Toward a Structure-Based Model of Salvinorin A Recognition of the κ-Opioid Receptor

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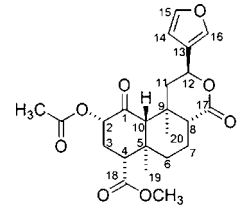
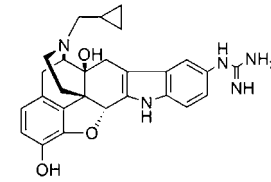
The structural basis to salvinorin A recognition of the κ-opioid receptor is evaluated using a combination of site-directed mutagenesis and molecular-modeling techniques. The results show that salvinorin A recognizes a collection of residues in transmembrane II and VII, including Q115, Y119, Y313, I316, and Y320. The mutation of one hydrophobic residue in particular, I316, was found to completely abolish salvinorin A binding. As expected, none of the residues in transmembrane III or VI commonly associated with opiate recognition (such as D138 or E297) appear to be required for ligand binding. On the basis of the results presented here and elsewhere, a binding site model is proposed that aligns salvinorin A vertically within a pocket spanning transmembrane II and VII, with the 2′ substituent directed toward the extracellular domains. The model explains the role that hydrophobic contacts play in binding this lipophilic ligand and gives insight into the structural basis to the μ-opioid receptor selectivity of 2′-benzoyl salvinorin (herkinorin).

Introduction

Salvinorin A is potent κ-opioid (KOP)[a] receptor agonist with unique physical properties.[1,2] This lipophilic ligand lacks an ionizable amine, which is known to be a key feature of most if not all opioid ligands. While much has been learned regarding the structural basis to recognition of opiates and related aminergic ligands,[3–14] our understanding of how salvinorin A and other nonbasic ligands recognize opioid receptors is limited.[15–17] Although several residues within transmembrane (TM) II and VII have been implicated in the KOP recognition of salvinorin A (including Q115, Y313, and Y320), site-directed mutagenesis studies have failed to produce a so-called "smoking gun" to pinpoint the ligand to a specific location.[15–17] This is in sharp contrast to opiate ligand recognition, which is dominated by a key salt-link interaction with a highly conserved aspartate in TM III (D138).[3,4] It has become fairly well-accepted that opiates bind within the central cavity of the receptor, spanning the conserved aspartate in TM III and key selectivity elements in TM VI and VII.[18,19] This model is best exemplified by the interaction of guanidinyl naltrindole (GNTI) with the KOP.[9,10] GNTI has been shown to take advantage of both D138 and E297 (in TM VI) in conferring selectivity and binding to the KOP.[9,10]

The data gathered on salvinorin A paints a very different picture. No evidence has surfaced to link salvinorin A binding or selectivity to D138 or E297 in the KOP. This, however, is not surprising because the ligand lacks a protonatable amino group required for salt-link formation. The handful of studies that have been reported suggest that salvinorin A binds in a very different orientation, involving numerous hydrophobic interactions in TM II and VII as well as hydrogen-bonding interactions with Q115.[2,15] One model that explains this data places the ligand in a unique binding epitope somewhat removed from the central cavity, spanning TM II and VII.[15] The orientation proposed aligns salvinorin A vertically within the receptor, allowing contacts with key residues both at the TM−extracellular interface and several turns into the TM helices.[15] The orientation also places the ligand in a position to interact with extracellular loops (EL) 2 and 3, which may play a pivotal role in conferring selectivity (Figure 1).





In this study, we describe and apply ligand-binding experiments using single-point mutant and chimeric opioid receptors to further build a binding site model for salvinorin A. In addition, we present corresponding data for the MOP-selective 2-salvinorinyl benzoate derivative (from here on out referred to as 2′-benzoyl salvinorin).[20] On the basis of comparisons of MOP and KOP residues, a model is developed to explain the structural basis to selectivity of these ligands.

Experimental Section

Single-Point Mutants and Chimeric Receptors. Rat KOP, MOP, and mouse DOP cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). Single-point mutants were constructed using primers purchased from Integrated DNA Technologies (Coralville, IA). In addition to containing the appropriate mutational codon, the primers were designed so that a restriction site was either created or eliminated. Polymerase chain reactions (PCRs)[21] were conducted with the high-fidelity DNA polymerase Pfu Turbo using a slightly modified procedure from that which is found in the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The PCR products were transformed into XL-1 Blue competent cells (Stratagene) and subsequently isolated using the Wizard Plus DNA purification system (Promega, Madison, WI). A restriction digest and electrophoresis on a 0.8% agarose gel (GibcoBRL, Invitrogen) was used to confirm the mutations. The

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[a] Abbreviations: κ-opioid receptor, KOP; μ-opioid receptor, MOP, δ-opioid receptor, DOP; transmembrane, TM; extracellular loops, EL; 6β-^{125}iodo-3,14-dihydroxy-17-cyclopropylmethyl-4,5α-epoxymorphinan, [^{125}I]-OXY; dynorphin A, Dyn A.

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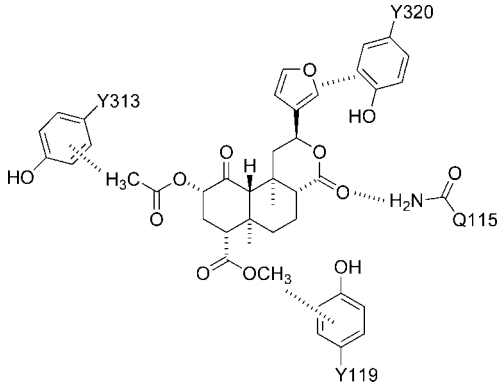

Figure 1. Proposed interactions of salvinorin A and the KOP.

mutated plasmid was purified using Qiafilter Plasmid Maxi Kit (Qiagen) or by cesium chloride/ethidium bromide-gradient centrifugation.[22] Sequences were verified by the BMGC DNA Sequencing and Analysis Facility (ABI PRISM 3100 Genetic Analyzer, University of Minnesota, Minneapolis, MN).

The Afl III and Bgl II chimeric receptors were constructed in our laboratory as described previously.[15] In short, the pcDNA sequences were digested with the appropriate restriction digest and electrophoresed on an agarose gel. Fragments were excised, purified using GENECLEAN II (MP Biomedicals, Irvine, CA), and religated using the LigaFast Rapid DNA ligation system (Promega). Aliquots from the ligation mixtures were transformed, screened, and amplified using similar procedures as those used for the single-point mutants (above). Similar procedures for construction of the MOP/KOP/MOP and KOP/MOP/KOP triple chimeras were applied.

Transient Transfection. Human embryonic kidney (HEK)-293 cells (ATCC, Manassas, VA) were maintained at 37 °C and 5% CO_2 with DMEM (high glucose, with L-glutamine) containing 10% bovine calf serum and 1% penicillin/streptomycin (all purchased from Gibco, Invitrogen). Cells were seeded at 20–30% confluence approximately 24 h before transfection. Fresh media was added 1–2 h before transfection. Cells were transfected with 10–20 μg of either the chimeric or mutant pcDNA using the calcium phosphate precipitation method.[23] Medium was changed 5 h later.

Receptor-Binding Assays. Transfected cells were harvested at 48–72 h post-transfection. The receptor-binding assays were conducted as previously described.[9] In short, cells were washed 3 times with 25 mM HEPES buffer (pH 7.4) and then resuspended with 8–12 mL of 25 mM HEPES/100 mm plate. Binding mixtures, containing [^{3}H]-diprenorphine (specific activity, 50 Ci/mmol; New England Nuclear) in a total volume of 0.5 mL, were incubated at room temperature for 90 min. The binding reactions were terminated via filtration through a Whatman GF/C filter presoaked in 0.25% poly(ethylenimine). Incubation mixtures were subsequently washed with 4 mL of ice-cold 25 mM HEPES buffer (3 times) to ensure complete transfer of the incubation contents. Scintillation cocktail was added, and vials were analyzed by a Beckman 3801 LS scintillation counter.

To determine the dissociation constants (K_d), saturation binding assays were conducted.[9] In short, eight different concentrations of [^{3}H]-diprenorphine (typically ranging from 25 pM to 3 nM) were used to determine total binding. Nonspecific values were established by the addition of 100 μM naloxone, nor-binaltorphimine (norBNI), or salvinorin A, depending upon which ligand showed the greatest inhibition. The data (collected in triplicate) were analyzed using the nonlinear regression analysis program in Prism (GraphPad Software, Inc., San Diego, CA). The K_d values for each mutant and chimera were similar to wild-type values (<10-fold difference), suggesting that no major changes in overall receptor structure occur.

Competitive binding experiments were conducted using a [^{3}H]-diprenorphine concentration of 0.5–1.0 times the K_d value for the particular mutant or chimera. Nine concentrations (also in triplicate) of salvinorin A were used in the displacement analysis. Again, naloxone, norBNI, or salvinorin A (100 μM) was used for nonselective binding. The Cheng−Prusoff equation allowed for the conversion of IC_{50} values to inhibition constants (K_i).[24]

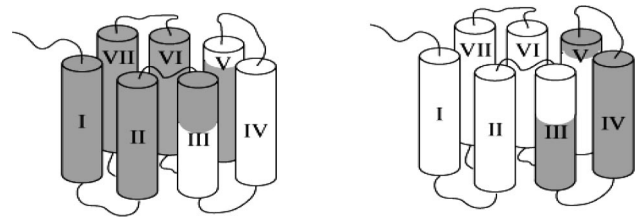

Figure 2. Schematic of the KOP/MOP/KOP and MOP/KOP/MOP chimeric receptors.

Isolation of Salvinorin A and 2′-Benzoyl Salvinorin. Salvinorin A was obtained by reported extraction and purification methods from *Salvia divinorum* leaves harvested from plants (Theatrum Botanicum, Laytonville, CA) propagated at the University of Mississippi. Purified, crystalline salvinorin A agreed with published characterization data.[25] The benzoyl derivative was synthesized semisynthetically from salvinorin B, in accordance with methods described by Harding et al.[20]

Molecular-Modeling Approach. Molecular-modeling studies were carried out using the Schrödinger software package.[26] The initial structures of salvinorin A and 2′-benzoyl salvinorin were created from the X-ray coordinates of a closely related analogue, salvinicin B (Cambridge Crystallographic Data Centre, 032341.cif).[27] The rigid *trans*-decalin core structure of this compound was applied as a common template in generating the coordinates of both ligands using simple model building techniques. The rKOP model was created on the basis of the previously described hKOP model,[28] by excluding the existing pentapeptide ligand and mutating the necessary amino acids to that of the rKOP. Salvinorin A was manually docked into the KOP. Amino acid side chains were rotated to minimize any gross steric overlap between salvinorin A and the KOP. Despite this, the model still contained unfavorable interactions between salvinorin A and the backbone atoms of the β hairpin of EL2. To alleviate these unfavorable interactions, a series of minimizations were conducted. While freezing the positions of the of KOP not in EL2, along with salvinorin A, 5000 steps of steepest descent followed by conjugate-gradient energy minimizations were carried out. Distance constraints were applied to retain the hydrogen bonds within the β hairpin. This protocol was repeated with the constraints being gradually decreased and eventually removed during successive runs. The resulting salvinorin A−KOP complex was devoid of clashes between salvinorin A and EL2. In addition, the total energy of the system converged (convergence gradient of 0.01). Once this steric-free model was obtained, additional energy minimizations were carried out on salvinorin A and the residues within 8 Å of salvinorin A. Atoms outside this region were fixed, while constraints were placed on backbone atoms within the region. Again, these constraints were gradually decreased and eventually removed during successive runs.

All minimizations were conducted using the OPLS-2005 force field.[29] The generalized Born model[30] with a dielectric constant of 1.0 was used to incorporate solvent effects during energy minimization. Long-range interactions were calculated with a nonbond cutoff distance of 12 Å. The final docked structure can be seen in Figure 5, and the PDB file can be obtained at http://opioid.pharmacy.umn.edu.

Results

Site-Directed Mutagenesis. Site-directed mutagenesis studies on the KOP were conducted, and the affinities for salvinorin A at these mutant receptors are summarized in Table 1. The most notable result is the lack of binding to the I316A mutant. When I316 is modified to an alanine, salvinorin A loses all appreciable affinity toward the mutant receptor (K_i > 10 000 nM). To date, this mutation has had by far the most significant impact on

Table 1. Affinity of Salvinorin A and 2′-Benzoyl Salvinorin at Receptor Mutants

salvinorin A	K_i (nM)[a]	F_{mut}[b]
KOP	17.5 ± 1.5 (3)	
KOP [I62A]	22 ± 2 (2)	1.26
KOP [Y66A]	18.1 ± 1.7 (4)	1
KOP [Y66F]	6.8 ± 0.5 (4)	0.39
KOP [K227A]	25.3 ± 5.2 (3)	1.45
KOP [I290A]	29.6 ± 8.6 (3)	1.69
KOP [I294A]	2.9 ± 0.6 (3)	0.17
KOP [I316A]	>10000 (3)	>500
Ref 15		
KOP [Q115A]	147 ± 47 (2)	8.4
KOP [Y119A]	67 ± 7.4 (3)	3.8
KOP [Y119F]	17.7 ± 3.9 (3)	1
KOP [D138A]	17.5 ± 4.4 (4)	1
KOP [Y139F]	9.5 ± 2.8 (5)	0.54
KOP [E297A]	19.5 ± 3.1 (4)	1.1
KOP [Y312A]	79 ± 28 (5)	4.5
KOP [Y312F]	16 ± 3.8 (3)	0.91
KOP [Y313A]	126 ± 48 (5)	7.2
KOP [Y313F]	37 ± 3.7 (4)	2.1
KOP [Y320A]	565 ± 49 (2)	32
KOP [Y320F]	71 ± 15 (3)	4.1
MOP	>25000 (2)	
DOP	>25000 (2)	
2′-benzoyl salvinorin		
MOP	245 ± 63 (3)	
KOP	1375 ± 165 (3)	
KOP [Y313A]	3870 ± 580 (3)	2.8
MOP [W318A]	426 ± 73 (3)	1.7

[a] The K_i values (±SEM) were determined in competition binding using [^{3}H]-diprenorphine in transiently expressed HEK-293 cells and analyzed by whole cell binding. The number of individual determinations is indicated in parentheses (n). [b] F_{mut} = mutational factor.

binding. In addition to this residue in TM VII, single-point mutants of TM I were analyzed. The residues believed to project toward TM II and VII, isoleucine 62 (I62) and tyrosine 66 (Y66), were both mutated to an alanine. In addition, Y66 was mutated to a phenylalanine (Y66F). The I62A and Y66A mutants yielded little to no change in binding (K_i = 22.0 and 18.1 nM, respectively), while Y66F resulted in a slight increase in binding affinity (K_i = 6.8 nM).

Residues on the opposite side of the receptor were also investigated. Specifically, the residues in TM V and VI that are postulated to be facing the traditional opiate-binding pocket were examined. In TM V, lysine 227 was mutated to an alanine (K227A). Results from this mutant show that binding of salvinorin A is only minimally affected (K_i = 25.3). In TM VI, the residues beneath the prototypical opiate selectivity site (E297) were examined. Both I290 and I294 were mutated to an alanine. Only minor changes in binding affinity were observed (K_i = 29.6 and 2.9 nM, respectively).

Limited site-directed mutagenesis studies on 2′-benzoyl salvinorin were conducted (Table 1). Control experiments on wild-type MOP and KOP revealed affinities of 245 and 1375 nM, respectively. In TM VII of MOP, W318 (the homologous residue to Y312) was mutated to an alanine. The 2′-benzoyl derivative bound to this mutant with similar to wild-type affinity (K_i = 426 nM). The benzoyl derivative was also tested at the KOP Y313A mutant. Here, the affinity for 2′-benzoyl salvinorin dropped approximately 3-fold (K_i = 3870 nM).

Chimeric Studies. In addition to the site-directed mutagenesis, chimeric studies were conducted. Previously, Bgl II and Afl III constructs were designed to probe regions of the receptors that were necessary for high-affinity binding.[15] From this study, it was found that salvinorin A showed greater affinity for both the KOP/MOP and KOP/DOP Bgl II constructs than the KOP/MOP and KOP/DOP Afl III constructs. Thus, it was speculated that the region of the KOP that is contained between these two restriction sites may be important for binding.[15] To better assess this, a three-piece chimera that interchanges sequences between these two sites was constructed (Figure 2). Results from these chimeras can be seen in Table 2. In each case, salvinorin A maintains moderate affinity for the chimeric receptor (K_i = 522 nM for KOP/MOP/KOP and 458 nM for MOP/KOP/MOP).

Binding affinities for 2′-benzoyl salvinorin at the chimeric receptors were also determined (Table 3). The data from the MOP/KOP Bgl II and Afl III chimeras (Figure 3) that exchange sequences only once show a general trend of decreased activity for 2′-benzoyl salvinorin. In addition to the MOP/KOP chimeras, 2′-benzoyl salvinorin was tested at the KOP/DOP Bgl II chimera. This chimeric receptor, which has been shown to bind to salvinorin A with an affinity slightly greater than that of the wild-type KOP, bound to 2′-benzoyl salvinorin with an affinity similar to that of the wild-type MOP (K_i = 450 nM). Meanwhile, 2′-benzoyl salvinorin did not show any appreciable activity at the KOP/DOP Afl III chimera (K_i > 10 000 nM). Chimeras in which the sequences exchange at the Afl III site and then exchange back at the Bgl II site were also evaluated. The KOP/MOP/KOP chimera shows a dramatic decrease in binding affinity ($K_i \sim$ 8300 nM), while at the converse chimera, MOP/KOP/MOP, the binding affinity is virtually unchanged ($K_i \sim$ 600 nM).

Discussion

Mutational Analysis. A number of binding site models have been reported to explain the structural basis to salvinorin A recognition at the KOP.[2,15–17] Despite a few differences, several commonalities exist among the current models. In all cases, it is speculated that tyrosine 313 (Y313) interacts with the 2′ substituent, mainly through hydrophobic effects.[15–17] Current models also place Y119 (TM II) and Y320 (TM VII) in favorable contacts with salvinorin A, further localizing the ligand to TM II and VII.[15–17] On the basis of structure-based models developed in our laboratory, we have postulated that, if tyrosines 313 and 320 play a role in recognition, then mutation of I316 may lead to decreased affinity for salvinorin A. This residue, which lies between the two tyrosines in the α-helical bundle, did just that. In fact, the I316A mutant not only decreases salvinorin A affinity but effectively abolishes it.

Further examination of our molecular models suggested that, if the TM II /TM VII interface is important for binding, then residues within TM I may be important as well. This helix, which lies in between TM II and VII in the counter-clockwise GPCR arrangement, contains two residues that point toward our putative salvinorin-A-binding pocket. These residues, I62 and Y66, were both subjected to site-directed mutagenesis. When the tyrosine was mutated to alanine, no change in binding was observed. Meanwhile, Y66F showed a slight increase in binding affinity for salvinorin A. We suggest that this result may be due to an alteration of the local steric environment of which salvinorin A interacts. Approximately one helical turn above Y66 is isoleucine 62 (I62). When this residue is mutated to an alanine, only minor changes in binding are observed. Together, the lack of significant changes in binding data for TM I mutants suggests that residues in this helix are not directly in contact with salvinorin A.

Residues in TM VI have also been implicated in previous binding site models of salvinorin A and the KOP.[16] One such residue, E297, is known to be a key site for selectivity of opiate ligands to the KOP. From mutagenesis studies, it is clear that

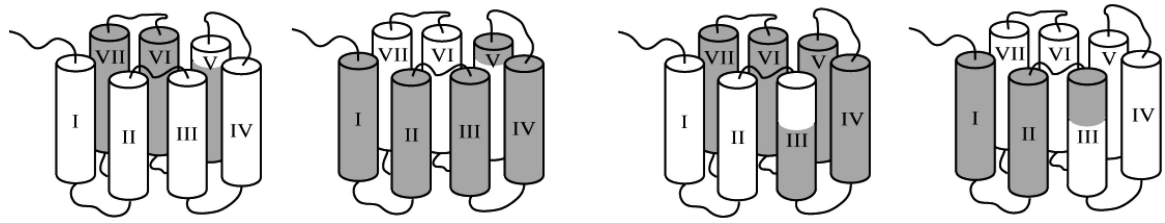

Figure 3. Schematic of the Afl III and Bgl II chimeric receptors.

Table 2. Binding Data of Salvinorin A at the KOP/MOP/KOP and MOP/KOP/MOP Chimeric Receptors

	K_i (nM)
KOP (1–141)/MOP (151–233)/ KOP (228–380)	522 ± 32 (3)
MOP (1–150)/KOP (142–227)/MOP (234–398)	458 ± 78 (3)

Table 3. Binding Data for 2′-Benzoyl Salvinorin at the Various Chimeric Receptors

	K_i (nM)
MOP (1–150)/KOP (142–380)	4110 ± 1300 (4)
MOP (1–233)/KOP (228–380)	2310 ± 470 (4)
KOP (1–141)/MOP (151–398)	1610 ± 570 (2)
KOP (1–227)/MOP (234–398)	9400 ± 650 (2)
KOP (1–141)/MOP (151–233)/KOP (228–380)	8320 ± 320 (2)
MOP (1–150)/KOP (142–227)/MOP (234–398)	615 ± 15 (2)
KOP (1–141)/DOP (132–372)	>10000
KOP (1–227)/DOP (215–372)	450 ± 35 (2)

this glutamate is not required for salvinorin A recognition.[15] Given the pivotal role that TM VI plays in conferring KOP selectivity, we have also examined I294 and I290 as potential hydrophobic sites of recognition. The first site, I294, which lies approximately one helical turn beneath E297, showed a moderate increase in affinity (~3 nM) when mutated to alanine. As in the case of Y66A, it is possible that this mutation alters local steric interactions, which allow salvinorin A to interact more favorably with adjacent residues. To further examine the significance of this result, isoleucine 290 (I290), which is located approximately one turn down the helical bundle beneath I294, was also mutated to alanine. No significant changes in binding, however, were observed. Taken together, the lack of significant changes in affinity of salvinorin A toward TM VI mutants suggests that this domain is not required for salvinorin A binding and selectivity.

Additional interactions between salvinorin A and the typical opiate-binding pocket were probed, specifically, interactions at TM V. This helix has been implicated in binding some of the larger opiate-based ligands. One such ligand, β-funaltrexamine (β-FNA),[31] is believed to interact irreversibly with a conserved lysine residue in the MOP (K233).[32] As can be seen in Figure 4, the KOP-selective agonist TRK-820 ((−)-17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl) acrylamido]morphinan hydrochloride) closely resembles this ligand.[33] To our knowledge, TRK-820 is the only other KOP-selective ligand that contains a furan ring. Although there is no precedence to suggest that the furan ring of salvinorin A and the furan ring of TRK-820 act in a similar manner, we decided to examine the possibility. Modeling studies suggest that the furan ring of TRK-820 would lie in close proximity to the lysine 227 (K227). Thus, if the furan rings interact at similar sites, then a decrease in salvinorin A binding would be expected at this lysine mutant. However, the K227A mutant revealed no such decreases in binding (K_i = 25 nM). Again, it appears that mutations made in the typical opiate-binding pocket do not affect salvinorin A binding.

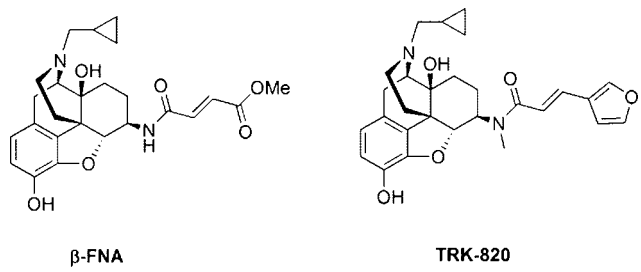

Figure 4. Structural comparison of β-funaltrexamine and TRK-820.

In addition to the data collected for salvinorin A, mutational data were also collected for 2′-benzoyl salvinorin (Table 1). Control experiments conducted on wild-type MOP and KOP receptors revealed affinities of 245 and 1375 nM, respectively. These values, which are higher than those reported by Harding et al., can be rationalized by the use of different binding conditions (whole cell assay versus membrane preparations) and/or the use of different radioligands ([^{3}H]-diprenorphine versus [^{125}I]-OXY).[20] Importantly, the relative difference between the two values is similar. Because the only difference between 2′-benzoyl salvinorin and salvinorin A is the replacement of an acetyl group with a benzoyl group (at the 2′ position), we examined the possibility that this analogue binds in a similar fashion as salvinorin A, using conserved mechanisms for the recognition of the diterpene core while using unique interactions near the 2′ substituent. A comparison of the transmembrane regions of MOP and KOP reveal that, with the exception of one residue, all of the proposed residues of our binding model are conserved. The residue not conserved, tyrosine 313 (in TM VII) is a histidine in the MOP (H319). Because we already had Y313A in hand, we decided to test the benzoyl derivative at this mutant. This mutant decreased the binding affinity of 2′-benzoyl salvinorin by approximately 3-fold, perhaps by the loss of a hydrophobic contact.

Chimeric Analysis. Prior studies using chimeric receptors have indicated that the regions between the Afl III and Bgl II restriction sites may be important for salvinorin A binding.[15] To further analyze this claim, triple chimeras (chimeric receptors that exchange sequences more than once) were constructed. For both the MOP/KOP/MOP and the KOP/MOP/KOP chimera, substantial decreases of salvinorin A binding were observed. This result suggests that the KOP region between these two restriction sites is in fact necessary for high-affinity binding. The chimeric receptors were also applied to evaluate the binding of the MOP-selective 2′-benzoyl salvinorin. Previously, it had been determined that the KOP/DOP Bgl II chimera binds to salvinorin A with extremely high affinity (~2 nM). When 2′-benzoyl salvinorin was tested at this chimera, it maintained an affinity similar to that of the MOP, despite the fact that the chimeric receptor was completely devoid of the MOP. This receptor, however, contains a histidine at position 313 as does the MOP/KOP/MOP trimeric receptor. A comparison of the

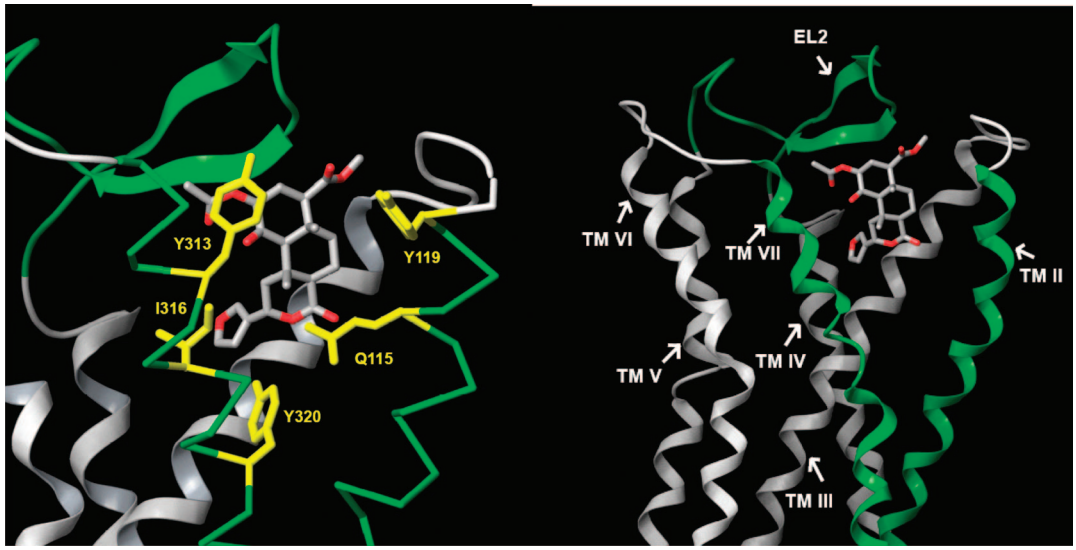

Figure 5. Close-up and distant view of salvinorin A docked to the KOP. The furan ring is positioned between residues I316 and Y320 of TM VII. The lactone carbonyl participates as a hydrogen-bonding acceptor with Q115 of TM II. The 4 position interacts with Y119, while the 2′ substituent interacts with Y313. The key residues are colored in yellow with TM II and VII, and EL-2 is colored in green.

KOP/MOP/KOP and MOP/KOP/MOP constructs indicates that the former lacks not only binding affinity for the ligand but a histidine at position 313 in TM VII. While provocative, the results should be applied with some caution. It is important to point out that the KOP/DOP AfI III chimeric receptor shows no affinity for 2′-benzoyl salvinorin, despite the presence of H319. This chimera, however, contains EL2 of the DOP, which, in previous work, has been shown to have a negative impact on salvinorin A binding.

As is typical in the analysis of mutational and chimeric data, there is some subjectivity as to what the results may actually signify. For chimeric receptors, one must always speculate as to whether or not the sum of the parts is equal to that of the whole. This concern is often addressed by examining the newly formed chimeric receptors ability to bind to a known radioligand, in this case [^{3}H]-diprenorphine. We measured such binding and found that [^{3}H]-diprenorphine does indeed bind to the chimeric receptors with similar affinities compared to that of the wild-type receptors. However, this does not rule out the possibility that topological changes do not occur outside of the opiate-binding pocket.

Site-directed mutagenesis studies also have some inherent uncertainties associated with them. In an attempt to minimize such uncertainties, controls were used for the residues that have been implicated in binding. In the cases of Y119, Y313, and Y320, previous studies have indicated that both U69,593 (5α,7α,8β-(–)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzene acetamide)[34] and dynorphin A (Dyn A) have binding constants similar to that of the wild-type receptor.[15] Binding affinities of U69,593 and Dyn A to other residues implicated in binding, namely, Q115 and I316, were examined here. With regard to the Q115A mutant, both U69,593 and Dyn A maintained similar to wild-type affinities (data not shown). Alternatively, U69,593 and Dyn A yielded decreased affinities at the I316A mutant. The latter result is not all that unexpected because I316 has been hypothesized to lie in the binding pocket for the arylacetamides as well as Dyn A. Even so, the extent of the binding change for salvinorin A was unmatched (~10-fold versus >500-fold). Consequently, we suggest that the results that we observed are not artifacts and that the conclusions based off the data are well-founded.

Nonetheless, it is possible that an alternative pharmacophore may exist. The site-directed mutagenesis data obtained for residues associated with the traditional opioid-binding pocket could be false negatives. The hydrophobic nature of salvinorin A inherently lends itself to recognition via hydrophobic interactions. These hydrophobic interactions are more difficult to probe experimentally because the decrease in affinity caused by a mutation of a residue that is believed to help stabilize the ligand may be in part compensated by a nearby residue. This is often not the case when dealing with mutations of the residue believed to be involved in a salt bridge.

Conclusions

This study has examined the binding of salvinorin A to a wide variety of single-point mutant and chimeric opioid receptors in an effort to further localize and refine the ligand-binding site of this unique lipophilic KOP agonist. The results presented here, coupled with those reported in previous work, suggest the binding pocket for salvinorin A is comprised of residues from TM II and VII, as opposed to those of TM III and VI traditionally associated with opiate recognition. We have also shown that the mutation of I316 results in a complete loss of salvinorin A binding. This result is consistent with the general hypothesis that hydrophobic interactions drive the binding and selectivity of this non-nitrogenous KOP ligand as opposed to hydrogen bonds or salt links to KOP residues (such as D138 or E297). While the data is limited, the results involving MOP-selective 2′-benzoyl salvinorin also point to TM VII as a key domain in ligand recognition. A sequence comparison of the MOP and KOP shows that position 313 (tyrosine) of the KOP is occupied by a histidine in the MOP (H319). The mutation of Y313 does, in fact, have an impact on the binding affinity of the benzoyl analogue. It is important to point out, however, that the chimeric data also points to EL2 as a probable factor in conferring selectivity of salvinorin A. This is not surprising given the role that EL2 has been proposed to play in opioid receptor selectivity and binding over the years.[4,35–38] Taken overall, the results imply that residues at or near the TM VII−extracellular interface are key to the binding and selectivity of salvinorin A and related analogues.

One model that is consistent with the data reported here and elsewhere is shown in Figure 5.[15,39] The vertical alignment of the ligand accounts for interactions along the helical face of TM VII as well as key contacts with residues in TM II (including residues Q115, Y119, Y313, I316, and Y320). Moreover, the 2′ substituent projects into the EL domain. As alluded to above, this may explain differences in the selectivity profiles of salvinorin A and 2′-benzoyl salvinorin both in terms of sequence variability across the opioid receptors and the differing steric requirements for these two derivatives. Additional support for this alternative binding site model can be found in recent substituted-cysteine-accessibility method (SCAM) studies.[40,41] These studies suggest the space surrounding TM VII of the KOP is more expansive than previously envisioned using homology built models and rhodopsin-based templates. As opposed to observing the normal trend of methanethiosulfonate (MTS)-associated inhibition on every third or fourth residue (suggestive of an α helix, of which only one face is water-accessible), the KOP shows a trend in which large increases in [^{3}H]-diprenorphine inhibition occur for numerous consecutive residues in TM VII.[40] It is reasonable to conclude that the increased sensitivity is due to a water-accessible pocket surrounding TM VII in the KOP. This further highlights the importance of hydrophobic interactions in driving the binding of salvinorin A to the KOP. It is quite possible that interactions with residues, such as I316, lead to very favorable binding free energies by displacing water from this putative binding site. Such effects are, of course, well-documented in the molecular recognition process and are undoubtedly key to the binding of this lipophilic ligand to the KOP.

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References

(1) Valdes, L. J., III; Diaz, J. L.; Paul, A. G. Ethnopharmacology of ska Maria Pastora (*Salvia divinorum*). *J. Ethnopharmacol.* **1983**, *7*, 287–312.
(2) Roth, B. L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K. C.; Steinberg, S.; Ernsberger, P.; Rothman, R. B. Salvinorin A: A potent naturally occurring nonnitrogenous κ opioid selective agonist. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11934–11939.
(3) Surratt, C. K.; Johnson, P. S.; Moriwaki, A.; Seidleck, B. K.; Blaschak, C. J.; Wang, J. B.; Uhl, G. R. μ-Opiate receptor. Charged transmembrane domain amino acids are critical for agonist recognition and intrinsic activity. *J. Biol. Chem.* **1994**, *269*, 20548–20553.
(4) Kong, H.; Raynor, K.; Resine, T. Amino acids in the cloned mouse kappa receptor that are necessary for high affinity agonist binding but not antagonist binding. *Regul. Pept.* **1994**, *54*, 155–156.
(5) Befort, K.; Tabbara, L.; Bausch, S.; Chavkin, C.; Evans, C.; Kieffer, B. The conserved aspartate residue in the third putative transmembrane domain of the δ-opioid receptor is not the anionic counterpart for cationic opiate binding but is a constituent of the receptor binding site. *Mol. Pharmacol.* **1996**, *49*, 216–223.
(6) Spivak, C. E.; Beglan, C. L.; Seidleck, B. K.; Hirshbein, L. D.; Blaschak, C. J.; Uhl, G. R.; Surratt, C. K. Naloxone activation of μ-opioid receptors mutated at a histidine residue lining the opioid binding cavity. *Mol. Pharmacol.* **1997**, *52*, 983–992.
(7) Mansour, A.; Taylor, L. P.; Fine, J. L.; Thompson, R. C.; Hoversten, M. T.; Mosberg, H. I.; Watson, S. J.; Akil, H. Key residues defining the μ-opioid receptor binding pocket: A site-directed mutagenesis study. *J. Neurochem.* **1997**, *68*, 344–353.
(8) Valiquette, M.; Vu, H. K.; Yue, S. Y.; Wahlestedt, C.; Walker, P. Involvement of Trp-284, Val-296, and Val-297 of the human δ-opioid receptor in binding of δ-selective ligands. *J. Biol. Chem.* **1996**, *271*, 18789–18796.
(9) Metzger, T. G.; Paterlini, M. G.; Ferguson, D. M.; Portoghese, P. S. Investigation of the selectivity of oxymorphone- and naltrexone-derived ligands via site-directed mutagenesis of opioid receptors: Exploring the "address" recognition locus. *J. Med. Chem.* **2001**, *44*, 857–862.
(10) Jones, R. M.; Hjorth, S. A.; Schwartz, T. W.; Portoghese, P. S. Mutational evidence for a common κ antagonist binding pocket in the wild-type κ and mutant μ[K303E] opioid receptors. *J. Med. Chem.* **1998**, *41*, 4911–4914.
(11) Hjorth, S. A.; Thirstrup, K.; Grandy, D. K.; Schwartz, T. W. Analysis of selective binding epitopes for the κ-opioid receptor antagonist norbinaltorphimine. *Mol. Pharmacol.* **1995**, *47*, 1089–1094.
(12) Subramanian, G.; Paterlini, M. G.; Larson, D. L.; Portoghese, P. S.; Ferguson, D. M. Conformational analysis and automated receptor docking of selective arylacetamide-based κ-opioid agonists. *J. Med. Chem.* **1998**, *41*, 4777–4789.
(13) Lavecchia, A.; Greco, G.; Novellino, E.; Vittorio, F.; Ronsisvalle, G. Modeling of κ-opioid receptor/agonists interactions using pharmacophore-based and docking simulations. *J. Med. Chem.* **2000**, *43*, 2124–2134.
(14) Kong, H.; Raynor, K.; Yano, H.; Takeda, J.; Bell, G. I.; Reisine, T. Agonists and antagonists bind to different domains of the cloned κ opioid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8042–8046.
(15) Kane, B. E.; Nieto, M. J.; McCurdy, C. R.; Ferguson, D. M. A unique binding epitope for salvinorin A, a non-nitrogenous κ opioid receptor agonist. *FEBS J.* **2006**, *273*, 1966–1974.
(16) Yan, F.; Mosier, P. D.; Westkaemper, R. B.; Stewart, J.; Zjawiony, J. K.; Vortherms, T. A.; Sheffler, D. J.; Roth, B. L. Identification of the molecular mechanisms by which the diterpenoid salvinorin A binds to κ-opioid receptors. *Biochemistry* **2005**, *44*, 8643–8651.
(17) Vortherms, T. A.; Mosier, P. D.; Westkaemper, R. B.; Roth, B. L. Differential helical orientations among related G protein-coupled receptors provide a novel mechanism for selectivity. Studies with salvinorin A and the κ-opioid receptor. *J. Biol. Chem.* **2007**, *282*, 3146–3156.
(18) McFadyen, I.; Metzger, T.; Subramanian, G.; Poda, G.; Jorvig, E.; Ferguson, D. M. Molecular modeling of opioid receptor−ligand complexes. *Prog. Med. Chem.* **2002**, *40*, 107–135.
(19) Kane, B. E.; Svensson, B.; Ferguson, D. M. Molecular recognition of opioid receptor ligands. *AAPS J.* **2006**, *8*, E126–E137.
(20) Harding, W. W.; Tidgewell, K.; Byrd, N.; Cobb, H.; Dersch, C. M.; Butelman, E. R.; Rothman, R. B.; Prisinzano, T. E. Neoclerodane diterpenes as a novel scaffold for μ opioid receptor ligands. *J. Med. Chem.* **2005**, *48*, 4765–4771.
(21) Mullis, K.; Faloona, F.; Scharf, S.; Saiki, R.; Horn, G.; Erlich, H. Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **1986**, *51*, 263–273.
(22) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning*, 2nd ed.; Cold Spring Harbor: Cold Spring Harbor Laboratory, NY, 1989.
(23) Chen, C.; Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **1987**, *7*, 2745–2752.
(24) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.
(25) Ortega, A.; Blount, J.; Manchand, P. Salvinorin, a new *trans*-neoclerodane diterpene from *Salvia divinorum*. *J. Chem. Soc., Perkin Trans.* **1982**, *1*, 2505–2508.
(26) Schrodinger, L. L. C. 2003. 32nd Floor, Tower 45, 120 West Forty-Fifth Street, New York, 10036.
(27) Harding, W. W.; Tidgewell, K.; Schmidt, M.; Shah, K.; Dersch, C. M.; Snyder, J.; Parrish, D.; Deschamps, J. R.; Rothman, R. B.; Prisinzano, T. E. Salvinicins A and B, new neoclerodane diterpenes from *Salvia divinorum*. *Org. Lett.* **2005**, *7*, 3017–3020.
(28) Przydzial, M. J.; Pogozheva, I. D.; Ho, J. C.; Bosse, K. E.; Sawyer, E.; Traynor, J. R.; Mosberg, H. I. Design of high affinity cyclic pentapeptide ligands for κ-opioid receptors. *J. Pept. Res.* **2005**, *66*, 255–262.
(29) Damm, W.; Halgren, T. A.; Murphy, R. B.; Smondyrev, A. M.; Friesner, R. A.; Jorgensen, W. L. OPLS 2002: A new version of the OPLS-AA force field, 224th ACS National Meeting, 2002.
(30) Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. J. Semianalytical treatment of solvation for molecular mechanics and dynamics. *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.
(31) Portoghese, P. S.; Larson, D. L.; Sayre, L. M.; Fries, D. S.; Takemori, A. E. A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. *J. Med. Chem.* **1980**, *23*, 233–234.
(32) Chen, C.; Yin, J.; Riel, J. K.; DesJarlais, R. L.; Raveglia, L. F.; Zhu, J.; Liu-Chen, L. Y. Determination of the amino acid residue involved in [^{3}H]β-funaltrexamine covalent binding in the cloned rat μ-opioid receptor. *J. Biol. Chem.* **1996**, *271*, 21422–21429.
(33) Nagase, H.; Hayakawa, J.; Kawamura, K.; Kawai, K.; Takezawa, Y.; Matsuura, H.; Tajima, C.; Endo, T. Discovery of a structurally novel opioid κ-agonist derived from 4,5-epoxymorphinan. *Chem. Pharm. Bull.* **1998**, *46*, 366–369.

(34) Lahti, R. A.; Mickelson, M. M.; McCall, J. M.; von Voigtlander, P. F. [^{3}H]U-69593 a highly selective ligand for the opioid κ receptor. *Eur. J. Pharmacol.* **1985**, *109*, 281–284.
(35) Meng, F.; Hoversten, M. T.; Thompson, R. C.; Taylor, L.; Watson, S. J.; Akil, H. A chimeric study of the molecular basis of affinity and selectivity of the κ and the δ opioid receptors. Potential role of extracellular domains. *J. Biol. Chem.* **1995**, *270*, 12730–12736.
(36) Wang, J. B.; Johnson, P. S.; Wu, J. M.; Wang, W. F.; Uhl, G. R. Human κ opiate receptor second extracellular loop elevates dynorphin's affinity for human μ/κ chimeras. *J. Biol. Chem.* **1994**, *269*, 25966–25969.
(37) Ferguson, D. M.; Kramer, S.; Metzger, T. G.; Law, P. Y.; Portoghese, P. S. Isosteric replacement of acidic with neutral residues in extracellular loop-2 of the κ-opioid receptor does not affect dynorphin A(1–13) affinity and function. *J. Med. Chem.* **2000**, *43*, 1251–1252.
(38) Paterlini, G.; Portoghese, P. S.; Ferguson, D. M. Molecular simulation of dynorphin A(1–10) binding to extracellular loop 2 of the κ-opioid receptor. A model for receptor activation. *J. Med. Chem.* **1997**, *40*, 3254–3262.
(39) Singh, N.; Cheve, G.; Ferguson, D. M.; McCurdy, C. R. A combined ligand-based and target-based drug design approach for G-protein coupled receptors: Application to salvinorin A, a selective κ opioid receptor agonist. *J. Comput.-Aided Mol. Des.* **2006**, *20*, 471–493.
(40) Xu, W.; Campillo, M.; Pardo, L.; Kim de Riel, J.; Liu-Chen, L. Y. The seventh transmembrane domains of the δ and κ opioid receptors have different accessibility patterns and interhelical interactions. *Biochemistry* **2005**, *44*, 16014–16025.
(41) Akabas, M. H.; Stauffer, D. A.; Xu, M.; Karlin, A. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science* **1992**, *258*, 307–310.

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