Switching Agonist/Antagonist Properties of Opiate Alkaloids at the δ Opioid Receptor Using Mutations Based on the Structure of the Orphanin FQ Receptor*

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In an earlier study, we have demonstrated that by mutating five amino acid residues to those conserved in the opioid receptors, the OFQ receptor could be converted to a functional receptor that bound many opioid alkaloids with nanomolar affinities. Surprisingly, when the reciprocal mutations, Lys-214 → Ala (TM5), Ile-277 → Val/His-278 → Gln/Ile-279 → Val (TM6), and Ile-304 → Thr (TM7), are introduced in the δ receptor, neither the individual mutations nor their various combinations significantly reduce the binding affinities of opioid alkaloids tested. However, these mutations cause profound alterations in the functional characteristics of the mutant receptors as measured in guanosine 3′-5′-O-(thio)triphosphate binding assays. Some agonists become antagonists at some constructs as they lose their ability to activate them. Some opioid antagonists are transformed into agonists at other constructs, but their agonistic effects can still be blocked by the peptide antagonist TIPP. Even the inverse agonist 7-benzylidenenaltrexone becomes an agonist at the mutant containing both the Ile-277 → Val/His-278 → Gln/Ile-279 → Val and Ile-304 → Thr mutations. Thus, although the mutated residues are thought to be part of the binding pocket, they are critically involved in the control of the δ receptor activation process. These findings shed light on some of the structural bases of ligand efficacy. They are also compatible with the hypothesis that a ligand may achieve high affinity binding in several different ways, each having different effects on receptor activation.

The mechanism underlying receptor activation has been intensively investigated by both empirical and theoretical approaches (1–12). In the opioid field, a study conducted soon after the cloning of opioid receptors showed that the Asp residue in the TM2 of the δ receptor was responsible for high affinity agonist binding (13). Agonist and antagonist were suggested to bind to different domains of the κ receptor (14). A study focused on the charged residues in the μ receptor revealed that His to Asn or Gln mutations increased the intrinsic activity of the μ receptor, and agonists such as naloxone, naltrexone, and diprenorphine activated those mutants in oocyte K+ channel modulation assays (15). Serendipitously, opioid antagonists were found to behave like agonists on the μ and the δ receptors with a mutation at a conserved Ser residue in TM4. The mutated receptors were not constitutively active, suggesting that Ser plays a specific role in the ligand-induced receptor activation process (16). A partial agonist to antagonist conversion was also observed in the δ receptor with the TM2 Asp mutation (17). Recently, there were two reports about the creation of constitutively active δ receptors through mutagenesis. One study demonstrated that the replacement of the Asp residue in TM3 with Ala, His, or Lys would endow the mutated receptors with constitutive activity. Whereas naltrindole was still an antagonist at the Asp to Ala mutant, it became an agonist at the receptor with the Ala to Lys mutation (18). Another study mutated the same Asp residue in TM3, the Tyr residue immediately below Asp in TM3, and a Tyr residue in TM7. All these receptor mutants exhibited constitutive activity, suggesting that the wild type δ receptor uses these residues to maintain its inactive state in the absence of agonists (19). Although these studies greatly advance our understanding of the opioid receptor activation process, many important questions, such as how factors determine a ligand to be an agonist or an antagonist and how the binding mechanism is related to the activation process, remain to be answered.

Agonist binding is the first step in ligand-induced receptor activation. Sometimes the same ligand can exhibit similar affinities but very different pharmacological properties on homologous receptors in the same gene family. Yet many other ligands will show the same agonist/antagonist properties across those homologous receptors. For example, (−)-ethylketocyclazocine and (−)-bremazocine are generally considered as agonists on the κ receptor but antagonists on the μ and the δ receptors, although their binding affinities toward the cloned opioid receptor subtypes are literally identical and the opioid receptors share many agonists and antagonists in different structural families. Such instances suggest that even if their binding affinities do not change on structurally and functionally similar receptors, ligands such as ethylketocyclazocine and bremazocine may functionally interact with different subtypes of opioid receptors in different ways. Conceptually, different receptor subtypes may share the same binding pocket for the non-selective ligands but have subtle differences in their activation “trigger.” It is also possible that the same ligand may interact with overlapping but different sets of structural elements in different opioid receptor subtypes. As a result, the

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‡ The abbreviations used are: TM, transmembrane; BNTX, 7-benzylidenenaltrexone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; OFQ, orphanin FQ/nociceptin; SNC80, (+)-4-[(α-R)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl-N,N-diethylbenzamide; GTP[S], guanosine 5′-3′-O-(thio)triphosphate.

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same ligand may differentially influence the functional state of different receptor subtypes through different mechanisms. Since many opioid ligands share the morphine backbone structure, it has been proposed for a long time that there is a "common opioid binding pocket" in different opioid receptor subtypes. It would be interesting to learn whether the hypothetical pocket really utilizes the same structural elements in different opioid receptors and to investigate how the same ligand exerts different effects on receptor activation.

In a previous study (20), we used a "gain-of-function" combinatorial mutagenesis approach to delineate the common opioid binding pocket shared by the \( \mu \), \( \delta \), and the \( \kappa \) opioid receptors (21, 22). Two other groups also reported similar results (23, 24). In our hands, the OFQ receptor was successfully converted to a functional "opioid" receptor by replacing five of its residues with the corresponding residues conserved in the opioid receptors (Ala-213 → Lys (TM5), Val-276 → Ile/Gln-277 → His/Val-278 → Ile (TM6), and Thr-302 → Ile (TM7)) (20). The OFQ receptor mutant bearing five conserved opioid receptor residues can bind opioid alkaloids such as (+)-bremazocine, naltrindole, naltxrene, and nor-binaltorphimine 2–3 orders of magnitude better than the wild type OFQ receptor. It can be activated by the opioid agonist etorphine and inhibited by the opioid antagonist naltrindole and naltxrene. In addition, that receptor also exhibits the same stereospecificity as the wild type opioid receptors. Such evidence strongly suggests that these five conserved residues are key elements of the common opioid binding pocket in the wild type opioid receptors. The goal for this study was to investigate further the properties of the common opioid binding pocket by conducting reciprocal mutations in the opioid receptors. All individual and combinatorial reciprocal mutations of those five residues were introduced into the wild type \( \delta \) receptor. The properties of these mutants as well as the wild type \( \delta \) receptor were analyzed side by side in both ligand and GTP-\( S \) binding assays. Our results were completely unexpected, and they might lead to a new way of understanding ligand-receptor interaction.

MATERIALS AND METHODS

The rat \( \delta \) opioid receptor (25) and the rat OFQ receptor (GenBank™ accession number U05239) used in this study were cloned in our laboratory. The \( \delta \) opioid receptor mutants were made using a double-stranded mutagenesis protocol (26). The presence of intended mutations in the \( \delta \) opioid receptor cDNAs was verified by sequencing the targeted regions. The wild type and mutant \( \delta \) opioid receptor cDNAs were subcloned into a pCMV-neo expression vector, courtesy of Dr. M. D. Uhler (27). For ligand binding assays, the standard calcium-phosphate transfection method (28) was used to express various receptor constructs in COS-1 cells. Each 10-cm plate of COS-1 cells was transfected with 25 \( \mu \)g of plasmid, and the transfected cells were harvested 48 h after washing away the calcium phosphate-DNA precipitates. Receptor binding assay was performed according to Goldstein and Naidu (29). The membrane preparations derived from the transfected cells were incubated with about 2 nM [\(^{3} \)H]bremazocine at room temperature for 1 h, and the free ligand and the receptor-bound ligand were separated using a 24-head Brandel cell harvester (Brandel, Gaithersburg, MD). All competition assays were conducted with nine different competing ligand concentrations at 1:5 dilution in duplicate. All data points represent the mean of three or four independent assays as indicated by the table legend. Binding data were analyzed with the Prism program (GraphPad Software Inc.).

For GTP-\( S \) binding assays, the transfection reagent FuGene 6 was used to transfet COS-1 cells in 10-cm plates according to the instructions of the manufacturer (Roche Molecular Biochemicals). Transfected cells were harvested 48 h later by scraping them off the plate in ice-cold phosphate-buffered saline. Cell pellets were collected by spinning at 5,000 rpm for 5 min at 4 °C. They were then resuspended in an ice-cold lysis buffer containing 5 mM Tris-HCl, pH 7.0, 5 mM EDTA, 2.5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride and homogenized. The cell homogenate was spun at 2,500 rpm for 10 min at 4 °C, and the supernatant was collected. It was centrifuged again at 40,000 \( \times \) g for 20 min at 4 °C. The pellet generated by the second spin was resuspended in 50 mM Tris-HCl, pH 7.0, 0.32 M sucrose and frozen at −80 °C if not used immediately. The protein concentration of the membrane preparation was measured by Bio-Rad protein assay.

[\(^{35} \)S]GTP-\( S \) binding was conducted according to Tian et al. (30) with some modifications. For each assay, 10 \( \mu \)g of membrane proteins were incubated in a final volume of 100 \( \mu \)l with various concentrations of opioid ligands and a buffer containing 50 mM Tris-HCl, pH 7.0, 6 mM MgCl\(_2\), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.03% CHAPS, 30 \( \mu \)M unlabeled and 0.1 nM [\(^{35} \)S]GTP-\( S \). Basal stimulation level was assayed in the absence of ligand, whereas nonspecific binding was measured in the presence of 20 \( \mu \)M GTP-\( S \). After 1 h of incubation at room temperature, membrane-bound GTP-\( S \) was separated from free GTP-\( S \) using a Brandel harvester by washing the membrane mixture 3 times with 4 ml of ice-cold buffer containing 50 mM Tris-HCl, pH 7.0, 5 mM MgCl\(_2\), and 50 mM NaCl through GF/B filters under vacuum. The ligand-stimulated GTP-\( S \) binding for all receptor constructs presented in each figure was measured side by side, and each assay was repeated for at least three independently transfected cell preparations. Bound radioactivity was quantified by liquid scintillation counting. EC\(_{50}\) values were determined using the Prism software.

RESULTS

Results from our laboratory as well as from other groups strongly suggested that the Lys residue at the top of TM5, the Ile-His-Ile residues in the middle of TM6, and the Ile residue in the upper half of TM7 were critically involved in the binding of opioid alkaloids (20, 23, 24). In the context of the OFQ receptor, the effects of these mutated residues are largely additive. The OFQ receptor carrying the full complement of mutations has the stereospecificity of the wild type opioid receptors, and it can stimulate GTP-\( S \) binding in the presence of opioid agonist etorphine. At that stage, we hypothesized that these five residues are critical components of the common opioid binding pocket, and we expected that the reciprocal mutations in the opioid receptors would greatly reduce their affinity toward the opioid ligands.

However, initial characterization with the reciprocal Lys-214 → Ala mutation in both the \( \delta \) and the \( \kappa \) receptor indicated that this mutation did not decrease the affinity of opioid alkaloids as expected, despite the fact that the Ala-213 → Lys mutation in the OFQ receptor increased the affinity of (+)-bremazocine, naltrindole, and naltxrene by around 100-fold. In order to analyze further this unexpected result, individual Lys-214 → Ala, Ile-277 → Val/His-278 → Val/Ile-304 → Thr mutations as well as all their permutations were introduced into the wild type \( \delta \) opioid receptor, and these receptor mutants were subjected to both binding and functional assays.

The competition binding results are summarized in Table I. It can be seen clearly that the affinity of the tested ligands toward various receptors is highly related to their structures. For the alkaloid ligands that possess an aromatic phenol ring, e.g. (+)-bremazocine, naltrindole, naltxrene, and etorphine, none of the mutants showed an affinity that deviated more than 5-fold from the wild type \( \delta \) receptor. Even in the \( \delta \) mutant that contains all five OFQ opioid receptor residue replacements, none of the alkaloids mentioned above showed a more than 3-fold decrease in binding affinity. This is in sharp contrast to its reciprocal OFQ receptor mutant, in which the affinity of the same opioid alkaloids was increased by 100–1000-fold. In fact, 30 \( \mu \)M in Table I would not support the idea that the five critical opioid receptor-specific residues identified through the OFQ receptor mutagenesis study have anything to do with the binding of opioid alkaloids. By contrast, SNC80, a very potent \( \delta \) selective agonist with a very different structure (31), showed a binding profile that is more or less in line with its behavior on the OFQ mutants.
The binding affinities of several other ligands were reduced by δ-fold or more in some of the mutants. The binding affinities of several δ peptide agonists, Leu-enkephalin, [δ-Pen²⁶]enkephalin, and deltorphin II, were reduced significantly by some of the individual mutations, and the combination of individual mutations frequently lead to even worse binding affinities. Such results suggest that the presence of all five opioid receptor-specific residues is important for the binding of peptide agonists. However, TIPP, a δ-selective peptide antagonist, exhibits a pattern that is different from that of the δ peptide agonists. Individual mutations at most decreased its affinity by δ-fold, and the combination of all the mutations did not lead to any further deterioration in affinity.

The intriguing nature of the alkaloid binding data prompted us to study the functional properties of these mutants in a GTPγS binding assay. Fig. 1 summarizes the effect of the non-selective opioid agonist etorphine and the δ-selective agonist SNC80 on GTPγS binding. Several tendencies are evident from the agonist stimulation data presented in this figure. First, the mutants with the Lys-214 → Ala mutation alone show greatly reduced maximum stimulation levels. Furthermore, the presence of the Lys-214 → Ala mutation also reduces the activation of all mutants that contain it, i.e. Lys-214 → Ala + Ile-277 → Val/His-278 → Gln/Ile-279 → Val and Lys-214 → Ala + Ile-304 → Thr. Second, the Ile-277 → Val/His-278 → Gln/Ile-279 → Val and Ile-304 → Thr mutations alone do not decrease and may possibly even increase the maximum stimulation level. Third, the combination of the Ile-277 → Val/His-278 → Gln/Ile-279 → Val and Ile-304 → Thr mutations seem to increase the maximum stimulation level of the mutant receptor over that of the wild type δ receptor. The high maximum stimulation level shown by the Lys-214 → Ala + Ile-277 → Val/His-278 → Gln/Ile-279 → Val + Ile-304 → Thr mutant may result from the dominance of Ile-277 → Val/His-278 → Gln/Ile-279 → Val + Ile-304 → Thrseffective effect over the Lys-214 → Ala detrimental effect on agonist-stimulated GTPγS binding. Finally, the EC₅₀ values for these receptors are very similar to each other. This is understandable given the fact that the binding affinity of these receptors toward etorphine and SNC80 are not significantly altered.

Whereas the maximum stimulation levels of these receptors suggest differences in the activation properties of various mutants, the results need to be interpreted cautiously due to the transiently transfected cells used in these assays. However, more striking qualitative differences were discovered when we studied the effect of classical opioid alkaloid antagonists on these receptors. It can be seen clearly from Fig. 2 A and B, that (−)-bremazocine and naltrindole, both of which are antagonists at the δ receptor, could significantly stimulate GTPγS binding at receptors bearing the Ile-277 → Val/His-278 → Gln/Ile-279 → Val and/or Ile-304 → Thr mutations. Furthermore, their stimulatory effects could be blocked by TIPP, a δ-selective peptide antagonist. In fact, (−)-bremazocine and naltrindole behaved in a manner fully comparable to the effect of the agonist etorphine on GTPγS binding assays. Such antagonist-
Data are expressed as mean ± S.E. with n = 3 or 4. B, dose-response curve for naltrindole. Data are expressed as mean ± S.E. with n = 3 or 4.

DISCUSSION

In the present study, we mutated several residues within the δ opioid receptor, converting them to the equivalent residues found in the OFQ receptor, with the working hypothesis that these residues are part of a common opioid binding pocket and that these mutations would therefore interfere with ligand binding. Our results demonstrate the following. 1) Contrary to expectations, the binding of opiate alkaloid ligands was not significantly altered by either the individual mutations or by various combinations. 2) By contrast, the binding of the more selective opioid peptide agonists was significantly reduced in receptors with multiple mutations. 3) The mutant receptors exhibited significantly altered activation properties, even by ligands that had unchanged binding affinities, indicating that the chosen sites were critical in determining ligand efficacy. 4) More specifically, the Lys-214 → Ala mutant could hardly be activated by opioid agonists although it could still couple to G protein(s) since inverse agonist BNTX now becomes an agonist at this mutant. The structure of BNTX is very similar to naltrindole and since naltrindole is an agonist on the Ile-277 → Val/His-278 → Gln/Ile-279 → Val + Ile-304 → Thr mutant as well as the wild type δ receptor in the high potassium buffer. All ligands tested showed the expected properties on the wild type δ receptor (Fig. 3). However, the Ile-277 → Val/His-278 → Gln/Ile-279 → Val + Ile-304 → Thr mutant demonstrated another unusual property; instead of reducing the basal GTPγS binding level as it did on the wild type δ receptor, BNTX stimulated GTPγS binding. In other words, the δ inverse agonist BNTX now becomes an agonist at this mutant. The structure of BNTX is very similar to naltrindole and since naltrindole is an agonist on the Ile-277 → Val/His-278 → Gln/Ile-279 → Val + Ile-304 → Thr mutant, it seems to be reasonable that BNTX is also an agonist at this receptor. Data presented in Fig. 3 also indicate that such inverse agonist to agonist change was highly related to ligand structure since the classical peptidergic δ inverse agonist ICI173864 still decreased the basal GTPγS binding on the Ile-277 → Val/His-278 → Gln/Ile-279 → Val + Ile-304 → Thr mutant.
At first glance, the results with alkaloid binding appear puzzling. After all, other groups and we (20, 23, 24) had made the reciprocal mutations in the context of the OFQ receptor and endowed this receptor with the ability to bind opiate alkaloids. Given the high degree of sequence homology between the OFQ and the opioid receptors, given that the chosen residues were conserved across all opioid receptors and divergent in the OFQ receptor which does not bind opioids, and given that changing these residues to their opioid equivalents was sufficient to endow the OFQ receptor with the ability to bind opioid alkaloids, it was logical to suppose that these sites were critical to the binding of these alkaloids to their own receptors and that they represented part of the common opioid binding pocket. The present results force us to revise this view and reconsider the very concept of a single common binding pocket. Yet, the results also show that these sites are far from irrelevant. They are important to the binding of the more selective peptide ligands, and more interestingly, they play an unexpected role in receptor activation and the determination of agonist/antagonist properties of various ligands.

If one believes that the OFQ receptor gain-of-function data is not coincidental, the earlier work together with the body of current data strongly suggest that there are multiple ways of generating what appears to be a common opioid binding pocket even in the same receptor. In other words, those small opioid alkaloid ligands that are non-selective and work well in the context of all three opioid receptors may be doing so because each of these receptors may be able to accommodate them in a variety of different ways. There are presumably a large number of residues that are critical in forming the opiate binding cavity. When we introduced a subset of these critical residues into a homologous receptor, the OFQ receptor, we introduced one of many possible configurations that can accommodate the opioid alkaloids, hence the gain of opioid alkaloid binding in those constructs. However, when we alter these very sites within the opioid receptors, we are altering only one of a set of possible high affinity binding pockets, and this cannot be detected with our ligand binding techniques.

This model of a more complex range of ligand-receptor interactions provides a way to understand the observed significant functional change in the absence of binding affinity change. Different ways of receptor interaction for the same ligand may have different effects on receptor activation. Therefore the agonist/antagonist property of a ligand can be viewed as the statistical average of the effects produced by different ways of interaction at a given receptor. How can such multiple ways of interaction with the same ligand coexist in one receptor? One way of achieving it is through the adoption of different receptor conformations. It has been proposed for a long time that a receptor may exist in multiple functional states, each with a different conformation (6, 33–35). Depending on their size and structure, ligands may fit one or more of these conformations and may stabilize primarily those that either can drive G protein coupling (agonists), or fail to drive it (antagonists), or prevent G protein coupling (inverse agonists). Our results would suggest that the residues we have identified participate in biasing the receptors into conformations that either favor agonism or antagonism. Thus, the constructs containing the Ala-213 → Lys mutation appear less likely to adopt a structure that can be driven to an activated conformation when bound by certain ligands typically classified as agonists. By contrast, the constructs with the simultaneous Ile-277 → Val/His-278 → Gln/Ile-279 → Val and Ile-304 → Thr mutations greatly favor agonist conformations after alkaloid ligand binding even when activated by alkaloid ligands that typically produce antagonism. For ligands such as TIPP and ICI174863, either they cannot drive their conformation to an agonistic state or they will selectively bind to a receptor conformation for antagonist and/or inverse agonist that is not significantly altered by the mutations introduced. Thus, our mutations seem to have biased the likely conformational patterns of the constructs for specific ligands either toward agonism or toward antagonism.

Alternatively, there is the possibility that even one particular conformational state of a receptor may contain multiple binding pockets for a given ligand. These binding pockets are most likely overlapping and therefore mutually exclusive. Each binding pocket may trigger the activation process differently. Upon ligand binding, some of these pockets may promote the coupling of G protein, some may inhibit G protein coupling, and yet others may have no effect on G protein coupling. A ligand will interact with different binding pockets dynamically, and the amount of time that a ligand spends in a given binding pocket will be proportional to its affinity toward that pocket. Therefore the apparent agonist/antagonist property of a ligand on a receptor will be the average of the properties of different binding pockets, weighted by their corresponding affinity value for that ligand. The elimination...
of an antagonistic binding pocket will increase the efficacy of the concerned ligand. Similarly, the deletion of an agonistic binding pocket will decrease the efficacy of a ligand. As a result, the observed ligand-specific qualitative change in agonist/antagonist property becomes very easy to understand. Most likely, the residues we mutated are critical elements for a high affinity antagonistic alkaloid binding pocket in the wild type δ receptor. At the same time, there must also be agonistic binding pocket(s) with equal or somewhat lower affinity that survives our mutation in the δ receptor. This can be the reason that classical alkaloid antagonists, even an alkaloid inverse agonist, become agonists at the δ receptor containing Ile-277 → Val/His-278 → Gln/Ile-279 → Val and Ile-304 → Thr mutations. This view is consistent with our previous OFQ receptor mutation results where alkaloid antagonists showed the most significant affinity increase (20). Furthermore, since the peptide antagonist TIPP and the peptide inverse antagonist IC174863 most likely use binding pocket(s) that are very different from those used by alkaloids, it is easy to understand that their antagonistic/inverse agonistic binding pocket(s) that are very different from those used by alkaloids, whereas ligands such as bremazocine and ethylketocyclazocine continue to bind with nanomolar affinities. With the results of our gain-of-function study wherein the altered OFQ constructs could bind a range of alkaloids of various selectivity but, with the exception of the dynorphins, could not bind the opioid peptides.

In summary, our results expand our view of how ligands of various classes interact within the δ opioid receptor in particular and this family of G protein-coupled receptors in general. They support a more complex view of the opioid binding pocket and identify a set of specific residues as representing key components of that pocket, capable of endowing a related receptor with alkaloid binding and responsible for biasing the state of efficacy of the opioid receptor.

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