A unique binding epitope for salvinorin A, a non-nitrogenous kappa opioid receptor agonist

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Salvinorin A is a potent kappa opioid receptor (KOP) agonist with unique structural and pharmacological properties. This non-nitrogenous ligand lacks nearly all the structural features commonly associated with opioid ligand binding and selectivity. This study explores the structural basis to salvinorin A binding and selectivity using a combination of chimeric and single-point mutant opioid receptors. The experiments were designed based on previous models of salvinorin A that locate the ligand within a pocket formed by transmembrane (TM) II, VI, and VII. More traditional sites of opioid recognition were also explored, including the highly conserved aspartate in TM III (D138) and the KOP selectivity site E297, to determine the role, if any, that these residues play in binding and selectivity. The results indicate that salvinorin A recognizes a cluster of residues in TM II and VII, including Q115, Y119, Y312, Y313, and Y320. Based on the position of these residues within the receptor, and prior study on salvinorin A, a model is proposed that aligns the ligand vertically, between TM II and VII. In this orientation, the ligand spans residues that are spaced one to two turns down the face of the helices within the receptor cavity. The ligand is also in close proximity to EL-2 which, based on chimeric data, is proposed to play an indirect role in salvinorin A binding and selectivity.

Salvinorin A is a potent kappa opioid receptor (KOP) agonist that is isolated from the leaves of *Salvia divinorum* [1]. It has been reported to produce similar behavioral effects to mescaline in mice and strong hallucinogenic activity in humans [2]. The psychoactive dose in humans is ~200–500 µg, making it one of the most potent hallucinogens known [2]. Traditionally, *S. divinorum* extracts have been used by the Mazatec Indians of north-eastern Oaxaca, Mexico primarily for its psychotropic activities to aid in spiritual rituals [3]. In addition, the extracts have been utilized for various ailments such as providing relief from headaches, and facilitating defecation and urination [3]. Throughout its use, salvinorin A has not shown any addictive potential and therefore, could serve as a template for

the development of non-addictive opioids as it has been shown to have analgesic activity in mice [4].

The structure of salvinorin A lacks key chemical features historically associated with opiate ligand activity [1]. A comparison of salvinorin A with morphine shows that the former lacks both the amino functionality and the phenolic moiety common to opiate-based ligands (Fig. 1). Moreover, the absence of a protonatable group places salvinorin A in a unique class of opioid ligand.

Over the years, a number of models have been proposed to explain the binding and selectivity of opioid ligands [5–9]. It has become fairly well established that KOP-selective opiates recognize three key elements within the opioid receptors: a highly conserved
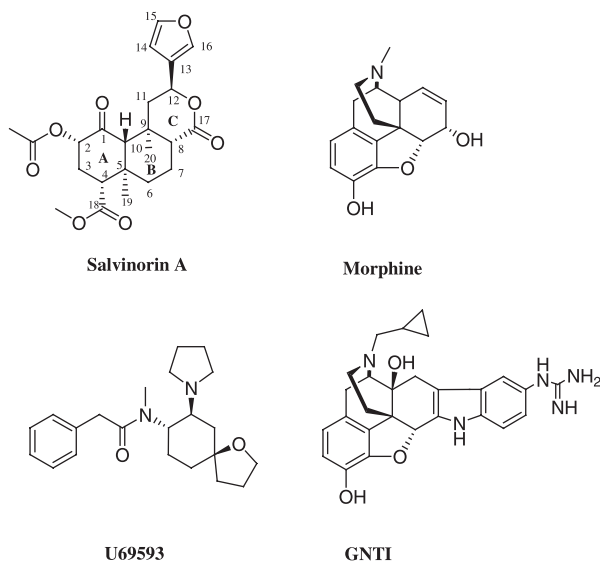
Fig. 1. Representative opioid ligands.

aspartate in transmembrane (TM) III (D138); a conserved aromatic pocket formed by TM V, VI, and VII; and a KOP-specific selectivity site at the extracellular boundary of TM VI (E297) [10–16]. This model was first proposed to explain the selectivity of a series of naltrexone-based ligands, and was subsequently applied in the design of the KOP-selective antagonist, guanidinyl naltrindole (GNTI) [13]. The pharmacophore established anchors the morphine-core of GNTI to the receptor cavity via a salt-link interaction with D138, while the guanidinyl group is projected toward E297 at the top of TM VI [13]. This model, however, has not found widespread application to other classes of opioid ligands, such as the KOP-selective agonist U69593 [17,18]. Although this ligand shares the common D138 salt-link anchor [19], ligand-binding experiments have shown that E297 is not required for high-affinity binding [12]. This is not surprising

because U69593 not only is too small to span D138 and E297, but also lacks a second ionizable group. Although the precise mechanism by which U69593 and other non-opiates attain selectivity is not known, most data points to involvement of residues at the extracellular end of TM VI and VII as well as indirect contacts with EL-2 [18,20–26].

Two binding models for salvinorin A at the KOP have recently been reported (Fig. 2) [1,27]. The initial model was based on structural similarities between salvinorin A and U69593 [1]. As Roth and co-workers point out, these compounds share very few similarities, significantly limiting comparative analysis [1]. Regardless, models were developed based on overlap of the furan ring of salvinorin A and the phenyl ring of the arylacetamides. The centroids of the aromatic rings and the carbonyl bonds were overlaid and docked [1]. From that study, it was concluded that the lactone carbonyl of salvinorin A interacts with the tyrosine (Y139) residue. It was also concluded that the furan ring points toward TM I and II, the 4-methoxycarbonyl points towards TM V and VI, the A ring toward the extracellular side, and the C ring toward the intracellular side. The investigators noted that although there was little atom-to-atom correspondence between salvinorin A and U69593, the two compounds occupy similar space. No mention of the conserved D138 residue and its role was implicated, probably due to the lack of a protonatable nitrogen in salvinorin A. Additional contact points for salvinorin A in the KOP-binding pocket were hypothesized [1]. Specifically, a glutamine in TM II (Q115) was believed to interact with the furan oxygen and a tyrosine in TM VII (Y313) was postulated to interact with the 2-acyl functionality.

A more recent model by Roth and co-workers has also been published [27]. The revised model places the furan ring in key interactions with Y119 and Y320,
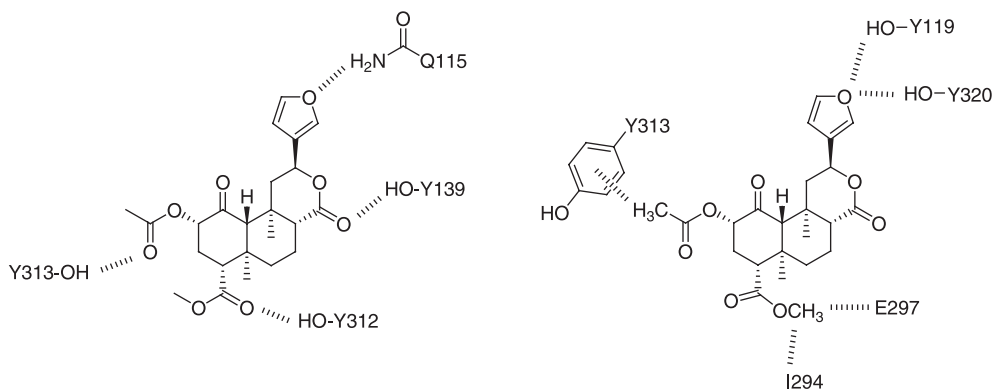

Fig. 2. Initial (left) and revised models of salvinorin A interacting with the KOP.

spanning TM helices II and VII. This places the 4-methyl ester in close proximity to the KOP address site, E297 in TM VI and I294. In this model, Y313 stabilizes the 2-acetyl group via a hydrophobic interaction with the aryl ring. The resulting binding site is comprised of residues from TM II, VI, and VII.

Although these models are insightful, they do not account for some of the more recently acquired structure–function data of salvinorin analogues [28,29]. Semi-synthetic work reported by Harding *et al.* revealed that modification of the 2′-position from the native acetate to a benzoyl ester affords a ligand that retains a high level of affinity for the KOP while also achieving high affinity at the mu opioid receptor (MOP) [29]. This suggests that the MOP and KOP may have overlapping regions that recognize salvinorin A. In addition, some structural features of the current model are hard to rationalize. In particular, the aromatic rings of residues Y119 and Y320 are ∼ 15 Å apart in the KOP-binding site. Given this distance, it is unlikely that both Y119 and Y320 participate in hydrogen bonding interactions with the furan oxygen. Such interactions are short-range effects and tail off quite quickly as a function of distance. Another key interaction proposed involves the KOP-specific site E297. As alluded to above, this residue is an established recognition site for the second cationic amine of opiate ligands such as norbinaltorphimine (norBNI) and GNTI. It is doubtful, however, that salvinorin A recognizes this site because the structure lacks cationic groups. The hypothesis is also inconsistent with the results of Harding *et al.* on MOP binding and selectivity.

This study explores potential binding-site interactions of salvinorin A with the KOP using a combination of wild-type, chimeric, and single-point mutant opioid receptors. The results are compared with previous work on salvinorin A in an effort to further refine current binding-site models and to gain insight into the design of additional salvinorin A analogues. A tentative model is also proposed to explain previous and new data on salvinorin A binding to the opioid receptors.

Results and Discussion

Chimera

Although the unique structure of salvinorin A, provides a novel scaffold for the design of new opioid ligands, it also provides a great challenge when attempting to create structural models. For most opioid ligands, the construction of structural models begins with docking the protonated amine to the conserved aspartate in TMIII [5–9]. Because such an interaction is not present in salvinorin A, an alternative approach must be undertaken. In these cases, a common starting point for examining nascent receptor–ligand interactions is by utilizing chimeric receptors. These chimeric receptors probe various regions of the receptors and generally lead to inferences about which regions are required for binding. These receptors are constructed around common restriction sites found within the receptors. The *Afl*III and *Bgl*II sites were chosen, first, because of their location (the *Afl*III and *Bgl*II sites are approximately one- and two-thirds of the way into the receptor, respectively), and second, because chimeric receptors utilizing these sites have been studied previously [22,30–33], allowing for the comparison of several data sets across multiple ligand classes. Schematics of these chimeric receptors are seen in Fig. 3. Before chimeric binding studies commenced, control experiments were conducted on wild-type KOP, delta opioid receptor (DOP), and MOP. The observed affinities for salvinorin A at these receptors were 17.5 nM > 25 000, and > 25 000 nM, respectively, as reported in Table 1. All chimera were also evaluated for their ability to bind diprenorphine. In all cases but one (the DOP/KOP *Afl*III chimera), the chimeras maintained a K_d value similar to wild-type values.
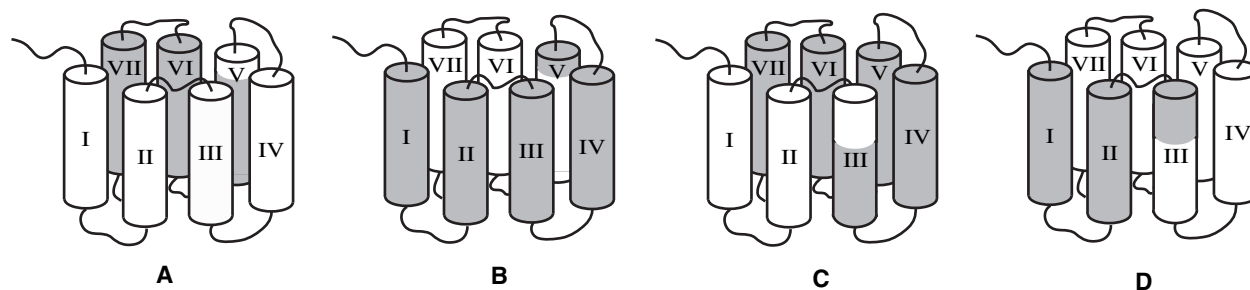

Fig. 3. Chimeric receptors utilizing restriction sites *Bgl*I (A, B) or *Afl*III (C, D). KOP fragments are represented by white while MOP and DOP are indicated by grey.

Table 1. *Bgl*II and *Afl*III chimeric data for salvinorin A.

Receptor	K_i (nM) [a] ± SEM
*Bgl*II	
KOP (wt)	17.5 ± 1.5 (3)
DOP (wt)	> 25 000 (2)
MOP (wt)	> 25 000 (2)
KOP (1–227)/DOP (215–372)	2.5 ± 0.4 (4)
KOP (1–227)/MOP (234–398)	2270 ± 880 (5)
DOP (1–214)/KOP (228–380)	> 25 000 (2)
MOP (1–233)/KOP (228–380)	1500 ± 210 (4)
*Afl*III	
KOP (1–141)/DOP (132–372)	910 ± 245 (5)
KOP (1–141)/MOP (151–398)	4650 ± 1400 (3)
DOP (1–131)/KOP (142–380)	N.D. [b]
MOP (1–150)/KOP (142–380)	351 ± 42 (3)

[a] The K_i values were determined in competition binding using [³H] diprenorphine in transiently expressed HEK293 cells and analyzed by whole-cell binding. The number of individual determinations is indicated in parentheses (*n*). [b] This chimera did not bind to [³H] diprenorphine.

Among the *Bgl*II chimeric-binding studies, one chimera stood out due to its increased ability to bind salvinorin A. The KOP/DOP chimera bound to salvinorin A with an affinity of 2.5 nM, or nearly 10 times the affinity of wild-type KOP. Meanwhile, the converse chimera (containing the DOP/KOP *Bgl*II sequence) did not bind salvinorin A (K_i > 25 000 nM). One possible explanation for this is that the recognition elements responsible for binding salvinorin A are found before the *Bgl*II restriction site in TM I–IV in EL-1, or in EL-2. If this is the case, then one might suspect that the KOP/MOP *Bgl*II chimera would bind salvinorin A with an affinity similar to that of wild-type KOP. However, as the data shows, there is a significant decrease in affinity for salvinorin A at the KOP/MOP chimera (2270 nM), but interestingly, a marked gain in affinity over the wild-type MOP.

The difference in affinity seen between the KOP/MOP chimera and the KOP/DOP chimera (2270 versus 2 nM) may be the result of 'selectivity by means of exclusion.' In other words, salvinorin A may exhibit decreased affinity for the KOP/MOP chimera not because of modifications to residues that stabilize its binding, but rather from other regions of the receptor that prevent the native interactions from occurring. In this case, residues of the MOP after the *Bgl*II site may block or disrupt binding through steric interactions or electronic effects. Such a mechanism has been proposed in the past by Metzger & Ferguson to explain selectivity among the KOP, MOP, and DOP [34].

The remaining *Bgl*II chimera (MOP/KOP), recognizes salvinorin A with a modest affinity of 1500 nM.

This result, when compared with the DOP/KOP *Bgl*II chimera (which does not recognize salvinorin A, K_i > 25 000) suggests that there may be regions of MOP before the *Bgl*II site that participate in the recognition of salvinorin A. This explanation seems very plausible because some salvinorin A analogues have been shown to have high affinity for MOP. Alternatively, there could be regions of the KOP in TM V, VI, and VII, and EL-3 that also help to stabilize binding.

The second set of chimeras was designed around an *Afl*III restriction site, found in the middle of TM III. Results from these chimeras are summarized in Table 1. Of considerable importance are results from the KOP/DOP *Afl*III chimera which show a marked loss in affinity compared with the KOP/DOP *Bgl*II chimera (910 versus 2.5 nM). Similarly, the KOP/MOP *Afl*III chimera also exhibits a decrease in binding compared with its *Bgl*II counterpart (∼ 4700 versus ∼ 2300 nM). Collectively, the data suggests that the KOP region between the *Afl*III and *Bgl*II sites (the bottom of TM III, IL-2, TM IV, and EL-2), may play a role in binding salvinorin A. Of these four regions, the bottom of TM III and IL-2 are unlikely sites of interaction due to their depth within the receptor. Of the remaining two regions, EL-2 has been implicated in past studies of KOP ligand binding and selectivity [20–25]. This loop interacts with EL-1 via a disulfide linkage and is thought to partially cover the receptor cavity. Given the data reported here, and past reports linking EL-2 to KOP binding and selectivity [20–25], it is reasonable to conclude that this loop plays some role in salvinorin A binding and selectivity. This hypothesis is further supported by the MOP/ KOP *Afl*III chimera, which also exhibits considerable affinity for salvinorin A (∼ 350 nM).

Single-point mutants

To compliment the chimeric studies, site-directed mutagenesis studies were conducted (results summarized in Table 2). The residues that had been suggested to interact with salvinorin A (as seen in Fig. 2) were examined. Point mutations revealed decreases in binding affinity for both Q115 and Y313. These residues are near the top of TM helices II and VII which lie across from each other in the TM bundle. Closer evaluation of the data for Y313 shows that the Y313F mutant retains affinity close to that of the wild-type, suggesting that Y313 may be involved in pi-stacking or other favorable hydrophobic interactions with the ligand. Similar arguments can be made for Y320. This residue, however, lies approximately two helical turns into the TM domain and may

Table 2. Binding values for salvinorin A.

Receptor	K_i (nM) ± SEM	F_{mut} [a][27]	K_i (nM)
KOP	17.5 ± 1.5 (3)		31.6
KOP [Q115A]	147 ± 47 (2)	8.4	
KOP [Y119A]	67 ± 7.4 (3)	3.8	342
KOP [Y119F]	17.7 ± 3.9 (3)	1	233
KOP [D138A]	17.5 ± 4.4 (4)	1	
KOP [Y139F]	9.5 ± 2.8 (5)	0.54	93
KOP [E297A]	19.5 ± 3.1 (4)	1.1	
KOP [Y312A]	79 ± 28 (5)	4.5	88.6
KOP [Y312F]	16 ± 3.8 (3)	0.91	65.1
KOP [Y313A]	126 ± 48 (5)	7.2	694
KOP [Y313F]	37 ± 3.7 (4)	2.1	63.3
KOP [Y320A]	565 ± 49 (2)	32	380
KOP [Y320F]	71 ± 15 (3)	4.1	301
MOP	> 25 000 (2)		
DOP	> 25 000 (2)		

[a] F_{mut} = mutational factor, K_i (mutant receptor)/K_i (wt receptor).

represent the 'floor' of the binding pocket. Because some activity is lost on mutation of this tyrosine to phenylalanine, it is not possible to rule out a hydrogen bond interaction for this group. There is some evidence (albeit weak) that Y119, which lies approximately one helical turn above Q115, may also be involved in a pi-stacking effect. Given the close proximity of these residues, it is fair to say that they may share a common pocket in stabilizing the binding of salvinorin A to the KOP.

The remaining residues proposed in Fig. 2 do not appear to have a significant impact on salvinorin A binding. Of particular interest is the lack of effect Y139 appears to have on binding. This residue is adjacent to the highly conserved aspartate D138 which is known to form an anchor-point for the cationic amine of many opioid ligands. Y139 has also been implicated in opioid ligand binding, mainly to the DOP [35]. Although our data tends to rule out a hydrogen bonding effect for Y139, we can not rule out pi-stacking effects as suggested by Ferguson and co-workers for SNC80 recognition at the DOP [36]. This type of effect, however, is unlikely because salvinorin A cannot form cation–pi interactions with Y139 (analogous to that proposed for SNC80). Unfortunately, attempts to express the Y139A mutant were unsuccessful. While our data does show some disparity with that reported by Yan *et al.* (as shown in Table 2) the trends are similar. Moreover, the majority of mutations result in less than tenfold changes in affinity, suggesting these residues play a minor role in salvinorin A binding.

Mutations were also performed to examine well-established sites of recognition in the KOP. In partic-

ular, we were very interested in examining the effect of mutating D138 and E297 on binding affinity. These two residues have been shown to form salt links with KOP-selective ligands such as GNTI [11–14,16]. In addition, a recent study has proposed E297 may also be involved in recognizing the 4-substituent of salvinorin A [27]. While D138 mutants typically show dramatic changes in binding affinities to opioid ligands [19,35], no change was noted for salvinorin A. This is also true of the E297A mutant. The results, however, are not surprising given the structure of salvinorin A, which lacks protonatable groups that are characteristic of aminergic ligands.

Conclusion

The results of this study suggest that salvinorin A recognizes the KOP through a unique binding epitope involving TM II, VII, and EL-2. This conclusion is somewhat provocative considering most opioid ligands are speculated to utilize recognition elements in TM VI to modulate selectivity. This general hypothesis is supported by site-directed mutagenesis data reported here and elsewhere that point to the involvement of Q115 and Y119 from TM II, and, Y312, Y313 and Y320 from TM VII in binding salvinorin A. While it is quite difficult to determine the specific role each residue plays in the stabilizing the ligand, this study suggests the tyrosines function through hydrophobic effects, either by pi-stacking or other electronic effects (e.g. charge transfer). In this case, Q115 most likely serves as a hydrogen bond donor. No support, however, was found for the involvement of D138 in TM III or E297 at the rim of TM VI in binding salvinorin A. The chimeric data suggest that elements of EL-2 may also be important to recognition. This is not surprising given the sequence variability of this loop among the opioid receptors and previous studies on the KOP highlighting the importance of EL-2. Prior work on EL-2, however, has failed to identify specific binding sites within the loop [24,25], suggesting that this domain may influence binding through indirect effects (such as long range electrostatics) or through an exclusion-type mechanism. In the latter case, EL-2 of the DOP and MOP function to inhibit salvinorin A binding, either by steric or electrostatic interactions. The chimeric data presented here is also consistent with this mechanism. Of course, some care must be taken in interpreting chimeric data. It is important to point out that most of the chimera containing KOP domains displayed some affinity for salvinorin A, indicating that there are elements from several KOP domains that effect binding. In light of the site-directed mutagenesis

data, such results are not surprising and further point to a binding-site model that involves multiple contacts as opposed to a single point of recognition.

One model that is consistent with the data presented here and elsewhere places salvinorin A vertically into the receptor cavity bridging TM II and VII as shown in Fig. 4 (The schematic is based on molecular docking of salvinorin A to the KOP using the Insight II Molecular Modeling System. The receptor structure was taken from our previous work on KOP-ligand receptor modeling and is available at http://opioid.pharmacy.umn.edu. The coordinates for salvinorin A were built interactively and subsequently optimized using the Discover Module of Insight II.) In this binding-site model, salvinorin A vertically spans residues Y119 and Y320, as well as Q115, Y313 and Y312. This is one of the few orientations that accounts for the spacing of these residues along the face of TM II and VII. As in the model proposed by Yan *et al.*, the ligand is still in close proximity to EL-2. This orientation also places the 2′-position of the ligand into the EL-3 domain which is highly variable in sequence across the opioid receptors. Given the importance of residues in this domain in conferring selectivity, the model may also help rationalize the MOP affinity reported by Harding *et al.* [29] for 2′-benzoyl salvinorin. Although the model is only qualitative, it does begin to explain the structural basis to differences in
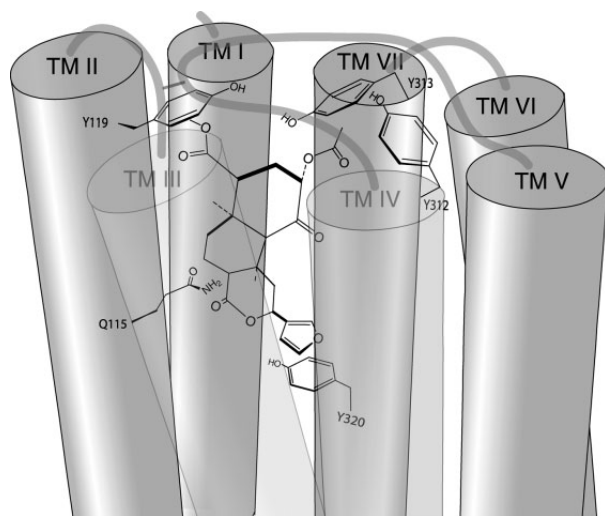
salvinorin A binding and selectivity and traditional opioid ligands that utilize stronger salt–link interactions with TM III and VI. The idea that salvinorin A would primarily take advantage of hydrophobic contacts within the KOP should come as no surprise. The determination of each contact involved and the precise orientation of salvinorin A in the KOP binding site, however, may prove quite challenging given the potential number of contacts and varied strength of hydrophobic forces.

Experimental procedures

Chimeric receptors and single point mutants

Rat KOP, MOP, and mouse DOP cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). Chimeric receptors were constructed by utilizing restriction sites to swap sequences between the receptor types. For the *Bgl*II chimeras, a restriction digest using *Bgl*II (New England Biolabs, Ipswich, MA), was followed by resolution of the fragments on a 0.8% agarose gel (GibcoBRL, ultraPURE, Invitrogen). The fragments were excised, purified using GENE-CLEAN II (BIO 101, Inc., Irvine, CA), and religated using LigaFast™ Rapid DNA Ligation System (Promega, Madison, WI). Note, a *Bgl*II site was introduced into the rMOP using methods described below. Similar procedures were utilized for the construction of the *Afl*III chimeras. However, in these cases, triple ligations using *Nde*I/*Afl*III, *Afl*III/*Apa*I, and *Apa*I/*Nde*I (New England Biolabs) fragments were conducted. Aliquots from the ligation mixtures were transformed into XL-1 Blue competent cells (Stratagene. La Jolla, CA). Colonies were screened for the correct chimera and then amplified using Qiafilter Plasmid Maxi Kit (Qiagen, Valencia, CA). The chimeric sequence was verified by the BMGC DNA Sequencing and Analysis Facility (University of Minnesota) on an ABI PRISM 3100 Genetic Analyzer.

For the single point mutants, primers were purchased from Integrated DNA Technologies (Coralville, IA). Mutations were introduced using the QuikChange Site-directed Mutagenesis Kit (Stratagene). Similar experimental procedures (as above) were conducted to purify and to confirm the correct mutant.

Fig. 4. Schematic depiction of salvinorin A docked to the KOP (The schematic is based on molecular docking of salvinorin A to the KOP using the Insight II Molecular Modeling System. The receptor structure was taken from our previous work on KOP-ligand receptor modeling and is available at http://opioid.pharmacy.umn.edu. The coordinates for salvinorin A were built interactively and subsequently optimized using the Discover Module of Insight II.)

Transient transfection

Human embryonic kidney (HEK293) cells (purchased from ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and in 10% CO_2. Cells were seeded to 20–30% approximately 24 h before transfection. Fresh media was added 1–2 h before transfection. Cells were transfected with plasmid cDNA (10–20 μg

per 100 mm plate) using the calcium phosphate precipitation method [37]. Medium was replaced 5 h later.

Receptor binding assays

Transfected cells were harvested at 48–72 h post transfection. These cells were washed three times with 25 mM Hepes buffer (pH 7.4) and then resuspended with 8–12 mL of 25 mM Hepes per 100 mm plate. K_d values were determined from saturation binding assays using [^{3}H]diprenorphine. Specifically, eight different concentrations of [^{3}H]diprenorphine (typically ranging from 25 pM to 3 nM) were used. Nonspecific values were established by the addition of 100 μM naloxone, norBNI, or salvinorin A, depending on which ligand showed the greatest inhibition. Three independent experiments (each in triplicate) were conducted, and a mean K_d was determined. All of the mutants and chimeras maintained a K_d value similar to wild-type values, suggesting that there were no major changes in overall receptor structure. Competitive binding experiments were conducted utilizing a [^{3}H] diprenorphine concentration of $0.5–1.0 \times K_d$. Nine concentrations of salvinorin A (in triplicate) were used in the displacement analysis. Again, naloxone, norBNI, or salvinorin A was used at 100 μM for nonselective binding. The Cheng–Prusoff equation allowed the conversion of IC$_{50}$ values to K_i [38].

Transfected cells were incubated at room temperature for 90 min in a total binding volume of 0.5 mL and were terminated by filtration through a Whatman GF/C filter (Brandel, Gaithersburg, MD) that had been presoaked in 0.25% poly(ethylenimine) (Sigma-Aldrich, St Louis, MO) immediately prior to filtration. Filters were washed three times with 4 mL of ice-cold 25 mM Hepes buffer, and scintillation counting was performed by a Beckman 3801 LS scintillation counter. In all cases, the data was fit to a simple one-site model using PRISM (GraphPad Software, Inc., San Diego, CA). [^{3}H]Diprenorphine (specific activity, 50 Ci·mmol^{-1}) was purchased at New England Nuclear (Boston, MA).

Isolation of salvinorin A

Salvinorin A was obtained by reported extraction and purification methods [39] from *S. divinorum* leaves harvested from plants (*Theatrum botanicum*, Laytonville, CA, USA) propagated at the University of Mississippi. Purified, crystalline salvinorin A agreed with published characterization data [40].

Acknowledgements

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