Determinants of Ligand Selectivity at the κ-Receptor Based on The Structure of the Orphanin FQ Receptor

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Abstract

It is unclear how opioid selectivity and activation are regulated within the receptor core. In previous studies, the OFQ receptor was converted into a functional opioid receptor by mutating five amino acids at three sites to the corresponding residues conserved across the μ-, κ-, and δ-opioid receptors, suggesting that these sites comprise an opioid binding pocket. To examine this hypothesis, the present study examines whether these conserved residues represent an opioid binding pocket in the context of the opioid receptors, i.e., does their removal from opioid receptors destroy opioid ligand binding? The reciprocal mutations K227A (transmembrane [TM]5), IHI290-292VQV (TM6), and I316T (TM7) were evaluated in the κ-opioid receptor. In terms of alkaloid binding, there were no changes in affinity for mutants K227A and IHI290-292VQV. At mutant I316T, antago-
nist binding was unaltered, but there was a trend toward slightly decreased agonist affinity. In contrast, the binding of peptides had a more complex pattern. Again, K227A and IHI290-292VQV did not decrease the binding affinity of dynorphin-related peptides. Mutant I316T had 10- to 20-fold decreased affinity for dynorphin-related peptides, suggesting that I316 is part of a critical dynorphin recognition site. In response to alkaloid stimulation, I316T activated more G-protein(s) than wild type, and similar levels were observed in response to dynorphin stimulation. Overall, these results suggest that ligands are capable of achieving high-affinity binding through interaction with multiple sites/conformations of the receptor. These different modes of interaction have different downstream results in terms of receptor activation and signal transduction.

Opioid receptors (μ, κ, and δ) belong to the family of seven TM, G-protein-coupled receptors (Meng et al., 1993; Thompson et al., 1993; reviewed in Akil et al., 1998) and are activated by a family of endogenous peptides, including the products of prodynorphin, proenkephalin, and pro-opiomelanocortin. Pharmacological studies indicate that no family of endogenous peptides is associated exclusively with a given receptor (e.g., Mansour et al., 1995). The functional architecture of the receptor family was evaluated by chimera and mutagenesis studies that suggest peptides and alkaloids bind to different structural features of the receptor. In the κ-receptor, TM4 (Meng et al., 1995) and negatively-charged EL2 (Xue et al., 1994; Meng et al., 1995) are key for binding prodynorphin products but not alkaloids. U-69,593 and U-50,488, arylacetamides, interact with multiple domains except EL2 (Xue et al., 1994; Hjorth et al., 1995), whereas the bimorphinan norBNI interacts with EL3. The delineation between peptide and alkaloid binding is demonstrated dramatically by the engineering of a κ-RASSL (receptor activated solely by synthetic ligands), recognized by non-peptide ligands only, and unresponsive to peptides (Coward et al., 1998).

Despite different requirements for peptide and alkaloid binding, there is evidence suggesting a “common opioid pocket”: 1) All endogenous peptides have an N-terminal Tyr1-Gly2-Gly3-Phe4 “message” representing the minimal structure for receptor activation; 2) there is high homology among the opioid receptors, particularly in TMs 2, 3, and 7 (82–92% identity); and 3) a number of structurally rigid ligands bind nonselectively and with high affinity to all three receptors. Nonetheless, the critical structural elements comprising the “common opioid pocket” within the receptor core remain unidentified.

An orphan receptor with high similarity to the classical opioid receptors was cloned (Meunier et al., 1995; Reinscheid et al., 1995). Highest similarity is in TMs 2, 3, and 7 (70–80% identity) with highest overall homology to the κ-receptor. In spite of the homology, the orphan receptor does not recognize opioids. The endogenous ligand, OFQ (Reinscheid et al., 1995) or nociceptin (Meunier et al., 1995), is a 17-amino acid peptide (FGGFTGARKSARKLANQ) that has significant homology to dynorphin A (YGGFLRRIRPKLKWDNSQ), sharing six identical amino acids (in bold). The first four amino acids...
of the N terminus differ from the canonical opioid core only by a single hydroxyl group. All opioid peptides require the Tyr-1-hydroxyl group for binding (Schwyzer, 1977), and its absence in OFQ is sufficient to prevent opioid receptor binding. Despite homology between the two systems, OFQ and dynorphin bind and activate their respective receptors by different mechanisms (Reinscheid et al., 1996, 1998; Lapalu et al., 1997, 1998).

Molecular modeling of the OFQ peptide/receptor complex suggests that the N-terminal Phe-Gly-Gly-Phe sequence fits a vestibial opioid pocket defined by two hydrophobic pockets in a cavity between TMs 3, 5, 6, and 7 (Topham et al., 1998). The aromatic side chains of Phe and Phe are proposed to occupy these pockets. In earlier studies, we converted this vestigial, nonfunctional pocket into a functional opioid pocket. Utilizing a "gain of function" approach, a bank of four residues was mutated to the corresponding residues conserved across opioid receptors (Val-Gln-Val-Thr→Ile-His-Ile [TM6] and Thr→Ile [TM7]) (Meng et al., 1996). Individually, these mutations endowed the OFQ receptor with the ability to bind dynorphin and alkaloids without affecting OFQ binding (Meng et al., 1998). Mutation of Ala<sub>213</sub> to Lys at the EL2/TM5 interface also dramatically improved the affinity of the receptor for opioid alkaloids (Meng et al., 1998). In combination, the triple mutant A→K+VQV→IHI+T→I lost affinity for OFQ but had an opioid-like functional profile, demonstrated by correct enantioselectivity for opioid alkaloids, activation by opioid alkaloids, and blockade by opioid antagonists (Meng et al., 1998). We proposed that the pocket generated in the OFQ receptor corresponds to a common opioid pocket present in the μ, δ, and κ-receptors.

To examine this proposed pocket, we mutated the conserved opioid residues in the κ-receptor to the divergent OFQ residues (Fig. 1) and examined ligand binding and receptor activation. We predicted that these mutations would not confer affinity for OFQ because they are not involved in its binding to the OFQ receptor (Meng et al., 1996; Mollereau et al., 1999) and other studies also suggest nonoverlapping domains (Lapalu et al., 1998; Reinscheid et al., 1998; Mollereau et al., 1999). Moreover, if the conserved residues form a common opioid binding pocket then their removal from opioid receptors should decrease the binding affinity and activation properties of opioid ligands (i.e., “loss of function”).

![Fig. 1. Schematic of the κ-opioid receptor showing relative location of mutations.](image)

### Materials and Methods

**Site-Directed Mutagenesis.** The rat κ-opioid receptor used in these studies was cloned in our laboratory (Meng et al., 1993; GenBank accession no. U00442). The receptor was subcloned into a pCMV-neo expression vector, courtesy of Dr. M. D. Uhler (Huggenvik et al., 1991). Receptor mutants were generated using a MORPH Site-Specific Plasmid DNA Mutagenesis kit (5 Prime—3 Prime, Inc., Boulder, CO). Mutations were confirmed by sequencing the targeted regions of the receptor cDNA. The mutant receptors were named using the following format: original κ-amino acid→mutated OFQ amino acid (e.g., K227TA, IHI290-292VQV, and I316T).

**Cell Culture and Transfection.** Monkey kidney epithelial (COS-1) cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells were seeded at a density of 1 × 10<sup>6</sup> on 10-cm plates 24 h before transfection. For radioligand binding studies, cells were transfected with 25 μg of Qiagen-purified DNA by the calcium phosphate precipitation method (Chen and Okayama, 1987) or with 8 μg of Qiagen-purified DNA using FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. For [<sup>35</sup>S]GTP·S binding studies, cells were transfected with 8 μg of Qiagen-purified DNA using FuGENE reagent.

**Radioligand Binding Assays.** For radioligand binding assays, transfected cells were washed twice with 50 mM Tris-HCl buffer (pH 7.0, 4°C), mechanically scraped from the plate in 5 ml of 50 mM Tris (pH 7.0, 4°C), then centrifuged for 5 min at 3000g. The pellet was resuspended in 5 ml of 50 mM Tris/plate then homogenized for 15 s at maximum speed. The homogenate was centrifuged for 30 min at 27,500g and the final pellet was suspended in an appropriate volume of 50 mM Tris (pH 7.0, 25°C). Approximately two 10-cm plates of confluent cells were used per curve.

For saturation binding assays, 50 to 200 μg of membrane protein was labeled with [<sup>3</sup>H](-)-EKC (specific activity = 18.1 Ci/mmol; New England Nuclear, Boston, MA) at concentrations ranging from 0.01 to 7.5 nM in 50 mM Tris (pH 7.0, 4°C) with 5 mM Mg<sup>2+</sup>. At each radioligand concentration, nonspecific binding was determined by the addition of 10 μM naltrexone or 10 μM (-)-bremazocine. Total assay volume was 250 μl. Tubes were incubated at room temperature for 60 min then harvested by vacuum filtration over GF/B filters, washed once with 5 ml of cold Tris buffer (pH 7.0, 4°C), and counted for tritium. Specific binding was calculated by subtracting nonspecific binding from total binding and counts per minute were converted to femtomoles per milligram of protein using a custom Excel spreadsheet (Microsoft, Redmond, WA). Data were plotted as specific binding (femtomoles per milligram of membrane protein) versus radioligand concentration (nanomolar) then analyzed as a one-site rectangular hyperbola to determine K<sub>i</sub> and B<sub>max</sub> (GraphPad Prism version 3.00, San Diego, CA).

Alkaloid competition assays were performed in 50 mM Tris (pH 7.0, 4°C) with 5 mM Mg<sup>2+</sup> and peptide competition assays were performed in 50 mM Tris (pH 7.0, 4°C) with 5 mM Mg<sup>2+</sup>, 0.03% BSA, and peptide inhibitors (final concentrations 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml aprotinin, 1 mM iodoacetamide, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). Alkaloid ligands were purchased from RBI (Natick, MA) and peptides were purchased from Peninsula (Belmont, CA) or Phoenix (Mountain View, CA). In a final volume of 250 μl, 50 to 125 μM of membrane protein was labeled with 1.5 nM [<sup>3</sup>H](-)-EKC. Total binding was determined in the absence of test ligand and nonspecific binding was defined by 10 μM naltrexone or 10 μM (-)-bremazocine. Test ligands were evaluated in duplicate at 10 concentrations ranging from 0.01 nM to 10 μM to span 2.5 log orders and tested across all mutants. Assay tubes were incubated at room temperature for 60 min then harvested as described for saturation binding. Data were plotted as percent specific bound versus log concentration of competing ligand then analyzed using a one-site competition model to determine IC<sub>50</sub> (GraphPad Prism version 3.00, San Diego, CA), and K<sub>i</sub> was determined accord-
ing to the Cheng-Prusoff (1973) equation. For all binding studies, experimental results are averaged from at least three independent experiments using independent transfections.

**Functional Studies.** For $[^{35}S]$GTP$^\gamma$S binding assays, COS-1 cells were transfected using FuGENE, as described. Cells were washed twice with cold 50 mM Tris (pH 7.0, 4°C), harvested by scraping, and then collected by centrifuging for 10 min at 3000g. Cells were homogenized with at maximum speed for 15 s in cold lysis buffer (5 mM Tris-HCl [pH 7.0], 5 mM EDTA, and 2.5 mM EGTA) and then centrifuged for 30 min at 35,500g. The pellet was homogenized again in 5 ml of lysis buffer and centrifuged at 35,500g for 30 min. The final pellet was resuspended in an appropriate volume of binding buffer (50 mM Tris [pH 7.0, 4°C], 5 mM MgCl$_2$, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT). In a total assay volume of 400 µl, 10 to 25 µg of membrane protein was incubated with 0.2 nM $[^{35}S]$GTP$^\gamma$S (specific activity = 1250 Ci/mmol, New England Nuclear, Boston, MA) and 30 μM GDP. Nonspecific activity was defined by the addition of 20 µM GTP$^\gamma$S. For each curve, 6 to 10 concentrations of ligand spanning at least 3 log units were evaluated, and each concentration was performed in duplicate or triplicate. Other test ligands were evaluated at saturating concentrations for screening purposes. Assay was incubated at room temperature for 60 min, then harvested by vacuum filtration over GF/B filters, and washed three times with 5 ml of cold 50 mM Tris (pH 7.0, 4°C). Radioactivity bound was determined by counting filter disks with liquid scintillation spectrophotometry. Basal activity was calculated by subtracting nonspecific activity from total activity. Data were plotted as percent stimulation versus log concentration of ligand and analyzed as a sigmoidal dose-response curve.

**Results**

Several residues that are conserved in all opioid were selected for mutagenesis based upon previous work in the OFQ receptor (Meng et al., 1996, 1998; Mollereau et al., 1996). Our goal was to examine the role of replacing the conserved opioid residues with the corresponding residues that are proposed to prevent opioid binding in the OFQ receptor. The $\kappa$-opioid receptor was selected for this study based on the homology of the receptors and endogenous peptides. Three $\kappa$-receptor mutants (Fig. 1) were engineered and evaluated in terms of binding affinity and functional activity for opioid peptides and alkaloid ligands. $\text{Lys}^{227}$ (TM5), $\text{Ile}^{298}$-$\text{His}^{291}$-$\text{Ile}^{292}$ (TM6), and $\text{Ile}^{316}$-$\text{Thr}$ (TM7) were mutated to the corresponding divergent OFQ residues (Ala, Val-Gln-Val, and Thr, respectively).

**Saturation Binding Studies.** Preliminary pharmacological characterization was carried out with saturation binding assays using $[^{3}H]$-($\neg$)-EKC as the radioligand (Table 1). Receptor binding was saturable and indicative of a homogenous population of sites. No specific binding was detected in mock-transfected cells. With the calcium phosphate precipitation method, $\kappa$-wild type and I316T expressed at much higher levels ($B_{\text{max}} = 2136$ and 2277 fmol/mg of protein, respectively) than K227A and IHI290-292VQV ($B_{\text{max}} = 506$ and 692 fmol/mg of protein, respectively). With FuGENE-transfected cells, higher levels of expression were obtained with $\kappa$-wild type and I316T ($B_{\text{max}} = 7935$ and 7978 fmol/mg of protein, respectively), but expression levels of K227A and IHI290-292VQV ($B_{\text{max}} = 452$ and 694 fmol/mg of protein, respectively) remained low. All mutants had high affinity for the $[^{3}H]$-($\neg$)-EKC ($K_D$ ranging from 0.25 to 1.69 nM), therefore this radioligand was used for more extensive pharmacological characterization. Method of transfection method did not affect $K_D$ (data not shown for FuGENE transfections).

**Peptide Competition Binding Studies.** A series of pro-dynorphin peptides were evaluated because these are the putative endogenous ligands for the $\kappa$-receptor and since the complementary mutations increased their binding affinity for the OFQ receptor (Meng et al., 1996). Similar to results from the alkaloid binding studies, no major changes in binding affinity of dynorphin-related peptides were observed for mutants K227A or IHI290-292VQV (Table 3). The complementary mutations in the OFQ receptor consistently increased dynorphin-related peptide binding by about 10- to 20-fold (Meng et al., 1996). In contrast, mutant I316T had significantly decreased affinity (10- to 25-fold; Table 3) for all

**Table 1**

<table>
<thead>
<tr>
<th>Construct</th>
<th>$K_D$ ± S.E.M.</th>
<th>Calcium Phosphate</th>
<th>FuGENE</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>fmol/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.49 ± 0.07</td>
<td>2136 ± 328</td>
<td>7935 ± 2293</td>
</tr>
<tr>
<td>K227A (TM 5)</td>
<td>0.26 ± 0.09</td>
<td>506 ± 147**</td>
<td>694 ± 221**</td>
</tr>
<tr>
<td>IHI290-292VQV (TM 6)</td>
<td>0.25 ± 0.04</td>
<td>692 ± 198**</td>
<td>1056 ± 78**</td>
</tr>
<tr>
<td>I16T (TM 7)</td>
<td>1.69 ± 0.01**</td>
<td>2277 ± 328</td>
<td>7978 ± 2729</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01.

*Binding affinity of dynorphin-related peptides were observed for mutants K227A or IHI290-292VQV (Table 3). The complementary mutations in the OFQ receptor consistently increased dynorphin-related peptide binding by about 10- to 20-fold (Meng et al., 1996). In contrast, mutant I316T had significantly decreased affinity (10- to 25-fold; Table 3) for all
TABLE 2
Binding of alkaloid ligands to \( \kappa \) mutants
Receptors were labeled with 1.5 nM of \(^{3}H\)-[\( \rightarrow \)]EKC (18.1 Ci/mmol). Test ligands were incubated with membranes (50–125 \( \mu \)g of protein) for 60 min at room temperature before harvesting by vacuum filtration. \( K_i \) values were determined from a minimum of three independent experiments using membranes prepared from independent transfections. Mutants were compared to \( \kappa \) wild type using an unpaired Student’s \( t \) test.

<table>
<thead>
<tr>
<th>Test Ligand</th>
<th>Apparent ( K_i ) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
</tr>
<tr>
<td>Nonselective</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>((-))-Bremazocine</td>
<td>1.58 ± 0.22</td>
</tr>
<tr>
<td>(±)-EKC</td>
<td>6.38 ± 1.41</td>
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<tr>
<td>Naltrexone</td>
<td>1.17 ± 0.33</td>
</tr>
<tr>
<td>Selective</td>
<td>8.05 ± 2.35</td>
</tr>
<tr>
<td>nBNI</td>
<td>2.78 ± 0.50</td>
</tr>
<tr>
<td>U-69,593</td>
<td></td>
</tr>
<tr>
<td>U-50,488</td>
<td></td>
</tr>
</tbody>
</table>

* \( p < 0.05; \) ** \( p < 0.01. \)

TABLE 3
Binding of peptide ligands to \( \kappa \) mutants
Receptors were labeled with 1.5 nM of \(^{3}H\)-[\( \rightarrow \)]EKC (18.1 Ci/mmol). Test ligands were incubated with membranes (50–125 \( \mu \)g of protein) for 60 min at room temperature before harvesting by vacuum filtration. Peptidase inhibitors were included to prevent degradation of peptides and bovine serum albumin was included to prevent adsorption of peptides to tubes (see Materials and Methods). Mean \( K_i \) values were determined from a minimum of three independent experiments using membranes prepared from independent transfections. Mutants were compared to \( \kappa \) wild type using an unpaired Student’s \( t \) test.

<table>
<thead>
<tr>
<th>Test Ligand</th>
<th>Apparent ( K_i ) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
</tr>
<tr>
<td>Prodynorphin peptides</td>
<td></td>
</tr>
<tr>
<td>Dynorphin A 1–9</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>Dynorphin A 1–11</td>
<td>0.94 ± 0.35</td>
</tr>
<tr>
<td>Dynorphin A 1–13</td>
<td>0.54 ± 0.14</td>
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<td>Dynorphin A 1–17</td>
<td>0.64 ± 0.06</td>
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<tr>
<td>Dynorphin B 1–9</td>
<td>1.79 ± 0.41</td>
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<tr>
<td>Dynorphin B/Rimorphin</td>
<td>3.11 ± 1.10</td>
</tr>
<tr>
<td>Proenkephalin peptides</td>
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</tr>
<tr>
<td>Leu-enkephalin</td>
<td>-2,000</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>-2,000</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Dynorphin A 2–17</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>OFQ</td>
<td>&gt;5,000</td>
</tr>
</tbody>
</table>

* \( p < 0.05; \) ** \( p < 0.01. \)

dynorphin peptides tested. This loss of affinity corresponds well to the gain in affinity associated with the corresponding T→I mutation in the OFQ receptor (Meng et al., 1996). As expected, leu- and met-enkephalin had low-affinity for \( \kappa \)-wild-type receptor (ca. 2000 nM; Table 3). Neither exhibited changes in affinity for mutants K227A or IHI290–292VQV, but similar to dynorphin peptides, both had decreased affinity (ca. 10-fold) for mutant I316T. The corresponding \( \delta \)-mutants exhibited a similar decrease in affinity for \( \delta \)-selective peptide agonists but not \( \delta \)-selective peptide antagonists (Meng et al., 2000).

Consistent with our predictions, these mutations did not confer affinity for OFQ, indicating that they are not part of the OFQ binding pocket. In addition, the des-tyrosine dynorphin analog dynorphin A 2–17 had no affinity for the mutants, emphasizing the importance of an N-terminal tyrosine for binding to opioid receptors.

**GTP\( \gamma \)S Functional Studies.** Functional activity of the \( \kappa \)-receptor mutants relative to wild type was assessed by measuring ligand-induced incorporation of \(^{35}S\)GTP\( \gamma \)S, a nonhydrolyzable analog of GTP. Because \(^{35}S\)GTP\( \gamma \)S binding is dependent on receptor expression levels, it is not possible to compare activity between mutant preparations with different levels of receptor expression. In our hands, potency was not affected by expression level although maximal stimulation was related to transfection efficiency. To ensure valid comparisons of basal activity and maximal levels of stimulation, we determined \( B_{max} \) values by incubating membrane samples with a saturating concentration of \(^{3}H\)-(\( \rightarrow \))EKC for each assay. Only preparations with similar \( B_{max} \) levels were used for direct comparisons of maximal stimulation (\( B_{max} \) ca. 8000 fmol/mg of protein, see \( B_{max} \) data for FuGENE transfections in Table 1).

**Basal Activity.** Basal incorporation of \(^{35}S\)GTP\( \gamma \)S for \( \kappa \)-wild type and mutant I316T was compared directly because these constructs had consistently similar levels of expression. No difference in basal activities was observed (data not shown), indicating that mutant I316T has no constitutive activity.

**Potency of Alkaloids.** We examined the functional activity of a select group of opioid agonists, including nonselective and selective alkaloids (Table 4). For \( \kappa \)-wild type, potencies of ligands tested in the GTP\( \gamma \)S assay were similar to other literature reports (Zhu et al., 1997 (CHO cells); Remmers et al., 1999 (C6 glioma cells)). In general, we found decreased affinity and potency for alkaloid agonists. The nonselective agonist (\( \rightarrow \))-EKC activated the \( \kappa \)-wild-type receptor with an \( EC_{50} \) of 1.84 nM. No significant changes in potency were...
observed for mutants K227A and IHI290-292VQ (EC50 = 0.84 and 0.96, respectively), whereas a small but significant decrease in potency was observed for I316T (10.2 nM). This decreased potency is similar to the slight decrease in binding affinity for (±)-EKC at mutant I316T. The κ-selective agonist (−)-U-50,488 potently activated κ-wild type, and a 22-fold decrease was observed at mutant I316T (EC50 = 6.18 and 155 nM, respectively). Although both potency and affinity of (−)-U-50,488 was decreased, the loss in potency was substantially greater.

Maximal Effect of Alkaloids. Absolute value of the observed stimulation was dependent on expression levels of receptor expression (data not shown). Therefore, we only compared the relative efficacies of κ wild type and I316T because they consistently demonstrated similar levels of expression (Bmax ca. 8000 fmol/mg of protein for FuGENE transfected cells; Table 1). At mutant I316T, alkaloid agonists produced a greater level of maximal stimulation relative to κ-wild type (Table 5). For example, the maximal stimulation for the nonselective alkaloid, (±)-EKC, was 149% greater at mutant I316T than the κ-wild-type receptor (Fig. 2). Greater maximal stimulation associated with mutant I316T also was observed with the κ-selective ligand, (−)-U-50,488 (158% increase, Fig. 3). With the I→T mutation in the δ-receptor, we observed a similar increase in efficacy for alkaloid ligands, including the nonselective agonist etorphine and the δ-selective agonist SNC-80 (Meng et al., 2000).

Potency of Peptides. The endogenous peptide dynorphin A 1-17 potently activated κ-wild type receptor (EC50 = 0.71 nM) and also was active at all the mutants (Table 4; Fig. 4). No change in potency was observed for mutants IHI290-292VQ and K227A (EC50 = 0.42 and 2.25 nM, respectively). However, dynorphin A 1-17 was 20-fold less potent at mutant I316T (EC50 = 14.3 nM). This decreased potency mirrors the decreased binding affinity of this dynorphin A 1-17. As expected, no functional effects were observed for OFQ at κ wild type or the mutants. These results are consistent with the lack of affinity of OFQ for these receptors.

Maximal Effect of Peptides. In contrast to the alkaloid agonists, dynorphin A 1-17 produced similar levels of maximal stimulation at both κ-wild type and mutant I316T
Previous studies from our lab demonstrated that a functional opioid binding pocket (for both peptide and nonpeptide ligands) could be engineered into the OFQ receptor by introducing amino acids conserved across µ-, κ-, and δ-receptors (Meng et al., 1996, 1998). The current study was undertaken to examine the hypothesis that these conserved residues serve the same role (i.e., comprise an opioid pocket) in the κ-receptor. Lys227 (TM5), Ile290-His291-Ile292 (TM6), and Ile316 (TM7) were mutated to the corresponding divergent OFQ residues (Ala, Val-Gln-Val, and Thr, respectively). Our results demonstrate several salient points: 1) contrary to our working hypothesis, affinity of alkaloids is not greatly altered although agonist affinity tends to increase at K227A and IHI290-292VQV and decrease at I316T, 2) binding of dynorphin products is significantly reduced at I316T, indicating that this site is part of a key opioid peptide binding pocket, and 3) functional properties of I316T in response to alkaloids are altered suggesting that this site regulates the agonist-induced interaction of the receptor with G-proteins. Overall, these results lead to the conclusion that the "common opioid pocket" is actually a complex structural feature of the receptor. They also provide insight into sites in the receptor core that are involved in potency and efficacy.

A number of factors suggested that the residues examined in this study might be critical components of a common opioid binding pocket. For example, the opioid receptors share a high degree of sequence homology to the OFQ receptor, but there is no cross talk between the two systems. Moreover, mutation of certain divergent OFQ receptor residues to the corresponding conserved opioid residues confers an opioid-like pharmacological profile (Meng et al., 1996, 1998; Mollereau et al., 1996). A logical postulation is that the conserved residues might also be critical for ligand binding and activation at the opioid receptors. However, the reciprocal mutations in the κ-receptor has minimal effects on the binding of most alkaloid ligands. Most significant is a slight but consistent decrease in alkaloid agonist binding at I316T. It is possible, but unlikely, that these sites are not critically involved in the binding of alkaloids to the opioid receptors and the opioid-like pharmacology introduced into the OFQ receptor was artifactual. The "gain of function" OFQ mutants displayed correct opioid enantioselectivity, activation by opioids, and blockade by opioid antagonists (Meng et al., 1998). A more intriguing interpretation is that the binding of small alkaloids is not greatly perturbed because ligands are capable of binding with high affinity to a receptor in more than one way. These multiple modes of binding in the native receptor (i.e., different pockets or receptor conformations with similar ligand binding affinities) may be indistinguishable with current technology. Based on this scenario, we postulate that only one of several possible opioid pockets was introduced into the OFQ receptor in our previous work (Meng et al., 1996, 1998).

Support for the second interpretation comes from the results of the functional studies. In the κ-receptor, I316T has no constitutive activity but increases the receptor's ability to activate G-proteins in response to alkaloid agonists. This mutation, near the interface of EL2/TM7, is far from the region of the receptor intimately involved in signal transduction yet it has a major impact on the receptor's ability to

**Effect of Antagonists.** Studies with the corresponding mutations in the δ-receptor (Meng et al., 2000) indicated that the κ-receptor residues examined in this study may be involved in signal transduction. With the δ-receptor, certain antagonists were converted to agonists at mutants I→T and IHI→VQV. In contrast, the nonselective antagonist naltrex-one and the κ-selective antagonist nabilontorphimine were completely devoid of agonist activity at all three κ-receptor mutants (Table 5).
couple to G-proteins. We postulate that Ile$^{316}$ may subtly alter alkaloid affinity for multiple pockets that have different capacities for eliciting a functional response in terms of potency (EKC versus U-50,488) and maximal stimulation (alkaloids versus peptides). The activity profiles of K227A and IHI290-292VQV also may be altered but these results are difficult to interpret due to low levels of receptor expression.

We recently reported that the corresponding mutations in the δ-receptor have little effect on alkaloid binding but exhibit dramatically altered functional activity as well (Meng et al., 2000). Based upon results from both the κ- and δ-mutagenesis, it appears that these pockets are similar from the perspective of ligand affinity but are functionally distinct.

Although the κ- and δ-mutants are similar in that the binding profile of alkaloids is not dramatically altered, there are key differences between the two receptor systems. At certain δ-mutants (i.e., IHI→VQV and I→T), several antagonists were converted to agonists (Meng et al., 2000) but no such changes are observed with the antagonists tested at the κ-mutants. These functional differences suggest distinct micromdomains in the δ- and κ-receptors that can be differentially unmasked. In other words, the multiple binding modes that we propose to exist in the opioid receptors do not appear to be equivalent across receptor types.

Receptors are dynamic structures capable of adopting multiple conformational states and these different conformations may represent potentially different functional states of the receptor (the simplest scenario being “on” or “off”). Basal activity of a receptor (i.e., functional activity in the absence of ligand) is the result of the equilibrium between active and inactive conformations. Low basal activity is the result of a higher receptor population in an inactive conformation, and high basal activity is the result of a higher receptor population in an active conformation. The physicochemical properties of a ligand are key factors for stabilizing or inducing receptor conformation thereby driving receptor activity. A ligand that stabilizes a conformation with high affinity for G-proteins is an agonist, a ligand that stabilizes a neutral conformation is an antagonist, and a ligand that stabilizes a conformation of that cannot interact with G-proteins is an inverse agonist. Although a ligand may preferentially stabilize one conformation versus another upon binding, we demonstrate here that removal of a binding mode (in the form of a unique conformation or binding pocket) can significantly alter the ligand’s functional profile.

This scenario becomes more complicated when considering the pharmacological profile of these mutants in response to peptide ligands. Larger peptide ligands potentially interact with many sites on the receptor and it is generally believed that these interactions direct receptor selectivity (Meng et al., 1995; Watson et al., 1996; reviewed in Akil et al., 1996). In terms of receptor binding, the larger number of interactions may serve to limit the number of ways that a peptide can interact with a receptor. In the current studies, only Ile$^{316}$ at the top of TM7 appears to be involved in the binding of opioid peptides as its removal consistently decreases affinity 10- to 20-fold for several dynorphin analogs. This trend also is observed for the enkephalin analogs tested, even though they have considerably lower affinity for the κ-receptor. This residue also is essential for peptide binding to the δ-receptor (Meng et al., 2000). Because this Ile is conserved across opioid receptors, these results suggest that we have identified a site critical for peptide agonist binding. It is unclear whether this site is directly involved in peptide binding or whether it is a structural component at the TM/EL interface that influences the conformation of a nearby binding site. In terms of biological activity, maximal stimulation at I316T in the κ-receptor is not altered in response to dynorphin. Presumably, more rigorous requirements for peptide binding limit the number of potential pockets that the peptide can occupy and thus fewer active conformations of the receptor may be available. Whereas smaller, exogenous ligands appear to be more promiscuous in terms of binding and activation, it is logical that endogenous ligands would be more tightly regulated. From an evolutionary standpoint, constraining the binding of the endogenous ligand may be an important mechanism for regulating biological activity of the opioid peptides.

The focus of these studies was to examine mechanisms of selectivity and activation in the opioid receptors using the κ-receptor as a model. These results, together with OFQ receptor mutagenesis studies (Meng et al., 1996, 1998) and δ-receptor mutagenesis studies (Meng et al., 2000), suggest that there may be more than one way to bind and activate a receptor because 1) in the OFQ receptor, it is possible to create an opioid-like binding and functional “pocket” by introducing opioid-like mutations (Meng et al., 1996, 1998); and 2) in the “mirror-image” κ-mutants, the corresponding OFQ residues do not destroy opioid binding but alter functional activity in terms of efficacy (i.e., maximal stimulation). We conclude that there is no “common” opioid binding pocket that accounts for the binding of nonselective ligands across opioid subtypes. Instead there appear to be multiple modes of binding within the receptor core that have similar (or indistinguishable) affinity for a number of ligands. These modes can be distinguished because they can have altered patterns of signal transduction. We postulate that the end result of ligand binding (i.e., agonism, partial agonism, or antagonism) is a function of the ligand’s affinity for the various binding pockets or conformations of the receptor. Understanding the molecular basis of opioid receptor function provides an important framework for understanding complex physiological issues, such as pain modulation, reward, and dependence.

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References


Huggenvik JI, Collard MW, Stofke RE, Seasholtz AF, and Uhler MD (1991) Regulation of the human enkephalin promoter by two isoforms of the catalytic subunit


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