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# Creating a Functional Opioid Alkaloid Binding Site in the Orphanin FQ Receptor through Site-Directed Mutagenesis

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## ABSTRACT

Although much has been learned about the mechanisms of ligand selectivity between different opioid receptor subtypes, little is known about the common opioid binding pocket shared by all opioid receptors. The recently discovered orphanin system offers a good opportunity to study the mechanisms involved in the binding of opioid versus nonopioid ligands. In the current study, we adopt a "gain of function" approach aimed at shifting the binding profile of the orphanin FQ receptor toward that of the opioid receptors. After two rounds of mutagenesis, several orphanin FQ receptor mutants can be labeled with the opiate alkaloid [$^3$H]naltrindole and show greatly increased affinities toward the opiate antagonists naltrexone, nor-binaltrophine HCl, and (−)-bremazocine. These orphanin FQ receptor mutants also display stereospecificity similar to that of opioid receptors. Furthermore, the orphanin FQ receptor mutant that has the best affinities toward the opioid alkaloids shows, in the presence of GTP and high salt concentration, an affinity-shift profile similar to that of the δ receptor. Most strikingly, the same mutant exhibits naltrindole-sensitive etorphine-stimulated [$^{35}$S]guanosine-5′-*O*-(3-thio)triphosphate binding, whereas the effect of etorphine on GTP binding cannot be inhibited by naltrindole in the wild-type receptor. Our results indicate that 1) several residues in the orphanin FQ receptor are critical to its selectivity against the opiate alkaloids, particularly antagonists; and 2) mutating these residues to those of the opioid receptor at the corresponding position preserves the agonist/antagonist nature of opiate alkaloids as they interact with the mutant receptor. It is reasonable to hypothesize that the corresponding residues in the opioid receptors may form a functional common binding pocket for opiate alkaloids. These findings may be helpful to medicinal chemists in designing ligands for the orphanin FQ receptor based on the structure of the opiate alkaloids.

Since the cloning of opioid receptors, there have been extensive structure-function analyses of the mechanism of binding selectivity for various opioid ligands. It is now generally accepted that the extracellular loops of the opioid receptors are critical for the binding selectivity of opioid ligands, especially the peptide ligands. For example, the second extracellular loop of the κ receptor was found to be critical for the high affinity binding of prodynorphin peptides (Wang *et al.*, 1994b; Meng *et al.*, 1995). The first and/or third extracellular loops of the μ receptor are involved in the binding of [D-Ala$^2$,*N*-MePhe$^4$,Gly-ol$^5$]-enkephalin under different conditions (Onogi *et al.*, 1995; Xue *et al.*, 1995; Watson *et al.*, 1996). The third extracellular loop of the δ receptor may be largely responsible for the high affinity binding of many δ ligands (Li *et al.*, 1996; Meng *et al.*, 1996b; Wang *et al.*, 1996). In addition, it was demonstrated that several residues in the transmembrane domains, especially the charged amino acids that are conserved across many families of G protein-coupled receptors, also play an important role in ligand binding and receptor activation (Kong *et al.*, 1993; Surratt *et al.*, 1994; Hjorth *et al.*, 1995).

Although these studies help us to understand how ligand selectivity between different subtypes of the opioid receptors is achieved via the extracellular loops and confirm the mechanisms shared by many families of receptors in the transmembrane domains, little is known about the residues involved in the binding of nonselective opiate ligands, especially nonselective opiate alkaloids. This is because many structure-function studies are based on chimeric receptors constructed between the opioid receptor subtypes. Subsequent mutagenesis studies were aimed at discovering the residues critical for the binding selectivity between different opioid receptor subtypes, with little emphasis on understand-

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**ABBREVIATIONS:** Dyn, dynorphin; nBNI, nor-binaltrophine HCl; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

ing the features required for the binding of nonselective opiate alkaloids. Yet, understanding the binding of alkaloids could be most valuable in that it would describe the structural features of a "common opiate binding pocket"; it would also greatly enhance our understanding of how nonpeptidergic ligands interact at a peptidergic receptor.

During the cloning of opioid receptors, many laboratories, including ours, also cloned a receptor that is highly homologous to the opioid receptors (Bunzow *et al.*, 1994; Chen *et al.*, 1994; Fukuda *et al.*, 1994; Mollereau *et al.*, 1994; Wang *et al.*, 1994a; Wick *et al.*, 1994; Lachowicz *et al.*, 1995; Pan *et al.*, 1995). However, its identity and endogenous ligand were not convincingly determined for 2 years. One group reported that a very high concentration of etorphine acted like an agonist at this receptor, and its effect could be blocked by high concentration of diprenorphine (Mollereau *et al.*, 1994). This would suggest that this receptor might have a low affinity but functional opioid binding pocket. Another group reported that this receptor may be related to the $\kappa_3$ opioid receptor based on an *in vivo* antisense mapping study (Pan *et al.*, 1995). Recently, the endogenous ligand for this receptor was identified independently by two groups based on a functional assay and the structural features of the receptor protein (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). It was named nociceptin by one group and orphanin FQ by the other. Here, we will refer to the endogenous peptide ligand as orphanin FQ and to the receptor as the orphanin FQ receptor. Although its receptor is most homologous to the opioid receptors, orphanin FQ also shares several structural features with opioid peptides, particularly DynA. However, despite the homology of this system to the opioid system at both the ligand and the receptor level, the orphanin FQ receptor does not bind any other opioid ligands with very high affinity, although it exhibits moderate affinities to DynA and some of its fragments (Meng *et al.*, 1996a). In addition, the orphanin FQ peptide has very low affinity toward all three opioid receptor subtypes (Civelli O, unpublished observations) and it seems to have a distinct structure-function profile as revealed by recent studies (Dooley and Houghten, 1996; Reinscheid *et al.*, 1996). It also has its own unique anatomical distribution (Nothacker *et al.*, 1996) and behavioral effects (Devine *et al.*, 1996a, 1996b). Thus, the orphanin and the opioid systems are highly related yet distinct and they provide us with an excellent opportunity to study the molecular mechanisms underlying ligand selectivity. In a previous study, we reported that individual mutations could endow the orphanin FQ receptor with a greatly enhanced ability to recognize products of the prodynorphin family (Meng *et al.*, 1996a). In this study, we use this "gain of function" mutagenesis approach to convert the orphanin FQ receptor to a receptor that can bind opiate alkaloids with good affinity. Our aim was to identify the residues that the orphanin FQ receptor uses to exclude the binding of opioid ligands. This may also help to reveal the basic "opioid pocket" in the opioid receptors.

## Materials and Methods

The rat orphanin FQ receptor used in this study was cloned in our laboratory (GenBank accession no. U05239). The iodination of the orphanin FQ peptide and the high performance liquid chromatography purification of the monoiodinated peptide were performed according to Reinscheid *et al.* (1996).

Orphanin FQ receptor mutants were made using a double-stranded mutagenesis protocol (Deng and Nickoloff, 1991). The presence of intended mutations in the orphanin FQ receptor cDNAs was verified by sequencing the targeted regions. The wild-type and mutant orphanin FQ receptors were subcloned into a pCMV-neo expression vector, courtesy of Dr. M. D. Uhler (Huggenvik *et al.*, 1991). Chen and Okayama's (1987) calcium-phosphate transfection method was used to express various receptor mutants, the wild-type orphanin FQ receptor, and the wild-type $\delta$ receptor 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 hr after washing away the calcium phosphate-DNA precipitates. Receptor binding of the membrane preparation derived from the transfected cells was performed according to Naidu and Goldstein (1989). About 50,000 cpm of $^{125}$I-orphanin FQ (corresponding to a final concentration of 50–80 pM) or around 1 nM [$^3$H]naltrindole were used in each tube in the binding assay, in the presence of a proteinase inhibitor cocktail. The final concentration of the components in the binding buffer was: 50 mM Tris, pH 7.4, 0.02% bovine serum albumin (radioimmunoassay grade), 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml aprotinin, 1 mM EDTA, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin A, and 1 mM iodoacetamide. The binding reactions were conducted at room temperature for 1 hr, and the free ligand and the receptor-bound ligand were separated using a Brandel cell harvester (Brandel, Gaithersburg, MD). To determine whether the opiate alkaloids interacted with the orphanin FQ receptor mutants like agonists or antagonists, ligand binding studies were conducted side by side in the presence and the absence of 50 $\mu$M GTP$\gamma$S and 120 mM sodium chloride. All binding assays were conducted in duplicate with nine different competing ligand concentrations at 1:5 dilution. All data points represent the mean of three or four independent binding assays as indicated by the table legends. Binding data were analyzed with the Ligand program (Munson and Rodbard, 1980).

The [$^{35}$S]GTP$\gamma$S assay on the transiently transfected COS-1 cells was conducted according to Befort *et al.* (1996) with some modifications in cell plating, electroporation, and [$^{35}$S]GTP$\gamma$S incubation. COS-1 cells were seeded at a density of $10^6$ cells/140-mm dish 72 hr before electroporation. Confluent cells from two plates were harvested and resuspended in 700 $\mu$l of electroporation buffer (1× = 50 mM $K_2HPO_4$, 20 mM $CH_3CO_2K$, 20 mM KOH, pH 7.4). They were incubated with 287 $\mu$l of 1× electroporation buffer containing 8 $\mu$g of receptor-encoding plasmid and 32 $\mu$g of pBluescript-SK(−) (Stratagene, La Jolla, CA) plus 13 $\mu$l of 1 M $MgSO_4$ for 10 min on ice. The cell/DNA mixture was transferred to a 1-ml cuvette, and electroporation was performed using the BRL Cellporater (BRL, Bethesda, MD) at a setting of 330 $\mu$F, 360 V, and low resistance. After electroporation, cells were immediately seeded into a 140-mm dish with 25 ml of Dulbecco's modified Eagle's medium and 10% fetal calf serum and grown for 72 hr. The transfection rate was about 50% as measured by 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside staining of a $\beta$-galactosidase reporter transfected cells.

Membrane preparation was performed according to Befort *et al.* (1996). A [$^{35}$S]GTP$\gamma$S binding reaction was incubated at room temperature for 1 hr after mixing various components on ice (Emmerson *et al.*, 1996). A final concentration of 0.0375% CHAPS was also included in the binding cocktail to reduce deviations among triplicates (data not shown). The percentage of stimulation was defined as the ratio of [$^{35}$S]GTP$\gamma$S binding in the presence and absence of a given concentration of ligand. Data from [$^{35}$S]GTP$\gamma$S binding assays were expressed as mean ± standard error in the figures and dose-response curves were created by fitting data to a three-parameter logistic equation using DeltaGraph (SPSS, Chicago, IL).

## Results

In the course of studying the effects of various orphanin FQ receptor mutations on the binding affinity of the endogenous opioid peptides, we noticed that some of the mutations, be-

sides increasing DynA (amino acid 1–17) affinity, can also improve the binding affinity of several opioid alkaloids and preserve their affinities toward the orphanin FQ peptide (Table 1). For example, a three-consecutive-amino-acid replacement in TM6 (Val276-Gln277-Val278 to Ile-His-Ile) improved the affinities of (−)-bremazocine, naltrexone, and naltrindole over 10-fold. A Thr302Ile mutation in TM7 significantly increased the affinity of naltrindole but had little effect on the binding of other ligands. Most strikingly, a single amino-acid mutation at the interface of EL2/TM5 (Ala213Lys) increased the affinities of (−)-bremazocine, naltrexone, and naltrindole by almost 2 orders of magnitude, whereas the binding affinity of the nonselective benzomorphan ethylketocyclazocine was increased by over 10-fold.

As indicated in Table 1, in this first set of mutants, we used residues conserved across the $\mu$, $\delta$, and $\kappa$ opioid receptors to replace the corresponding residues in the orphanin FQ receptor. Except for the Leu-to-Ser mutation in TM1, all the other mutations still bound the orphanin FQ peptide with very high affinities. This suggests that in most cases studied, the mutants still maintained a reasonably good orphanin FQ binding pocket and therefore a good receptor conformation. The observed increase in opioid alkaloid affinities after changing the orphanin FQ receptor residues to those of the opioid receptor is probably achieved through increasing the similarity of the orphanin FQ receptor to the opioid receptors. If this is truly the case, one may expect that the combination of these mutations would further increase the affinities of the opiate ligands, although the combined effects of these mutations may not be strictly additive.

A second round of mutagenesis was carried out based on the results of the first round study. Because three of the mutants mentioned above showed the most significant increases in binding affinities for the opioid alkaloids, all permutations of these mutants were made in the orphanin FQ receptor: A − K + VQV − IHI, A − K + T −I, VQV − IHI + T − I and A − K + VQV − IHI + T − I. For reasons that are not clear to us, only one of the new constructs, VQV − IHI + T − I could still bind the orphanin FQ peptide with an affinity comparable to that of the wild-type orphanin FQ receptor (0.042 ± 0.024 nM versus 0.063 ± 0.018 nM), whereas all the other mutants could no longer be labeled by $^{125}$I-orphanin FQ (Meng *et al.*, 1996a). Surprisingly, when we used various radioactive opioid alkaloids to screen these mutants, three of them could be labeled by [$^3$H]naltrindole. The only construct that bound neither $^{125}$I-orphanin FQ nor [$^3$H]naltrindole was A − K + VQV − IHI. Further pharmacological characterization was conducted on the three constructs that could be labeled by [$^3$H]naltrindole along with the wild-type $\delta$ receptor. The results are summarized in Table 2.

It can be seen that the binding profiles of the second-round mutants correspond pretty well to the combined effects of those of the first-round mutations. Although none of these mutants exhibits good affinities toward the $\mu$-agonist morphine and the $\delta$-agonist SNC80 [(+)-4-[($\alpha$-*R*)-$\alpha$-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide] the construct with A − K + VQV − IHI + T − I mutations demonstrates very good affinities toward alkaloid antagonists that are subtype-specific on the wild-type opioid receptors. Thus, it seems that by combining all three mutations, we created a generic opioid receptor that bound opioid alkaloid antagonists particularly well.

Because a ligand may bind these orphanin FQ receptor mutants in a different way than it does on the wild-type opioid receptors, we further tested the stereoselectivity of these receptor mutants. It is well known that the (−)-enantiomer of an opioid alkaloid usually has much higher affinity toward the opioid receptors than its (+)-enantiomer (Naidu and Goldstein, 1989). Interestingly, these receptor mutants also exhibited much higher affinity toward (−)-bremazocine than toward (+)-bremazocine (Table 2). This suggests that these mutants may bind opioid alkaloids in a way similar to that of the opioid receptors.

To determine if the opioid alkaloid binding pocket created in the current study is also functionally similar to that in the opioid receptors, we first chose to use the affinity shift assay in the presence and absence of GTP and high salt concentration (Blume, 1978) to analyze the functional roles of these alkaloids on the best mutant, A − K + VQV − IHI + T − I (Table 3). In control experiments we demonstrated that the presence of 50 $\mu$M GTP$\gamma$S and 120 mM sodium chloride could reduce the binding affinity of $^{125}$I-orphanin FQ by an order of magnitude (from 0.19 ± 0.03 nM to 1.6 ± 0.3 nM) when 140 pM of $^{125}$I-orphanin FQ was used to label the wild-type orphanin FQ receptor. Similarly, the affinities of $\delta$ agonist BWB373 [(±)-4-[[$\alpha$-*R*]-$\alpha$-[[2*S*,5*R*]-4-allyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide] toward the wild-type $\delta$ opioid receptor were 14 ± 3 nM and 0.88 ± 0.10 nM in the presence and absence of 50 $\mu$M GTP$\gamma$S and 120 mM NaCl, respectively. These findings demonstrate that the GTP/NaCl combination does produce the expected shift in agonist binding affinity in these receptors. However, it is clear from Table 3 that the affinities of (−)-bremazocine, naltrexone, naltrindole, and nBNI toward both the A − K + VQV − IHI + T − I mutant and the $\delta$ opioid receptor were not changed signif-

TABLE 1

Pharmacological profile of first round orphanin FQ receptor mutants (apparent $K_i$, nM)

Bold-underlined letters denote residues that are conserved across the $\mu$, $\delta$, and $\kappa$ opioid receptors. About 50 pM $^{125}$I-Tyr$^{-14}$-orphanin FQ peptide (1200–1500 Ci/mmol) was used to label the receptors. Data are expressed as mean ± SD of three or four receptor experiments.

| | Location and mutation | | | | | | | Wild-type orphanin receptor |
|---|---|---|---|---|---|---|---|---|
| | TM1 L-**S** | TM2 LL-**T**T | TM3 TVIA-IVI**S** | TM4 SA-V**T** | ECL2/TM5 A-**K** | TM6 VQV-**IHI** | TM7 T-**I** | |
| Orphanin FQ | N.S.B. | 0.053 ± 0.011 | 0.24 ± 0.04 | 0.22 ± 0.07 | 0.096 ± 0.02 | 0.12 ± 0.04 | 0.092 ± 0.038 | 0.069 ± 0.02 |
| DynA(1–17) | | 11 ± 5 | 35 ± 15 | 43 ± 18 | 2.9 ± 1.4 | 6.6 ± 4.5 | 3.8 ± 2.3 | 57 ± 46 |
| Bemazocine | | >5000 | >5000 | >5000 | 38 ± 16 | 150 ± 50 | 1100 ± 430 | >5000 |
| Ethylketocylazocine | | >5000 | >5000 | >5000 | 350 ± 220 | 1900 ± 380 | 2600 ± 1200 | >5000 |
| Naltrexone | | >5000 | >5000 | >5000 | 22 ± 9 | 400 ± 90 | 1700 ± 800 | >5000 |
| Naltrindole | 830 ± 390 | 1200 ± 790 | 1200 ± 630 | 17 ± 5.7 | 16 ± 4.7 | 55 ± 5.1 | 1000 ± 170 | |

TM = transmembrane domain; N.S.B., no specific binding.

icantly under such conditions. The behavior of these alkaloids on both receptors corresponds very well with the previous pharmacological knowledge that (−)-bremazocine, naltrexone, naltrindole, and nBNI are opioid receptor antagonists, with the exception that (−)-bremazocine is probably an agonist on the κ opioid receptor and an antagonist at the other opioid receptors. In comparison, the affinities of the nonselective opioid agonist etorphine, which acts as an agonist on the wild-type orphanin FQ receptor at high concentration (Mollereau *et al.*, 1994), can be significantly reduced on both the A − K + VQV − IHI + T − I mutant and the wild-type δ receptor. Therefore, it seems that the A − K + VQV − IHI + T − I mutant preserves the agonist/antagonist nature of opiate alkaloids.

Although affinity-shifting assays are used in many circumstances to study the functionality of various G protein-coupled receptors, a direct answer from a functional assay is most desirable to determine whether mutant receptors created in this study are functionally coupled to G proteins. Thus, we also conducted the [$^{35}$S]GTPγS binding assay to test the A − K + VQV − IHI + T − I mutant and the wild-type orphanin FQ receptor side by side. In control experiments, orphanin FQ could stimulate [$^{35}$S]GTPγS binding up to 300% with an $EC_{50}$ of about 4 nM at the wild-type orphanin FQ receptor. However, the orphanin FQ dose-response curve for the A − K + VQV − IHI + T − I mutant is largely flat (Fig. 1A). Surprisingly, the presence of etorphine can increase [$^{35}$S]GTPγS binding by 20-fold through the wild-type orphanin FQ receptor and up to 40-fold through the A − K + VQV − IHI + T − I mutant. But $EC_{50}$ values of etorphine on both receptors are very similar (Fig. 1B). However, the [$^{35}$S]GTPγS binding stimulated by 100 nM etorphine can be inhibited by 40% in the presence of 10 nM naltrindole at the A −K + VQV − IHI + T − I mutant, whereas naltrindole does not show any inhibitory effect on the wild-type orphanin FQ receptor (Fig. 2). These results strongly suggest that the binding pocket created in the A − K + VQV − IHI + T − I mutant is indeed functional.

## Discussion

The current study shows that the affinities of the orphanin FQ receptor toward some opiate antagonists can be increased dramatically by changing two to five orphanin FQ receptor residues to corresponding residues conserved across all three subtypes of opioid receptors. These mutant orphanin FQ receptors exhibit an opioid receptor-like stereospecificity. Furthermore, the GTP/NaCl affinity shift assay and the [$^{35}$S]GTPγS binding assay demonstrate that the orphanin FQ receptor mutant A − K + VQV − IHI + T − I interacts with opiate alkaloids to activate or to inhibit the activation of the receptor in the same way as the opioid receptors do. Most pronounced affinity increases are observed for the alkaloid antagonists. Regardless of their selectivity in the native opioid receptors, naltrindole, naltriben, naltrexone and nBNI display two to three orders of magnitude increases in binding affinity toward the mutated orphanin FQ receptors. Indeed, for the orphanin FQ receptor construct with simultaneous mutations in TM5, TM6 and TM7, none of the wild-type opioid receptors could match its high affinities toward all these opioid antagonists. Although somewhat surprising, such a binding profile may be explained by the notion that the orphanin FQ receptor lacks the structural elements responsible for subtype-selectivity toward various alkaloids in the wild-type μ, δ, and κ opioid receptors. In other words, by changing several orphanin FQ receptor residues to the residues conserved across the opioid receptors, we may have created in the mutant orphanin FQ receptors a common opioid alkaloid binding pocket with a preference for antagonists. This conclusion is reinforced by the fact that these mutants show opioid receptor-like stereospecificity and that

TABLE 2

Alkaloid binding profile of orphanin FQ receptor mutants (apparent $K_i$, nM)

The receptors were labeled with about 1 nM [$^3$H]naltrindole (32.0 Ci/mmol). Each $K_i$ value was determined by three independent assays. Data were expressed as mean ± SD of three receptor experiments.

| | A − K + T − I | VQV − IHI + T − I | A − K + VQV − IHI + T − I | δ Receptor |
|---|---|---|---|---|
| Naltrindole | 4.2 ± 1.0 | 7.2 ± 1.3 | 0.93 ± 0.22 | 0.43 ± 0.26 |
| (−)-Bremazocine | 53.2 ± 4.4 | 250 ± 150 | 19.2 ± 4.2 | 4.9 ± 1.3 |
| (+)-Bremazocine | >10,000 | >10,000 | >10,000 | >10,000 |
| Morphine | 730 ± 160 | >10,000 | 750 ± 100 | 1,940 ± 300 |
| SNC80 | 1,220 ± 530 | 1,500 ± 220 | 600 ± 230 | 9.4 ± 2.4 |
| Naltriben | 4.6 ± 1.8 | 26 ± 12 | 1.0 ± 0.2 | 0.35 ± 0.08 |
| nBNI | 19 ± 13 | 45 ± 11 | 7.5 ± 1.2 | 98 ± 26 |
| Naltrexone | 35 ± 18 | 690 ± 220 | 12.6 ± 4.3 | 120 ± 30 |

TABLE 3

Apparent binding affinity of opioid alkaloid in the presence and the absence of GTPγS and NaCl ($K_i$, nM)[a]

Data are expressed as mean ± SD ($n$ = 3 or 4). About 1 nM [$^3$H]naltrindole was used to label the receptors. Final concentration of GTPγS was 50 μM and that of NaCl was 120 mM. It should be noted that the binding assays were conducted approximately 6 months after the binding assays shown in Table 2. Thus the variations in affinity values (e.g., up to 2-fold for bremazocine) between these two tables are more likely attributable to the errors in binding assays rather than the changes in receptor properties.

| Akaloid | Orphanin FQ receptor mutant, A − K + VQV − IHI + T − I | | Wild-type δ receptor | |
|---|---|---|---|---|
| | 50 mM Tris only | +GTPγS +NaCl | 50 mM Tris only | +GTPγS +NaCl |
| Bremazocine | 17 ± 2 | 21 ± 1 | 10 ± 2 | 14 ± 3 |
| Naltrexone | 7.3 ± 1.2 | 6.9 ± 1.8 | 81 ± 19 | 80 ± 10 |
| Naltrindole | 0.66 ± 0.18 | 0.67 ± 0.10 | 0.29 ± 0.30 | 0.30 ± 0.26 |
| nBNI | 3.4 ± 0.2 | 5.5 ± 0.3 | 67 ± 5 | 62 ± 10 |
| Etorphine | 46 ± 3 | 420 ± 60 | 8.1 ± 0.4 | 200 ± 20 |

the opiate alkaloids preserve their agonist/antagonist profile on the A − K + VQV − IHI + T − I mutant.

The major advantage of using the orphanin FQ receptor/peptide system to investigate the binding mechanism of opioid receptors is that such an approach allows us to use a "gain of function" strategy, which is preferable to the more common "loss of function" mutagenesis approach (Schwartz, 1994). However, further assumptions must be made to conclude that these residues are indeed the critical ones in the binding of these opioid alkaloids in the opioid receptors. A convenient working hypothesis is that when an opiate alkaloid binds the opioid receptors, it only adopts one orientation in terms of its spatial relationship with the receptor. In other words, there is only one way that a ligand can bind a receptor with high affinity. Indeed, this assumption is widely adopted in the structure-function analysis of both receptors and ligands as well as in the prevailing pharmacological models of receptors. If we accept this hypothesis, we can expect that two highly homologous receptor systems sharing many structural features would bind a given ligand in a very similar manner. Such logic would suggest that the binding pocket created in our study is very likely similar to that in the opioid receptors.

Nonetheless, gain of function mutagenesis cannot exclude the possibility that these mutations may have created a fortuitous binding pocket in the orphanin FQ receptor, which is different from the pocket of the opioid receptors. This brings up the important question of whether a ligand can bind its receptor with high affinity through many different modes of interaction. Indeed, the presence of multiple binding pockets in a receptor is logically complementary to the widely accepted idea that a receptor can adopt several different conformations when it interacts with a ligand (De Lean *et al.*, 1980; Kenakin, 1995). It has been concluded that a single molecule of growth hormone has two sets of structural elements for interaction with two identical growth hormone receptor molecules (de Vos *et al.*, 1992). In a dopamine $D_2$ receptor mutagenesis study conducted in our laboratory, it was discovered that the presence of either Ser194 or Ser197 is necessary and sufficient for high affinity N-0437 binding, therefore N-0437 could fit in the binding pocket in at least two different ways (Mansour *et al.*, 1992). We have also proposed the possibility of multiple binding pockets based on the chimeric study of the δ receptor ligand binding (Meng *et al.*, 1996b).

If this were the case, the explanation of the mutagenesis results would be more complicated. If a ligand can indeed bind a receptor by interacting dynamically with different sets of amino acid residues and protein backbone structures, the

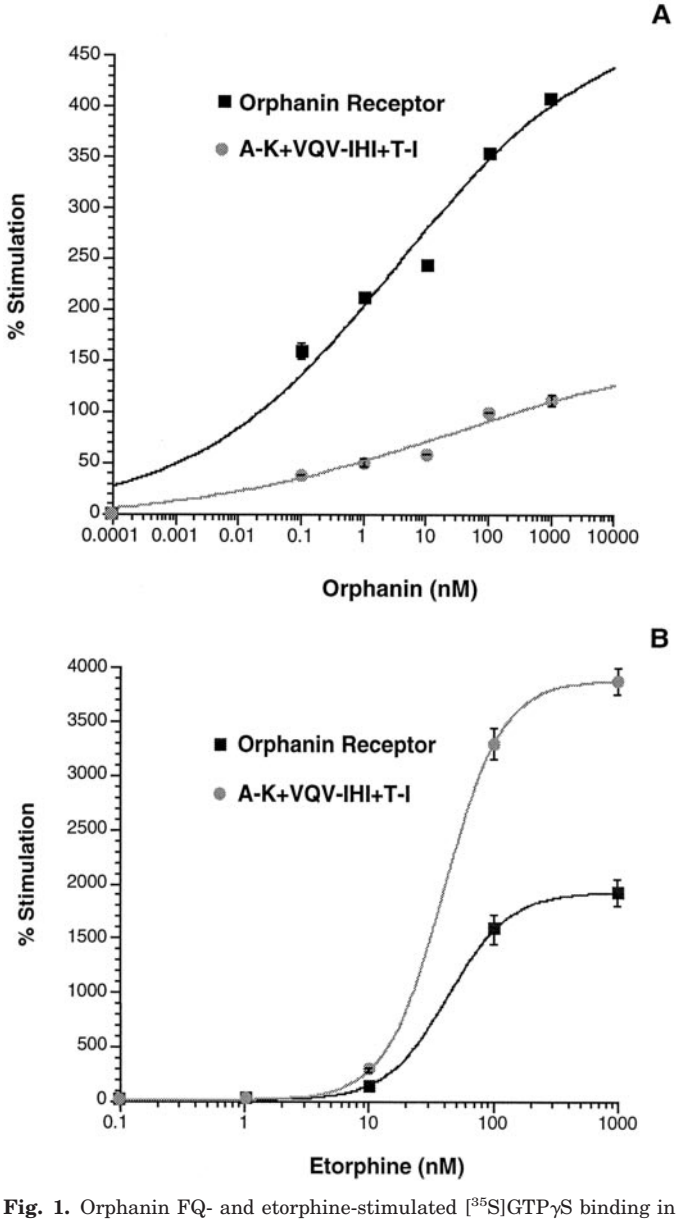


**Fig. 1.** Orphanin FQ- and etorphine-stimulated [$^{35}$S]GTPγS binding in transiently transfected COS-1 cells. The *x* axis indicates drug concentration as nanomolar. The *y* axis is the percentage of ligand stimulated [$^{35}$S]GTPγS binding. The level of [$^{35}$S]GTPγS binding in the absence of the ligand is defined as 100%. A, Orphanin FQ-stimulated [$^{35}$S]GTPγS binding; B, etorphine-stimulated [$^{35}$S]GTPγS binding.

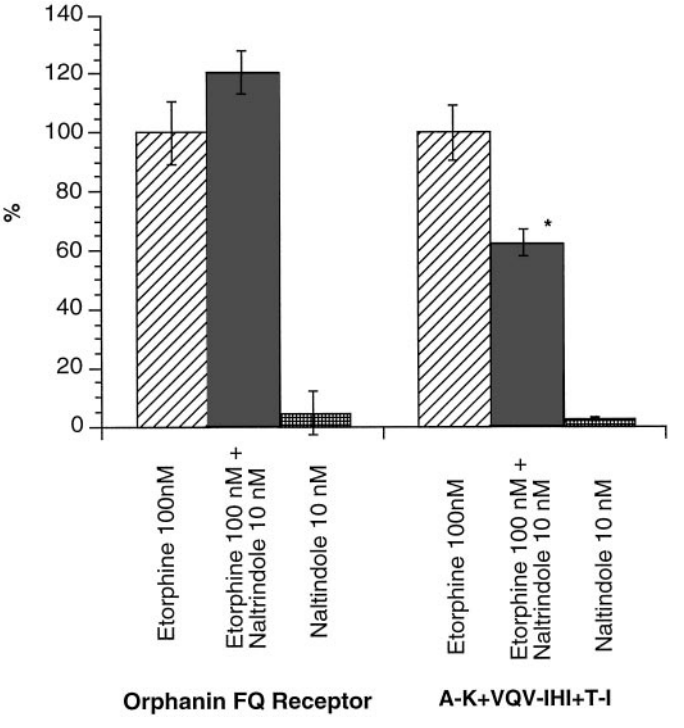


**Fig. 2.** Naltrindole inhibits the etorphine-stimulated [$^{35}$S]GTPγS binding in the A − K + VQV − IHI + T − I-transfected cells. One hundred percent is defined as the stimulation level of 100 nM etorphine in the corresponding mutant or wild-type orphanin FQ receptors.

influence of a mutation on all the possible binding pockets may not be even. As a result, although some of the mutants here show a dramatic increase in their affinities toward several opioid alkaloids, one cannot exclude the possibility that such mutations in the orphanin FQ receptor only generate an incidental pocket, rather than the primary alkaloid binding pocket present in the opioid receptors.

Given the complexity in the explanation of the mutagenesis results, additional experimental data are necessary. Nevertheless, the results of the present study are helpful in beginning to reveal some of the mechanisms whereby the orphanin FQ receptor avoids the opioid ligands, as well as suggesting some key residues critical to generic opioid binding.

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