:DOR when specific binding was determined with CTOP and TIPP (Fig. 1A). Thus, the putative heterodimerization did not affect the antagonists binding affinities.

The activation of DOR has been reported to have a synergistic effect on the MOR functional activities (14, 40). We re-examined the abilities of the MOR-selective agonists to inhibit forskolin-stimulated adenylyl cyclase activity in our model system. The abilities of the agonists to control intracellular cAMP levels were measured first in EcR293 cells cultured in the absence of PA. The same agonist abilities to control cAMP levels in EcR293 cells cultured in the presence of 2 µM PA were measured again. This current paradigm should reflect any difference in the opioid responses in similar backgrounds with only the level of DOR changed. As summarized in Table 1, all opioid agonists tested exhibited adenylyl cyclase inhibition with IC50 values much lower than their respective affinities for the RH. With the exception of endomorphin-2, which showed increased potency paralleling the increase in this peptide affinity in the presence of DOR, the presence of DOR did not affect other opioid agonists potencies tested. This is not unexpected for as agonist such as PL017, which has no affinity for DOR. The inhibition of intracellular cAMP levels by PL017 should reflect the activation of MOR. However, the absence of changes in the IC50 values for agonists such as DAMGO and morphine, which have been shown to activate DOR in various in vitro cell lines, suggests there is no measurable synergism in the MOR and DOR activation. Nevertheless, the observed increase in endomorphin-2 potency in the presence of DOR suggested the putative heterodimers of MOR/DOR could have different functional properties than the corresponding homodimers or monomers.

The presence of DOR did not alter the opioid receptor-G protein coupling. Earlier studies indicated that the presence of DOR allowed the coupling of the heterodimers to pertussis toxin (PTX)-insensitive G proteins. However, as shown in Fig. 2, this does not appear to be the case. DAMGO inhibited the forskolin-stimulated intracellular cAMP production in EcR293 cells expressing MOR alone or MOR/DOR heterodimers in a dose-dependent manner. Pretreating the cells with 100 ng/ml PTX for 24 h blocked the DAMGO inhibition. At 100 µM, DAMGO inhibited the cAMP production by 13 ± 3.6% in EcR293 cells cultured at 0 PA after PTX treatment, whereas the same concentration of DAMGO did not inhibit the cAMP production in cells cultured in 2 µM PA for 48 h (Fig. 2). Hence, the putative MOR/DOR heterodimers control intracellular cAMP levels via the G/Gs proteins, as in the case of the homodimers or monomers.

The existence of unique binding and function phenotypes when MOR was expressed in the presence of DOR supports the notion of heterodimers for these receptors. Heterodimers could form during the trafficking from endoplasmic reticulum (ER), facilitating the transport of the receptors to the plasma membrane. For example, GABAB1i is retained in the ER during homomeric expression, and GABAB2i is responsible for the trafficking of GABAB1 to the cell surface and the formation of high

**Table 1**

Affinities and potencies of various opioid ligands for MOR and DOR expressed in EcR293 or HEK293 cells

| Ligands      | Ks, EcR293 | IC50, EcR293 | Ks, HEK-DOR |
|--------------|------------|-------------|-------------|
|              | 0 PA       | 2 µM PA     |             |             |
| DAMGO        | 0.34 ± 0.12| 4.9 ± 2.5   | 210 ± 50    |
| (26 ± 2.2%)  | (22 ± 5.5%)|             | (55 ± 2.6%) |
| 98 ± 12      | 220 ± 48   |             | (62 ± 2.2%) |
| 0.28 ± 0.09  | 3.5 ± 1.8  | 120 ± 26    |
| (42 ± 3.3%)  | (25 ± 6.6%)|             | (60 ± 2.0%) |
| 37 ± 8       | 150 ± 40   | 100 ± 16    |
| 3.0 ± 0.8    | 8.1 ± 3.9  | 11 ± 8      |
| Endomorphin-1| (39 ± 3.1%)| 30 ± 6.9    |
| 250 ± 55     | (26 ± 6.1%)| (51 ± 1.9%) |
| 3.4 ± 1.5    | 0.33 ± 0.19| 76 ± 1.6    |
| (38 ± 5.8%)  | (24 ± 4.1%)| (43 ± 1.7%) |
| 210 ± 65     | 550 ± 160  | 15 ± 2.9a   |
| (76 ± 1.9%)  | (48 ± 1.7%)| >10,000     |
| PL017        | 150 ± 28   | 490 ± 190   |
| (34 ± 4.1%)  | (52 ± 4.0%)| >10,000     |
| Naloxone     | 3.1 ± 0.2  | 1.4 ± 0.85  |
| (28 ± 13%)   | (27 ± 8)   |             |
| 0.56 ± 0.24  | 0.56 ± 0.24|             |
| Naltrexone   | 0.78 ± 0.31| (40 ± 14)   |
| (11 ± 1.7)   |            |             |

* p < 0.05, when compared with 0 µM PA.

**Fig. 2.** Effect of PTX on DAMGO inhibition of forskolin-stimulated intracellular cAMP production. EcR293 cells were cultured in the presence or absence of 2 µM PA for 48 h. Eighteen hours before the assays the cells were treated with 100 ng/ml PTX. Then the abilities of DAMGO to inhibit 10 µM forskolin-stimulated intracellular cAMP production in cells cultured without PA (○) and treated with PTX (●) were compared with the cells cultured in the presence of 2 µM PA (△) for 48 h and treated with PTX (▲). The values represent the averages ± S.E. of four separate dose-response curves.

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affinity GABA binding complexes (41, 42). Truncation mutants of D_2_–dopamine receptor could inhibit the cell surface expression of the wild type, thus suggesting the formation of receptor dimers within the cells (43). MOR and DOR likewise could form heterodimers during the trafficking from ER. To examine such a possibility, the traffic-deficient mutants of MOR were utilized. Our previous studies indicated that deletion of a MOR sequence within the third intracellular loop (Δ^289RLSKY^2862, i3–1 mutant) or a sequence residing at the proximal carboxyl tail (Δ^344KRCFR^348, C-2 mutant) resulted in the retention of the mutants at ER (44). As in the case with wild type DOR, where the cell surface expression could be increased with ligands (45), the cell surface expression of these mutants could be rescued by incubating with hydrophobic ligands such as naloxone. If MOR and DOR heterodimerize during the receptor export, it is reasonable to surmise that the wild type DOR should be able to rescue these traffic-deficient mutants of MOR.

When MOR C-2 mutant was transiently expressed in HEK293 cells, the cell surface expression was monitored with FACS analyses using the anti-HA antibodies. As expected, there was 64 ± 7% increase in the cell surface fluorescence when compared with the cells that were mock-transfected (Fig. 3C). Treatment of the HEK293 cells with 10 μM naloxone for 48 h after transfection resulted in a 3.6 ± 0.01-fold increase in cell surface fluorescence when compared with mock transfection. This naloxone-induced increase in cell surface expression of MOR C-2 mutant was similar to those previously reported with HEK293 cells stably expressing the mutant (44). On the other hand, co-transfection of the MOR C-2 mutant with wild type DOR did not increase the cell surface expression of MOR C-2. As shown in Fig. 3C, there was no significant difference in cell surface fluorescence between the cells transfected with receptors and the mock control. Naloxone treatment increased the cell surface fluorescence by 2.1 ± 0.07-fold. The failure to increase MOR C-2 cell surface expression was not due to the absence of DOR expression. When DOR expression was monitored with the FLAG M-2 antibodies, co-expression of wild type DOR with MOR C-2 mutant resulted in a 9.3 ± 0.04-fold increase in cell surface fluorescence when compared with mock control (Fig. 3D). This level of DOR cell surface expression was significantly lower than that observed when HEK293 cells were transfected with DOR alone (12.3 ± 0.7-fold increase in fluorescence). Because culturing of the cells transfected with MOR C-2 mutant and DOR or with DOR alone with naloxone resulted in a similar increase (~15-fold) in the cell surface fluorescence (Fig. 3D), the reduced level of cell surface DOR expression in the presence of MOR C-2 could be due to the intracellular retention of wild type DOR by the traffic-deficient MOR. However, similar reduction in the cell surface expression of the wild type receptors was observed when both MOR and DOR were transiently transfected into the HEK293 cells. Tagging was reversed to demonstrate receptor trafficking was unaffected by the epitope used. As shown in Fig. 3, A and B, co-expression of the MOR and DOR in the same cells together reduced the level of cell surface expression of these opioid receptors when they were expressed in the HEK293 cells separately. Similar to earlier reports (45), naloxone treatment could increase the levels of DOR or MOR expressed in the cell surface. Hence, the observed decrease in the wild type DOR cell surface expression in the presence of MOR C-2 mutant was not due to the retention of the wild type receptor by the traffic-deficient mutant.

Wild type DOR could not rescue the traffic-deficient i3–1 mutant of MOR also. As shown in Fig. 3E, there was a basal level of cell surface expression of MOR i3–1 (2.3 ± 0.09-fold increase from mock transfection) that could be increased by culturing the HEK293 cells in the presence of 10 μM naloxone (4.1 ± 0.13-fold). Co-transfection of MOR i3–1 with wild type DOR did not result in the rescuing of the cell surface expression of the traffic-deficient mutant. In contrast, the cell surface fluorescence of the cells thus transfected was similar to that of mock-transfected cells. Naloxone treatment increased the cell surface fluorescence of MOR i3–1 in these cells by 2.0 ± 0.1-fold over mock control. Again, the failure to rescue the traffic-deficient mutant was not due to the absence of wild type DOR expression, as indicated by the cell surface fluorescence detected and summarized in Fig. 3F. Similar deletion of the third intracellular loop sequence of DOR, Δ^289RLSR^242 (DOR i3–1), also resulted in a traffic-deficient mutant of DOR. This suggests that the cell surface expression of this mutant could be rescued by naloxone but not by co-expression with wild type MOR (data not shown). Thus, the inability of wild type DOR receptor to enhance the export of the traffic-deficient mutant suggests that the observed heterodimer phenotypes must be the results of MOR and DOR oligomerization after receptor insertion in the plasma membrane.

Previous studies with DOR and its carboxyl tail-truncated mutant (11) and with MOR and the MOR/DOR receptor chimera (46) have suggested that the opioid receptors internalize as dimers. If the model of domain swapping is the basis for the heterodimers formation, then it is reasonable to assume that MOR and DOR will internalize as a complex. As shown in Fig. 4A, removal of the insect hormone PA from the culturing medium will result in the time-dependent decrease in the receptor level. For the EcR293 cells expressing DOR under the inducible promoter, t_{50} for the disappearance of the cell surface binding site was determined to be 6.1 ± 0.9 h, whereas t_{50} for MOR was determined to be 9.7 ± 1.6 h. Thus, by inducing the expression of either MOR or DOR in the presence of 2 μM PA for 48 h followed by culturing the EcR293 cells in the absence of PA for additional 24 h, we examined whether the turnover of the induced receptors at the cell surface influences the receptor level that is constitutively expressed. Fig. 4, B–E, summarized the data obtained with the FACS analyses experiments. In Fig. 4, B and C, the EcR293 cells stably expressing the PA-inducible HA-tagged DOR (HA-DOR) were transiently transfected with either FLAG-tagged DOR or FLAG-tagged MOR. As shown in these figures, removal of PA in cells transfected with FLAG-DOR resulted in the reduction the HA-DOR level to 28 ± 1.0% that observed in cells treated with 2 μM PA for 48 h. In cells transfected with FLAG-MOR, the HA-DOR level was reduced to 46 ± 1.0% of the initial receptor level. Such data suggest that FLAG-MOR increased the retention time of HA-DOR at the cell surface after PA removal. However, the >50% decrease in the cell surface HA-DOR did not affect the amount of FLAG-DOR or FLAG-MOR detected at the cell surface (Fig. 4, B and C). It was striking to observe that the amount of FLAG-MOR transiently expressed at the cell surface was reduced in the presence of HA-DOR-induced expression. The cell surface amount of FLAG-MOR was increased after the induced level of HA-DOR was decreased upon PA removal (Fig. 4C). Similar observations were obtained with the EcR293 cells expressing inducible HA-MOR and transiently transfected FLAG-DOR or FLAG-MOR. Fig. 4, D and E, indicated that the removal of PA after 48 h of induction resulted in a 70% reduction of the initial HA-MOR cell surface level. Again, the reduction in HA-MOR level did not decrease the FLAG-MOR or FLAG-DOR steady-state level at the cell surface. Thus, these data suggested that the internalization of the HA-DOR or HA-MOR leading to the turnover of these receptors did not affect the steady-state levels of FLAG-DOR or FLAG-MOR that were constitutively expressed. Hence, MOR and DOR endocy-
Anti-HA

(A)

HA-DOR+pCDNA3

FLAG-MOR+HA-DOR

MOCK

Cell Surface Fluorescence, RFU

0 500 1000 1500

(B)

Anti-FLAG

Cell Surface Fluorescence, RFU

0 250 500 750 1000 1250

Inability of wild type opioid receptor to rescue traffic-deficient mutants. MOR or DOR wild type receptor tagged with FLAG epitope was co-transfected into HEK293 cells with the MOR i3–1 (Δ258RLSKV262, HA-MORΔRLKSV) or the MOR C-2 (Δ344KRCFR348, HA-MORΔKRCFR) as described under “Experimental Procedures.” The cell surface expressions of the receptors after culturing the cells for 48 h after transfection were determined by FACS analyses (open bars) and compared with those cells cultured for 48 h in the presence of 10 μM naloxone (filled bars). The cell surface fluorescence of these cells was also compared with those that were transfected with the plasmid pCDNA3, mock transfection (hatched bars). In panels A, C, and E, the cell surface expressions of HA-tagged receptors were determined, whereas in panels B, D, and F, the cell surface expression of FLAG-tagged receptors were determined. In panels A and B, HEK293 cells were transfected with FLAG-tagged MOR and HA-tagged DOR. In panels C and D, HEK293 cells were transfected with HA-MORΔKRCFR and FLAG-tagged DOR. In panels E and F, the cells were transfected with HA-MORΔRLKSV and FLAG-tagged DOR. The values represent the averages ± S.D. of three separate transfections and FACS analyses. RFU, relative fluorescence units.

tosis do not appear to require the formation of homo- or heterodimers.

It is possible that the measurement of the steady-state cell surface receptor levels does not reflect the internalization kinetics of MOR and DOR heterodimers. Thus, the endocytosis of the cell surface receptor pools was measured with biotinylation.
studies. Previous studies indicated that DOR could internalize constitutively, whereas MOR could not (47). Thus, if MOR and DOR form domain-swapping heterodimers, such dimers should internalize as complexes. As shown in Fig. 5, the EcR293 cell surface-located MOR could be biotinylated, and there were similar amounts of receptor detected in cells cultured in the presence of 0 or 2 μM PA by Western analyses. However, when the cells were incubated at 37 °C for 1 h after biotinylation and the cell surface biotin labels were stripped before immunoprecipitation, a minimal amount of receptor was detected (2.1 ± 0.5%). These data suggest that the majority of cell surface-located MOR did not internalize during incubation. Similar levels of biotinylated MOR (1.4 ± 0.2%) were detected after stripping the biotin labels from EcR293 cells that were cultured in the presence of 2 μM PA for 48 h, and then cultured in the absence of PA for 24 h. The cell surface receptors were then determined by FACS analyses. The open bars represent the averages ± S.D. of three separate FACS analyses using anti-HA, whereas the filled bars represent the averages of three separate FACS analyses using anti-FLAG. In panels D and E, EcR293 cells expressing PA-inducible HA-MOR were transfected with either FLAG-MOR or FLAG-DOR and were treated with PA as described above. Again, the open bars represent the averages of three separate FACS analyses using anti-HA, and the filled bars represent the averages ± S.D. of three separate FACS analyses using anti-FLAG.

**FIG. 4. MOR and DOR do not endocytose as dimers.** Whether or not MOR and DOR endocytose as dimers was examined by determining the turnover of the cell surface receptors. In A, EcR293 cells expressing PA-inducible HA-DOR (A) or inducible HA-MOR (E) were cultured in the presence of 2 μM PA for 48 h. Then the cells were washed to remove the PA, and the amount of receptor remained on the cell surface at different time periods after PA removal was determined with [3H]diprenorphine binding. The values represent the averages from 3 separate 60-mm plates of cells thus treated. In panels B and C, EcR293 cells expressing the PA-inducible HA-DOR were transfected with either FLAG-DOR or FLAG-MOR in pCDNA3, cultured in the presence of 2 μM PA for 48 h, and then cultured in the absence of PA for 24 h. The cell surface receptors were then determined by FACS analyses. The open bars represent the averages ± S.D. of three separate FACS analyses using anti-HA, whereas the filled bars represent the averages of three separate FACS analyses using anti-FLAG. In panels D and E, EcR293 cells expressing PA-inducible HA-MOR were transfected with either FLAG-MOR or FLAG-DOR and were treated with PA as described above. Again, the open bars represent the averages of three separate FACS analyses using anti-HA, and the filled bars represent the averages ± S.D. of three separate FACS analyses using anti-FLAG.

**FIG. 5. MOR does not constitutively endocytose and is not affected by DOR constitutive internalization activity.** The constitutive internalization of MOR and DOR was examined. The EcR293 cells expressing MOR and inducible DOR were cultured in the absence or presence of 2 μM PA for 48 h. The cell surface located receptors were biotinylated as described. After biotinylation, the cells were incubated at 37 °C for 1 h, and then the cell surface biotin labels were stripped. The receptors were extracted and immunoprecipitated with streptavidin, and the amount of receptors remaining biotinylated were determined by Western analyses using the HA epitope and DOR-specific antibodies as described under “Experimental Procedures.”

Although turnover studies and constitutive endocytosis studies have indicated that MOR and DOR do not internalize as heterodimers, earlier studies suggested that receptor oligomerization was a prerequisite for agonist-induced receptor internalization (11). Therefore, the ability of etorphine, an opioid receptor non-selective agonist to induce MOR internalization in the presence or absence of DOR, was examined. As reported in
produced the FLAG-DOR to internalize 85.18% receptor internalized). At the same time, etorphine in-
terorhine (or maximal level of HA-MOR internalized in the presence of
separate FACS analyses.

Etorphine concentrations of etorphine so as to induce the internalization of HA-MOR in cells cultured in the absence (\( \text{EC}_{50} \)) or in the presence \( (2 \mu M \text{ PA}) \) to induce the internalization of FLAG-DOR in cells cultured at 2 \( \mu M \text{ PA} \). The values in both panels are the averages ± S.D. from three separate FACS analyses.

earlier studies (46–48), agonist induced rapid internalization of DOR, but slower MOR endocytosis rate was observed due to the recycling of MOR. When the kinetics of etorphine-induced HA-MOR internalization were measured in Ecr293 cells cultured without PA, a rate of \( t_{1/2} = 8.0 \pm 3.4 \text{ min} \) was observed, with a maximal level of 46 ± 4.2% receptor internalized (Fig. 6A). Inducing the expression of FLAG-DOR by culturing in the presence of 2 \( \mu M \text{ PA} \) for 48 h did not significantly alter the rate or maximal level of HA-MOR internalized in the presence of etorphine \( (t_{1/2} = 9.1 \pm 1.1 \text{ min}) \), and maximal level = 52 ± 1.8% receptor internalized). At the same time, etorphine induced the FLAG-DOR to internalize 85 ± 12% maximally with a \( t_{1/2} = 22 \pm 10 \text{ min} \) (Fig. 6A). The absence of DOR effect on agonist-induced MOR internalization was not due to the high concentration of etorphine used in the studies. Etorphine \( \text{EC}_{50} \) to induce MOR endocytosis in the absence or presence of DOR was determined to be 0.56 ± 0.16 and 0.71 ± 0.17 \( nM \) respectively, with maximal receptor internalized at 47 ± 1.8 and 40 ± 1.4% (Fig. 6B). Meanwhile, the \( \text{EC}_{50} \) value of etorphine to induce DOR internalization was determined to be 2.2 ± 0.03 \( nM \), with the maximal receptor internalized to be 74 ± 1.4%. Because both the concentration-dependent and the endocytosis kinetics studies did not reveal any changes in agonist-induced MOR internalization in the presence of DOR, it is likely MOR and DOR endocytosed separately.

A traffic-deficient mutant of MOR was used to further demonstrate the failure of DOR to promote the internalization of MOR in the presence of agonist. Deletion of the amino acid sequence in the third intracellular loop (iL3) of MOR, \( \Delta^{276RRTR280} \), resulted in a receptor mutant \( (\Delta i3–5) \) that could not be down-regulated by agonist treatment (49). As shown in Fig. 7, FACS analyses of HEK293 cells transiently transfected with the HA-MOR \( \Delta i3–5 \) mutant and treated with 1 \( \mu M \) etorphine did not result in the reduction of the cell surface fluorescence. Expression of this traffic-deficient mutant in Ecr293 cells expressing the FLAG-MOR did not result in agonist-induced endocytosis of the mutant (Fig. 7). Meanwhile, the rate \( (t_{1/2} = 18 \pm 6.3 \text{ min}) \) and the maximal receptor internalized level \( (32 \pm 7.7\%) \) observed with the wild type FLAG-MOR appeared to be attenuated by the presence of this mutant. Again, the expression of this MOR \( \Delta i3–5 \) mutant in Ecr293 cells expressing the FLAG-DOR did not result in the etorphine-induced internalization of the mutant (Fig. 7). The rate \( (t_{1/2} = 10 \pm 0.3 \text{ min}) \) and maximal level of DOR internalized \( (75 \pm 7.3\%) \) were similar to those observed when DOR was co-expressed with wild type MOR (Fig. 6). Parallel results were obtained with the \( \Delta^{275RRTR280} \) deletion of the iL3 sequence of DOR \( (\Delta i3–5) \). Co-expression of wild type MOR with DOR \( \Delta i3–5 \) mutant receptor did not result in the agonist-induced endocytosis of the mutant (data not shown). Hence, the failure to rescue the traffic-deficient mutant by wild type receptor suggests that agonist-induced receptor internalization does not involve the heterodimerization of the receptors.

The absence of MOR and DOR trafficking together likely indicates that the observed heterodimers phenotypes in the
binding assays could not be the consequences of domain swapping. Furthermore, the trafficking data suggest that the putative heterodimers only exist at the plasma membrane. Interestingly, the heterodimer phenotypes were observed only with the agonists tested. For antagonists such as naloxone and naltrexone, the binding affinities for MOR in the EcR293 cells when DOR was induced reflected the affinities of the ligands when these two receptors were expressed alone (Table I). These data suggest that the MOR and DOR heterodimers could be the contact dimers formed involving G proteins, i.e. receptor-G protein interactions are required for the formation of dimers. If this is the case, then pretreatment of PTX, i.e. ADP-ribosylating the G/G_{α}-subunits and uncoupling the receptors from the G proteins, should attenuate the formation of heterodimers. To demonstrate that PTX pretreatment might affect the MOR/DOR heterodimer formation and to minimize the co-immunoprecipitation artifacts, an N terminus His_{6}-epitope-tagged MOR was co-expressed with FLAG-DOR in HEK293 cells. After the receptor complex was extracted with Triton X-100 as described (14), the heterodimers were retained with a Ni^{2+} column and eluted from the column with 10 mM imidazole, pH 8.0. As shown in Fig. 8A, similar to the co-immunoprecipitation experiments, FLAG-DOR could be retained by the Ni^{2+} resin in the presence of His_{6}-MOR. Interestingly, a similar Ni^{2+} column retained drastically lower amounts of FLAG-DOR from HEK293 cells expressing both opioid receptors but previously treated with 100 ng/ml PTX for 24 h. Such a reduction in the amount of DOR retained was not due to the difference in the level of His_{6}-MOR bound to the Ni^{2+} resin or the amount of FLAG-DOR expressed in the HEK293 cells (Fig. 8A). When

### Table II

**Agonists affinities for the opioid receptors expressed in EcR293 cells pretreated with pertussis toxin**

| Ligands      | 0 PA     | 2 μM PA |
|--------------|----------|---------|
| DAMGO        | 140 ± 36 | 300 ± 46 |
| Morphine     | 28 ± 15  | 6.3 ± 3.5 |
| (29 ± 13%)   |          | (17 ± 3.4%) |
| Endomorphin-2| 390 ± 110| 760 ± 120 |
| PL017        | 210 ± 31 | 300 ± 61  |
|              | 990 ± 340| 1100 ± 140|

PTX pretreatment attenuated the formation of MOR/DOR heterodimers. A, Triton X-100 extracts from HEK293 cells co-expressing His_{6}-MOR and FLAG-DOR or similar cells pretreated with 100 ng/ml PTX for 24 h were partially purified with the Ni^{2+} columns. Opioid receptors were eluted from the columns with 250 mM imidazole, pH 8.0. Western analyses were carried out with guinea pig antibodies against the carboxyl tail domain of DOR and with rabbit antibodies against the carboxyl tail domain of MOR to detect the opioid receptors retained by the Ni^{2+} columns. Total amounts of DOR expressed in these cells were determined by immunoprecipitation (IP) of FLAG-DOR using monoclonal anti-FLAG M2 and immunoblotting (IB) with guinea pig (GP) anti-DOR. In B, [3H]diprenorphine competition binding studies with morphine were carried out with membrane prepared from EcR293 cells treated with 0 PA (●) and with membranes isolated from cells treated with 2 μM PA (○) for 48 h. In C, similar competition binding studies were carried out with membranes isolated from EcR293 cells treated with (●) or without (○) 2 μM PA that were pretreated with 100 ng/ml PTX 24 h before harvesting the cells. The values represent the averages of three separate competition binding experiments.
membranes were prepared from the PTX-treated cells, the induction of DOR by 2 μM PA did not decrease the morphine affinity for the opioid receptor to the same extent as with membranes isolated from control cells (Fig. 8, B and C). When the affinities of other opioid agonists were determined, the induction of DOR expression in EcR293 cells did not alter the affinities of these agonists significantly also (Table II). With the exception of morphine, all agonists tested exhibited a single affinity state binding after PTX treatment. Instead of a 10-fold decrease in the high affinity binding observed in control membranes (Table I), the induction of DOR expression in cells with MOR only reduced the DAMGO affinity 2-fold. The affinities after PTX treatment for endomorphin-2 and PL017 in membranes containing both MOR and DOR remained similar to those expressing MOR alone (Table II). All these data suggested that the observed MOR-DOR heterodimer phenotypes required the coupling of the receptors with G proteins.

The involvement of the opioid receptor iL3 domain in the activation of G proteins has been well documented with mutational analyses and peptide competition studies (50). Deletion of the RRRITR260 sequence within the iL3 domain of MOR resulted in the complete blockade of the agonist effect (49). If interaction and subsequent activation of G proteins are prerequisites for the observed heterodimer phenotypes, then the disruption of G protein interaction by the deletion of RRRITR sequence should also result in the blunting of the heterodimers formation. Hence, we subcloned the Δ257RRITR261 DOR mutant receptor (DORΔi3–5) into the pINDsp1 vector system and established a stable EcR293 cell line that expressed the MOR constitutively and the inducible DORΔi3–5 mutant receptor.

As shown in Fig. 9A, the clone we identified expressed a low level of DOR in the absence of PA, and the level of DORΔi3–5 increased in the presence of PA. Thus, the EcR293 cells expressing the MOR and DORΔi3–5 were cultured in the presence of PA for 48 h, and the agonist competition binding studies were carried out at various ratios of DORΔi3–5:MOR. As shown in Fig. 9A, at the low or equal level of DORΔi3–5 to MOR, the DAMGO or PL017 affinities were similar. When the cells were cultured in the absence of PA, i.e., the DORΔi3–5: MOR ratio was ~1:6, DAMGO interacted with MOR with mul-
DPDPE affinity for the receptor and its ability to inhibit the forskolin-stimulated intracellular cAMP production in EcR293 cells expressing either wild type FLAG-DOR or PA-inducible HA-DORΔ3−5 (DORΔ3–5) were carried out as described under “Experimental Procedures.” The values represent the average ± S.E. from three separate competition curves or dose-response curves. In competition binding studies, either 5 mM Mg2+ or 100 mM Na+ and 5 μM GTPγS were present in the assay mixtures. The values in parentheses for the receptor affinity determinations represent the percentage of receptor retained in high affinity states. The values in parentheses for the potency determinations represent the calculated maximal inhibition levels.

| Receptor | $K_d$ (nM) | +Mg2+ | +Na+ | GTPγS | IC50 (nM) |
|----------|------------|-------|------|-------|-----------|
| DOR      | 0.32 ± 0.07 | (43 ± 2.2%) | 150 ± 15 | 0.19 ± 0.05 | (55 ± 1.3%) |
|          | 27 ± 3.7   |       |      |       |           |
| DORΔ3−5  | 1.2 ± 0.32  | (67 ± 6.1%) | 170 ± 39 | 1.5 ± 0.4  | (61 ± 2.5%) |
| DORΔ257RRITR261 | 37 ± 14 |       |      |       |           |

The presence of heterodimeric phenotypes at the high ratio of DORΔ3−5:MOR suggests that, unlike the MORΔ3−5 deletion mutant, this DOR mutant could interact and activate the G protein. The ability of DORΔ3–5 to interact with the G proteins was demonstrated by agonist competition binding experiments. As expected, the DOR agonist DPDPE exhibited multiple affinity states binding to the wild type receptor expressed in HEK293 cells (Table III). When the competition binding assays were carried out in the presence of 100 mM Na+ and 5 μM GTPγS, resulting in the uncoupling of the receptor from G proteins, DPDPE was observed to exhibit single low affinity binding to the receptor. Although there was a reduction in the DPDPE affinity for the mutant receptor, a similar decrease in this agonist binding to DORΔ3−5 in the presence of Na+ and GTPγS was observed (Table III). As a matter of fact, in the presence of 100 mM Na+ and 5 μM GTPγS, which uncouple the receptor from G protein, the DPDPE affinity for the DORΔ3−5 mutant was similar to that of wild type DOR. This suggests that deletion of the RRITR sequence from the IL-3 domain did not affect the binding domain of this opioid peptide. The ability of DORΔ3−5 to interact and activate G protein could be demonstrated further by examining the ability of DPDPE to inhibit the forskolin-stimulated increase in the intracellular cAMP level. As shown in Table III, the potency and the maximal inhibition level of DPDPE were similar in EcR293 cells expressing either the wild type DOR or DORΔ3−5. The potency of DPDPE to inhibit the forskolin-stimulated increase of intracellular cAMP production was lower in the DORΔ3–5 cells. Such a decrease in the agonist affinity reflected the parallel decrease in the agonist affinity for the mutant receptor (Table III).

DISCUSSION

In the current studies, by controlling the expression of DOR with the mammalian ecdysone-inducible expression system, the functions of MOR were measured at different DOR levels in the same cells. Similar to previous reports (14, 15) in which the functions of MOR when expressed independently were compared with different cells co-expressing MOR and DOR, the presence of DOR reduced the affinities of various MOR-selective agonists for the receptor (Table I). However, such a reduction in affinities was restricted to agonists and was not uniform with all the agonists tested. To our surprise, the MOR-selective agonist, endomorphin-2, actually exhibited an increase in its affinity for the receptor when DOR was present. This is in contrast with another MOR-selective agonist, PL017, which exhibited a reduction in its affinity for the receptor. Because these peptide agonists have minimal affinities for DOR, such alterations in the agonists’ affinities suggest an interaction between MOR and DOR; hence, the probable existence of the receptor heterodimers. The physical interaction between MOR and DOR was demonstrated in our expression system by the co-immunoprecipitation studies (Fig. 1B), similar to the studies reported earlier (14, 15). In contrast to previous reports (14, 40), the presence of DOR did not potentiate MOR activities. With the exception of endomorphin-2, the abilities of the MOR-selective agonists to inhibit the forskolin-stimulated adenyl cyclase activity were the same in the presence or absence of DOR expression. In the presence of DOR, the potency of endomorphin-2 was increased 5-fold (Table I), which was within the range of increase in the agonist affinity for the receptor (10-fold). Furthermore, regardless of the agonists used in the assays, pretreatment of EcR293 cells expressing MOR alone or MOR and DOR together with PTX resulted in a complete loss of opioid agonist-mediated inhibition of adenyl cyclase activity (Fig. 2). This is in direct contrast to the previous report by George et al. (15) in which the ability of MOR/DOR heterodimers to activate PTX-insensitive G proteins was suggested. The induction of DOR in the same cells expressing MOR did not alter the coupling of MOR with respective G proteins. Because the agonists tested have selectivity toward MOR, it is not obvious from our current studies whether the DOR activities could be affected by the presence of MOR.

The prevailing models for heterodimers are either the domain-swapping dimers or the contact dimers (32–35). In either case, the individual receptors within the heterodimers should have similar cellular trafficking patterns. With other GPCRs, effects of one type of the receptor monomer on the trafficking of the other monomer type have been documented (41–43). The dimerization of DOR as the prerequisite for agonist-induced receptor internalization had been suggested (11). The ability of morphine to induce the endocytosis of the wild type MOR in the presence of a MOR/DOR receptor chimera was reported (46). These studies suggested that opioid receptor could internalize as homo- or heterodimers. However, from our current studies on the trafficking of MOR and DOR, the results do not support such hypothesis. Using the traffic-deficient mutants, such as the MOR C-2, MOR i3–1, or DOR i3–1, the corresponding wild types or wild types of other opioid receptors could not rescue the cell surface expression of the these mutant receptors, whereas the opioid antagonist naloxone could (Fig. 3). Although the co-expression of the wild type and mutant receptors has resulted in a decrease in the cell surface expression of the wild type receptors, this was not due the intracellular retention of the wild type receptors as a consequence of the heterodimerization between the wild type and traffic-deficient mutant receptors. Parallel decreases in the cell surface expression of the wild type receptors were observed when both MOR and DOR
were co-expressed in the same cells. Similarly, by monitoring the rapid constitutive endocytosis of the receptors (Fig. 5) and by determining the turnover rate of the cell surface receptor content with the removal of the insect hormone inducer ponasterone A (Fig. 4), we demonstrated that MOR and DOR do not endocytose constitutively as homo- or heterodimers. Furthermore, our data also suggest that agonists can induce MOR and DOR to internalize as monomers. In addition, the presence of DOR did not alter the internalization kinetics of MOR (Fig. 6), and wild type receptors could not rescue the endocytosis-deficient mutants (Fig. 7). All these data together suggest that the observed heterodimers phenotypes in the binding assays exist only when MOR and DOR are localized at the cell surface.

In some of our receptor trafficking studies, mutant receptors with deletion in the intracellular domains were used. One could argue that the failure of wild type receptors to rescue the trafficking of these mutant receptors is due to the involvement of intracellular domains in the formation of the opioid receptor heterodimers. The participation of the intracellular domains of the two GABA\(_B\) receptors in the signaling of the agonist has been reported (36, 37). The carboxyl tail of GABA\(_B\) interacts with the carboxyl tail of GABA\(_A\), thus masking the ER retention signals within the GABA\(_A\) and resulting in the proper trafficking of the receptors (51–53). The heterodimerization between the adrenergic and muscarinic receptors appeared to involve the third intracellular loop (34). Thus, the deletion of amino acid residues within the intracellular loops or the carboxyl tail domains of the opioid receptors could prevent the dimerization of these receptors. Failure to dimerize could contribute to the inability of wild type to rescue the traffic-deficient mutants (\(i3–1\) and \(C-2\)) or the endocytosis-deficient mutants (\(i3–5\)). However, this could not be the case for the putative MOR and DOR heterodimers. If \(iL3\) domain participates in the homo- or heterodimerization of these opioid receptors, then the respective wild type receptors should be able to rescue the MOR C-2 deletion mutant. The converse is true if the carboxyl tail domain participates in the dimers formation.

An argument against such a conclusion could be made if both \(iL3\) and carboxyl domains are involved in the opioid receptor heterodimerization. Such a scenario is not supported by the rhodopsin model. Furthermore, the observation that the presence of wild type DOR does not affect the agonist-dependent and -independent endocytosis of MOR also argues against the notion that \(iL3\) domain (in particular, the \(i3–5\) (RRITR) sequence or carboxyl tail domain, \(i.e\), the \(C-2\) (KRCFR) sequence) is essential for the formation of homo- or heterodimers.

If indeed the formation of the heterodimers of MOR and DOR could be observed only at the cell surface, probably other cellular structures or proteins (such as the cytoskeleton or proteins with multiple protein interaction motifs) could serve as the scaffold for the oligomers. An excellent example is the ability of the third loop of D\(_\beta\) or D\(_\alpha\)-dopamine receptor to interact with filamin A (ABP-280) of actin, thereby causing an increase in the clustering of receptors at cell surface and the efficiency of effector coupling (54, 55). Also, cellular proteins such as \(\beta\)-arrestin that are recruited to the vicinity of the GPCR could serve as scaffolding proteins (56). Because the alterations in the ligand binding affinities were observed only with the opioid agonists and not the antagonists, we surmise that such alteration is due to the interaction of the receptors with the G protein molecules. As indicated by our PTX studies, the uncoupling of the MOR and DOR from the G proteins attenuated the observed heterodimers phenotypes. George et al. (15) reported similar observations with their binding studies. We further demonstrated with the DOR\(i3–5\) deletion mutant, that the putative heterodimer phenotypes were the results of receptors-G protein interactions. The DOR\(i3–5\) deletion receptor mutant has a phenotype distinct from that of MOR\(i3–5\). With the deletion of the RRITR sequence from the \(iL3\) domain of MOR, the \(\mu\)-opioid agonists could not inhibit adenylyl cyclase activity, and the MOR receptor mutant exists in a low affinity G protein-uncoupled state (49). However, the deletion of the same sequence from DOR resulted in a reduction of the high affinity agonist binding, with the retention of the ability of the agonist DPDPE to inhibit the adenylyl cyclase activity (Table III). These data suggest that DOR remained coupled to the G protein after the deletion of the RRITR sequence from the \(iL3\) domain, albeit with a lower affinity. Thus, it was not surprising to observe that at a high DOR\(i3–5\) to MOR ratio the heterodimer phenotypes persisted, whereas at the equal DOR\(i3–5\) to MOR ratio, the heterodimer phenotypes were not observed (Fig. 9). These data together with the PTX pretreatment data strongly suggest that the observed heterodimers phenotypes were due to the interactions between the opioid receptors and their respective G proteins.

If the formation of MOR/DOR heterodimers requires interaction with G proteins, then what could be a possible model that involves the participation of G proteins? In two recent reports, using atomic force microscopy, which allows vertical resolution of \(\pm 2\) \(\text{Å}\) or higher in the imaging of the rhodopsin in native disks, Fotiadis et al. (57) and Liang et al. (58) demonstrate that native rhodopsin or opsin proteins are densely packed in the membranes of rod outer segment as rows of oligomers. The individual units of rhodopsin or opsin exists as dimeric structures, with interactions between transmembrane domains 4 and 5 constituting the strongest interaction between the monomers (58). The dimers form rows stemming from the contact between \(iL3\) and transmembrane domains 1 and 2 of the adjacent dimers (58). An interesting observation derived from these studies is that the rhodopsin dimers, in contrast to the monomers with cytoplasmic surfaces that are too small to anchor both the \(\alpha\)- and \(\beta\)-subunits of the G\(_T\), provide sufficiently large platforms for such interactions. Hence, a model of 2:1 rhodopsin-G\(_T\) interaction is proposed (58). A similar model could apply for the MOR/DOR heterodimers or their respective homodimers. Similar to the reported rhodopsin dimers, computational analyses of putative structures of the opioid receptors dimers have suggested transmembrane domains 4 and 5 as possible contact sites (35). Thus, similar to rhodopsin, a 2:1 receptor-G protein interaction could exist for the opioid receptors. Then, the interaction between the G/G\(_T\) heterotrimers and the platforms formed by the receptor dimers could offer an explanation for our current observations. Tight interaction between opioid receptor and G proteins was demonstrated by the co-immunoprecipitation of the G proteins with receptor-specific antibodies (59). Because motifs within the Go subunits serve as receptor recognition sites (60, 61), the distinct interaction between receptor and G proteins could define the GPCR function. For the homodimeric receptors, the individual receptor unit has equal affinity for the Go subunit, and agonist binding would only reflect the high affinity state (the G protein interacting unit) and the low affinity state (the G protein non-interacting unit). However in the case of the MOR/DOR heterodimers, in which DOR has been shown to remain coupled to G proteins after PTX treatment, whereas MOR does not (62), the receptor selective agonist binding should reflect whether the receptor interacts with the Go subunit or not. The G protein higher affinity for DOR would dictate the decrease of the MOR-selective agonist binding to the receptor with increasing DOR concentration, \(i.e\), the increase level of heterodimers. This is exactly what our data and others (15) have indicated. Our data further appear to indicate that the interaction with the G
proteins precede the formation of the heterodimers. The deletion of the RRITR sequence from the IL3 domain of MOR resulted in the reduction in the DPDPE affinity and potency (Table III). Hence, a higher DORΔ3–5 mutant receptor level as compared with wild type was needed to reduce agonist affinities, indicative of heterodimeric phenotypes. Furthermore, the failure to observe heterodimeric phenotypes after PTX pretreatment suggests that the interaction with the G proteins as the critical step in formation of heterodimers. Such a model is also in agreement with the data in which receptor monomers and not heterodimers are the units in the constitutive or agonist-induced receptor endocytosis. The absolute requirement for β-arrestin for opioid receptor internalization has been demonstrated (22, 63). The replacement of G protein molecule with β-arrestin within the binding platform of the dimers should be the initial step for the endocytosis process. Such binding of β-arrestin could have a similar effect on the stability of the heterodimers as the PTX pretreatment.

Regardless of whether the interaction with G proteins precedes the formation of heterodimers or vice versa, our data clearly suggest that the model of domain swapping cannot be the basis for the opioid receptor heterodimerization. If domain swapping is the basis, then we should be able to detect an alteration in the trafficking of MOR proteins in the presence of DOR. Under all the experimental paradigms used in current studies, the MOR trafficking was not affected by the presence of DOR or the activation of DOR. Thus, the MOR/DOR heterodimers are contact dimers, with transmembrane domains 4 and 5 the most likely candidates for interaction sites as suggested by the modeling studies. The interaction between the G proteins and receptors stabilizes such contact dimers.

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