Moving from the Orphanin FQ Receptor to an Opioid Receptor Using Four Point Mutations*

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It is unclear how receptor/ligand families that are evolutionarily closely related can achieve functional separation. To address this question, we focus on the newly discovered Orphanin FQ, a peptide homologous to the opioid peptide Dynorphin, and its receptor, the Orphanin FQ receptor, which is highly homologous to the opioid receptors. In spite of this high degree of homology in terms of both ligands and receptors, there is little direct cross-talk between the Orphanin FQ system and the endogenous opioid system. Thus, the opioid peptides show either relatively low affinity or no affinity toward the Orphanin FQ receptor; conversely, Orphanin FQ has no affinity toward any of the opioid receptors. We sought to investigate the molecular basis of such discrimination by attempting to reverse it and endowing the Orphanin FQ receptor with the ability to bind opioids. We report that by mutating as few as four amino acids, we can produce a receptor that recognizes pro-Dynorphin products with very high affinity and yet still binds Orphanin FQ as well as the wild-type receptor. This suggests that the Orphanin FQ receptor has developed features that specifically exclude the opioids and that these features are distinct from those required for the high affinity binding of its own endogenous ligand.

The three major types of opioid receptors, μ, δ, and κ, have been cloned and shown to belong to the seven transmembrane domain, G protein-coupled family (1, 2). In addition, several laboratories have cloned a protein highly homologous to these opioid receptors but that, nevertheless, does not bind with high affinity any known opioid peptides or alkaloids (3). The endogenous ligand for this opioid-like orphan receptor has recently been isolated by two independent groups (3, 4). Interestingly, it is a 17-amino acid peptide with a significant degree of sequence homology to DynA-(1–17). This novel peptide has been termed Nociceptin by Meunier et al. (3) to denote its ability to increase pain responsiveness and Orphanin FQ by Civelli and coworkers (4). We shall refer here to the ligand as Orphanin FQ and to its receptor as the Orphanin FQ receptor.

It is clear from ongoing anatomical studies that Orphanin FQ and its receptor represent a novel and distinct peptidergic system with a unique anatomical distribution within the central nervous system and the gastrointestinal system. Behavioral results have already demonstrated a novel profile for Orphanin FQ in pain and place preference tests and in patterns of tolerance development (5, 6). Orphanin FQ has at its amino terminus the sequence Phe-Gly-Gly-Phe, which only differs from the common opioid core (Tyr-Gly-Gly-Phe) by a single OH group. Yet, since the N-terminal tyrosine is critical for the binding of all opioid peptides (7), this change is sufficient to preclude Orphanin FQ from binding to any of the opioid receptors. Conversely, reciprocal events must have taken place to preclude the opioid peptides from being recognized by the Orphanin FQ receptor. Thus, this system offers a most interesting example of how, through evolution, sets of receptors and their endogenous ligands can diverge to achieve a clear separation of function.

In addition, the existence of the Orphanin system provides an excellent opportunity to address the issue of the structural requirements of an opioid receptor. Based on the examination of the similarities and differences between the three types of opioid receptors, we can suggest some elements common to all of them that may help define an opioid binding pocket and other elements that may be critical for the discrimination by ligands between receptors, i.e. the structural features of high affinity and of selectivity. To date, the body of evidence suggests that some specific residues near the interface of the extracellular loop and the α-helical transmembrane domains may play important roles in ligand binding through ionic or hydrogen bonding interactions (8–10). The extracellular loops, which are highly divergent, clearly play a role in ligand selectivity, especially for the peptides, allowing them to differentiate between the κ, μ, and δ receptors (11–15). However, most of the mutagenesis studies aiming at defining a common opioid binding pocket rely on loss of binding as an indicator of a potential role of a given residue in ligand interaction. This is often difficult to discern from a loss of binding due to a nonspecific conformational distortion of the receptor, which points to the critical nature of the residue but does not necessarily implicate it in direct ligand interactions. A complementary approach would be to endow a receptor, previously incapable of opioid binding, with the ability to bind opioid ligands. We, therefore, undertook to use the Orphanin FQ receptor as our starting point and attempted to endow it with opioid binding properties. One question of interest was whether or not the same structural elements responsible for excluding the opioids from binding the Orphanin FQ receptor would also be critical for the high affinity binding of Orphanin FQ to its own receptor.

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EXPERIMENTAL PROCEDURES

The cDNA encoding the rat Orphanin FQ receptor was cloned in our lab (GenBank™ accession number U05239). Its coding region is identical to the clone that other groups reported. The preparation of monomiodinated Tyr14-orphanin FQ, was used to label the receptors. BWB373, (+/−)-4-((α-R)-α-(25S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-N,N-diethylbenzamide; CTOP, n-pen-cyclic [Cys-Tyr-d-Trp-Orn-Thr-Pen]-Thr-NH; DP-DPE, cyclic (α-penicillamine, α-penicillamine) enkephalin; EKC, ethylketocyclazocine; nBNI, nor-binaltorphine HCl; NTB, naltriben methanesulfonate; SNC 80, (+/−)-4-((α-R)-α-(25S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-N,N-diethylbenzamide; TIPP, Tyr-Tic-Phe-Phe; U63,639, spiradoline(+)-enantiomer; U63,640, spiradoline(−)-enantiomer.

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### RESULTS

We first characterized the binding profile of the Orphanin FQ receptor, using 125I-labeled Tyr14-Orphanin FQ as the labeling ligand, and examining the binding (or lack of such) of various endogenous or exogenous opioids under a standard opioid receptor binding condition (20). The results in Table I show that, with one exception, none of the opioid ligands tested could displace the binding of 125I-Orphanin FQ with concentrations up to 1000 nM under the testing conditions used. The only deviation from this pattern is the binding of DynA and some of its fragments, which displaced 125I-Orphanin FQ binding in the range of 10–100 nM. While these affinities are substantial, they are approximately 2 orders of magnitude lower than the affinities of Dynorphin A and its fragments to the κ receptor, as the latter are in the subnanomolar range under the same assay condition (20).

We then identified residues that were conserved in all three opioid receptors but were divergent in the Orphanin FQ receptor. This was based on the argument that if a residue is critical for defining a common opioid core that is distinct from the Orphanin FQ binding site, it should be present in all opioid receptors and absent in the Orphanin FQ receptor. Furthermore, based on structure-function studies of numerous G protein-coupled receptors including the opioids (8–15, 22), we focused on the residues that are near the top half of the proposed transmembrane domains. In our first round of study, seven mutations were individually introduced into the Orphanin FQ receptor (Fig. 1). Of these mutations, three (TM2 LL-TT; TM3 AA-TT; and TM4 SA-VT) did not bring about any significant changes in either Orphanin FQ binding or competition by a number of opioid peptides and alkaloids (data not shown). A fourth mutation that converted a leucine to a serine in transmembrane one (TM1 L-S) destroyed the binding of iodinated Orphanin FQ. However, there were three mutations that showed a substantial increase in the affinity of the new construct toward DynA and other opiate drugs that were screened (the non-selective alkaloid bremazocine, the κ-selective agonist ethylketocyclazocine, the partially μ-selective antagonist naltrindole, and the highly δ-selective antagonist naltrindole). 1) A Thr302 to Ile (T-I) mutation near the top of TM7 substantially increased the binding affinity of DynA (1–17) and naltrindole. 2) A bank of three residues, VQQ726–728 in TM6 of the Orphanin FQ receptor, when mutated to IHI, also increased the affinities of the screening ligands including DynA, by one

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**TABLE I**

**Opioid ligand binding profile of the wild type Orphanin FQ receptor**

| Ligand       | K<sub>a</sub> (nM) |
|--------------|------------------|
| DynA (1–13)  | 530              |
| DynA (1–11)  | 19               |
| DynA (1–9)   | 80               |
| DynA (1–7)   | 80               |
| DynA (2–17)  | >10,000          |
| nBNI         | 780              |
| U63,640      | >10,000          |
| Non-selective ligands | |
| Bremazocine  | 2600             |
| EKC          | >10,000          |
| β-endorphin  | >10,000          |
| Delta Ligands|                 |
| Leu Enkephalin| >10,000         |
| DP-DPE       | >10,000          |
| DSLET        | >10,000          |
| Dermenkephalin| >10,000         |
| TIPP         | >10,000          |
| Naltrexone   | 640              |
| NTI2         | 2500             |
| BWB 373      | >10,000          |
| Mu Ligands   |                 |
| CTOP         | >10,000          |
| Dermorphin   | >10,000          |
| Naltrexone   | 5200             |
| Cyprodine    | >10,000          |
| U63,639      | >10,000          |
| Fentanyl     | 1600             |

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5 The abbreviation used is: Dyn, Dynorphin
order of magnitude. It should be noted that this region contains the histidine site in the opioids that was previously proposed to be critical to opioid binding (9).

3) Most strikingly, an Ala213 to Lys (A-K) mutation brought the affinity of bremazocine, naltrexone, and naltrindole to 10^{-8} and DynA-(1-13) to 10^{-9} M ranges. This Ala213 is located near the predicted top of TM5, adjacent to extracellular loop 2 (EL2). Interestingly, the rat somatostatin receptor subtype 1, which is also closely related to the opioid receptor family but does not recognize opioid ligands, has an Ile residue instead of a Lys residue at this position, also showing a deviation from the three opioid receptors.

The results from this first round of study suggested that we had identified some critical residues that are important in preventing opioids from binding the Orphanin FQ receptor and that may conceivably be important in forming the opioid pocket in the context of the opioid receptors. Interestingly, none of them interfered with the binding of Orphanin FQ itself, suggesting their role in selectivity but not high affinity binding of this peptide to its receptor. To test this idea further, we carried out the next round of study, extending the findings in two directions. 1) We combined the mutations at each of the three sites identified above, such that we created dual and triple mutants. 2) We tested a larger number of ligands, focusing primarily on the endogenous opioids, as we were most interested in the discrimination among the naturally occurring ligands. All studies were repeated 3 times. The results, displayed in Table II, demonstrate again that single mutations at these sites were able to yield receptors with excellent affinity for the Orphanin FQ. In addition, one of the double mutants shown (VQV-IHI + T-I) was also capable of recognizing Orphanin FQ with excellent affinity. By contrast, when the A-K mutation was combined with either of the others or with both, binding affinity of Orphanin FQ was lost (not shown). This appears to result from a change in binding affinity toward Orphanin FQ rather than from a more fundamental loss in protein expression, folding, or membrane insertion. This can be ascertained because the double or triple mutant receptors that included the A-K change acquired the ability of being labeled with the δ-opioid ligand, [3H]naltrindole (not shown).

The mutants displayed in Table II demonstrate that the combination of the mutations at the T-I and VQV-IHI sites (i.e. a total change of four residues) yielded a receptor that now recognizes both Orphanin FQ and DynA-(1-17) with subnanomolar affinity. This construct also binds the highly κ-selective fragment of Dynorphin, DynA-(1-13), with subnanomolar affinity, whereas it has intermediate affinity toward DynA-(1-8), which has lesser selectivity across the κ and δ receptors (20, 23). Leucine-enkephalin, which represents DynA-(1-5), but is highly δ-selective, is still unable to bind this mutant receptor. Interestingly, other pro-Dynorphin products known to be κ-selective, including α-neoendorphin and DynB exhibit nanomolar affinities for this construct, whereas the endogenous opioid β-endorphin, which is μ- and δ-selective and does not bind κ, remains unable to bind this construct. Finally, it is clear that this construct requires the N-terminal tyrosine of the opioids, as the non-opioid DynA-(2-17) is unable to bind either this construct or the wild-type Orphanin FQ receptor.

**DISCUSSION**

The results of this series of studies have shown the following. (a) The wild-type Orphanin FQ receptor strongly discriminates against all the endogenous opioid ligands except DynA and some of its fragments. These latter peptides exhibit appreciable affinities to the wild-type Orphanin FQ receptor, albeit 30–100-fold lower than their affinities for the κ opioid receptor. (b) By changing as few as four amino acids in the Orphanin FQ receptor, it is possible to create a protein that simultaneously recognizes, with nanomolar or better affinities, the Orphanin FQ peptide and the κ-selective members of the pro-Dynorphin family. (c) The entire set of constructs continues to discriminate against the non-κ endogenous opioids. (d) These constructs, like the wild-type Orphanin FQ receptor, appear to require an N-terminal phenyl residue for high affinity binding as des-Tyr Dynorphin, a non-opioid form of Dynorphin A, fails to recognize them. These findings suggest that, in the wild-type Orphanin FQ receptor, a handful of residues are sufficient to prevent the binding of the κ-selective opioids to the Orphanin FQ receptor. Yet these residues are, at least in part, distinct from those that are required for high affinity binding of Orphanin FQ since the latter continues to bind when they are mutated.

The binding profile of the wild-type Orphanin FQ receptor reveals a pattern of selectivity that favors pro-Dynorphin A and its fragments. Moreover, the observed pattern is reminiscent of the interaction of these same peptides with the wild-type κ receptor. Thus, the longer forms, DynA-(1-17), DynA-(1-13), and DynA-(1-11), exhibit the highest affinities toward the Orphanin FQ receptor while the shortened forms DynA-(1-7) and DynA-(1-5) (or leucine-enkephalin) exhibit a substantial loss in affinity to that receptor. This is very similar to the behavior of the DynA peptides toward the κ receptor, suggesting that the C-terminal tail of the DynA fragments may be largely responsible for their limited affinity toward the Orphanin FQ receptor. Using the message-address concept (7, 23) as applied to the opioid peptides, we can see that multiple messages exist in the C-terminal half of DynA-(1-17). One of these messages, DynA-(8–13), promotes the binding with the Orphanin FQ receptor; however, another message, found in the DynA-(14–17) region, appears to discriminate against the Orphanin FQ receptor as evidenced by the observation that DynA-(1-13) has better affinity than DynA-(1-17) toward the Orphanin (but not the κ) receptor. It should be noted here that the C-terminal part of the
Orphanin FQ peptide was also found to be important for binding and biological activity (16). Since none of the opioid peptides tested including DynA and its fragments showed truly high affinities toward the wild-type Orphanin FQ receptor, we would suggest that the binding pocket formed by the transmembrane domains of the Orphanin FQ receptor selects against the binding of the N-terminal opioid core Tyr-Gly-Gly-Phe. Furthermore, the same wild-type receptor selects against all the opioid alkaloids tested. Thus, the wild-type Orphanin FQ receptor has structural features that discriminate against the vast majority of opioid drugs and opioid peptides.

The mutation of the bank of three residues in TM6 with histidine at the center brought about a significant change in the binding profile of the Orphanin FQ receptor, moving it substantially toward a $\kappa$ opioid profile and thereby confirming an important role of this region of TM6 in the binding of opioids to their receptors. The threonine-isoleucine single point mutation in TM7 was even more effective at improving the affinity toward the $\kappa$-selective endogenous ligands deriving from the pro-Dyn precursor. However, the most remarkable single change was the A-K mutation at the interface of TM5 and the second extracellular loop. This residue endowed the Orphanin FQ receptor with an opioid profile that included not only binding the pro-Dynorphin peptides but greatly improved affinity toward several small alkaloids (data not shown). This mutation also demonstrates that, while the histidine in TM6 is important for opioid binding, it is not critical to the binding of some ligands since the A-K mutant achieves good binding affinities in the absence of the TM6 histidine. Interestingly, none of these mutations interfered with the binding of Orphanin FQ itself. When the mutations were combined, one of the constructs (VQV-IHI$^+$T-I) retained the ability of binding Orphanin FQ while exhibiting affinities for the pro-Dyn peptides comparable with those seen with the $\kappa$ receptor. Thus, this construct represents the creation of a true pro-Dyn/Orphanin dual receptor.

It is notable that, although the residues chosen for mutations were conserved across all three opioid receptors, they only enhanced the binding of pro-Dyn-derived $\kappa$-selective peptides. Indeed, the affinity of the double mutant toward these peptides is fully predicted by the $\kappa$ profile and the wild-type Orphanin FQ receptor profile, with DynA-(1–17) and DynA-(1–13) showing extremely high affinities, DynA-(1–8) showing intermediate affinity, and leucine-enkephalin showing extremely low affinity. While DynA-(1–17) and DynA-(1–13) already had substantial affinities toward the Orphanin FQ receptor, and while $\alpha$-neoendorphin (another pro-Dyn product) also exhibited affinity in the 100 nM range, another pro-Dyn derived peptide, DynB, had an affinity to the wild-type Orphanin FQ receptor close to the micromolar range. Yet DynB exhibited a hundredfold increase in binding the double mutant, showing that our mutations enhanced the binding of pro-Dyn products regardless of their starting affinities for the wild-type receptor. Nevertheless, it is evident that among the endogenous opioid peptides tested, it is only the $\kappa$-selective products of pro-Dyn that show any improvement with these mutations. We interpret these observations to mean that the sites we mutated may be necessary for binding of opioids in general, but they are obviously not sufficient. A key element critical for discrimination in favor of $\kappa$ ligands must be already present in the Orphanin FQ receptor. We suggest that this element may be the highly negatively charged second extracellular loop. While the EL2s of the $\kappa$- and the Orphanin FQ receptors are clearly divergent in terms of strict peptide sequence, they share the distinct feature of possessing a large number of negative charges. Work from our laboratory and that of others has shown that this second extracellular loop of opioids is critical in discriminating between the endogenous peptides and endowing them with $\kappa$ versus $\delta$ selectivity (11, 24). We had previously argued that these extracellular loops may not discriminate via direct point to point interactions but via general patterns of attractions and repulsion based on their general secondary structures and hydrophobic properties. The present findings tend to support this view.

It is notable that the analog of DynA that lacks the N-terminal tyrosine is incapable of binding either the wild-type Orphanin FQ receptor or any of the mutants. This indicates that the phenyl group present in both Orphanin FQ (Phe) and in all the endogenous opioid peptides (Tyr) is critical for interaction with this receptor. However, the present studies do not allow us to pinpoint the exact site of this interaction nor the site that allows a possible discrimination between a Phe and Tyr in the N-terminal position. It should be noted however that the conversion of Phe$^1$ to Tyr$^1$ in the context of the Orphanin FQ sequence is not sufficient to endow it with opioid properties in spite of the fact that such a change now reconstitutes the critical Tyr-Gly-Gly-Phe core, emphasizing again the complex nature of peptide receptor binding selectivity.

It was not possible to predict, a priori, whether a given set of mutations would simultaneously endow the Orphanin FQ receptor with opioid recognition and rob it of Orphanin FQ binding or whether these two events could be separated. The present results clearly demonstrate that discrimination against opioids resides at somewhat different sites from the key sites of Orphanin FQ recognition, as evidenced by the fact that we created a dual receptor that could recognize both Orphanin FQ and the peptidergic $\kappa$ ligands. However, the sites may not be completely separable as the combination of the AK mutation with others, while leading to opioid alkaloid binding (data not shown), substantially decreases the binding of Orphanin FQ.

In summary, we have shown that a small number of specific residues are responsible for excluding $\kappa$ peptide ligands from binding to the Orphanin FQ receptor. Since the introduction of these residues in the Orphanin FQ receptor is sufficient to endow it with the ability to bind $\kappa$-selective endogenous peptides, we would propose that these very residues are critical for peptide binding in the context of the opioid receptors. However, we also suggest that other elements of these receptors, such as the extracellular loops, participate in binding selectivity. The reported results demonstrate the value of using related members of a given receptor family to understand the structural basis of high affinity and high selectivity ligand binding. They also demonstrate how small but coordinate changes during evolution of the receptor and the ligands may be sufficient to derive an entirely novel neurotransmitter system.

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