Comparison of the Amino Acid Residues in the Sixth Transmembrane Domains Accessible in the Binding-Site Crevices of μ, δ, and κ Opioid Receptors[†]

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ABSTRACT: We have mapped the residues in the sixth transmembrane domains (TMs 6) of the μ, δ, and κ opioid receptors that are accessible in the binding-site crevices by the substituted cysteine accessibility method (SCAM). We previously showed that ligand binding to the C7.38S mutants of the μ and κ receptors and the wild-type δ receptor was relatively insensitive to methanethiosulfonate ethylammonium (MTSEA), a positively charged sulfhydryl-specific reagent. These MTSEA-insensitive constructs were used as the templates, and 22 consecutive residues in TM6 (excluding C6.47) of each receptor were mutated to cysteine, 1 at a time. Most mutants retained binding affinities for [^{3}H]diprenorphine, a nonselective opioid antagonist, similar to that of the template receptors. Treatment with MTSEA significantly inhibited [^{3}H]diprenorphine binding to 11 of 22 mutants of the rat μ receptor and 9 of 22 mutants of the human δ receptor and 10 of 22 mutants of the human κ receptor. Naloxone or diprenorphine protected all sensitive mutants, except the A6.42(287)C μ mutant. Thus, V6.40, F6.44, W6.48, I6.51, Y6.54, V6.55, I6.56, I6.57, K6.58, and A6.59 of the μ receptor; F6.44, I6.51, F6.54, V6.55, I6.56, V6.57, W6.58, T6.59, and L6.60 of the δ receptor; and F6.44, W6.48, I6.51, F6.54, I6.55, L6.56, V6.57, E6.58, A6.59, and L6.60 of the κ receptor are on the water-accessible surface of the binding-site crevices. The accessibility patterns of residues in the TMs 6 of the μ, δ, and κ opioid receptors are consistent with the notion that the TMs 6 are in α-helical conformations with a narrow strip of accessibility on the intracellular side of 6.54 and a wider area of accessibility on the extracellular side of 6.54, likely due to a proline kink at 6.50 that bends the helix in toward the binding pocket and enables considerable motion in this region. The wider exposure of residues 6.55−6.60 to the binding-site crevice, combined with the divergent amino acid sequences, is consistent with the inferred role of residues in this region in determining ligand binding selectivity. The conservation of the accessibility pattern on the cytoplasmic side of 6.54 suggests that this region may be important for receptor activation. This accessibility pattern is similar to that of the D2 dopamine receptor, the only other GPCR in which TM6 has been mapped by SCAM. That opioid receptors and the remotely related D2 dopamine receptor have similar accessibility patterns in TM6 suggest that these segments of GPCRs in the rhodopsin-like subfamily not only share secondary structure but also are packed similarly into the transmembrane bundle and thus have similar tertiary structure.

Opiate and opioid compounds act on opioid receptors to produce pharmacological and physiological effects, most notably analgesia. Multiple opioid receptors (μ, δ, κ, ϵ) have been demonstrated, and these have unique ligand specificities, anatomical distributions, and physiological functions (*1*, *2*). The opioid receptors are coupled through G proteins to affect a variety of effectors, which include adenylate cyclase, potassium channels, calcium channels (*3*), and a mitogen-activated protein kinase pathway (*4*). In 1992, Kieffer et al. (*5*) and Evans et al. (*6*) cloned a mouse δ opioid receptor by expression cloning. Subsequently, μ and κ receptors were cloned from several species [(*7*) and references cited therein]. Deduced amino acid sequences of these clones display the motif of seven transmembrane domains (TMs)[1] connected

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[1] Abbreviations: buffer A, 25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, and 0.006% BSA, pH 7.4; GPCR, G protein-coupled receptor; HEK293 cells, human embryonic kidney cells; MTS reagents, methanesulfonate reagents; MTSEA, MTS ethylammonium, $CH_3SO_2SCH_2CH_2NH_3^+$; SCAM, substituted cysteine accessibility method; TEL buffer, 50 mM Tris-HCl buffer, 1 mM EGTA, and 10 μM leupeptin, pH 7.4; TM, transmembrane domain.

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by alternating intracellular and extracellular hydrophilic loops, that is characteristic of GPCRs. Based on the arrangement of seven α-helices from a 9 Å projection map of bovine rhodopsin (*8*, *9*) and electron micrographs of frozen-hydrated two-dimensional frog rhodopsin crystals (*10*), several groups have constructed molecular models of opioid receptors (*11*−*14*). Recently, Palczewski et al. (*15*) reported a crystal structure of bovine rhodopsin from X-ray diffraction data at 2.8 Å resolution.

Many drugs act on μ, δ, and κ opioid receptors, yet there are selective ligands for each receptor. Studies have been performed to characterize the interaction of ligands with opioid receptors. Chimeric receptor studies have revealed that the region including TMs 6 and 7 and the third extracellular (e3) loop plays important roles in ligand binding selectivity of μ, δ, and κ opioid receptors (*16*−*29*).

By examining the irreversible binding of [^{3}H]β-funaltrexamine (β-FNA), an irreversible μ antagonist and a reversible κ agonist (*30*), to four chimeric μ/κ receptors, we demonstrated that the region of TM6/e3 loop/TM7of the μ receptor was essential for the covalent binding of this ligand (*21*). However, by peptide mapping of [^{3}H]β-FNA-labeled fragments and site-directed mutagenesis, we found that covalent incorporation of β-FNA occurred not within the region of TM6/e3 loop/TM7, but at Lys5.39(233) (*31*). Lys5.39(233) is located at the extracellular end of the TM5 and conserved among the μ, δ, and κ opioid receptors. These results suggest that the TMs 6 and 7 and the e3 loop of the μ receptor confer "type-specific" binding.

Two possible scenarios may account for "type-specific" binding. One is that the α-carbon backbone structures are the same in the three opioid receptors and the selectivity is due to the presence of different amino acid side chains in the binding pocket. The other is that there are more global differences among the three receptors in this particular region; i.e., not only are there variations in amino acid side chains in the binding pocket, but also there are differences in the packing, rotation, kink, and/or tilt of the α-carbon backbone. In this study, we applied the substituted cysteine accessibility method (SCAM) [for reviews, see (*32*, *33*)] to the TMs 6 of opioid receptors to address this important issue in the functional selectivity of the receptors.

Various experimental probes have indicated that binding pockets of GPCRs involve the seven TMs and are accessible from the extracellular medium. Specific water-accessible residues within the binding pocket can directly interact with ligands. Javitch and his colleagues have applied SCAM to map residues within the TMs 2, 3, 4, 5, 6, and 7 of the D2 dopamine receptor accessible in the binding-site crevice (*34*−*41*). SCAM [for reviews, see (*32*, *33*)] was originally developed by Karlin and Akabas and his colleagues to identify the residues that lined the channel of the nicotinic acetylcholine receptor (*35*−*40*, *42*). Small charged methanethiosulfonate (MTS) reagents that react specifically with reduced sulfhydryl groups were employed (*42*, *43*), including MTS ethylammonium, $CH_3SO_2SCH_2CH_2NH_3^+$ (MTSEA). When reacted with cysteine, these reagents form mixed disulfide bonds with the -SH group of cysteine. MTS reagents react 10^9 times faster with ionized thiolates (S^-) than with un-ionized thiols (SH) (*44*), and ionization of cysteine is expected to occur to a significant extent only in the aqueous medium (*32*). Thus, the reaction rate of charged sulfhydryl-specific reagents with cysteine residues is expected to be much faster with cysteine residues at the water-accessible surface than with those in the interior of proteins or facing lipid. For SCAM analysis, each residue within a transmembrane domain is mutated to cysteine, one at a time, in an MTS reagent-insensitive background. The microenvironment of the engineered cysteine residue is probed using the MTS reagents. If binding to a mutant containing the engineered cysteine is sensitive to MTS reagents, we infer that the residue is on the water-accessible surface of the receptor. If this reaction is retarded by the presence of ligand, we further infer that the residue forms the surface of the binding-site crevice.

We have recently shown that μ, δ, and κ opioid receptors are differentially sensitive to MTSEA and the conserved Cys7.38 is largely responsible for the MTSEA sensitivity in each receptor (*45*). Thus, C7.38 in the μ and κ receptors is substantially more reactive with MTSEA than C7.38 in the δ receptor (*45*). In the present study, we used the MTS-insensitive C7.38S mutants of μ and κ opioid receptors and the wild-type δ receptor as the templates to generate cysteine substituted mutants and apply SCAM to identify the amino acid residues within the TM6 that are accessible in the binding-site crevice. By comparing conserved and nonconserved residues that are accessible in the binding-site crevices among the three opioid receptors, we identified structural features that are related to pharmacological type specificity of the opioid receptors. In addition, we compared the opioid receptors with the D2 dopamine receptor, thus far the only GPCR that has been systematically mapped with SCAM, to determine whether these distantly related GPCRs within the rhodopsin-like Class A subfamily are similar in the accessibility patterns of TM6 residues. These comparative results serve to identify common structural features in the GPCRs, and relate them to functional specificity and receptor type selectivity.

MATERIALS AND METHODS

Materials. [^{3}H]Diprenophine (58 Ci/mmol) was purchased from NEN Life Science (Boston, MA). Diprenorphine was provided by the National Institute on Drug Abuse. Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). MTS reagents were 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 opioid receptor were identified by their sequence numbers. In addition, the generic numbering scheme of amino acid residues in GPCRs proposed by Ballesteros and Weinstein (*46*) and recently applied to opioid receptors (*45*, *47*) was used in order to relate the results to equivalent positions in other GPCRs. According to this nomenclature, amino acid residues in TMs were assigned two numbers (*N*1.*N*2). *N*1 refers to the TM number. For *N*2, the most conserved residue in each TM was assigned 50, and the other residues were numbered in relation to this conserved residue, with numbers decreasing toward the

N-terminus and increasing toward the C-terminus. The generic numbering allows for easy comparison among the three opioid receptors and cross-reference to cognate SCAM data in the D2 dopamine receptor and to the published literature on other GPCRs. The most conserved residue in the TM6 of the rat μ, human δ, and human κ opioid receptors is Pro6.50 in TM6 (μ, Pro295; δ, Pro276; κ, Pro289). The boundary for the extracellular end of TMs 6 in the μ, δ, and κ opioid receptors is at position 6.60 (μ, L305; δ, L286; κ, L299), consonant with the models of Strahs and Weinstein (*11*) and with the recent X-ray structure of rhodopsin (*15*).

Oligodeoxynucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed on the C7.38S mutants of the rat μ and the human κ receptors and the wild-type human δ opioid receptor with the overlap PCR method described by Higuchi et al. (*48*). FLAG-tagged wild-type and mutant rat μ receptors were subcloned into *Hin*dIII and *Xba*I sites of the mammalian expression vector pcDNA3 (*45*). FLAG-tagged human wild-type and mutant δ receptors were subcloned into *Hin*dIII and *Xba*I sites of the vector pcDNA3 (*45*). FLAG-tagged human wild-type and mutant κ receptors were subcloned into *Hin*dIII and *Xho*I sites of the vector pcDNA3 (*45*). DNA sequence was determined with the method of Sanger et al. (*49*) to confirm the presence of desired mutations and the absence of unwanted mutations.

Transfection of HEK293 Cells. HEK293 cells were grown in 100 mm culture dishes in minimum essential medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C. Cells were transfected with the wild-type or a mutant of the μ, δ, or κ opioid receptor cDNA (5 μg/dish plus 15 μg of vector) using the calcium phosphate method (*50*). Sixty to seventy-two hours after transfection, cells were harvested for experiments by detaching with Versene solution.

Determination of K_d and B_{max} Values of [^{3}H]Diprenorphine Binding. Membranes were prepared from transfected HEK cells as described previously (*51*). Saturation binding of [^{3}H]-diprenorphine to the wild-type and mutant μ, δ, and κ receptors was performed with at least six 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 μM leupeptin (pH 7.4) (TEL buffer) at room temperature for 1 h in duplicate in a volume of 1 mL with 10−20 μg of membrane protein. Naloxone (10 μM) was used to define nonspecific binding. Binding data were analyzed with the EBDA program (*52*).

Reaction with MTSEA. The experiments were performed using a procedure modified from that of Javitch et al. (*39*). Sixty to seventy-two hours after transfection, cells were detached by use of Versene solution, pelleted at 1000*g* for 1 min at room temperature, and washed with buffer A (NaCl 140 mM, KCl 5.4 mM, EDTA 1 mM, HEPES 25 mM, and 0.006% BSA, pH 7.4) and centrifuged again. In some experiments, 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, pH 7.4) was used in place of buffer A, which yielded similar results. The pellets were resuspended in buffer A, 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 slowed by dilution with 0.5 mL of 0.8% BSA solution, and the cell suspensions were pelleted and washed once with buffer A. After centrifugation, the pellets were resuspended in 1 mL/dish of buffer A, and 200 μL aliquots were used for [^{3}H]diprenorphine binding to intact cells at room temperature for 1 h as described previously (*53*). Naloxone (10 μM) was used to define nonspecific binding. The fractional inhibition was calculated as [1 − (specific binding after the MTS reagent/specific binding without the reagent)] × 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 constant of interaction between the mutants of μ, δ, or κ opioid receptor and MTSEA was determined to gain quantitative information on MTSEA sensitivity, according to Javitch et al. (*40*) with modifications. Each receptor was incubated with the indicated concentrations of MTSEA for 5 min. The results were fit to the equations:

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

$$\text{maximal extent of inhibition} + \text{plateau} = 1.0$$

Y is the fraction of the initial binding, k is the second-order rate constant (M^{-1} s^{-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 the indicated concentrations of (−)-naloxone or (+)-naloxone for the μ and κ receptors or with diprenorphine for the δ receptor for 20 min for binding 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 3 times by centrifugation and then resuspended in buffer A and assayed for [^{3}H]diprenorphine binding. Protection was calculated as 1 − [(inhibition in the presence of naloxone or diprenorphine)/(inhibition in the absence of drug)].

Determination of Protein Content. Protein contents of membranes were determined by the bicinchoninic acid method of Smith et al. (*54*) with bovine serum albumin as the standard.

RESULTS

Effect of Cysteine Substitution in TM6 on [^{3}H]Diprenorphine Binding. We previously showed that [^{3}H]diprenorphine binding to the C7.38S mutants of the rat μ and human κ opioid receptors and the wild-type human δ receptor was relatively insensitive to MTSEA (*45*). Using these receptor constructs as the templates, we mutated 22 consecutive amino acid residues (excluding C6.47) in the TMs 6 of the 3 receptors to cysteine, 1 at a time. The residues mutated were from position 6.38 to position 6.60, i.e., from L283 to L305 of the μ receptor, from L264 to L286 of the δ receptor, and from L277 to L299 of the κ receptor. Each mutant receptor was transiently expressed in HEK293 cells, and K_d and B_{max} values of binding of [^{3}H]diprenorphine, a nonselective opioid antagonist, to each mutant were determined (Table 1A−C). For all three receptors, P6.50C and H6.52C mutants exhibited no detectable [^{3}H]diprenorphine binding.

Table 1: K_d and B_{max} Values of [^{3}H]Diprenorphine Binding to the Cysteine-Substituted Mutants of (A) the Rat μ, (B) the Human δ, and (C) the Human κ Opioid Receptors Transiently Expressed in HEK293 Cells[a]

(A) The Rat μ Opioid Receptor

mutant	K_d (nM)	$K_{d_{mut}}/K_{d_{C7.38S}}$	B_{max} (pmol/mg of protein)	n	mutant	K_d (nM)	$K_{d_{mut}}/K_{d_{C7.38S}}$	B_{max} (pmol/mg of protein)	n
L6.60(305)C	0.14 ± 0.03	1.0	0.87 ± 0.20	3	W6.48(293)C	0.41 ± 0.10	3.1	0.12 ± 0.04	4
A6.59(304)C	0.14 ± 0.05	1.0	0.66 ± 0.14	4	C6.47(C7.38S)	0.13 ± 0.03	1.0	1.73 ± 0.70	5
K6.58(303)C	0.21 ± 0.06	1.6	0.38 ± 0.06	4	V6.46(291)C	0.22 ± 0.01	1.6	2.12 ± 0.47	5
I6.57(302)C	0.16 ± 0.03	1.2	0.77 ± 0.25	4	I6.45(290)C	0.20 ± 0.03	1.5	1.29 ± 0.31	4
I6.56(301)C	0.34 ± 0.03	2.6	0.56 ± 0.12	8	F6.44(289)C	0.25 ± 0.03	1.9	0.10 ± 0.02	5
V6.55(300)C	0.20 ± 0.03	1.5	2.03 ± 0.52	3	V6.43(288)C	0.16 ± 0.05	1.2	2.25 ± 0.23	4
Y6.54(299)C	0.87 ± 0.04	6.7	0.41 ± 0.02	3	A6.42(287)C	0.24 ± 0.01	1.8	0.18 ± 0.04	3
I6.53(298)C	0.17 ± 0.03	1.3	2.42 ± 0.39	4	V6.41(286)C	0.18 ± 0.05	1.4	1.42 ± 0.17	8
H6.52(297)C	*b*			3	V6.40(285)C	0.15 ± 0.02	1.4	0.12 ± 0.01	4
I6.51(296)C	0.33 ± 0.04	2.5	0.15 ± 0.02	4	L6.39(284)C	0.23 ± 0.05	1.8	3.16 ± 0.68	4
P6.50(295)C	*b*			3	L6.38(283)C	0.20 ± 0.03	1.5	0.22 ± 0.08	3
T6.49(294)C	0.18 ± 0.02	1.4	1.16 ± 0.29	6					

(B) The Human δ Opioid Receptor

mutant	K_d (nM)	$K_{d_{mut}}/K_{d_{Wt}}$	B_{max} (pmol/mg of protein)	n	mutant	K_d (nM)	$K_{d_{mut}}/K_{d_{Wt}}$	B_{max} (pmol/mg of protein)	n
L6.60(286)C	0.56 ± 0.09	1.6	2.47 ± 0.37	3	W6.48(274)C	0.42 ± 0.09	1.2	0.13 ± 0.07	2
T6.59(285)C	0.29 ± 0.03	0.83	1.36 ± 0.32	3	C6.47(wt)	0.35 ± 0.07	1.0	1.28 ± 0.20	5
W6.58(284)C	0.73 ± 0.12	2.1	3.02 ± 0.44	3	V6.46(272)C	0.31 ± 0.08	0.87	0.94 ± 0.11	3
V6.57(283)C	0.62 ± 0.10	1.8	2.13 ± 0.40	3	V6.45(271)C	0.29 ± 0.05	0.83	0.26 ± 0.03	3
L6.56(282)C	0.39 ± 0.04	1.1	0.30 ± 0.03	3	F6.44(270)C	0.45 ± 0.11	1.3	0.11 ± 0.02	2
V6.55(281)C	0.95 ± 0.18	2.7	2.48 ± 0.31	3	A6.43(269)C	0.38 ± 0.07	1.1	0.10 ± 0.04	2
F6.54(280)C	0.78 ± 0.20	2.2	1.71 ± 0.15	3	G6.42(268)C	0.49 ± 0.10	1.4	3.21 ± 0.28	3
I6.53(279)C	0.67 ± 0.13	1.9	1.77 ± 0.17	3	V6.41(267)C	0.58 ± 0.14	1.7	2.12 ± 0.30	3
H6.52(278)C	*b*			3	V6.40(266)C	0.34 ± 0.41	0.97	2.02 ± 0.21	3
I6.51(277)C	1.16 ± 0.21	3.3	1.61 ± 0.21	3	V6.39(265)C	0.57 ± 0.12	1.6	1.92 ± 0.22	3
P6.50(276)C	*b*			3	L6.38(264)C	0.40 ± 0.09	1.1	0.38 ± 0.03	3
A6.49(275)C	0.55 ± 0.15	1.6	1.30 ± 0.14	3					

(C) The Human κ Opioid Receptor

mutant	K_d (nM)	$K_{d_{mut}}/K_{d_{C7.38S}}$	B_{max} (pmol/mg of protein)	n	mutant	K_d (nM)	$K_{d_{mut}}/K_{d_{C7.38S}}$	B_{max} (pmol/mg of protein)	n
L6.60(299)C	2.61 ± 0.92	7.5	2.39 ± 0.57	3	W6.48(287)C	0.28 ± 0.07	0.8	0.21 ± 0.09	3
A6.59(298)C	0.25 ± 0.07	0.7	0.80 ± 0.11	3	C6.47(C7.38S)	0.35 ± 0.03	1.0	1.51 ± 0.37	4
E6.58(297)C	0.45 ± 0.08	1.3	0.31 ± 0.07	3	V6.46(285)C	0.41 ± 0.12	1.2	0.80 ± 0.15	3
V6.57(296)C	2.80 ± 0.50	8.0	1.15 ± 0.51	3	V6.45(284)C	0.40 ± 0.15	1.1	0.72 ± 0.12	3
L6.56(295)C	1.50 ± 0.61	4.3	0.11 ± 0.05	3	F6.44(283)C	0.15 ± 0.04	0.4	0.42 ± 0.08	3
I6.55(294)C	0.27 ± 0.05	0.8	0.31 ± 0.45	3	V6.43(282)C	0.18 ± 0.01	0.5	1.31 ± 0.06	3
F6.54(293)C	1.64 ± 0.41	4.7	1.41 ± 0.36	3	A6.42(281)C	0.15 ± 0.02	0.4	0.68 ± 0.04	3
I6.53(292)C	3.60 ± 0.81	10.3	2.90 ± 0.61	3	V6.41(280)C	0.55 ± 0.14	1.6	1.53 ± 0.35	3
H6.52(291)C	*b*			3	V6.40(279)C	0.21 ± 0.05	0.6	0.74 ± 0.04	3
I6.51(290)C	0.24 ± 0.06	0.7	2.61 ± 0.56	3	V6.39(278)C	0.19 ± 0.02	0.5	0.69 ± 0.03	3
P6.50(289)C	*b*			3	L6.38(276)C	0.15 ± 0.01	0.4	0.78 ± 0.08	3
T6.49(288)C	0.62 ± 0.05	1.8	0.48 ± 0.02	3					

[a] Each residue within the TM6 was mutated one at a time to Cys using the rat μ C7.38S mutant, the wild-type human δ, or the human κ C7.38S mutant as the template. Saturation binding of [^{3}H]diprenorphine to each of the mutants and the templates was performed, and K_d and B_{max} values were calculated. Data are expressed as mean ± sem. n represents the number of experiments performed in each duplicate. [b] No binding was detected.

For the μ receptor, the K_d values of 16 cysteine-substitution mutants were between 1.0- and 2.0-fold that of the C7.38S mutant. The K_d values of I6.56C, Y6.54C, I6.51C, and W6.48C mutants were 2.6-, 6.7-, 2.5-, and 3.1-fold that of the C7.38S mutant μ receptor, respectively. For μ mutants showing detectable [^{3}H]diprenorphine binding, the B_{max} values ranged from 6% to 183% of that of the C7.38S mutant (Table 1A).

The K_d values of 18 mutants of the δ receptor ranged from 0.8- to 2.2-fold that of the δ receptor, whereas those of I6.51C and V6.55C were 3.3- and 2.7-fold that of the δ receptor, respectively. The B_{max} values of these mutants were between 7% and 193% of that of the δ receptor except for P6.50C and H6.52C (Table 1B).

For the κ receptor, the K_d values of 15 cysteine-substitution mutants of the C7.38S κ opioid receptor varied from 0.4- to 2-fold that of the C7.38S mutant. In contrast, the K_d values of I6.53C, F6.54C, L6.56C, V6.57C, and L6.60C were 10.3-, 4.7-, 4.3-, 8.0-, and 7.5-fold that of the C7.38S mutant. The B_{max} values of these 22 mutants ranged from 9% to 251% of that of the C7.38S κ receptor (Table 1C).

Effect of MTSEA on [^{3}H]Diprenorphine Binding to Cysteine-Substitution Mutants. Treatment with MTSEA (2.5 mM for μ and κ, 1 mM for δ) for 5 min at room temperature followed by washing significantly inhibited [^{3}H]diprenorphine (0.5 nM) binding to 11 of 22 mutants of the rat μ receptor (V6.40C, A6.42C, F6.44C, W6.48C, I6.51C, Y6.54C, V6.55C, I6.56C, I6.57C, K6.58C, A6.59C), 9 of 22 mutants of the human δ receptor (F6.44C, I6.51C, F6.54C, V6.55C, I6.56C, V6.57C, W6.58C, T6.59C, L6.60C), and 10 of 22 mutants of the human κ receptor (F6.44C, W6.48C, I6.51C, F6.54C, I6.55C, L6.56C, V6.57C, E6.58C, A6.59C, and L6.60C) (Figure 1).

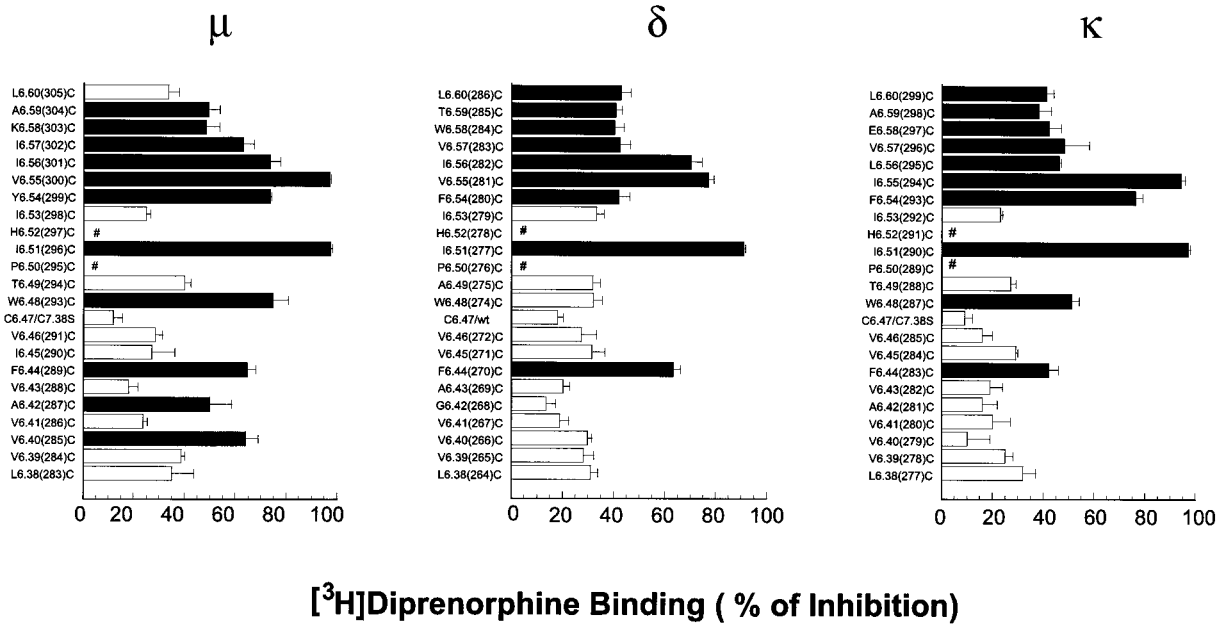

FIGURE 1: SCAM analysis of the TM6 of μ, δ, and κ opioid receptors: Inhibitory effects of MTSEA on [^{3}H]diprenorphine binding to the substituted cysteine mutants expressed in HEK293 cells. Each residue within the TM6 of the μ, δ, and κ opioid receptors was mutated to Cys, one at a time, using the C7.38S mutants of the μ and κ opioid receptors and the wild-type δ opioid receptor as the templates. Each mutant as well as the template was transiently expressed in HEK293 cells, treated with MTSEA (2.5 mM for μ and κ, 1.0 mM for δ), and [^{3}H]diprenorphine binding was determined. Each point represents the mean ± sem of 4−8 experiments in duplicate. Filled bars indicate mutants for which inhibition was significantly different ($p < 0.05$) from the template by one-way ANOVA followed by post hoc Sheffe F test. #: [^{3}H]Diprenorphine binding was undetectable for P6.50C and H6.52C mutants of the μ, δ, and κ opioid receptors.

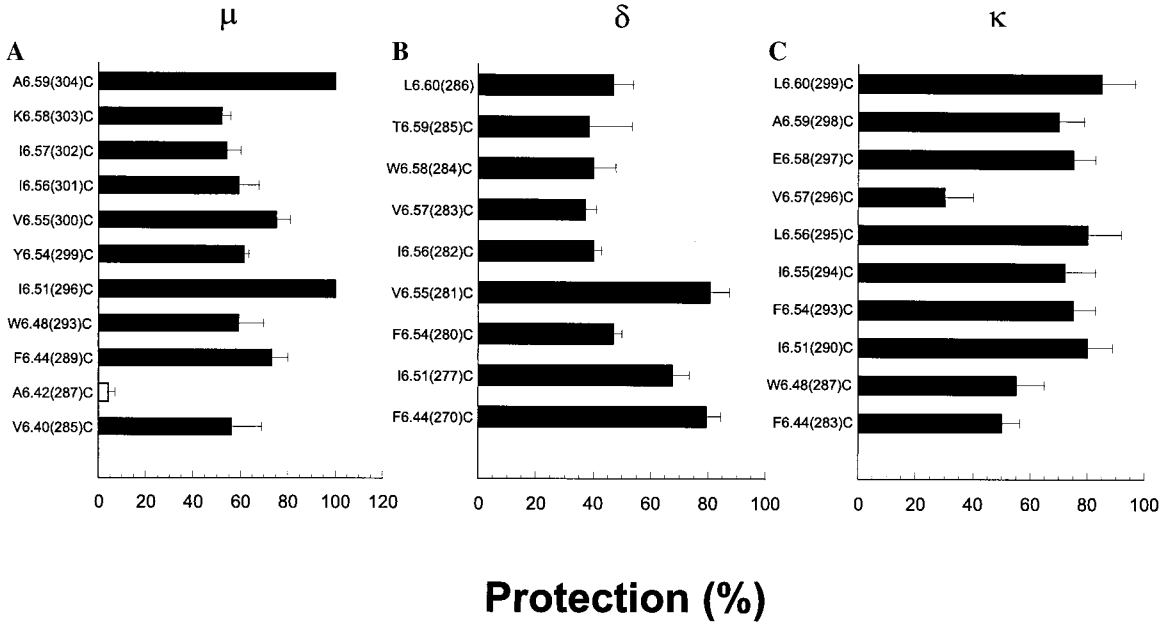

FIGURE 2: Protection by naloxone or diprenorphine against inhibitory effects of MTSEA on [^{3}H]diprenorphine binding to MTSEA-sensitive mutants. Each MTSEA-sensitive cysteine-substituted mutant (identified in Figure 1) expressed in HEK293 cells was preincubated with 20 μM naloxone (for the μ and κ receptors) or 1 μM diprenorphine (the δ receptor) 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 the Table 2 experiments. The cells were washed 3 times and assayed for [^{3}H]diprenorphine binding as described under Materials and Methods. Each datum represents the mean ± sem of 4−7 experiments in duplicate. Naloxone or diprenorphine provided significant protection ($p < 0.05$, by paired *t*-test) for all of the mutants (filled bars) except A6.42(287)C of the μ opioid receptor.

Protective Effect of Naloxone or Diprenorphine against the Inhibitory Action of MTSEA. The residues that constitute the surface of the binding-site crevice are a subset of the water-accessible residues. To determine whether substituted cysteine residues reacted with MTSEA are indeed within or in the vicinity of the binding pockets, we examined whether the reversible opioid receptor antagonist naloxone or diprenorphine could protect the substituted cysteine from the inhibitory action of MTSEA on [^{3}H]diprenorphine binding. Based on experiments for determination of the second-order rate constants, we chose a concentration of MTSEA for each mutant that was just sufficient to produce maximal inhibition

Table 2: Second-Order Rate Constants of Reaction of $MTSEA^+$ (M^{-1} s^{-1}) with Cysteine-Substituted Mutants of the Rat μ Opoid Receptor, the Human δ Opioid Receptor, or the Human κ Opioid Receptor Transiently Expressed in HEK293 Cells[a]

	μ	δ	κ
6.60C	nd[c]	9.8 ± 5.2	3.7 ± 0.18
6.59C	14.6 ± 9.7	24.1 ± 7.1	3.5 ± 0.16
6.58C	3.3 ± 0.6	11.6 ± 7.0	2.5 ± 0.13
6.57C	13.4 ± 5.0	15.8 ± 4.3	3.1 ± 0.15
6.56C	4.0 ± 0.6	24.8 ± 1.8	6.1 ± 0.70
6.55C	61.6 ± 4.6	108.2 ± 7.0	235.2 ± 11.5
6.54C	3.1 ± 0.4	4.9 ± 0.2	145.5 ± 10.2
6.51C	51.2 ± 4.1	192.3 ± 10.0	120.5 ± 9.5
6.48C	30.2 ± 8.6	(1.50 ± 0.02)[b]	50.1 ± 2.3
6.45C	nd	(1.24 ± 0.03)	nd
6.44C	4.6 ± 0.8	5.7 ± 0.5	3.0 ± 0.12
6.40C	13.9 ± 1.2	(1.70 ± 0.01)	nd
6.39C	(0.50 ± 0.11)	nd	nd
6.38C	nd	nd	(0.59 ± 0.10)
background construct	(<0.4)	(0.42 ± 0.12)	(<0.4)

[a] Cells were treated with at least four concentrations of MTSEA, and [^{3}H]diprenorphine binding was performed on washed cells. In most cases, 0.1, 0.25, 1, and 2.5 mM MTSEA were used. The second-order rate constant (k) for each mutant was calculated as described under Materials and Methods. Rate constants of all accessible mutants and some inaccessible mutants (see Figure 1) were determined. Data represent mean ± sem of three dependent experiments in duplicate. [b] Rate constants for selected mutants termed "insensitive" because they were not significantly more inhibited than the background construct by the screening concentration of MTSEA (Figure 1) are shown in parentheses. [c] nd: not determined.

(see below). Pretreatment of MTSEA-sensitive cysteine-substituted mutants of the μ, δ, or κ opioid receptor with naloxone (for the μ and κ mutants) or diprenorphine (for the δ mutants) significantly reduced the inhibitory effects of MTSEA on [^{3}H]diprenorphine binding (Figure 2A−C), except for the A6.42C μ mutant. The extent of protection ranged from ~50% to 100% for the μ mutants (Figure 2A), from ~30% to 80% for the δ mutants (Figure 2B), and from ~30% to ~85% for the κ mutants (Figure 2C). For comparison, naloxone at 1 μM protected the wild-type μ, δ, and κ opioid receptors by 70−90% (*45*).

Determination of Second-Order Reaction Rate Constants of MTSEA Reaction with Sensitive Mutants. To quantitatively express the reactivity of the MTSEA-sensitive mutants of the three opioid receptors with MTSEA, we determined the second-order rate constants (Table 2). An example of the effects of different concentrations of MTSEA is given in Figure 3 for one mutant each of the μ, δ, and κ opioid receptors.

We also determined the reaction rate constants of several "insensitive" mutants that were not significantly inhibited by the screening concentration of MTSEA (see Figure 1). For the κ receptor, even L6.38(276)C, which was inhibited most by MTSEA among the insensitive mutants, had a rate constant of only 0.59 ± 0.10 M^{-1} s^{-1} (mean ± sem, $n = 3$). Other insensitive mutants (6.39, 6.40, 6.41, 6.42, 6.43, 6.45, 6.46, 6.49, 6.53) were inhibited less by all four concentrations of MTSEA (0.25, 1, 2.5, and 10 mM) than L6.38(276)C, and rate constants could not be determined for these mutants due to lack of MTSEA inhibition. Similarly, the L6.39(284)C mutant of the μ opioid receptor had a rate constant of 0.5 ± 0.11 M^{-1} s^{-1} (mean ± sem, $n = 3$), and rates of other insensitive Cys mutants could not be determined. For the δ receptor, the second-order rate constants of some MTSEA-insensitive mutants were as follows: V6.40(266)C, 1.70 ± 0.10 M^{-1} s^{-1} ; V6.45(271)C, 1.24 ± 0.03 M^{-1} s^{-1}; W6.48-(274)C, 1.50 ± 0.02 M^{-1} s^{-1} (mean ± sem, $n = 3-5$). These rate constants should be compared with that of the background construct, the wild-type δ receptor, for which the rate constant was 0.42 ± 0.12 M^{-1} s^{-1} (*45*).

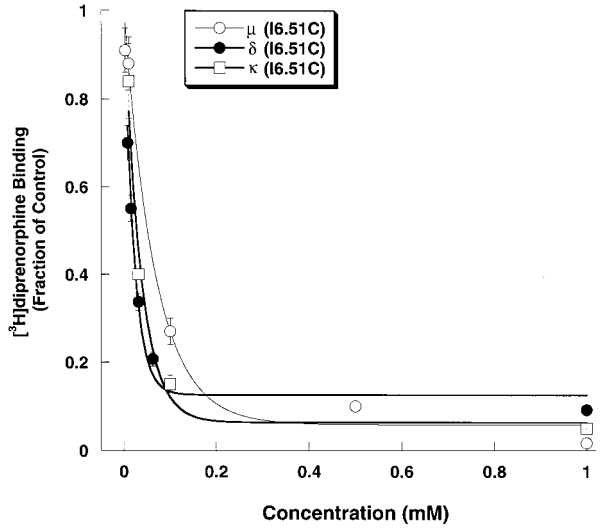

FIGURE 3: Concentration dependence of MTSEA effect on one mutant each of the μ, δ, and κ opioid receptors. HEK293 cells transiently transfected with the I6.51C mutant were treated with four concentrations of MTSEA, and [^{3}H]diprenorphine binding was performed on washed cells. The second-order rate constant (k) for each mutant was calculated as described under Materials and Methods (see Table 2). Each value represents mean ± sem of three independent experiments in duplicate.

Helical wheels and helical nets of the TM6 residues of the μ, δ, and κ opioid receptors are shown in Figure 4. Based on the rate constants of the reactions with MTSEA, residues that are accessible in the binding-site crevices are divided into three groups shaded differently in the representation: >50 M^{-1} s^{-1}, >9−50 M^{-1} s^{-1}, 2.5−9 M^{-1} s^{-1}, to show different degrees of reactivities. A molecular model of TM6 of the rat μ opioid receptor incorporating the information on the residues accessible in the binding-site crevice is shown in Figure 5.

DISCUSSION

Effects of Cysteine Substitutions on [^{3}H]Diprenorphine Binding. In the present study, most of the cysteine-substitution mutants in TM6 of three subtype opioid receptors had K_d values for [^{3}H]diprenorphine within 3-fold of those of the corresponding μ or κ C7.38S mutant or the wild-type δ receptor. However, it is noteworthy that in the κ opioid receptor, unlike in the μ or δ receptor, several cysteine-substitution mutants on the extracellular side of the His6.52 had K_d values for [^{3}H]diprenorphine 4−10-fold greater than that of the C7.38S mutant. This may indicate a more direct involvement of the region from 6.53 to 6.60 in the κ opioid receptor in the binding of [^{3}H]diprenorphine. In addition, mutations in this region may cause a perturbation in the binding site and indirectly affect binding.

We found that W6.48(274)C mutation did not alter [^{3}H]-diprenorphine binding affinity for the δ opioid receptor, whereas W6.48(274)A mutation in the δ opioid receptor

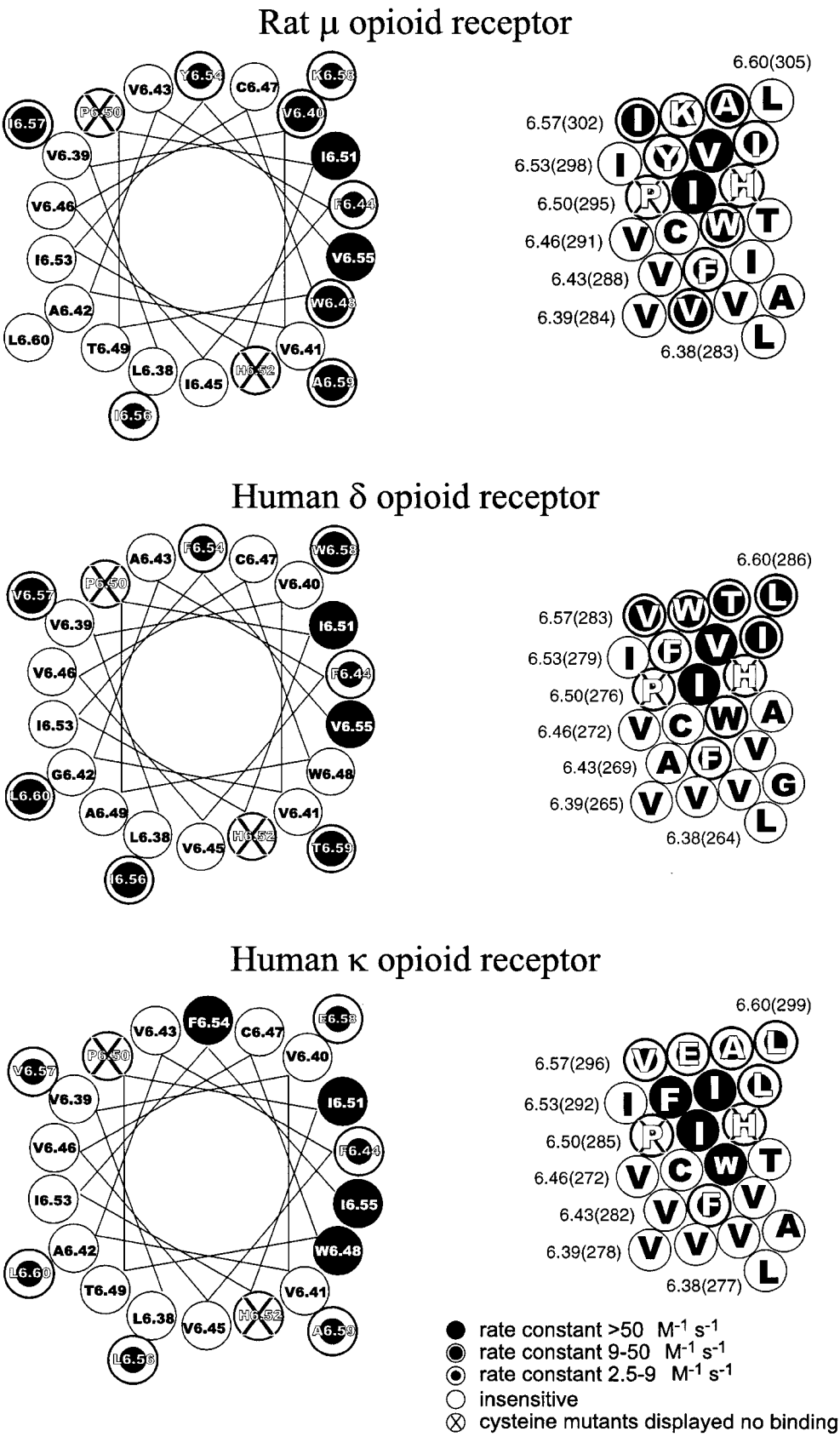

FIGURE 4: (Left) Helical net and (right) helical wheel representations of the residues in the TM6 of the rat μ, human δ, and human κ opioid receptors. Filled circles represent residues accessible in the binding-site crevice, whereas open circles indicate those that are not accessible. Filled circles are divided into three groups depending on the rate constants of MTSEA reaction: >50, >9−50, and 2.5−9 M^{-1} s^{-1}.

reduced binding for nonselective alkaloids up to 65-fold, yet enhanced TIPPψ binding by 5-fold (*55*). This difference may relate to different modes of binding of various ligands. Another possible explanation for these results is a preferred interaction of a Cys, but not an Ala, with aromatic residues. Such an interaction would preserve the properties of the

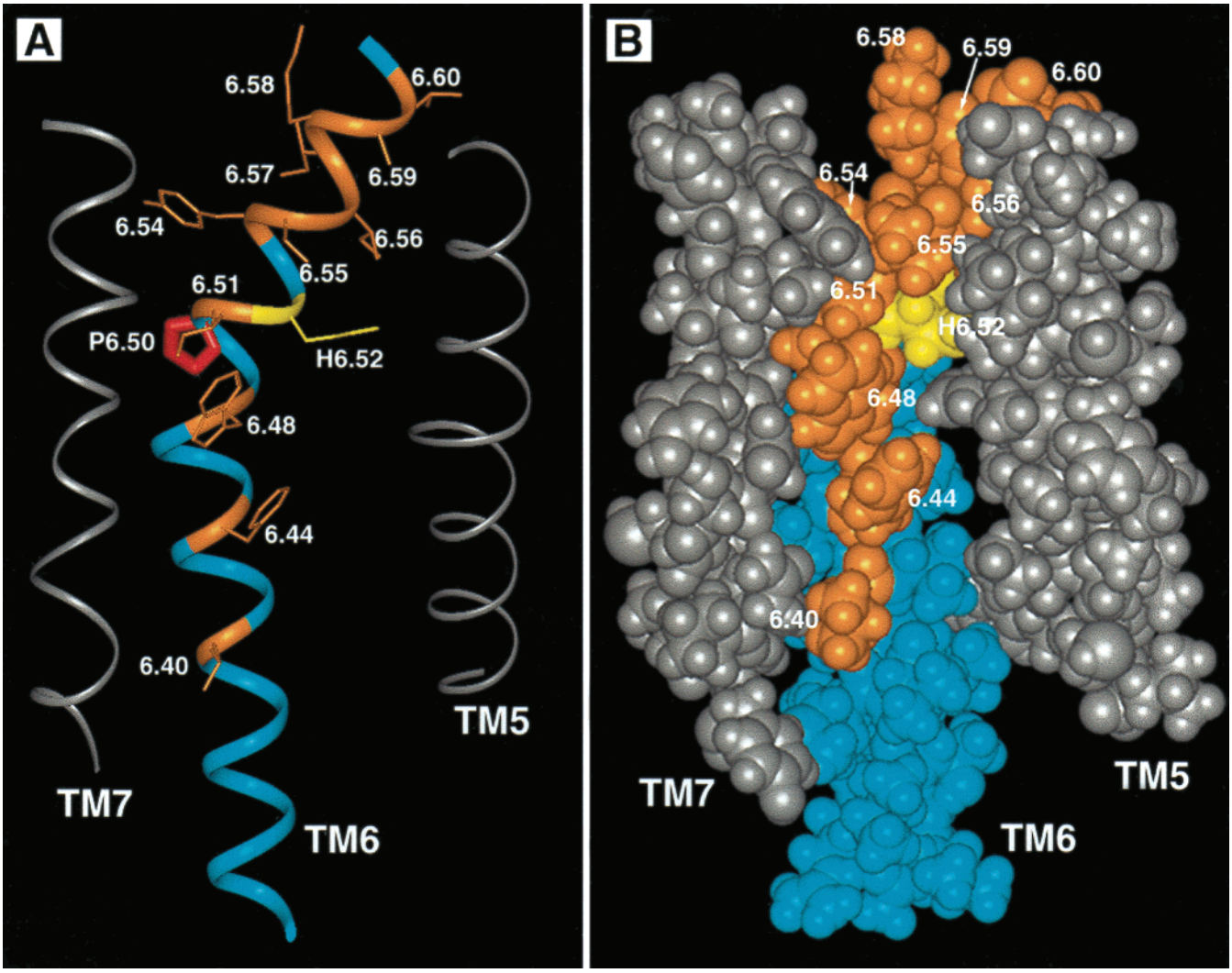

FIGURE 5: Molecular model of TM6 of the rat μ opioid receptor shown in the context of the neighboring TMs 5 and 7. The model of the rat μ opioid receptor was obtained as described recently (*64*) with reference to the crystal structure of rhodopsin (*15*). The views are perpendicular to the membrane and the central axis of the transmembrane bundle. In panel A, the Cα ribbons of TMs 5 and 7 are shown in gray. For TM6, the stick representations of side chains of accessible residues are shown in orange; the side chains of highly conserved Pro6.50 (red), which is responsible for helix kink, and His6.52 (yellow), which has been found to be involved in ligand binding, are also shown in sticks; the Cα ribbons are colored according to the colors of the side chains at the corresponding positions, while the portions not found to be accessible are shown in light blue. In panel B, the color scheme is same as panel A, but all the residues of TMs 5, 6, and 7 are shown in van der Waals space-filling representation.

aromatic cluster (*39*, *64*), a finding consistent with the known propensity of Cys to interact with aromatic residues and with previous observations in the D2 receptor (*39*).

TM6 Residues That Are Accessible in the Binding-Site Crevices. We have shown from SCAM results that in TM6, binding to 11 of 22 mutants of the rat μ opioid receptor, 9 of 22 mutants of the human δ opioid receptor, and 10 of 22 mutants of the human κ opioid receptor were sensitive to MTSEA as determined by [^{3}H]diprenorphine binding. Naloxone or diprenorphine protected the binding of [^{3}H]diprenorphine to all of the sensitive cysteine mutants from the inhibitory action of MTSEA, except for the A6.42(287)C mutant of the μ receptor. We chose to use naloxone or diprenorphine for these protection experiments to decrease the possibility of conformational changes that can be induced by an agonist. Also, naloxone and diprenorphine are readily dissociated from the receptor by washing. Naloxone or diprenorphine could protect directly substituted cysteine residues on the surface of the binding pocket from reaction with MTSEA, as well as protect substituted cysteine residues deeper in the binding-site crevice by blocking the passage of MTSEA from the extracellular medium. These results indicate that V6.40, F6.44, W6.48, I6.51, Y6.54, V6.55, I6.56, I6.57, K6.58, and A6.59 of the μ receptor; F6.44, I6.51, F6.54, V6.55, I6.56, V6.57, W6.58, T6.59, and L6.60 of the δ receptor; and F6.44, W6.48, I6.51, F6.54, I6.55, L6.56, V6.57, E6.58, A6.59, and L6.60 of the κ receptor are on the water-accessible surface of the binding-site crevices (Figures 4 and 5).

Secondary Structures of the TMs 6. Overall, the amino acid homology among μ, δ, and κ opioid receptors is about 60%, and within TM6, 13 of 23 residues are identical. Comparison of the patterns of residues that are accessible in the binding-site crevice in the TMs 6 of the three opioid receptors revealed several interesting similarities and differences. F6.44, I6.51, F/Y6.54, V/I6.55, I/L6.56, I/V6.57, K/W/E6.58, and A/T6.59 in all three receptors are accessible in the binding-site crevice. W6.48 is accessible in the μ and κ, but not the δ, receptors. V6.40 is accessible in the μ, but not the δ and κ, opioid receptor. The patterns of residues in

TM6 accessible in the binding-site crevices of the μ, δ, and κ opioid receptors are consistent with TM6 of these receptors having an α-helical conformation with narrow strips of accessibility on the intracellular side of 6.54 and a proline kink at 6.50, which bends the extracellular portion of the TM6 in toward the binding pocket. This Pro kink may permit considerable motion of this region (*56*), thereby allowing a wider area of accessibility on the extracellular side of 6.54 (Figures 4 and 5). The wider exposure of residues 6.55−6.60 to the binding-site crevice, coupled with the divergent amino acid sequences, suggests the potential importance of this region for ligand binding selectivity. These results are consistent with the recent high-resolution X-ray diffraction results showing that the TM6 of rhodopsin has an α-helical structure with a strong proline kink (*15*).

Distinction between MTSEA-Sensitive and Insensitive Residues. In the δ receptor, the extent of inhibition by 1 mM MTSEA at some of the sensitive loci (6.54, 6.57−6.60) appears only slightly higher than for some insensitive loci (6.38, 6.39, 6.40, 6.45, 6.48, 6.49, 6.53) (see Figure 1). We determined the second-order rate constants for the V6.40-(266)C, V6.45(271)C, and W6.48(274)C mutants and found substantially lower rate constants than for the sensitive mutants (Table 2). In addition, we determined the reaction rate constants of several μ and κ mutants that were not significantly inhibited by the screening concentration of MTSEA. L6.38(276)C in the κ receptor and L6.39(284)C in the μ opioid receptor, the mutants among the insensitive mutants that were inhibited most by MTSEA, had much lower rate constants than those of the sensitive mutants. The results, shown in Table 2, provide support for the distinction between the sensitive and insensitive mutants (Figure 1) using a screening concentration of MTSEA.

SCAM relies upon the comparison of the effects of reaction with MTSEA of each Cys mutant with the effect on the background construct. The background constructs of the opioid receptor subtypes were differentially sensitive to MTSEA. The second-order reaction rate constant for the δ background construct (the wild-type δ receptor) was 0.42 $\pm$ 0.12 M^{-1} s^{-1} (*45*). Compared to the δ receptor, the κ and μ background constructs (the C7.38S mutants) were substantially less inhibited by MTSEA in the concentration range of 0.25−10 mM, and because of the lack of significant MTSEA inhibition, the rate constants in the κ and μ background constructs could not be determined.

Differences in the Rate Constants of MTSEA Reaction among the Three Receptors. Although the three opioid receptors appear to have similar general accessibility patterns, the accessible residues did not always have similar rate constants. Across the three receptors, both 6.51C and 6.54C had rate constants >50 M^{-1} s^{-1}, and 6.44C had rate constants in the lowest group (2.5−9 M^{-1} s^{-1}). In contrast, 6.40C is accessible in the μ, but not in the δ or κ receptor. 6.54C in the κ receptor had a rate constant >50 M^{-1} s^{-1}, whereas in the μ and δ receptors the rate constants belonged to the lowest group. From 6.56C to 6.60C, in general, the rate constants were in the order of $\delta > \mu > \kappa$. The differences in the rate constant of a substituted cysteine with MTSEA may reflect differences in many parameters, some of which are interrelated, including steric factors, the ionization state of the sulfhydryl group, the electrostatic potential in the local microenvironment, and local differences in dynamic fluctuations of the structure.

Residues on the Intracellular Side of F6.44. In the δ and κ opioid receptors, [^{3}H]diprenorphine binding to each cysteine-substitution mutant of the amino acid residues on the intracellular side of F6.44 was unaffected by MTSEA treatment, although V6.40 proved accessible in the μ receptor. Several different factors may explain the lack of effect of an MTS reagent treatment on ligand binding in the δ and κ opioid receptors. First, since we use ligand binding as an indirect measure of the MTS effect, it is possible that the MTS reagent reacts with a cysteine residue without inhibiting binding (*37*). Second, it can indicate a lack of reaction due to the inaccessibility of the cysteine in the binding-site crevice, perhaps because of tighter packing of TMs of the δ and κ opioid receptors in the vicinity of V6.40. Third, the cysteine residue may be accessible in the binding-site crevice, but prevented from reacting with the MTS reagent by steric hindrance or unfavorable charge in the microenvironment surrounding the residue. Another possibility for V6.40 is greater dynamic fluctuations in the structure of the μ opioid receptor.

F6.44 and W6.48. F6.44 and W6.48 are part of the highly conserved aromatic cluster in the TM6 of GPCRs, which was suggested to act as a toggle switch during activation of the 5-HT2A receptor (*57*) and likely other GPCRs. In addition, photoactivation of rhodopsin results in part from conformational changes at W6.48(265) (*58*). In rhodopsin, F6.44(261) and W6.48(265) along with E3.37(122) form a major part of the binding pocket covering the β-ionone ring of 11-*cis*-retinal (*15*). In the D2 dopamine receptor, F6.44-(382) and W6.48(386) are part of the aromatic cluster that is on the water-accessible surface of the binding-site crevice (*39*). Our results that F6.44 in all three opioid receptors and W6.48 in the μ and κ opioid receptors are accessible in the binding-site crevices are consistent with all of these findings. Curiously, although W6.48C of the μ and κ receptors was sensitive to MTSEA, W6.48C of the δ receptor was not. Because the aromatic cluster is highly conserved and involved in receptor activation, it is likely that W6.48 is accessible in the binding-site crevice of all three opioid receptors. The reduced MTSEA sensitivity in the δ receptor may be due to steric hindrance, to suppressed ionization of the cysteine, or to subtle differences in the local conformation of the helix backbone in this region.

I6.51. Our finding that the I6.51C mutant of each opioid receptor is highly reactive with MTSEA indicates that I6.51 is readily accessible in the binding-site crevice. This observation, coupled with the exposure of His6.52 in the binding pocket (*59*) (see below), is consistent with reports that these residues are significantly involved in ligand recognition and/or maintaining high-affinity binding pockets in opioid receptors. For example, by mutating four or five amino acids in the orphanin FQ receptor to those of opioid receptors, including VQV6.51−6.53IHI, Meng et al. (*60*) generated receptor mutants that recognized opioid ligands with high affinity. These results are also in accord with the X-ray crystal structure of rhodopsin showing that Y6.51(268) and A6.52(269) are part of the binding pocket which surrounds the β-ionone ring and C9-methyl of 11-*cis*-retinal (*15*).

H6.52. Although we could not draw any conclusion as to the accessibility of His6.52 in the binding-site crevice due

to the lack of [^{3}H]diprenorphine binding activity to the H6.52C mutants, this residue was demonstrated to be exposed in the binding pocket. Spivak et al. (*59*) showed that treatment of the rat μ opioid receptor with diethyl pyrocarbonate (DEPC), a histidine-specific alkylating agent, substantially decreased ligand binding. Mutation of His6.52(297) to Gln or Asn greatly reduced the effect of DEPC, indicating that the reaction with His6.52(297) is largely responsible for its effect. Incubation with opioid ligands protected the receptor from the inhibitory effect of DEPC, supporting the notion that the side chain of His6.52(297) of the rat μ opioid receptor is positioned in or near the binding cavity. Our observation that the H6.52(297)C mutant of the rat μ opioid receptor exhibited no detectable [^{3}H]diprenorphine binding is consistent with those of Surratt et al. (*61*) and Mansour et al. (*62*), who found that H6.52(297)A mutation greatly reduced or completely abolished ligand binding (*62*). In contrast, mutation of His6.52(297) to Gln or Asn retained reasonable affinity for several ligands and afforded agonist-like signal transduction mediated by the alkaloid antagonists (*59*). These results are consistent with a role for the His6.52-(297) in maintaining the integrity of high-affinity binding sites and in determining whether a specific ligand triggers receptor activation.

Residues on the Extracellular Side of H6.52. The notion that because of wider exposure and sequence divergence, residues on the extracellular side of P6.50 in the TM6 of the opioid receptors play important roles in ligand binding selectivity is supported by results from chimeric receptor and site-directed mutagenesis studies. Chimeric receptor studies have revealed that the region encompassing TM6, the third extracellular loop, and TM7 is important for binding of selective agonists in the μ receptor (*16, 17*), for the selectivity in the δ receptor (*23*−*26*), and for binding of the selective antagonist norbinaltorphimine to the κ receptor (*22, 28, 29*). The 6.58 locus was shown to be important for ligand binding selectivity. The E6.58(297)K mutation reduced the binding affinity of norbinaltorphimine for the κ receptor 142-fold (*29, 63*). Mutation of Trp6.58(284) to Ala significantly decreased the binding of δ-selective ligands (*23*). A κ opioid receptor mutant in which Glu6.58 (297), Ser7.33 (310), Tyr7.35(312), and Tyr7.36(313) were changed to the corresponding residues in the μ receptor, Lys, Val, Trp, and His, respectively, bound DAMGO with high affinity and efficiently mediated the inhibitory effect of DAMGO adenylate cyclase (*18*). Since the extracellular portion of TM6 is involved in interactions with peptide as well as nonpeptide ligands, it is likely that this region has a significant degree of conformational flexibility, which may be reflected in its wide accessibility in the binding-site crevice.

Comparison between the μ Opioid and the D2 Dopamine Receptors. The accessibility patterns of the μ opioid and the D2 dopamine receptors are very similar, with only minor differences. Javitch et al. (*39*) have shown that 10 of 22 residues in TM6 of the D2 dopamine receptor are on the surface of the binding site (V6.40, F6.44, W6.48, P6.50, F6.51, F6.52, T6.54, H6.55, I6.56, and I6.59). On the cytoplasmic side of P6.50, the residues accessible in the binding-site crevice are identical between the D2 dopamine receptor and the μ opioid receptor, namely, V6.40, F6.44, and W6.48. The conservation of the secondary structures within this region between the D2 dopamine receptor and the μ opioid receptor, which have structurally distinct ligands, suggests its importance in receptor activation, as shown in rhodopsin (*58*) and the serotonin 5-HT2A receptor (*57*). On the extracellular side of P6.50, the accessibility patterns of the μ opioid receptors appear to be similar to, but wider than, the D2 dopamine receptor. The μ opioid receptor and the D2 dopamine receptor are remotely related members of the rhodopsin-like subfamily of GPCRs. Such a high degree of similarity in the secondary structures of the μ opioid receptor and the D2 dopamine receptor suggests that TMs 6 of the rhodopsin-like subfamily of GPCRs are likely not only to have similar secondary structures, but also to be packed similarly into the transmembrane bundle and thus have similar tertiary structure.

Effects of cysteine substitution of P6.50 and H6.52 in the μ, δ, and κ opioid receptors on [^{3}H]diprenorphine binding differ from those of the analogous mutations in the D2 dopamine receptor. Thus, P6.50C and H6.52C mutations in the opioid receptors abolished binding, while P6.50C and F6.52C mutations only reduced the binding affinity of the antagonist [^{3}H]-*N*-methylspiperone to the D2 dopamine receptor by about 3- and 4-fold, respectively. These results indicate that the 6.50 and 6.52 loci play more important roles in [^{3}H]diprenorphine binding or in maintaining the integrity of the binding pocket of the opioid receptors. Site-directed mutagenesis studies on H6.52 in opioid receptors support this notion (*59, 61, 62*). In contrast, whereas W6.48(386)C and F6.51(389)C mutations in the D2 dopamine receptor greatly reduced the affinities of the two antagonists [^{3}H]-*N*-methylspiperone and (−)-sulpiride, the W6.48C and I6.51C mutants of opioid receptors bound [^{3}H]diprenorphine with similar affinities as the wild-type or the C7.38S mutant. Thus, the 6.48 and 6.51 loci appear to be more important for the binding of antagonists to the D2 dopamine receptor than for diprenorphine binding to the opioid receptors.

Limitations of SCAM. The residues were defined as "sensitive" if the effect of reaction of our screening concentration of MTSEA significantly inhibited binding relative to the background by ANOVA and post hoc testing (see Figure 1). This is a statistical method, and it is imperfect, given that each position will have a different reactivity that is affected by local steric factors, electrostatic potential, ionization of the cysteine sulfhydryl, and dynamics of the protein. Moreover, the extent of the inhibition caused by modification of a substituted Cys can vary, and, therefore, this indirect method of assaying for reaction, although necessary, is imperfect as well. Among the sensitive mutants, if naloxone or diprenorphine could prevent the reaction with MTSEA, we called the substituted cysteine, and hence the residue mutated, "accessible" in the binding site-crevice.

There are considerable variations in the second-order rate constants among the sensitive cysteine mutants of each of the μ, δ, and κ opioid receptors. For example, among the sensitive κ mutants, there is an almost 100-fold difference between the most reactive one [I6.55(294)C, 235.2 ± 15 M^{-1} s^{-1} (mean $\pm$ sem, $n = 3$)] and the least reactive one [E6.58-(297)C, 2.5 ± 0.13 M^{-1} s^{-1}]. Such a large difference has also been observed in the SCAM analyses of the D2 dopamine receptor (*34*−*41*), and results from the factors are discussed above. When comparing across receptor subtypes, an issue that did not exist for the original work in the D2 receptor, we must compare not only the overall pattern of

accessibility but also the reactivity of the substituted Cys in order to make a fair and sensible comparison. 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.

Concluding Remarks. The patterns of accessibility of residues in the TM6 of three opioid receptors are consistent with α-helical secondary structures with a narrow strip of accessibility on the intracellular side of 6.54; on the extracellular side of 6.54, there is a wider exposure of residues in the binding pocket, most likely due to a proline kink at 6.50, allowing bending of the helix in toward the binding pocket and enhancing the mobility of this portion of the TM6. The highly conserved α-helical structure on the intracellular side of 6.54 suggests its importance in receptor activation, while the wide exposure on the extracellular side of 6.54 coupled with divergent amino acid sequences supports a role of this region in ligand binding selectivity. The accessibility pattern is similar to that in the D2 dopamine receptor (*39*). The inferred secondary structure is similar to the recently published structure of rhodopsin (*15*). Such similarity among rhodopsin, the D2 dopamine receptor, and opioid receptors suggests that GPCRs of the rhodopsin-like subfamily are likely to have similar secondary and tertiary structures.

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