Role of Aromatic Transmembrane Residues of the δ-Opioid Receptor in Ligand Recognition*

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In the present study we examine the role of transmembrane aromatic residues of the δ-opioid receptor in ligand recognition. Three-dimensional computer modeling of the receptor allowed to identify an aromatic pocket within the helices bundle which spans transmembrane domains (Tms) III to VII and consists of tyrosine, phenylalanine, and tryptophan residues. Their contribution to opioid binding was assessed by single amino acid replacement: Y129F and Y129A (Tm III), W274A (Tm VI), Y308F (Tm VI). Scatchard analysis shows that mutant receptors, transfected into COS cells, are expressed at levels comparable with that of the wild-type receptor. Binding properties of a set of representative opioids were examined. Mutations at position 129 most dramatically affected the binding of all tested ligands (up to 430-fold decrease of deltorphin II binding at Y129A), with distinct implication of the hydroxyl group and the aromatic ring, depending on the ligand under study. Affinity of most ligands was also reduced at Y308F mutant (up to 10-fold). Tryptophan residues seemed implicated in the recognition of specific ligand classes, with reduced binding for endogenous peptides at W274A mutant (up to 40-fold) and for nonselective alkaloids at W274A mutant (up to 65-fold). Phenylalanine residues in Tm V appeared poorly involved in opioid binding as compared with other aromatic amino acids examined. Generally, the binding of highly selective δ ligands (TIPPδ, naltrindole, and BW337U86) was weakly modified by these mutations. Noticeably, TIPPδ binding was enhanced at W274A receptor by 5-fold. Conclusions from our study are: (i) aromatic amino acid residues identified by the model contribute to ligand recognition, with a preponderant role of Y129; (ii) these residues, which are conserved across opioid receptor subtypes, may be part of a general opioid binding domain; (iii) each ligand-receptor interaction is unique, as demonstrated by the specific binding pattern observed for each tested opioid compound.

Opiates are strong analgesic compounds currently used in the treatment of pain. They also have euphoriant action and strong addictive properties. Opiates, as well as endogenous opioid peptides, exert their biological action through three classes of membrane receptors, known as μ, δ, and κ. The recent cloning of three genes encoding these receptors (for a review, see Ref. 1) has shown that opioid receptors belong to the G protein-coupled receptors (GPRs)1 superfamily with a seven transmembrane domain topology and share high protein sequence identity, particularly in transmembrane and intracellular regions. The availability of opioid receptor clones allows to examine the mechanisms of action of opioid ligands at the molecular level, and here we investigate the structural basis of receptor-opiate interactions in the mouse δ-opioid receptor (mδOR, see Refs. 2 and 3).

It is believed that the seven putative transmembrane domains of GPRs are folded as helices and tightly associated within the membrane to form the receptor protein core. The binding site of GPRs which recognize small biogenic amines has been best characterized (4). Three-dimensional computer modeling relying on bacteriorhodopsin structure as a scaffold and the analysis of amino acid conservation in sequence alignments were used in conjunction with site-directed mutagenesis data to identify residues which interact with small nonpeptide ligands. The binding site was shown to lie within the helical bundle, about 15 Å from the extracellular surface and spanning Tms II to VI, with a central role for Tm III. Other GPRs bind peptide ligands with sizes ranging from tripeptides (e.g. N-formyl peptide or thyrotropin-releasing hormone) to proteins (follicle-stimulating, luteinizing, and thyroid-stimulating hormones). Mutagenesis experiments demonstrated that, in addition to transmembrane residues, extracellular domains participate in ligand recognition (for reviews, see Refs. 5 and 6), indicating that the peptide binding site extends beyond the transmembrane pocket for these receptors.

Opioid receptors are GPR family members endowed with an extremely large ligand repertoire (7). They bind a wide variety of structurally diverse molecules, including small rigid alkaloid compounds, synthetic peptides, as well as a family of endogenous peptides. Opioid receptors are therefore expected to interact with their ligands at multiple sites, both extracellular and intramembranous, an assumption which is supported by initial point mutagenesis and chimeric studies (6–18). We have focused on the role of transmembrane domains in the δ receptor. In a previous study, we have demonstrated that the conserved Asp residue in Tm III, although not interacting directly with the positive charge of opioid ligands, is critically involved in maintaining intact binding properties of the receptor (19). Other reports have shown implication of charged residues found in Tm II and Tm VI in opioid recognition (8, 9). In this study, we have used our knowledge from studies of other GPRs

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and receptor modeling to establish a putative topology of the receptor binding site. We have identified a number of aromatic amino acid residues as candidates for opiate recognition and we have examined the contribution of their side chains to the binding of a large set of structurally distinct opiate molecules, using a site-directed mutagenesis approach. We provide evidence that a cluster of aromatic residues may represent a major area of interaction for most ligands. In addition our results demonstrate a distinct mode of interaction for each ligand under investigation, ruling out the possibility of a unique opioid binding site.

EXPERIMENTAL PROCEDURES

Materials—All ligands tested were obtained from Sigma unless otherwise stated. Bremazocine was from Research Biochemicals International and BW373U86 was kindly provided by K. J. C. Chang (Burroughs Wellcome Co., Research Triangle Park, NC); TIPP and TIPPe were provided by Astra Pain Control (Montreal, Canada); (3H)naltrindole (specific activity, 44.5 Ci/mmol) was provided by A. Borsodi (Szegued, Hungary), and (3H)diprenorphine (specific activity, 37 Ci/mmol) was obtained from Amersham Corp.

Modeling—The modeling procedure has been previously described in detail for the modeling of the angiotensin AT1 receptor (20). Briefly, the mDOR three-dimensional model was constructed following a three-step strategy: (i) a model of the human rhodopsin receptor was built from the resolved structure of bacteriorhodopsin (21); (ii) from that, a model of the best well studied hamster β2-adrenergic receptor was built from the obtained human rhodopsin model using a sequence alignment that results from homologies existing between all GPR Tm regions. Many amino acid guide points could be retained in each Tm helix to allow next pertinent use of homology building procedures. As for the building of the opsin receptor, only the seven Tm were built. (iii) This β2-adrenergic receptor model was finally used as a template for the modeling of the mouse δ-opioid receptor itself. The whole modeling was performed with the BIOVIA software using the “Insight Discover,” and “Homology” modules (Biosym Technologies, San Diego, CA).

Site-directed Mutagenesis of mDOR—mDOR cDNA (3) was subcloned in HindIII–BamHI sites of the mammalian expression vector pCDNAI/Amp (Invitrogen). Single stranded DNA for mutagenesis was prepared from pCDNA–mDOR using the helper phage M13KO7 (Promega) and the Escherichia coli strain NM 522 (Stratagene) and was used as template for site-directed mutagenesis. Sequences of oligodeoxynucleotides were chosen to replace the following residues of mDOR receptor either by phenyalanine: Tyr129, Trp173, Trp218, Trp222, Trp274, and Tyr308 (Tm III), Trp173 (Tm IV), Phe218 and Phe222 (Tm V), Trp274 (Tm VI). Oligodeoxynucleotides containing the mutant codon were annealed to the single-stranded template, followed by an elongation with the Klenow fragment and ligase. Heteroduplex plasmid DNA was then used to transform the repair-deficient E. coli strain BMH71-18 mutS (Clontech). Transformants were selected by growth on LB plates supplemented with ampicillin (100 μg/ml), and mutations were confirmed by manual DNA sequencing (Sequenase kit; U. S. Biochemical Corp.) in both directions. Sequences were also confirmed by automated DNA sequencing (373A DNA, Perkin-Elmer) using fluorescently labeled nucletedixides (Taq DyeDeoxy terminator cycle sequencing kit, Perkin-Elmer).

Expression of Wild-type mDOR and Mutant Receptors in COS Cells—Ligand Binding—COS cells (1.5 × 106 cells/10-mm dish) were transected with purified plasmids (35 μg/dish) using the DEAE-dextran method. After 72 h growth in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, the cells were harvested and membranes were prepared as described previously (3). For binding experiments, various amounts of membrane proteins of mDOR and mutant receptors, ranging from 20 to 100 μg, were diluted in 50 mM Tris-HCl, pH 7.4, and incubated for 1 h at 25°C with opioid ligands in a final volume of 0.5 ml. For saturation experiments, eight concentrations of [3H]diprenorphine ranging from 0.05 to 10 nM (for WT, Y129F, W173A, Y308F) and eight concentrations of [3H]naltrindole ranging from 0.1 to 1.2 nM (for WT, Y129F, F222A, W274A) were used. Nonspecific binding was determined in the presence of 2 μM (for WT and F218A), 0.1 μM (for Y129F, W173A, and Y308F), or 0.5 μM (for Y129F, F222A, and W274A) naltrexone. For competition studies, membrane preparations were incubated with [3H]diprenorphine (1 nM for WT, Y129F, F222A, or Y308F and 3 nM for W173A and Y308F) or [3H]naltrindole (2 nM for Y129F, F222A, Y308F), in the presence of variable concentrations of opioid competing ligands. When using endogenous peptides as competitors, assays were conducted in the presence of a mixture of protease inhibitors (leupeptin, pepstatin, aprotinin, antipain, and chymostatin, each at 2.5 μg/ml). Kd, Ki, and Bmax values were calculated using the EBDA/Ligand program (G. A. McPherson, Biosoft, Cambridge, United Kingdom).

RESULTS

Opioids are cationic compounds with strong hydrophobic character. Structure-function studies have shown that a positively charged nitrogen atom located at a fixed distance from a phenolic ring, a common structure between alkaloids and opioid peptides, represents the active or “message” part of opioid molecules (see Ref. 22). The positive charge being an absolute requirement for ligand binding, the existence of a salt bridge has long been postulated to be the major interaction between the ligand and its receptor site. In preliminary work (19), we tested the hypothesis that this interaction occurs at Asp129 in mDOR. This negatively charged residue is present in Tm III of all GPRs that bind small cationic neurotransmitters and is known to interact directly with catecholamines in the β2-adrenergic receptor. Our results showed that, although this Asp
residue is a structural component of the binding site, it does not interact directly with opioid ligands. Another study has shown that Asp\(^95\) (Tm II), the only other anionic residue present in transmembrane domains, does not represent a common attachment point for opioids (8). The lack of evidence for a main electrostatic interaction between opioids and the \(\delta\) receptor strengthens the hypothesis that non-ionic interactions are predominantly responsible for ligand chelation in the binding site.

**Three-dimensional Modeling of mDOR and the Role of Aromatic Residues**—We have used three-dimensional computer modeling to identify intramembranous amino acid residues that may face the inner side of the helices bundle and account for ligand binding. Interestingly, we found that the best candidates are predominantly aromatic residues, and their side chains are presented on Fig. 1. On the model we observe that Trp\(^{173}\), Phe\(^{218}\), Phe\(^{222}\), and Trp\(^{274}\) are clustered and form an aromatic pocket spanning Tms IV, V, and VI. The putative binding site extends over Tms III and VII, where residues facing the pore acquire a more hydrophilic character. Among these, the amphiphilic Tyr\(^{308}\) residue seems to bridge Tm VII and Tm III. Hydrophilic residues have also been identified as putative anchor points for ligand on this model, such as Asn\(^{131}\) and His\(^{278}\) (not shown) or Asp\(^{128}\) (Tm III), which we have investigated earlier. In the empty receptor, some of the identified aromatic residues seem to be in close contact with other amino acid side chains. Trp\(^{274}\) may interact with Phe\(^{270}\) within Tm IV through aromatic-aromatic interaction. Polar interhelices interactions may also be seen with a possible interaction of the OH group of Tyr\(^{308}\) or the indole NH group of Trp\(^{274}\) with the carbonyl of Asp\(^{128}\) via hydrogen bonds.

Residue Tyr\(^{129}\), which is adjacent to the critical Asp 128 residue, does not face the binding pocket in this model, but rather participates in reinforcing helical III intrinsic stability through aromatic-aromatic interactions occurring between Tyr\(^{129}\), Phe\(^{133}\), and Phe\(^{137}\). Interestingly, in another model of mDOR derived from bovine opsin crystallographic data, the side chain of Tyr\(^{129}\) seems more likely to be located inside the pore due to a different position of Tm III relative to adjacent helices. In addition, this tyrosine residue is present in muscarinic m3 and TRH receptors and was shown to play a critical role in ligand recognition (23, 24). We therefore have included examination of the role of Tyr\(^{129}\) in our study despite its outside positioning in our model.

**Mutagenesis of the Receptor and Choice of Opioid Ligands**—Point mutant receptors constructed in this study are shown on Fig. 2. We have replaced the identified tryptophan (Tms IV and VI) and phenylalanine (Tm V) residues by alanine (W173A, F218A, F222A, and W274A), thus removing the side chains which seem to constitute the aromatic pocket proposed by the model. We have modified the tyrosine residues in Tms III and VII into phenylalanines (Y129F and Y308F) to evaluate the

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2 M. Hibert, personal communication.
possible contribution of the hydroxyl groups. Finally, we have replaced tyrosine 129 by alanine (Y129A) to remove the entire phenol moiety and ascertain the role of this aromatic ring in Tm III. We then have examined the binding properties of each mutant receptor. Since opioids and opioid peptides constitute a group of compounds with high structural diversity, we have tested a repertoire of ligands with representative molecules in each class, including endogenous peptides, synthetic peptide analogues, or alkaloid compounds, agonists or antagonists, nonselective or δ-selective ligands (Fig. 3).

Expression of Wild-type (WT) and Mutant Receptors—Plasmids encoding WT and mutant receptors were transiently expressed in COS cells and their ability to bind \[3H\]diprenorphine, a nonselective antagonist, was assessed by saturation experiments performed on membrane preparations. For three mutant receptors (Y129A, F222A, and W274A) \[3H\]diprenorphine binding appeared weak. Thus, \[3H\]naltrindole, another alkaloid antagonist that exhibited nanomolar affinity at these mutants, was used as a radiolabeled ligand. The examination of mutant receptors (Y129A, F222A, and W274A) \[3H\]diprenorphine binding remained nevertheless easily detectable. We further established a detailed binding profile for each mDOR mutant receptor. Table II summarizes \(K_d\) values obtained for each ligand and affinity changes relative to the WT receptor are illustrated in Fig. 4. Each mutant receptor bound at least one ligand with an affinity comparable with that of the WT receptor, suggesting that the general receptor conformation is maintained in the mutated receptors.

Tyrosine 129 Critically Contributes to the Opioid Binding—Mutant Y129F exhibits reduced affinities for the ligands under study, with effects being very modest (2.8-fold for DPDPE) or more significant (12-fold for bremazocine). This result suggests a general contribution of a hydrogen bond to ligand binding. Remarkably, a stronger reduction (91-fold) is observed for deltorphin II, a δ-selective agonist isolated from frog skin which does not contain the canonical YGGF N-terminal sequence typical of opioid peptides and therefore may bind in a distinct manner. For this particular peptide, the OH group of Tyr129 may represent a major anchor point. Further replacement of tyrosine by alanine shows contributions of both the hydroxyl group and the aromatic ring of the tyrosine side chain. Binding of long endogenous peptides (β-endorphin and dynorphin A), a synthetic peptide (TIPPP) or δ-selective alkaloids (BW373U86 and naltrindole) at Y129A mutant is affected similarly to binding at Y129F mutant, suggesting that the aromatic ring does not contribute to recognition of these compounds. In contrast short endogenous peptides (Leu- and Met-enkephalin), synthetic peptides (DADLE and DPDPE) as well as nonselective alkaloids (bremazocine and naloxone) exhibit affinities for Y129A up to 90-fold lower than those for Y129F. Deltorphin II binding is also reduced 5-fold more and appears as the most affected ligand, with a total decrease in affinity of 430-fold relative to the WT receptor. For these latter compounds, therefore, the aromatic ring of Tyr129 participates in ligand binding. Altogether, these results underscore a general involvement of Tyr129 side chain in ligand recognition, with a distinct contribution of both the OH group and the aromatic ring depending on the ligand under study.

Mutations of Tryptophan Residues in Tms IV and VI Strongly Affect the Binding of Specific Ligand Classes—Binding of the endogenous peptides β-endorphin, dynorphin A, Leu- and Met-enkephalin at W173A mutant receptor is strongly reduced (21–43-fold), whereas TIPPP, BW373U86, naltrindole, and bremazocine affinities remain unaffected. \(K_d\) values for DADLE and DPDPE significantly decrease, but to a lesser extent.

| Location | \(B_{max}\) pmol/mg |
|----------|------------------|
| mDOR     |                  |
| Y129F    | Tm III 7.29 ± 0.98 |
| Y129A    | Tm III 14.6 ± 5.29 |
| W173A    | Tm IV 2.43 ± 0.99  |
| F218A    | Tm V 4.39 ± 0.82  |
| F222A    | Tm V 0.59 ± 0.26  |
| W274A    | Tm VI 2.16 ± 0.69  |
| Y308F    | Tm VII 5.25 ± 2.58 |

**TABLE II**

Binding potency of opioid ligands at WT and mutant receptors

Inhibition constants were obtained from competition experiments using transfected COS cell membranes (see “Experimental Procedures”). Experiments were done in duplicate and repeated or three times for each compound. ND, not determined.

| Ligand       | \(K_d\) pmol/mg |
|--------------|----------------|
| mDOR    | Y129F | Y129A | W173A | F218A | F222A | W274A | Y308F |
| β-Endorphin | 19.3  | 137   | 235   | 592   | 110   | 59.9  | 144   | 91.4  |
| Dynorphin A | 21.6  | 195   | 309   | 836   | 87.3  | 153   | 192   | 175   |
| Leu-enkephalin | 6.52  | 40.3  | 872   | 137   | 7.5   | 38.4  | 33.1  | 28.3  |
| Met-enkephalin | 5.01  | 39.5  | 868   | 215   | 5.2   | 29.2  | 43.8  | 4.6   |
| DADLE    | 10.0  | 107   | 2280  | 55    | 6.01  | 146   | 9.49  | 52.7  |
| DPDPE    | 31.7  | 87.5  | 7880  | 430   | 21.9  | 167   | 496   | 166   |
| Deltorphin II | 9.23  | 843   | 3964  | 90.9  | 6.9   | 124   | 4.5   | 64    |
| TIPPP    | 2.41  | 24    | 63.5  | 19.5  | 3.46  | 7.23  | 0.76  | 10.3  |
| TIPPP    | 10.6  | 64.1  | 99.9  | 21.7  | 8.98  | 17    | 2.03  | 10.2  |
| BW373U86 | 1.63  | 13.8  | 8.63  | 3.22  | 5.17  | 2.55  | 17.9  | 13    |
| Naltrindole | 0.42  | 3.72  | 2.28  | 0.91  | 0.56  | 0.61  | 3.59  | 4.3   |
| Kd       | ND    | ND    | 1.21  | ND    | ND    | 0.3   | 1.86  | ND    |
| Bremazocine | 2.79  | 32.8  | 245   | 3.33  | 6.39  | 18.4  | 180   | 6.58  |
| Naloxone | 30.6  | 122   | 1450  | 172   | 145   | 150   | 1575  | 286   |
| Diprenorphine (Kd) | 0.6   | 3.34  | ND    | 6.99  | 2.15  | ND    | ND    | 8.43  |
FIG. 4. Binding profile of mutant receptors. The abbreviations used are: β-endorphin; Dyn A, dynorphin A; Leu-enk and Met-enk, Leu- and Met-enkephalin, respectively; Del II, deltorphin II; BW, BW373U86; NTI, naltrindole; Brm, bremazocine; and Nalox, naloxone. Histograms represent effect of the mutation on ligand binding with black bars showing the ratio of \( K_i \) values at mutant and WT receptors. Value 1 means that the mutation does not modify ligand binding. Results are presented by the model (see "Results") is important to maintain helix III pointing inward. The significant contribution of Tyr129 in ligand binding seems to be in contradiction with its outward orientation in the proposed model (see Fig. 1B). One hypothesis may be that the possible implication of Tyr129 in aromatic stacking suggested by the model (see "Results") is important to maintain helix III conformation and that Tyr129 plays a structural role. This aromatic ring of Tyr129 does not seem to be involved in the binding of some ligands (β-endorphin, dynorphin A, TIPP, BW373U86, and naltrindole), which rather interact with the OH group of the tyrosine residue. A more likely possibility to explain the decreased binding potency of opioid ligands at Y129F and Y129A mutants is that the amino acid side chain...
faces the inner core of the helices bundle and act as an anchor point for these ligands. Refinement of the model is now required to assess this hypothesis. In particular, revision of the alignment procedure and consideration of mutagenesis data on β2 receptor may lead to repositioning of helix III.

Although less drastically involved in receptor binding properties, Tyr308 (Tm VII) contributes significantly to the binding of most ligands tested in this study. This residue is also found in TRH receptor, bradykinin receptor, and in amine binding GPRs and was shown important for agonist and antagonist binding in m3 muscarinic receptor (23). Also a histidine residue is present at homologous position in adenosine A1 receptor and mutation into leucine demonstrated its critical role in ligand recognition (25). Finally, position 308 in mDOR corresponds to that of a lysine residue in the opsins family, where it represents the attachment point of retinal, the covalently bound ligand of this receptor family. Thus, the OH group of Tyr308, which seems to be generally involved in opioid binding at the δ receptor, appears to play a role similar to that of homologous residues in other GPRs.

Role of the Aromatic Cluster Spanning Tms IV, V, and VI—Tryptophan residues 173 (Tm IV) and 274 (Tm VI) play a critical role in the binding of specific groups of opioid compounds. These amino acids are highly conserved among GPRs. Site-directed mutagenesis studies have shown their involvement in ligand recognition at m3 muscarinic receptor for both residues (26), as well as at bovine rhodopsin and angiotensin AT1 receptors for tryptophan residue in Tm VI (27, 28). Our study shows that these two conserved tryptophan residues also play a role in binding at mDOR, consistent with the idea that these residues may represent common binding site determinants in GPRs despite the wide diversity of the ligands.

Mutations of phenylalanine residues of Tm V poorly affect ligand binding compared with other mutations examined in this study. Particularly, F218A represents the only mutation in this study that barely affects ligand binding. A phenylalanine residue is also present at this position in other GPRs. At present a role for this amino acid has not been documented, but the involvement of a serine residue, which is found at homologous position in receptors that bind small biogenic amines (Ser204 in β2-adrenergic, Ser199 in D1 dopamine, Ser201 in α2-adrenergic), has been clearly demonstrated. The serine amino acid residue was suggested to interact with the catechol moiety of the ligand, both from three-dimensional modeling studies (4).
and mutagenesis experiments where a 25-fold affinity decrease is described following serine to alanine substitution (29, 30). Our results indicate that Phe\(^{222}\) in the \(\delta\) receptor does not participate to ligand binding to the same extent as the homologous serine residue in catecholamine receptors. Modification of Phe\(^{222}\) into alanine alters binding potency of a larger number of ligands compared with F218A mutation. Taken together, results from mutations of the two phenylalanine residues in Tm V suggest that Phe\(^{222}\) is located closer to the binding site than Phe\(^{218}\), which is consistent with our model (Fig. 1A). Furthermore, the overall weak effect of both F218A and F222A mutations, relative to the important alterations of ligand binding arising from modifications at Tms III, IV, and VI, indicates that residues identified in this study constitute major anchor points for nonselective opioids, while additional specific interactions may take place for \(\delta\)-selective compounds in other regions of the receptor.

Our study does not provide evidence for the existence of distinct agonist and antagonist binding sites in the region embedded between Tms III and VII, that we have investigated. Each amino acid substitution affects the binding of at least one agonist and one antagonist, suggesting largely overlapping sites for both ligand types in the transmembrane aromatic domain.

Altogether these observations suggest that the set of aromatic residues proposed by our model is not involved in agonist/antagonist- or \(\delta\) selectivity, but rather constitute a general binding domain for opioids. Consistent with this idea is the fact that all six aromatic amino acids, identified by three-dimensional modeling as potential key residues, are conserved across \(\delta\), \(\mu\)- and \(\kappa\)-opioid receptors. We might therefore expect these residues to play similar roles in \(\mu\) and \(\kappa\) receptors, and further mutagenesis experiments may confirm this assumption. Finally, the suggestion that transmembrane aromatic residues examined here are part of a general binding domain raise the possibility that these residues interact with the “message” part of opioid molecules (22), which is common to all tested compounds. Further ligand-docking studies on a refined three-dimensional model should provide precise indications in support of this hypothesis.

Distinct Binding Fingerprints for Each Opioid Ligand—We have used a schematic representation of the putative aromatic site proposed by three-dimensional modeling to illustrate effects of mutations for the binding of each ligand under investigation (Fig. 5). An interesting observation is that the point mutations, altogether, alter the binding of every ligand with a specific pattern. This allows to define a unique binding fingerprint for each ligand and indicates that every opioid compound interacts at the \(\delta\) receptor using a distinct combination of interactions with aminooacidic residues of the binding domain. Indeed, and although two of our mutations allow to group structurally related ligands (W137A and W274A), we clearly observe specific features which make each ligand-receptor interaction unique. These include the striking reduction of deltorphin II binding at Y129F mutant as compared with other ligands, the equal involvement of both tryptophan residues for DPDPE binding only, and the unique property of TIPP\(\beta\) in binding at W274A mutant with increased affinity. In line with this idea also is the observation of different interaction modes for the two highly similar peptides Leu- and Met-enkephalin. Binding of Met-enkephalin is more reduced at the W173A mutant relative to Leu-enkephalin, while its affinity remains unchanged at Y308F, a mutation that significantly affects Leu-enkephalin binding. Thus, our study demonstrates for the first time that there is no unique binding site for opioids.

Conclusion—Evidence has accumulated that aromatic residues may interact with various chemical groups and undergo intra- or intermolecular bonds. Aromatic-aromatic interactions have been suggested to be important in maintaining receptor structure and driving conformational changes in GPRs (31). Also, the existence of amine-aromatic interactions between a ligand and its receptor site has been postulated based on crystallographic data for both acetylcholinesterase (32) and the phosphotyrosine recognition SH2 domain of v-src oncogene (33), as well as from mutagenesis studies of the tachykinin NK1 receptor (34). Numerous aromatic residues are present in transmembrane domains of opioid receptors, some of them being conserved in all GPRs (35). Our study shows that the side chains of Tyr\(^\text{129}\), Tyr\(^\text{173}\), Phe\(^{222}\), Trp\(^\text{274}\), and Tyr\(^\text{308}\) residues provide favorable interaction sites for opioid compounds and that their contribution may be distinct from that of their homologous counterparts in other GPRs. Our results further suggest that these transmembrane aromatic residues, which are conserved across opioid receptor subtypes, may represent common anchor points for opioid ligands in the \(\delta\) receptor. Finally, the overall picture of opioid binding at the mutated receptors supports the idea of a general transmembrane binding domain with no single binding site. Studies of complementary modifications of both receptor and ligand structures, in \(\delta\) as well as in \(\mu\) and \(\kappa\) receptors, may help in the future to elucidate the precise mechanisms of ligand-receptor interaction at aromatic residues in transmembrane domains of opioid receptors.

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REFERENCES
1. Kieffer, B. L. (1995) Cell. Mol. Neurobiol. 15, 615–635
2. Evans, C. J., Keith, D. E., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) Science 258, 1952–1955
3. Kieffer, B. L., Befort, K., Depoortere, I., De Jaegere, P., and Hirth, C. G. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 12048–12052
4. Hibert, M. F., Trumpf-Kallmeyer, S., Bruinvels, A., and Hoffack, J. (1991) Mol. Pharmacol. 40, 8–15
5. Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995) FASEB J. 9, 745–754
6. Schwartz, T. W. (1994) Curr. Opin. Biotech. 5, 434–444
7. Peck, D. C., and Hunter, J. J. (1990) Opioid Receptors (Emmet, J. C., ed) pp. 805–846, Pergamon Press, New York
8. Kong, H., Raynor, K., Yasuda, K., Moe, S. T., Porthoghese, P. S., Bell, G. I., and Uhl, G. R. (1994) J. Biol. Chem. 269, 23055–23058
9. Surratt, C. K., Hjorth, S. A., Thirstrup, K., Grandy, D. K., and Schwartz, T. W. (1995) J. Biol. Chem. 270, 72730–72736
10. Xue, J. C., Chen, C., Zhu, J., Kunapuli, S., Deriel, J., Kim, L., and Liuchen, L. Y. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 8042–8046
11. Hjorth, S. A., Thirstrup, K., Grandy, D. K., and Schwartz, T. W. (1995) Mol. Pharmacol. 47, 1089–1094
12. Fukuda, K., Kato, S., and Mori, K. (1995) J. Biol. Chem. 270, 6702–6709
13. Minami, M., Onogi, T., Nakagawa, T., Kato, Y., Aoki, Y., and Katsumata, S. (1994) FEBS Lett. 354, 242–247
14. Xue, J. C., Chen, C., Zhu, J., Kunapuli, S. K., Kim, L., Kieffer, B. L., DeVies, J., Yu, L., and Uhl, G. R. (1994) J. Biol. Chem. 269, 25986–25993
15. Fukuda, K., Kato, S., and Mori, K. (1995) J. Biol. Chem. 270, 6702–6709
16. Minami, M., Onogi, T., Nakagawa, T., Kato, Y., Aoki, Y., and Katsumata, S. (1994) FEBS Lett. 354, 242–247
17. Minami, M., Onogi, T., Nakagawa, T., Kato, Y., Toya, T., Katsumata, S., and Satoh, M. (1995) FEBS Lett. 357, 93–97
18. Wang, J. B., and Uhl, G. R. (1994) J. Biol. Chem. 269, 25986–25993
site-directed mutagenesis of the mouse δ receptor

10168

Liu-Chen, L.-Y. (1995) J. Biol. Chem. 270, 12977-12979

19. Beftor, K., Tabbara, L., Bausch, S., Chavkin, C., Evans, C., and Kieffer, B. (1996) Mol. Pharm. 49, 216-223

20. Joseph, M., Maigret, B., Bonnafous, J.-C., Marie, J., and Scheraga, H. A. (1995) J. Protein Chem. 14, 381-398

21. Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Bednman, E., and Downing, K. H. (1990) J. Mol. Biol. 213, 899-929

22. Barnard, E. A. (1993) Curr. Biol. 3, 211-214

23. Wess, J., Maggio, R., Palm, J. R., and Vogel, Z. (1992) J. Biol. Chem. 267, 19313-19319

24. Perlman, J. H., Thaw, C. N., Laakounen, L., Bowers, C. Y., Osman, R., and Gershengorn, M. C. (1994) J. Biol. Chem. 269, 1610-1613

25. Olah, M. E., Ren, H., Ostrowski, J., J. acetob, K. A., and Stiles, G. L. (1992) J. Biol. Chem. 267, 10764-10770

26. Wess, J., Nanavati, S., Vogel, Z., and Maggio, R. (1993) EMBO J. 12, 331-338

27. Nakayama, T. A., and Khorana, H. G. (1991) J. Biol. Chem. 266, 4269-4275

28. Yamano, Y., Ohwana, K., Kikyo, M., Sano, T., Nakagomi, Y., Inoue, Y., Nakamura, N., Morishima, I., Guo, D.-F., Hamakubo, T., and Inagami, T. (1995) J. Biol. Chem. 270, 14024-14030

29. Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S., and Dixon, R. A. F. (1989) J. Biol. Chem. 264, 13572-13578

30. Pollock, N. J., Manelli, A. M., Hutchins, C. W., Steffey, M. E., Mackenzie, R. G., and Frail, D. E. (1992) J. Biol. Chem. 267, 17780-17786

31. Trumpf-Kallmeyer, S., Hoflack, J., Brunvends, A., and Hiber, M. (1992) J. Med. Chem. 35, 3448-3462

32. Sussman, J. L., Harel, M., Frolov, F., Oehn, C., Goldman, A., Toker, L., and Sillman, I. (1991) Science 253, 872-879

33. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., Resh, M. D., Ris, C. B., Silverman, L., and Kuriyan, J. (1992) Nature 358, 646-653

34. Fong, T. M., Cascieri, M. A., Yu, H., Bansal, A., Swain, C., and Strader, C. D. (1993) Nature 362, 350-353

35. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992) DNA Cell Biol. 11, 1-20
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