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THE SEVENTH TRANSMEMBRANE DOMAINS OF THE δ AND κ OPIOID RECEPTORS HAVE DIFFERENT ACCESSIBILITY PATTERNS AND INTERHELICAL INTERACTIONS[†]

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Abstract

We applied the substituted cysteine accessibility method (SCAM) to map the residues of the transmembrane helices (TMs) 7 of δ and κ opioid receptors (δ OR and κ OR) that are on water-accessible surface of the binding-site crevices. Twenty five consecutive residues (except C7.38) in the TMs7 were mutated to Cys, one at a time, and each mutant was expressed in HEK 293 cells. Most mutants displayed similar binding affinity for [³H]diprenorphine, an antagonist, as the wildtypes. Pretreatment with (2-aminoethyl) methanethiosulfonate (MTSEA) inhibited [³H]diprenorphine binding to eight δ OR and eight κ OR mutants. All mutants except δ OR L7.52(317)C were protected by naloxone from MTSEA effect, indicating that the side chains of V7.31(296), A7.34(299), I7.39(304), L7.41(306), G7.42(307), P7.50(315) and Y7.53(318) of δ OR and S7.34(311), F7.37(314), I7.39(316), A7.40(317), L7.41(318), G7.42(319), Y7.43(320) and N7.49(326) of κ OR are on water-accessible surface of the binding pockets. Combining the SCAM data with rhodopsin-based molecular models of the receptors led to the following conclusions. *i*) The residues of the extracellular portion of TM7 predicted to face TM1 are sensitive to MTSEA in κ OR, but are not in δ OR. Thus, TM1 may be closer to TM7 in δ OR than in κ OR. *ii*) MTSEA-sensitive mutants start at position 7.31(296) in δ OR and at 7.34(311) in κ OR, suggesting that TM7 in δ OR may have an additional helical turn (from 7.30 to 7.33). *iii*) There is a conserved H-bond network linking D2.50 of the NLxxxD motif in TM2 with W6.48 of the CWxP motif in TM6. *iv*) The NPxxY motif in TM7 interacts with TM2, TM6, and helix 8 to maintain receptors in inactive states. To the best of our knowledge, this represents the first such comparison of the structures of two highly homologous GPCRs.

More than one thousand G protein-coupled receptors (GPCRs) are present in the human genome. GPCRs play important roles in physiological functions, including smell, taste, light perception, neurotransmission, functions of endocrine and exocrine glands and immune functions. In addition, GPCRs are targets of numerous clinically used drugs. It is, therefore, of great interests to understand their structures at the molecular level. Based on structural characteristics, GPCRs are classified into multiple families and the rhodopsin family is by far the largest in number. To date, rhodopsin is the only GPCR of which high-resolution crystal

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structures have been elucidated (1-3). Rhodopsin has an extracellular N-terminal domain, seven transmembrane helices (TMs) connected by alternating intracellular and extracellular hydrophilic loops and an intracellular C-terminal domain. A short helix, helix 8 (H8), parallel to the plane of plasma membranes is present in the proximal region of the C-terminal domain. GPCRs of the rhodopsin family are thought to have similar 7-TM bundles (4,5).

Opioid receptors mediate effects of opiate and opioid compounds, which are important therapeutic agents for pain management. Multiple opioid receptors (at least μ , δ and κ) have been defined pharmacologically. Following the cloning of the mouse δ opioid receptor, μ and κ receptors were cloned [(6,7) and references therein]. These receptors belong in the rhodopsin family (family A) of GPCRs. These opioid receptors are coupled through pertussis-toxin sensitive G proteins to affect a variety of effectors [for a review, (8)]. Several groups have constructed molecular models of opioid receptors [for example, (9-12)].

Results from experimental probes have indicated that binding pockets of GPCRs involve the seven TMs and are accessible from the extracellular medium. Some water-accessible residues within the binding pocket directly interact with ligands. Javitch and his colleagues have used charged, hydrophilic methanethiosulfonate (MTS) reagents, which react specifically with reduced sulfhydryl groups, to identify Cys residues accessible in the binding pocket of the D₂ dopamine receptor and applied substituted cysteine accessibility method (SCAM) to map residues within the TMs of this receptor exposed in the binding-site crevice [for reviews, (5, 13)]. We have also employed SCAM to identify and compare residues accessible in the binding-site crevice in the TMs 6 of the μ , δ and κ opioid receptors (14). MTS reagents react 10^9 times faster with ionized thiolates (S^-) than with unionized thiols (SH) (15) and ionization of cysteine occurs to a significant extent only in the aqueous medium (16). Thus, the reaction rate of these MTS reagents is expected to be much faster with cysteine residues at the water-accessible surface than with those that are not. SCAM analysis involves substitution of each residue with cysteine within a TM one at a time and the effects of reaction with MTS reagents on ligand binding were determined. Those that results in binding inhibition are inferred to be on the water-accessible binding site crevice.

Studies have been performed to characterize interaction of ligands with opioid receptors. The region of the TMs 6 and 7 and the third extracellular (e3) loop was demonstrated to play important roles in ligand binding selectivity of μ , δ and κ opioid receptors by site-directed mutagenesis and chimeric receptor studies [for example, (17-20)].

TMs 7 of rhodopsin family of GPCRs have been documented to play important roles in maintaining receptors in inactive states. Disruption of an ionic interaction between a positively charged residue in TM7 and a negatively charged residue in TM3 in rhodopsin (21), α_{1b} -adrenergic (22), and δ opioid (23) receptors leads to constitutive activation. Hydrogen bonding interactions between TM2 and TM7 has been suggested to be important for the activity of gonadotropin-releasing hormone, 5-hydroxytryptamine_{2A} (5-HT_{2A}), and μ opioid receptors (24-26). In addition, there is an interaction between Y7.53 in TM7 and an aromatic residue F/Y7.60 in H8 of the NPXXYX₅₋₆F/Y7.60 motif in the inactive states of rhodopsin (27) and the 5HT_{2C} receptor (28).

We have shown that μ , δ and κ opioid receptors are differentially sensitive to MTSEA, CH₃SO₂SCH₂CH₂NH₃⁺, in the order of $\kappa > \mu > \delta$ and the conserved Cys7.38 largely confers the MTSEA sensitivity in each receptor (29). While C7.38 in the μ and κ receptors is readily accessible from the binding-site crevice, this conserved residue in the δ receptor appears to be much less accessible (29). In the present study, we applied the SCAM to further explore the differential exposure of C7.38 to the binding site crevice and identify and compare the amino acid residues within the TM 7 of the δ and κ opioid receptors that are accessible from the

extracellular medium. In addition, the experimental results are interpreted in the context of rhodopsin-based computational models of the δ OR and the κ OR. These comparative results serve to identify unique features in the δ and κ ORs as well as common structural patterns in GPCRs. Comparison of amino acid sequences of TMs 7 of the δ and κ ORs and rhodopsin is shown in Fig. 1.

EXPERIMENTAL PROCEDURES

Materials

[3 H]Diprenorphine (58 Ci/mmol) was purchased from Perkin-Elmer Co. (Boston, MA). Naloxone was a gift from the former DuPont/Merck Co. (Wilmington, DE). MTSEA was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Enzymes and chemicals used in molecular biology and mutagenesis experiments were purchased from Life Technologies Co. (Gaithersburg, MD), Promega (Madison, WI), Boehringer-Mannheim Co. (Indianapolis, IN) and Qiagen Co. (Valencia, CA).

Numbering schemes for amino acid residues in opioid receptors

Two numbering schemes were used. Amino acid residues in the δ OR and κ OR are identified by their sequence numbers. In addition, the generic numbering scheme of amino acid residues in GPCRs according to Ballesteros and Weinstein (30) is used. According to this nomenclature, the most conserved residue in the TM7 of human δ and human κ ORs is Pro7.50 (δ , Pro315; κ , Pro327). The boundary for the TMs 7 of δ and κ ORs are 7.31–7.56 (δ , V296-L321; κ , A308-L333) based on the models of Strahs and Weinstein (9) with modifications. The generic numbering allows easy comparison among the opioid receptors and cross-reference to other GPCRs in the literature.

Oligodeoxynucleotide-directed mutagenesis

Site-directed mutagenesis was performed on the C7.38(315)S mutants of the human κ receptors and the wildtype human δ OR with the overlap PCR method described by Higuchi *et al.* (31). FLAG-tagged human wildtype and mutant δ receptors were subcloned into EcoR I and BamH I sites of the vector pIRESneo (originally described as pCIN4). FLAG-tagged human wildtype and mutant κ receptors were subcloned into HindIII and XhoI sites of the vector pcDNA3 (29). DNA sequence was determined to confirm the presence of desired mutations and the absence of unwanted mutations.

Transfection of HEK293 cells

HEK 293 cells were grown in 100-mm culture dishes in Minimum Essential Medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37°C. Cells were transfected with the wildtype or a mutant of κ OR DNA (5 μ g/dishes plus 15 μ g vector) using the calcium phosphate method (32). Sixty to 72 h after transfection, cells were harvested for experiments by detaching with Versene solution. Transfection of HEK cells with the DNA clones of the wildtype or a mutant of the human δ OR in pIRESneo was performed with Lipofectamine according to the manufacturer's instructions, cells were grown under the selection pressure of geneticin (0.8 mg/ml) and nearly all surviving colonies stably expressed the receptor.

Determination of K_d and B_{max} value of [3 H]diprenorphine binding

Membranes were prepared from transfected HEK cells as described previously (33). Saturation binding of [3 H]diprenorphine to the wildtype and mutant δ and κ receptors was performed with at least 6 concentrations of [3 H]diprenorphine (ranging from 25 pM to 2 nM) and K_d and B_{max} values were determined. Binding was carried out in 50 mM Tris-HCl buffer containing

1 mM EGTA and 10 μ M leupeptin (pH 7.4) (TEL buffer) at room temperature for 1 h in duplicate in a volume of 1 ml with 10~20 μ g membrane protein. Naloxone (10 μ M) was used to define non-specific binding. Binding data were analyzed with the EBDA programs(34).

Reaction with MTSEA

The experiments were performed according to our published procedure (14). Transfected cells were detached by use of Versene solution, pelleted at 1,000 x g for one min at room temperature and washed with Krebs's solution (NaCl 130 mM, KCl 4.8 mM, KH₂PO₄ 1.2 mM, CaCl₂ 1.3 mM, MgSO₄ 1.2 mM, glucose 10 mM, and HEPES 25 mM, pH7.4) and centrifuged again. The pellets were re-suspended in Krebs's solution and aliquots of cell suspension were incubated with freshly prepared MTSEA at the stated concentration in a final volume of 0.5 ml at room temperature for 5 min. The reaction was stopped by adding 0.5 ml of 0.8 % BSA solution. The cell suspensions were pelleted and washed once with Krebs's solution. After centrifugation, the pellet were re-suspended in 1 ml/dish Krebs's solution and 50 μ l aliquots were used for [³H] diprenorphine binding to intact cells at room temperature for 1 h as described previously (35). Naloxone (10 μ M) was used to define nonspecific binding. The % inhibition was calculated as [1-(specific binding after the MTS reagent/specific binding without the reagent)] x 100%. Data were analyzed by one-way ANOVA followed by post hoc Sheffe F test using $p < 0.05$ as the level of significance.

Determination of second-order rate constants

The second-order rate constants of interaction between the δ OR or κ OR mutants and MTSEA was determined to gain quantitative information on MTSEA sensitivity, according to our published method (36). Cells expressing a mutant receptor were incubated with indicated concentrations of MTSEA for 5 min. The results were fit to the equations:

$$Y = (\text{extent of inhibition}) e^{-kct} + \text{plateau}; \quad \text{extent of inhibition} + \text{plateau} = 1.0$$

Y is the fraction of the initial binding, k is the second-order rate constant ($\text{M}^{-1}\text{sec}^{-1}$), c is the concentration of MTSEA (M) and t is the incubation time (300 s).

Protection by naloxone against MTSEA reaction

Dissociated cells were incubated with indicated concentrations of naloxone for 20 min for binding to the δ and κ receptors to reach equilibrium. Cells were then treated with a concentration of MTSEA that was just sufficient to achieve maximal inhibition of binding to each receptor. Cells were washed three times by centrifugation and then re-suspended in Krebs's solution and assayed for [³H]diprenorphine binding.

Determination of protein content

Protein contents of membranes were determined by the bicinchoninic acid method of Smith *et al.* (37) with bovine serum albumin as the standard.

Construction of molecular models of the δ and κ ORs

Models of the δ OR and the κ OR were constructed by homology modeling using the crystal structure of bovine rhodopsin (PDB code 1GZM) (2) as template. Included in the models are the TMs 1–7, the intracellular loops 1–3, the extracellular loops 1–3, the second extracellular (e2) loop forming the disulfide-bonded cysteine to TM5, and the H8 that expands parallel to the membrane. The residues considered to be most conserved in the Class A family of GPCRs were aligned and assigned as #.50, in which # represents the TM according to the nomenclature of Ballesteros and Weinstein (30). These include D2.50 (rhodopsin-83, δ 95, κ -105), R3.50

(rho-135, δ -146, κ -156), W4.50 (rho-161, δ -173, κ -183), P5.50 (rho-215, δ -225, κ -238), P6.50 (rho-267, δ -276, κ -289), P7.50 (rho-303, δ -315, κ -327). Opioid receptors contain the T2.56-X-P2.58 motif in TM2. This motif orients the extracellular moiety of TM2 toward TM3 (38). Therefore, TM2 and TM3 were modeled as has been suggested for the chemokine CCR5 receptor (39). Water molecules 2, 7, and 9 observed in the D2.50/N7.49/Y7.53 environment of rhodopsin (2) are also included in the model. SCWRL-3.0 was employed to add the side chains of the non-conserved residues based on a backbone-dependent rotamer library (40). These molecular models were placed in a rectangular box ($\sim 80 \text{ \AA} \times 93 \text{ \AA} \times 75 \text{ \AA}$ in size) containing a lipid bilayer (~ 95 molecules of palmitoylcholine and ~ 13000 molecules of water resulting in a final density of $\sim 1.0 \text{ g cm}^{-3}$).

The receptor-lipid bilayer systems were subjected to 500 iterations of energy minimization and then heated to 300 K in 15 ps. This was followed by an equilibration period (15 to 250ps) and a production run (250 to 500ps) at constant pressure with anisotropic scaling, using the particle mesh Ewald method to evaluate electrostatic interaction. During the processes of minimization, heating, and equilibration a positional restraint of $10 \text{ kcal mole}^{-1} \text{ \AA}^{-2}$ was applied to the C_{α} atoms of the receptor structure. This simulation protocol seems adequate to adapt the rhodopsin template to the structural requirements of the opioid receptors side chains, with the aim of understanding the local helix-helix interactions. Structures were collected for analysis every 10 ps during the production run (25 structures per simulation). Representative structures for each trajectory were selected by automatically clustering the collected geometries into conformationally-related subfamilies with the program NMRCCLUS (41). The molecular dynamics (MD) simulations were run with the Sander module of AMBER 8 (42), the ff99 force field (43), a 2 fs integration time step, and constant temperature of 300 K.

RESULTS

Effect of cysteine substitutions on the affinity of the δ OR and κ OR for [^3H]diprenorphine

Each residue in the TMs 7 was mutated to cysteine, one at a time, and mutant receptors were expressed in HEK293 cells. Saturation binding of [^3H]diprenorphine to each mutant was performed on intact cells with naloxone to define nonspecific binding and K_d and B_{max} values were determined (Table 1 and Table 2). It should be noted that both [^3H]diprenorphine and naloxone can penetrate cell membranes; therefore, the binding assay detected both cell surface and intracellular receptors. S7.46(311)C mutation in the δ OR and P7.50(327)C mutation in the κ OR abolished [^3H]diprenorphine binding although immunoblotting using anti-FLAG antibody showed that both mutants were expressed (data not shown). K_d values of [^3H]diprenorphine binding to all other κ OR and δ OR mutants ranged from 0.3- to 2.1-fold that of the δ OR wild-type and 0.5- to 4.3- fold that of the κ OR C7.38S mutant, respectively (Table 1 and Table 2), indicating that these mutants have similar affinities for [^3H]diprenorphine as the wild-type. We also generated S7.46(311)A mutant of the δ OR and found that this mutant bound [^3H]diprenorphine with similar affinity as the wildtype. Thus, most mutations did not affect the binding pockets substantially. The mutants were expressed to different levels (Table 1 and Table 2). These results suggest that most substituted cysteine side chains may have similar orientations as the residues being replaced at the same positions, which is the basis for SCAM analysis.

Cell surface expression of the wildtype and mutant δ and κ ORs

Intact cells were used in the study since it is necessary to limit the MTSEA reaction to -SH groups accessible from the extracellular medium. Whether receptor constructs were expressed on cell surface is an important issue. We determined the % of each wildtype and mutant receptor expressed on cell surface by ligand binding. Total and cell surface receptor binding were performed with 0.2–0.4 nM [^3H]diprenorphine. For total receptors, nonspecific binding was

defined as binding in the presence of 10 μ M naloxone. For cell surface receptor, nonspecific binding was defined by 10 μ M dynorphin A(1–17) and 10 μ M DPDPE for the κ and δ receptors, respectively (35,44). Naloxone, being highly hydrophobic, is able to penetrate cell membranes, whereas both dynorphin A(1–17) and DPDPE are hydrophilic and thus unable to go through plasma membranes. We found that for all mutant and wildtype receptors that exhibited binding activities, 70% to 80% of the receptors were expressed on cell surface. It should be noted that each of the wildtype and mutant FLAG- κ OR and FLAG- δ OR constructs contains an amino terminal cleavable signal peptide sequence, which enhances translocation of the receptor into the endoplasmic reticulum membrane and thus facilitates expression of functional receptor in plasma membranes (45).

Effect of MTSEA pretreatment on [3 H]diprenorphine binding to the cysteine mutants of the δ OR and the κ OR

Eight of the 25 cysteine-substituted mutants of the δ OR were sensitive to MTSEA: V7.31(296), A7.34(299), I7.39(304), L7.41(306), G7.42(307), P7.50(315), L7.52(317) and Y7.53(318) (Fig. 2). In addition, pretreatment with MTSEA significantly inhibited [3 H]diprenorphine binding to eight of the 25 cysteine substituted mutants of the κ OR: S7.34(311), F7.37(314), I7.39(316), A7.40(317), L7.41(318), G7.42(319), Y7.43(320) and N7.49(326) (Fig. 2).

Protective effect of naloxone against the inhibitory action of MTSEA

All the MTSEA-sensitive mutants (from Fig. 2), except the δ OR L7.52C mutant, were protected by naloxone from MTSEA alkylation (Fig. 3), indicating that V7.31(296), A7.34(299), I7.39(304), L7.41(306), G7.42(307), P7.50(315) and Y7.53(318) of the δ OR and S7.34(311), F7.37(314), I7.39(316), A7.40(317), L7.41(318), G7.42(319), Y7.43(320) and N7.49(326) of the κ OR are on the water-accessible surface of the binding-site crevice. In addition, we showed previously that C7.38(318) of the κ OR was exposed in the binding pocket, but C7.38(303) of the δ OR was not (29).

Determination of second-order rate constants of MTSEA reactions with sensitive mutants

To quantitatively express the inhibitory potency of MTSEA on [3 H]diprenorphine binding to MTSEA-sensitive mutants of the δ OR and κ OR, we determined the second-order rate constants (Table 3). The κ OR mutants, except the 7.42C mutant, have much higher reaction rates than the δ OR mutants. For the κ OR mutants, the rate constants were in the order of $7.39C \gg 7.40C > 7.34C$, $7.37C$, $7.41C > 7.43C > 7.42C > 7.49C$. For the δ OR mutants, the rank order of the rate constant was $7.39C > 7.42C$, $7.50C > 7.31C$, $7.34C$, $7.41C > 7.53C$.

Molecular models of the δ OR and the κ ORs

Fig.4A shows the result of superimposing the initial structure of the molecular dynamic simulation of the δ OR (in tube ribbon) and the rhodopsin template. The κ OR is similar to the δ OR and is not depicted for clarity. Opioid receptors contain the T2.56-X-P2.58 motif in TM2, as the CC chemokine and angiotensin receptors. This motif generates structural differences in the extracellular part of TMs 2 (goldenrod) and 3 (dark red) of the receptor, relative to rhodopsin, without modifying its more compact cytoplasmic surface (38,39). Thus, TM 2 (goldenrod) is predicted to be in close contact with TM3 (dark red) and not with TM1 (crimson) as in rhodopsin. This also induces the relocation of TM3 (dark red) toward TM5.

Fig. 4B shows the amino acid side chains of TM7 (blue) of the δ OR and the κ OR. *Green* residues represent Cys substitutions that were sensitive to MTSEA and protected from MTSEA by naloxone pretreatment. *Grey* residues represent Cys substitutions that MTSEA did not cause any binding inhibition or MTSEA caused binding inhibition, but were not protected by naloxone.

DISCUSSION

In this study, we identified by SCAM analysis the residues in the TMs 7 of the δ OR and the κ OR accessible in the binding site crevices and interpreted the data in the context of rhodopsin-based molecular models of the receptors. To the best of our knowledge, this represents the first study in which such comparison is made on two closely related receptors. Despite their high sequence homology within the segment examined (17 of 26 residues are identical), the MTSEA sensitivity patterns and reaction rates are very different between the two receptors. The differences are most likely due to the differential interhelical interactions in the δ OR and the κ OR.

κ OR and δ OR have different SCAM sensitivity patterns

A cluster of consecutive residues in the middle of TM7 (7.37–7.43) of the κ OR are accessible and many of the residues are not on the same face of the helix (Fig. 4B). Among the residues accessible in the binding-site crevice of the δ OR, V7.31(296), I7.39(304), G7.42(307), P7.50(315) and Y7.53(318) are on the same face of the helix, but A7.34(299) and L7.41(306) are not (Fig. 4B). Since this region is α -helical in the rhodopsin structure, such accessibility patterns are likely due to conformational changes and dynamic movements in this region over the course of the reaction with MTSEA, facilitated by the proline kink at 7.50. In addition, in the κ OR, the distance between TM1 and TM7 may be larger (see below), likely rendering residues facing TM1 accessible to MTSEA. MTSEA, the smallest of the MTS reagents, can conceivably penetrate the κ OR TM1-TM7 interface, to react with substituted cysteines and interfere with ligand binding. MTSEA has been shown to cross membranes to interact with intracellular cysteines (46). For this study, we used intact cells and a 5-min pretreatment period for all experiments to minimize the entry of MTSEA into cells. A 5-min incubation was chosen since the $t_{1/2}$ of MTSEA in aqueous media is about 12 min and 5-min incubation is close to the reaction plateau (16).

The κ OR mutants have much higher reaction rates than the δ OR mutants, except for G7.42C

The overall higher reaction rates of the κ OR mutants over the δ OR mutants indicate that the previously observed higher rate of C7.38 in the κ OR than the δ OR (29) is part of an overall pattern, but not a localized effect. The e2 loops of rhodopsin and the D2 dopamine receptor have been shown to dip into the binding pockets (1,47). It is possible that the e2 loop of the δ and κ receptors may be similarly positioned in the binding pockets. We (19), Wang et al. (48) and Meng et al. (18) demonstrated previously that the e2 loop of the κ OR, containing several Glu and Asp residues, was essential for the high affinity binding of dynorphin peptides. We have shown that the negative charges in the e2 loop of the κ OR may play a role in the higher reaction rates of the κ OR by attracting the positively charged MTSEA and accelerating its reaction with the substituted cysteines (29). In addition, a longer distance between TM1 and TM7 in the κ OR than in the δ OR is also an important factor (see below).

Similar % of the wildtype and mutant receptors is expressed on cell surface

It has become evident in recent years that a portion of newly synthesized mutant receptors, and even wildtype receptors, may not be able to exit the endoplasmic reticulum quality control mechanism to varying extents and are retained in the endoplasmic reticulum (49,50). One may argue that the observed differential sensitivity may be due to differential surface expression. We thus determined the % of receptors expressed on cell surface. Seventy to 80% of each of the wildtype and mutant receptors was found on cell surface. Therefore, the differential sensitivity to MTSEA observed for the wildtype and mutant receptors is not due to their different subcellular localization. The most likely reason for the similar extents of cell surface expression is that each construct contains a signal peptide in the N-terminal domain that converts Type IIIa integral membrane protein to Type IIIb and thus enhances insertion of

receptors into endoplasmic reticulum and thus facilitates transport to Golgi apparatus and plasma membranes (45). In studies of Petaja-Repo et al. (49,50), no such a signal peptide was added to the δ opioid receptor constructs, which may explain the retention of a high percentage of the receptor in the endoplasmic reticulum they have observed.

The interfaces between TM1 and TM7 are different in the δ OR and the κ OR

There are large differences in MTSEA sensitivity and reaction rates in the extracellular portion of the TM7 between the δ OR and the κ OR, indicating significant divergences between the two receptors (Fig. 5A and Fig. 5B). The δ OR contains C1.43(60) in TM 1 while the κ OR has F1.43(70). We found that the δ OR S7.46(311)C mutant did not bind [3 H]diprenorphine, but the S7.46(311)A and the wildtype S7.46(311) did. These results suggest that a disulfide bond may form between C7.46 and C1.43 in the δ OR S7.46(311)C mutant, which may distort the binding pocket and thus prevent [3 H]diprenorphine from binding. In addition, in the wildtype δ receptor, C1.43 and S7.46 may be sufficiently close to form a hydrogen bond, which is much weaker than a disulfide bond and disruption of this hydrogen bond did not affect binding. In contrast, the F1.43(70) side chain in the κ OR is positioned in the face-to-edge orientation (T-shaped) to the aromatic rings of Y1.39(66) and Y7.43(320) (see below) whereas S7.46(323) remains uncoordinated.

The extracellular portion of TM1 is closer to TM7 in the δ OR than in the κ OR

The presence of F1.43(70) in the κ OR modifies, relative to the δ OR, the TM1–7 interface at the extracellular domain of the receptor. The aromatic moiety of F1.43(70) in the κ OR interacts in the face-to-edge orientation with both Y1.39(66) and Y7.43(320) (Fig. 5B). In contrast, the absence of F1.43(60) in the δ OR causes the side chains of Y1.39(56) and Y7.43(308) to form a direct interaction (Fig. 5A). This type of π - σ aromatic-aromatic interaction has been described as an important element of protein structure stabilization (51). Fig. 8A shows the inter-atomic distance between the C $_{\alpha}$ atoms of Y1.39 and Y7.43 in δ OR (solid line) and κ OR (broken line) as the computer simulation progresses. Clearly, TM1 in the δ OR is located closer to TM7 (average C $_{\alpha}$ -C $_{\alpha}$ distance of 6.9 Å, see gray line in Fig. 8A) to establish the direct aromatic interaction between Y1.39(56) and Y7.43(308) than the κ OR, in which TM1 is more distant to TM7 (average C $_{\alpha}$ -C $_{\alpha}$ distance of 8.2 Å) to accommodate the side chain of F1.43(70) between Y1.39(66) and Y7.43(320). In addition, the hydrogen bond capabilities of the -OH moiety of Y1.39 and Y7.43 in both the δ OR and the κ OR are satisfied by the interactions with the polar side chains of Q2.60 and D3.32. Based on their mutagenesis results, Kieffer and colleagues (23,52) suggest that in the δ OR D3.32(128) interacts with Y7.43(308), which is part of the interhelical interactions maintaining the δ OR in inactive conformations. The TM1-TM7 distance correlates with the kind of aromatic and polar side chains placed at position 7.36. While the δ OR possesses the shorter H7.36(301) side chain to form a hydrogen bond interaction with the proposed closer T1.36(63) side chain (Fig. 5A), whereas the κ OR possesses the longer Y7.36(313) side chain to interact with the more distant T1.36(63) (Fig. 5B). Consequently, the average C $_{\alpha}$ -C $_{\alpha}$ distance between T1.36 and H7.36 in δ OR is smaller (9.4 Å, Fig. 8B) than the distance obtained between T1.36 and Y7.36 in κ OR (10.3 Å, Fig. 8B). H7.36C in the δ OR and Y7.43C in both the δ OR and the κ OR are poorly expressed (see B $_{\max}$ in Tables 1 and 2), indicating the significant role of these side chains in the stability of the TM1–7 interface. Therefore, the patterns of residues in the extracellular portion of TM7 that are accessible to the binding-site crevice are very different in the δ OR and the κ OR (Fig. 2 and Tables 3 and 4). The residues of TM7 in the κ OR in the interface with TM1 are sensitive to MTSEA (Fig. 4C) and possess, in addition, much higher reaction rates than the δ mutants (Table 3) because of the proposed more distant TM1 in this receptor. In contrast, the residues of TM7 in the δ OR that are predicted to face TM1 are not sensitive to MTSEA (Fig. 4B) probably due to the closer proximity of TM1.

TM7 may be longer in δ OR than in κ OR

Making the assumption that TM6 extends to position 6.62 (included) and TM7 starts at position 7.33 (included), as observed in the three-dimensional structure of rhodopsin (2), the e3 loop is formed by the I6.63(289)DRRDPLV7.32(297) sequence in the δ OR and by the T6.63(302)SHSTAAL7.32(309) sequence in the κ OR. Thus, the putative number of amino acids forming the e3 loop is one residue longer in the δ OR than in the κ OR. In addition, Pro residues are mostly located in loop regions, acting as helix-breaker (53). It is, thus, tempting to suggest that TM7 starts at L7.30(295) in the δ OR, due to the presence of P7.29(294), and at S7.33(310) in the κ OR as in rhodopsin. Therefore, TM7 in the δ OR would have an additional helical turn (from 7.30 to 7.33) relative to the κ OR and other rhodopsin-like GPCRs (see TM7 in Fig. 4B). This is consistent with the finding that MTSEA-sensitive mutants start at position 7.31 in the δ OR and at position 7.34 in the κ OR (Table 4). In AT1 angiotensin II receptor, MTSEA-sensitive mutants start at A7.28(277) (54), whereas in the D₂ dopamine and A₁ adenosine receptors start at L7.34(407) and T7.35(270) (55,56), respectively. Thus, the TM7 may start at different positions in different GPCRs. The finding that Val7.31(296), Val7.32(297), and Leu7.35(300) are crucial in selectivity of the δ OR ligand binding (57,58) is consistent with these residues being part of the binding pocket.

N7.45 has different environment in the δ OR and in the κ OR: A conserved hydrogen bond network linking D2.50 and W6.48

Fig. 6A shows a detailed picture of the N7.45(310) environment of the molecular model of the δ OR. The O _{δ 1} atom of N7.45(310) acts as a hydrogen bond acceptor in the interaction with the side chains of both C6.47(273) and W6.48(274). A conformational rearrangement of C6.47 and W6.48 has been associated with the process of activation of monoamine receptors (4,59). The conformational change of W6.48 upon receptor activation has been observed in the structure of metarhodopsin I, determined by electron crystallography (60). Thus, the role of N7.45 is to restrain C6.47 (χ_1 *trans*) and W6.48 (χ_1 *gauche*+) in these rotamer conformations, pointing towards TM7, in the inactive state of the receptor as has been shown for the histamine H₁ receptor (61). Notably, this hydrogen bond network of interactions is different in the κ OR due to the presence of T7.44(321) instead of A7.44(309) in the δ OR (Fig. 6B). In the κ OR, T7.44(321) forms a hydrogen bond with C6.47(286) (as observed in the crystal structure of rhodopsin that lacks N7.45, see below), whereas the O _{δ 1} atom of N7.45(322) is dedicated to W6.48(287). While both C6.47 and W6.48 are constrained in the inactive conformation by the single N7.45(310) amino acid in the δ OR, this task is shared by T7.44(321) and N7.45(322) in the κ OR. This different hydrogen bond network between both types of receptors explains the different levels of B_{max} values observed in the N7.45C mutant receptor (Table 1 and 2). The N7.45(310)C δ OR mutant expressed poorly, whereas the N7.45(322)C κ OR mutant expressed well. It is noteworthy that rhodopsin contains C6.47(264), W6.48(265), and T7.44(297) in its sequence but lacks N7.45(298). Rhodopsin fulfills the interactions with C6.47(264) and W6.48(265) through T7.44(297) and water molecule #10 (2) (see Fig. 6C).

On the other hand, the N _{δ 2}-H₂ moiety of N7.45 interacts with N3.35 in both the δ OR and the κ OR (Fig. 6A and 6B). N3.35 bridges N7.45 and D2.50, acting as a hydrogen bond donor in the interaction with the side chain of D2.50, and as a hydrogen bond acceptor in the interaction with N7.45. N3.35 is partly conserved and only present in opioid, bradykinin, formyl-met-leuphe, somatostatin, angiotensin, C5a anaphylatoxin, and proteinase-activated families of GPCRs. This Asn in the AT1 receptor for angiotensin II plays a critical role in stabilizing the inactive conformation of the receptor (62). Importantly, rhodopsin possesses water molecule #12 at the same position as opioid receptors have N3.35, linking D2.50 and water molecule #10 (2) (see Fig. 6C).

Thus, there is a network of interactions between D2.50 and W6.48 (present in 94% and 71%, respectively, of the sequences among family A of GPCRs). However, N3.35 and N7.45 are only conserved in 29% and 67% of the sequences, respectively. Thus, we hypothesize that opioid receptors or other N3.35/N7.45-containing receptors form the D2.50·N3.35·N7.45·W6.48 network of interactions; GPCRs lacking N3.35 form a similar network through D2.50·water#12·N7.45·W6.48 interactions (61); and if both N3.35 and N7.45 are absent in the sequence, as in rhodopsin, the D2.50·water#12·water#10·W6.48 (Fig. 6C) network links D2.50 and W6.48 (2).

Modeling the environment of the NPxxY motif in TM7

The environment of the NPxxY motif in the κ OR is similar to that in the δ OR and only the δ OR is depicted. Fig. 7A shows a detailed view of the D2.50(95)/N7.49(314) environment of the molecular model of the δ OR. Water molecules #2 and #9 found in the recent structures of rhodopsin are included in the model (2,3). These structural waters mediate a number of interhelical interactions through the highly conserved D2.50(95) and N7.49(314) amino acids. Water #2 is located between TM2 and TM7, linking the backbone carbonyl of N7.45(310), the backbone N-H amide of N7.49(314), the side chain of D2.50(95), and the side chain of N7.49(310). Water #9 bridges the side chain of N7.49(310) and the backbone carbonyl of V6.40(266), keeping the Asn side chain towards TM6. N7.49 plays a central role in the process of receptor activation, acting as an on/off switch by adopting two different conformations in the inactive and active states (63,64). The role of this active water molecule #9 is to restrain N7.49 in the inactive *gauche*+ conformation, pointing toward TM6 (3). We have shown previously that the interaction of D2.50(114) and N7.49(332) in the μ opioid receptor is important for ligand binding and receptor activation (26).

Fig. 7B shows the interactions of Y7.53(318) with the residues located in TM2 and H8. The aromatic moiety of Y7.53(318) interacts with F7.60(325) in the H8 whereas the hydroxyl group of Y7.53(318) forms hydrogen bonds with the N δ 2-H $_2$ group and the carbonyl oxygen (via water molecule #7) of the partly conserved N2.40(85) in TM2 [40% of the sequences among family A of GPCRs]. The Y7.53C substitution in both the δ OR and the κ OR leads to poorly expressed mutant receptors (Table 1 and 2). It has been suggested that the Y7.53-F7.60 interaction is disrupted during receptor activation, leading to a proper realigning of helix8 (27,28). In accord with this model, Decaillet et al. (52) demonstrated that the δ OR Y7.53(318) H mutant displayed enhanced constitutive activity.

Interpretation of SCAM results

SCAM analysis has been applied to TMs 7 of D $_2$ dopamine, AT $_1$ angiotensin II and A $_1$ adenosine receptors (54-56), in addition to the δ OR and κ OR in the current study. The residues determined to be accessible in the binding-site crevices in the TMs7 of these receptors are listed in Table 4.

Although the TMs 7 of rhodopsin family GPCRs are thought to be helical (4,5), these receptors have different accessibility patterns and none of them strictly conforms to a pattern consistent with a helical structure. Even between TMs 7 of the δ OR and the κ OR, which are highly homologous with 17 of 26 residues identical, the SCAM sensitivity patterns and MTSEA reaction rates are very different. This is most likely due to different interhelical interactions among these receptors as discussed above. Thus, there is a need to broaden our interpretation of the residues identified in SCAM analysis as being sensitive to MTS reagents. These residues, in addition to those exposed in the binding site crevices, should also include those accessible from the extracellular medium, but not in the binding pocket. Substituted cysteines at latter positions react with MTS reagents resulting in inhibition of ligand binding indirectly by local steric factors, electrostatic potential and dynamics of the protein. Therefore, some residues in

the TM-TM interface in the outer portion of TMs may be accessible to the extracellular medium. In this region, there are sufficient dynamic movements and/or conformational changes during the course of MTS reagent incubation to allow the reactions to occur. The presence of a ligand such as naloxone probably reduces the dynamic movements and/or conformational changes to decrease reactivity with MTSEA.

Limitations of SCAM

The residues were defined as "sensitive" if the MTSEA reaction at the screening concentration significantly inhibited binding relative to the background, as determined by ANOVA and post hoc testing (see Fig.2). This is a statistical method, and it is imperfect, given that each position will have a different reactivity that is governed by local steric factors, ionization of the cysteine sulfhydryl, electrostatic potential, and dynamics of the protein. In addition, the extent of the inhibition on ligand binding caused by MTSEA modification of a substituted Cys can vary, and, therefore, this indirect method of detecting reaction, although necessary, is also imperfect. Among the sensitive mutants, if naloxone could prevent the reaction with MTSEA, we called the substituted cysteine and, hence the residue mutated, "accessible" in the binding site-crevice.

There are substantial variations in the second-order rate constants among the sensitive cysteine mutants of the δ or κ opioid receptor. For example, among the sensitive κ mutants, there is more than 150-fold difference between the most reactive one [I7.39(316)C, $472.0 \pm 5.1 \text{ M}^{-1}\text{s}^{-1}$ (mean $\pm$ s.e.m., $n = 3$)] and the least reactive one [N7.49(326)C, $2.8 \pm 0.5 \text{ M}^{-1}\text{s}^{-1}$]. Such a large difference has also been observed in the SCAM analyses of the D2 dopamine receptor [for example, (65)], and results from the factors discussed above. In the case of the least reactive sensitive mutants, only a small margin of reactivity may separate them from mutants deemed insensitive. This is to be expected whenever one uses a statistical definition of accessibility, but this screening method is nonetheless consistent with the rates."

Although using SCAM to probe receptor structure has its shortcomings, the method has been applied to a number of GPCRs, including D2 dopamine receptor, A1 adenosine receptor and AT1 angiotensin II receptors [for example, (5,13,54-56)], for which no X-ray crystal structures are available. Coupled with rhodopsin-based molecular modeling, SCAM analysis has proven to be very useful for elucidating the structures of GPCRs.

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ABBREVIATIONS

TM, transmembrane helix
H8, the helix 8
 δ OR and κ OR, δ and κ opioid receptors
SCAM, substituted cysteine accessibility method
MTS reagents, methanethiosulfonate reagents
MTSEA, (2-aminoethyl)methanethiosulfonate
GPCR, G protein-coupled receptor
TEL buffer, 50 mM Tris-HCl buffer containing 1 mM EGTA and 10 μ M leupeptin (pH 7.4)
Kreb's solution, NaCl 130 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, CaCl_2 1.3 mM, MgSO_4 1.2 mM, glucose 10 mM, and HEPES 25 mM, pH7.4
e3 loop, the third extracellular loop
e2 loop, the second extracellular loop.

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brho 7.33(286) IFMTIPAFFAKTSAVYNPVIYIMM 7.56(309)
hdor 7.31(296) VVAALHLCIALGYANSSLNPVLYAFL 7.56(321)
hkor 7.31(308) ALSSYYFCIALGYTNSSLNPILYAFL 7.56(333)

Figure 1.

Amino acid sequence alignment of TMs 7 of the human δ OR and κ OR and bovine rhodopsin (brho).

Figure 2. SCAM analysis of TM 7 of the δ OR and the κ OR: Inhibition by MTSEA of [³H]diprenorphine binding to the substituted cysteine mutants

Each residue within the TM 7 was mutated to Cys, one at a time, using the wild-type δ OR and the κ OR C7.38S mutant as the templates. Each of the mutants as well as the templates was expressed in HEK293 cells and treated with indicated concentrations of MTSEA. Cells were washed and assayed for [³H]diprenorphine binding to the receptor in intact cells. Each point represents the mean $\pm$ s.e.m of 3–8 experiments in duplicate. Filled bars indicate mutants for which inhibition was significantly different ($p < 0.05$) from the template by one ANOVA followed by *post hoc* Sheffe F test. ++: [³H]diprenorphine binding was undetectable for S7.46(311)C and P7.50(327)S mutants of the δ OR and the κ OR, respectively.

Figure 3. Protection by naloxone against inhibitory effects of MTSEA on [3 H]diprenorphine binding to MTSEA-sensitive mutants

Each MTSEA-sensitive cysteine-substituted mutant (identified in Fig. 2) expressed in HEK293 cells was pre-incubated with 20 μ M naloxone for 20 min at room temperature and then reacted with MTSEA for 5 min at a concentration that caused about 50% of the maximal extent of inhibition for each mutant as determined in Table 3 experiments. Cells were washed and assayed for [3 H]diprenorphine binding as described in Experimental Procedures. Each value represents the mean $\pm$ s.e.m. of 4–7 experiments in duplicate. Protection was calculated as 1-(inhibition in the presence of naloxone)/(inhibition in the absence of naloxone). The inhibition in the presence of naloxone was significantly decreased ($p < 0.05$, by paired Student's t test) for all the mutants except L7.52(317)C of the δ OR compared with that in the absence of naloxone.

Figure 4. Computational models of δ and κ opioid receptors

(A) The α -carbon ribbons of transmembrane helices 1 (crimson), 2 (goldenrod), 3 (dark red), 4 (grey), 5 (grey), 6 (orange), and 7 (blue) of rhodopsin (2); and transmembrane helices 2 (goldenrod) and 3 (dark red) of the δ OR (shown as a tube ribbon). Transmembrane helices 1, 4–7 are identical in δ opioid receptor and rhodopsin. Figures 5, 6 and 7 use the same color scheme. The κ OR is similar to the δ OR. (B) The side chains in TMs 7 for the δ OR and the κ OR depicted in ball-and-stick representation. Residues ranging from V7.31(296) to L7.56(321) of the δ OR and from S7.34(311) to L7.56(333) of the κ OR are shown. Based on our data, we propose that TM7 starts at 7.31(296) in the δ OR, but 7.34(311) in the κ OR. *Green* residues depict Cys substitutions that were sensitive to MTSEA and protected from MTSEA by naloxone pretreatment. *Grey* residues depict Cys substitutions that MTSEA incubation did not inhibit binding or MTSEA caused binding inhibition, but naloxone did not give sufficient protection. P7.50 (*Green*) in the δ OR facing away is not visible from this angle. These figures were created using MolScript v2.1.1 (66) and Raster3D v2.5 (67).

Figure 5. The interface between TMs 1 and 7 in the extracellular domain of the δ OR (A) and the κ OR (B)

TM1 may be closer to the extracellular half of the TM7 in the δ OR than in the κ OR because of the presence of C1.43(60)-S7.46(311) hydrogen bond in the δ OR and the need to accommodate the side chain of F1.43(70) between Y1.39(66) and Y7.43(320) in the κ OR. This correlates with the shorter side chain of H7.36(301) in the δ OR and the longer side chain of Y7.36(313) in the κ OR, each forming a hydrogen bond with T1.36. Only polar hydrogens are depicted to offer a better view.

Figure 6. The proposed hydrogen bond network linking D2.50 and W6.48 in the inactive conformation of the δ OR (A) and κ OR (B) opioid receptors and rhodopsin (2) (C)
 A conserved hydrogen bond network linking D2.50 and W6.48 includes D2.50 - N3.35 - N7.45 - C6.47 and W6.48 in the δ OR and κ OR . An additional T7.44(321) is present in the κ OR. The crystal structure of rhodopsin has T7.44 and two water molecules (#12 and #10) in place of N3.35 and N7.45.

Figure 7. The micro-environment of N7.49(314) and Y7.53(318) of the NPXXY motif of the δ OR
 (A) A detailed view of the proposed hydrogen bond network at the D2.50/N7.49 environment in the inactive conformation. N7.49 is linked to D2.50 and N7.45 by water #2 and to V6.40 by water #9. (B) The interactions of Y7.53 of the NPxxY motif with the residues located in TM2 and helix 8. Y7.53(318) interacts with Y7.60(325) in the helix 8 by an aromatic interaction and links with N2.40(75) by water #7.

Figure 8. Evolution of the interface between TMs 1 and 7 monitored by the inter-atomic distances between the C_{α} atoms of (A) Y1.39 and Y7.43 and (B) T1.36 and H/Y7.36 in δ OR (solid line) and κ OR (broken line)

Gray lines show the average C_{α} - C_{α} distance obtained during the production phase of the computer simulations.

Table 1

K_d and B_{max} of [³H]diprenorphine binding to the cysteine-substituted δOR mutants expressed in HEK 296 cells

Saturation binding of [³H]diprenorphine to the cysteine-substituted δOR mutants were performed and K_d and B_{max} values were calculated as described in **Experimental Procedures**.

mutant	<i>K_d</i> (nM)	<i>K_m</i> / <i>K_{C7.38S}</i>	<i>B_{max}</i> (pmole/mg protein)
V7.31(296)C	0.38±0.03	1.1	10.2±0.18
V7.32(297)C	0.26±0.03	0.7	4.5±0.32
A7.33(298)C	0.21±0.06	0.6	0.39±0.05
A7.34(299)C	0.26±0.05	0.7	0.11±0.02
L7.35(300)C	0.49±0.08	1.4	1.1±0.04
H7.36(301)C	0.25±0.01	0.7	0.05±0.01
L7.37(302)C	0.28±0.02	0.8	2.4±0.31
C7.38(303)/WT	0.35±0.07	1.0	3.5±0.20
I7.39(304)C	0.16±0.01	0.4	1.06±0.04
A7.40(305)C	0.21±0.04	0.6	0.33±0.03
L7.41(306)C	0.21±0.02	0.6	0.52±0.14
G7.42(307)C	0.61±0.11	1.7	0.62±0.05
Y7.43(308)C	0.69±0.19	1.9	0.06±0.01
A7.44(309)C	0.28±0.01	0.8	2.0±0.08
N7.45(310)C	0.68±0.15	1.9	0.20±0.02
S7.46(311)C		No binding was detected	
S7.47(312)C	0.28±0.01	0.8	3.1±0.06
L7.48(313)C	0.23±0.03	0.6	0.43±0.03
N7.49(314)C	0.32±0.03	0.9	0.42±0.01
P7.50(315)C	0.74±0.12	2.1	0.42±0.21
V7.51(316)C	0.23±0.03	0.6	3.9±0.37
L7.52(317)C	0.23±0.03	0.6	0.35±0.03
Y7.53(318)C	0.12±0.01	0.3	0.07±0.01
A7.54(319)C	0.31±0.01	0.8	4.2±0.07
F7.55(320)C	0.20±0.02	0.5	1.5±0.09
L7.56(321)C	0.35±0.02	1.0	0.57±0.02

Table 2

K_d and B_{max} of [³H]diprenorphine binding to the cysteine-substituted κOR mutants expressed in HEK 296 cells

Saturation binding of [³H]diprenorphine to the cysteine-substituted κOR mutants were performed and K_d and B_{max} values were calculated as described in **Experimental Procedures**.

mutant	<i>K_d</i> (nM)	<i>K_m</i> / <i>K_{C7.38S}</i>	<i>B_{max}</i> (pmole/mg protein)
A7.31(308)C	1.43±0.07	4	1.2
L7.32(309)C	0.48±0.03	1.4	2.1
S7.33(310)C	0.32±0.12	0.9	1.5
S7.34(311)C	0.28±0.15	0.8	1.9
Y7.35(312)C	0.17±0.04	0.5	0.22
Y7.36(313)C	0.88±0.01	2.5	1.2
F7.37(314)C	0.18±0.02	0.5	0.8
C7.38(315)S	0.35±0.03		1.5
I7.39(316)C	0.68±0.14	1.9	2.1
A7.40(317)C	0.23±0.05	0.7	0.52
L7.41(318)C	0.33±0.02	0.9	0.15
G7.42(319)C	0.66±0.01	1.9	0.8
Y7.43(320)C	0.37±0.09	1.1	0.03
T7.44(321)C	0.48±0.37	1.4	1.6
N7.45(322)C	0.68±0.15	1.9	2.4
S7.46(323)C	1.10±0.12	3.1	1.7
S7.47(324)C	0.29±0.08	0.8	1.6
L7.48(325)C	1.35±0.06	3.9	1.8
N7.49(326)C	0.70±0.04	2	0.93
P7.50(327)C		No binding was detected	
I7.51(328)C	0.48±0.08	1.4	2.0
L7.52(329)C	0.39±0.03	1.1	0.62
Y7.53(330)C	1.52±0.05	4.3	0.74
A7.54(331)C	0.53±0.06	1.5	1.35
F7.55(332)C	0.28±0.05	0.8	1.14
L7.56(333)C	0.31±0.07	0.9	0.59

Table 3

The second-order rate constants of reaction of MTSEA⁺ (M⁻¹s⁻¹) with cysteine-substituted mutants of the human δ OR or the human κ OR transiently expressed in HEK 293 cells

Each mutant receptor was incubated with at least four concentrations (in most cases, 0.1, 0.25, 1 and 2.5 mM) of MTSEA for 5 min and [³H]diprenorphine binding was performed on washed cells. The second-order rate constant (*k*) for each mutant was calculated as described in **Experimental Procedures**. Data represent mean $\pm$ s.e.m. of three to six independent experiments in duplicate.

	δ OR	κ OR
7.31C	5.3 $\pm$ 0.3	nd
7.34C	6.0 $\pm$ 1.2	53.6 $\pm$ 6.3
7.37C	nd	62.3 $\pm$ 10.2
7.39C	20.16 $\pm$ 6.6	472.0 $\pm$ 5.1
7.40C	nd	109.1 $\pm$ 47.7
7.41C	6.2 $\pm$ 3.7	58.6 $\pm$ 17.2
7.42C	10.0 $\pm$ 0.46	7.3 $\pm$ 3.3
7.43C	nd	32.1 $\pm$ 5.1
7.44C	nd	nd
7.49C	nd	2.8 $\pm$ 0.5
7.50C	10.5 $\pm$ 0.6	nd
7.53C	3.6 $\pm$ 0.7	nd
Background construct	(0.42 $\pm$ 0.12)	(<0.4)

nd: indicate mutants that are not significantly sensitive to MTSEA as shown in Fig. 2.

Table 4

Comparison of TM7 amino acids accessible in the binding pockets of the δ and κ opioid (current study), D₂ dopamine, A₁ adenosine and AT₁ angiotensin II receptors (54–56) as determined by SCAM analyses.

δ opioid receptor	κ opioid receptor	D ₂ dopamine receptor	A ₁ adenosine receptor	AT ₁ angiotensin II receptor
				A7.28(277)
V7.31(296)				V7.31(280)
				T7.33(282)
A7.34(299)	S7.34(311)	L7.34(407)		A7.34(283)
			T7.35(270)	
	F7.37(314)			I7.37(286)
	C7.38(315)	F7.38(411)	A7.38(273)	
I7.39(304)	I7.39(316)	T7.39(412)	I7.39(274)	
	A7.40(317)	W7.40(413)		
L7.41(306)	L7.41(318)			
G7.42(307)	G7.42(319)		T7.42(277)	A7.42(291)
	Y7.43(320)	Y7.43(416)	H7.43(278)	
		N7.45(418)		
	N7.49(326)	N7.49(422)	N7.49(284)	
P7.50(315)		P7.50(423)		
				F7.52(301)
Y7.53(318)		Y7.53(426)	Y7.53(288)	