The Conserved Cysteine 7.38 Residue Is Differentially Accessible in the Binding-Site Crevices of the μ, δ, and κ Opioid Receptors[†]

Wei Xu,[‡] Chongguang Chen,[‡] Peng Huang,[‡] Jin Li,[‡] J. Kim de Riel,[§] Jonathan A. Javitch,[||] and Lee-Yuan Liu-Chen*,[‡]

Department of Pharmacology and Center for Substance Abuse Research and Fels Institute for Molecular Biology and Cancer Research, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and Center for Molecular Recognition, Columbia University College of Physicians and Surgeons, New York, New York 10029

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ABSTRACT: Binding pockets of the opioid receptors are presumably formed among the transmembrane domains (TMDs) and are accessible from the extracellular medium. In this study, we determined the sensitivity of binding of [^{3}H]diprenorphine, an antagonist, to μ, δ, and κ opioid receptors to charged methanethiosulfonate (MTS) derivatives and identified the cysteine residues within the TMDs that conferred the sensitivity. Incubation of the μ opioid receptor expressed in HEK293 cells with MTS ethylammonium (MTSEA), MTS ethyltrimethylammonium (MTSET), or MTS ethylsulfonate (MTSES) inhibited [^{3}H]-diprenorphine binding with the potency order of MTSEA > MTSET > MTSES. Pretreatment of μ, δ, and κ opioid receptors with MTSEA dose-dependently inhibited [^{3}H]diprenorphine binding with MTSEA sensitivity in the order of $\kappa > \mu \gg \delta$. The effects of MTSEA occurred rapidly, reaching the maximal inhibition in 10 min. (−)-Naloxone, but not (+)-naloxone, prevented the MTSEA effect, demonstrating that the reaction occurs within or in the vicinity of the binding pockets. Each cysteine residue in the TMDs of the three receptors was mutated singly, and the effects of MTSEA treatment were examined. The mutants had similar affinities for [^{3}H]diprenorphine, and C7.38(321)S, C7.38(303)S, and C7.38-(315)S mutations rendered μ, δ, and κ opioid receptors less sensitive to the effect of MTSEA, respectively. These results indicate that the conserved Cys7.38 is differentially accessible in the binding-site crevice of these receptors. The second extracellular loop of the κ receptor, which contains several acidic residues, appears to play a role, albeit small, in its higher sensitivity to MTSEA, whereas the negative charge of Glu6.58(297) did not. To the best of our knowledge, this is the first report to show that a conserved residue among highly homologous G protein-coupled receptors is differentially accessible in the binding-site crevice. In addition, this represents the first successful generation of MTSEA-insensitive mutants of μ, δ, and κ opioid receptors, which will allow determination of residues accessible in the binding-site crevices of these receptors by the substituted cysteine accessibility method.

Structure−activity relationships of G protein-coupled receptors (GPCRs)[1] have been intensively studied. Lack of high-resolution three-dimensional structural information on GPCRs makes it necessary to probe structure−activity relationships by site-directed mutagenesis, chimeric receptors, and affinity labeling approaches. These studies have provided insights into the potential functional roles of specific residues and domains of receptors as well as the mechanisms of receptor binding and activation (for reviews, see refs *1*−*4*). Identification of the residue forming a covalent bond with an affinity ligand provides the most definitive evidence that a residue lines the binding pocket. In contrast, alterations by mutation or in chimeric receptors can be due to changes in direct ligand−receptor interaction, global conformational changes in the receptor, and local conformational changes in or around the binding pocket, which may complicate the interpretation of data.

Karlin and his colleagues (*5*, *6*) developed three small and charged methanethiosulfonate (MTS) reagents that react specifically with reduced sulfhydryl groups: MTS ethylam-

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* Correspondence should be sent to this author. Phone: (215) 707-4188. Fax: (215) 707-7068. E-mail: lliuche@astro.temple.edu.

[‡] Department of Pharmacology and Center for Substance Abuse Research, Temple University School of Medicine.

[§] Fels Institute for Molecular Biology and Cancer Research, Temple University School of Medicine.

[||] Center for Molecular Recognition, Columbia University College of Physicians and Surgeons.

[1] Abbreviations: buffer A, 25 mM HEPES buffer, 140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, and 0.006% BSA, pH 7.4; GPCR, G protein-coupled receptor; HA, hemagglutinin peptide sequence YPY-DVPDYA; hdor, human δ opioid receptor; HEK293 cells, human embryonic kidney cells; hkor, human κ opioid receptor; MTS reagents, methanethiosulfonate reagents; MTSEA, MTS ethylammonium or (2-aminoethyl)methanethiosulfonate hydrobromide, $CH_3SO_2SCH_2CH_2NH_3^+$ Br^-; MTSET$^+$, MTS ethyl trimethylammonium or [2-(trimethylammonium)ethyl]methanethiosulfonate bromide, $CH_3SO_2SCH_2CH_2N(CH_3)_3^+$ Br^-; MTSES$^-$, MTS ethylsulfonate or sodium (2-sulfonatoethyl)methanethiosulfonate, $CH_3SO_2SCH_2CH_2SO_3^-$ Na^+; NEM, *N*-ethylmaleimide; rmor, rat μ opioid receptor; SCAM, substituted cysteine accessibility method; TEL buffer, 50 mM Tris-HCl buffer, 1 mM EGTA, and 10 μM leupeptin, pH 7.4; TMD, transmembrane domain.

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monium, $CH_3SO_2SCH_2CH_2NH_3^+$ ($MTSEA^+$), MTS ethyltrimethylammonium, $CH_3SO_2SCH_2CH_2N(CH_3)_3^+$ ($MTSET^+$), and MTS ethylsulfonate, $CH_3SO_2SCH_2CH_2SO_3^-$ ($MTSES^-$). When reacted with cysteine, $-SCH_2CH_2NH_3^+$, $-SCH_2CH_2N(CH_3)_3^+$, and $-SCH_2CH_2SO_3^-$ of these reagents form mixed disulfide bonds with the −SH group of cysteine. MTS reagents react 10^9 times faster with ionized thiolates than with un-ionized thiols (*7*), and ionization of cysteine is likely to occur to a significant extent only for water-accessible residues (*8*). The reaction rate of charged sulfhydryl-specific reagents with cysteine residues would be expected to be highest with water-accessible cysteine residues and much less with those in the interior of proteins or facing lipid. When reacted with cysteine residues within the pore of the nicotinic acetylcholine receptor, these reagents disrupt the ion transport function of the channels (*5*). A method was developed, named the substituted cysteine accessibility method (SCAM) (for reviews, see refs *8* and *9*), to identify the residues that lined the channel of the nicotinic acetylcholine receptor (*5*). Each residue within the transmembrane domain was mutated to cysteine, one at a time, and the microenvironment of the engineered cysteine residue was probed using these MTS reagents. If the mutant containing an engineered cysteine is sensitive to MTS reagents, it is inferred that the residue being substituted lines the pore.

Binding pockets of GPCRs are presumably formed by the seven putative TMDs and are accessible to the extracellular medium. Within the binding pocket, water-accessible residues can directly interact with ligands. Javitch et al. (*10*) used the MTS reagents to identify Cys118 being exposed in the binding-site crevice of the D_2 dopamine receptor. Subsequently, Javitch and his colleagues have applied SCAM to map residues accessible in the binding-site crevice within the TMDs 2, 3, 5, 6, and 7 of the D_2 dopamine receptor (*11*−*16*).

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, which have unique ligand specificities, anatomical distributions, and physiological functions (*17*, *18*). These opioid receptors are coupled through G proteins to affect a variety of effectors, which include adenylate cyclase, potassium channels, calcium channels (*19*), and a mitogen-activated protein kinase pathway (*20*). In 1992, Kieffer et al. (*21*) and Evans et al. (*22*) cloned a mouse δ opioid receptor by expression cloning. Subsequently, μ and κ receptors were cloned from several species (*23* and references cited therein). The deduced amino acid sequences of these clones display the motif of seven putative transmembrane domains (TMDs) connected by alternating intracellular and extracellular hydrophilic loops, which is characteristic of GPCRs. On the basis of the arrangement of seven α-helices from a 9 Å projection map of bovine rhodopsin (*24*, *25*) and electron micrographs of frozen-hydrated two-dimensional frog rhodopsin crystals (*26*), several groups have constructed molecular models of opioid receptors (*27*−*29*).

Ligand binding to opioid receptors was shown to be inhibited by sulfhydryl alkylating agents, such as *N*-ethylmaleimide (NEM), iodoacetamide, and *p*-hydroxymercuribenzoate (*30*−*39*). The presence of an agonist or antagonist protected the receptor from alkylation by these sulfhydryl reagents, indicating that the sensitive −SH group(s) is (are) within the binding site or in close proximity to the binding site. NEM inhibited binding by at least two mechanisms: direct inhibition by alkylation of the receptor or indirect inhibition by uncoupling G proteins and the receptor (*37*−*39*).

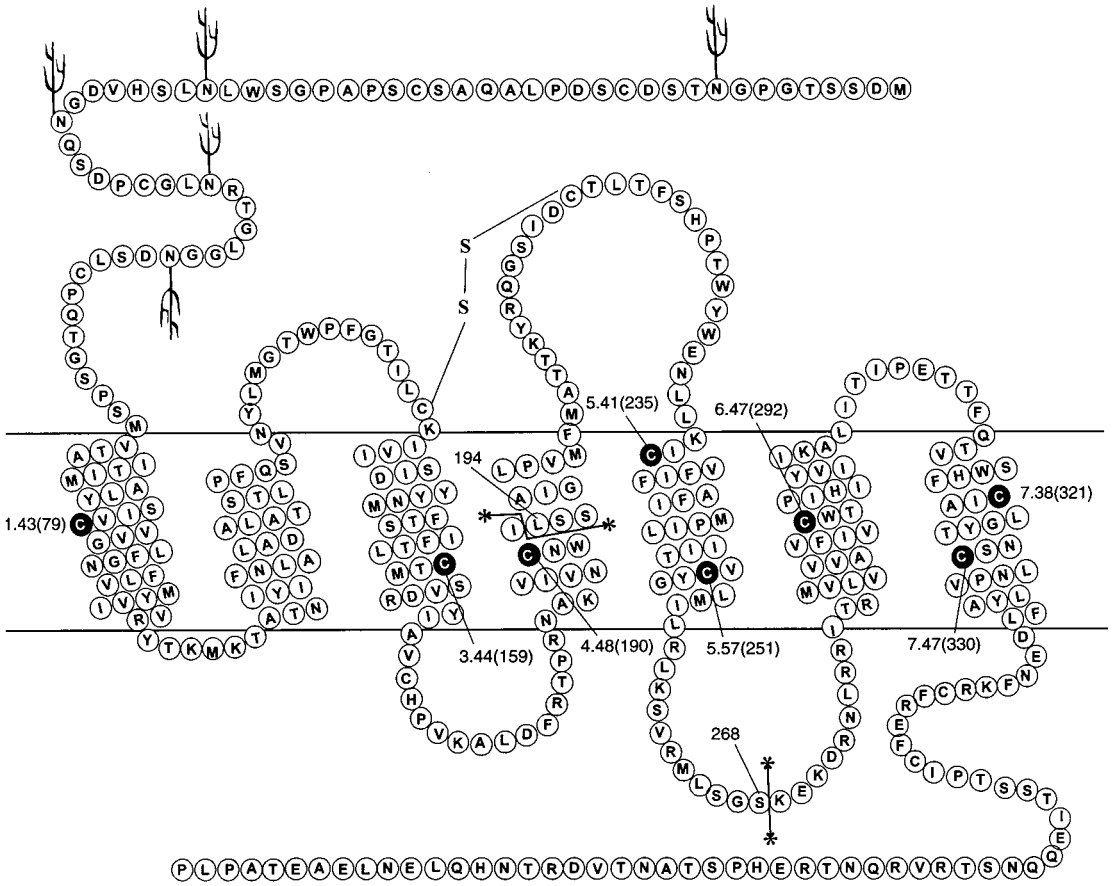
Within the putative seven TMDs of the μ, δ, and κ receptors, there are eight, six, and five cysteine residues, respectively (Figure 1, Table 1). Of these cysteine residues, five are conserved among the three receptors (C4.48, C5.41, C5.57, C6.47, and C7.38), one is present in both μ and δ receptors (C1.43), and two are unique in the μ receptor (C3.44 and C7.47) (Table 1) (see Materials and Methods for numbering schemes). Effects of MTS reagents on ligand binding to the μ and δ opioid receptor in membrane preparations and to purified μ opioid receptors have been reported (*36*, *40*, *41*). Since MTS reagents react readily with water-accessible reduced −SH groups, using membranes or purified receptors allows reactions with accessible cysteine residues in intracellular domains of the receptor molecules, which may complicate the interpretation of the data. In addition, no MTS-insensitive mutants of the μ and δ opioid receptors have been identified under the published experimental conditions. In the present study, we used intact cell preparations and short incubation periods with MTS reagents to examine their effects on [^{3}H]diprenorphine binding to μ, δ, and κ receptors. Using such a paradigm, similar to that of Javitch et al. (*16*), we sought to minimize penetration of MTSEA into cells and hence its reactions with cysteine residues in intracellular domains. The objectives of this study are twofold. First, we examined whether the wild-type μ, δ, and κ opioid receptors were sensitive to MTS reagents. Second, if the receptors were sensitive to these reagents, we then determined the cysteine residues in the binding pocket that conferred the sensitivity. Three criteria were used to establish that a cysteine was accessible in the binding-site crevice (*10*): (1) MTSEA added extracellularly irreversibly inhibited ligand binding, (2) binding activity was protected from MTSEA by pretreatment with opioid ligands, and (3) mutation of cysteine in the binding pocket to serine reduced sensitivity to the MTS reagent.

MATERIALS AND METHODS

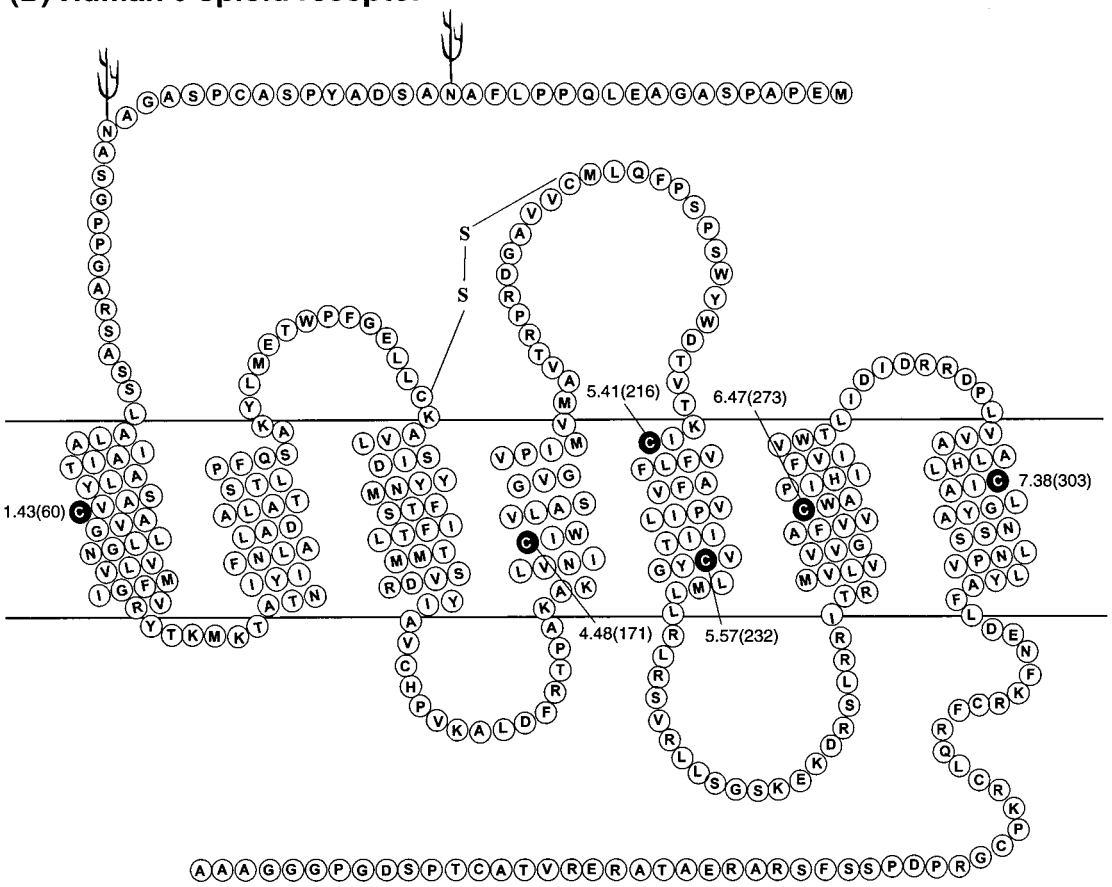
Materials. [^{3}H]Diprenophine (58 Ci/mmol) and [^{3}H]-bremazocine (26.2 Ci/mmol) were purchased from NEN Life Science (Boston, MA). Diprenorphine and (+)-naloxone were provided by the National Institute on Drug Abuse. (−)-Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). MTSEA [(2-aminoethyl)methanethiosulfonate hydrobromide], MTSES [sodium (2-sulfonatoethyl)methanethiosulfonate], and MTSET [[2-(trimethylammonium)ethyl]-methanethiosulfonate bromide] were purchased from Toronto Research Chemicals (North York, Ontario, Canada).

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 (*2*) was used. According to this nomenclature, amino acid residues in putative TMDs were assigned two numbers (N1.N2). N1 refers to the TMD number. For N2, the most conserved residue in each TMD

(A) Rat μ opioid receptor



(B) Human δ opioid receptor



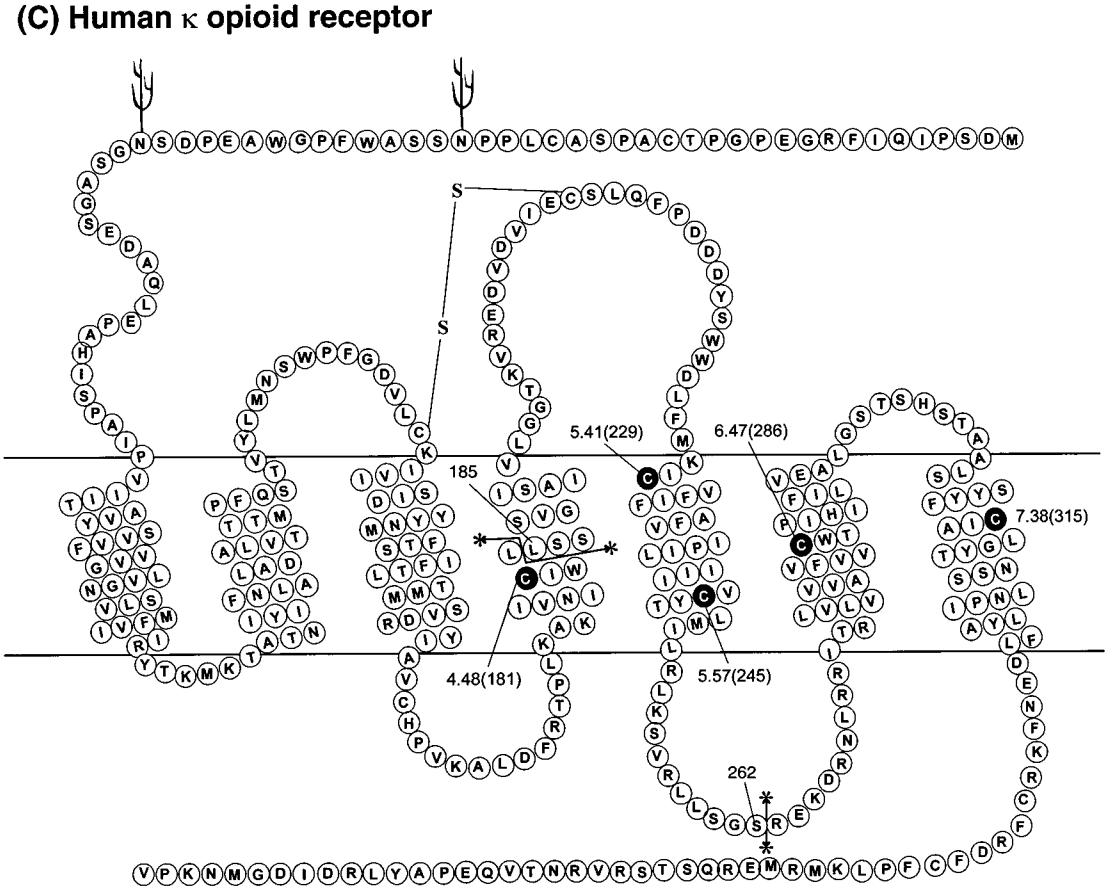


FIGURE 1: Schematic representation of amino acid sequences of the rat μ, human δ, and human κ opioid receptors and cysteine residues within the putative transmembrane domains. The single letter amino acid codes are used for the amino acid sequences. The numbers refer to the positions of the residues within the protein sequences. The dark circles indicate cysteine residues in the transmembrane domains that were mutated in the present study. Fragments that were exchanged in μ/κ chimeras I and II (I = κ1−184/μ194−268/κ263−380 and II = μ1−193/κ185−262/μ269−398) are indicated by the symbol (∗−∗) at both ends.

Table 1: Mutation of Each Cysteine Residue to Serine or Methionine within the TMDs of the Rat μ, the Human δ, and the Human κ Opioid Receptors: Identification by Generic Numbers and Sequence Numbers[a]

	1.43	3.44	4.48	5.41	5.57	6.47	7.38	7.47
μ	C79S	C159S	C190S	C235S	C251S	C292S	C321S	C330M
δ	C60S		C171S	C216S	C232S	C273S	C303S	
κ			C181S	C229S	C245S	C286S	C315S	

[a] Each cysteine residue within the TMDs of the rat μ, the human δ, and the human κ opioid receptors was mutated to serine or methionine, one at a time. The first row shows generic numbers of all cysteine residues mutated according to Ballesteros and Weinstein (*2*). The second, third, and fourth rows represent the corresponding sequence number of each cysteine and its mutant in the rat μ, the human δ, and the human κ opioid receptors, respectively.

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 the published literature on other GPCRs. The following are residues that correspond to the most conserved residues in each TMD of the rat μ, human δ, and human κ opioid receptors: Asn1.50 in TMD1 (μ, Asn86; δ, Asn67; κ, Asn77), Asn2.50 in TMD2 (μ, Asn114; δ, Asn95; κ, Asn105), Arg3.50 in TMD3 (μ, Arg165; δ, Arg146; κ, Arg156), Trp4.50 in TMD4 (μ, Trp192; δ, Trp173; κ, Trp183), Pro5.50 in TMD5 (μ, Pro244; δ, Pro226; κ, Pro238), Pro6.50 in TMD6 (μ, Pro295; δ, Pro276; κ, Pro289), and Pro7.50 in TMD7 (μ, Pro333; δ, Pro315; κ, Pro327).

Construction of Epitope-Tagged μ, δ, and κ Opioid Receptors. The rat μ receptor (*42*) was epitope-tagged with the hemagglutinin (HA) sequence (YPYDVPDYA) immediately after the first methionine residue and subcloned into *Hin*dIII and *Xba*I sites of the mammalian expression vector pcDNA3 as described previously (*43*).

An ~130-bp fragment containing the FLAG tag sequence was excised with *Hin*dIII and *Nco*I from a β_2-adrenergic receptor construct in pcDNA3, which was FLAG-tagged 5′ to the initiation codon (*44*). The human κ opioid receptor in the *Sac*I and *Pst*I sites of the vector pBK/CMV (*45*) was digested with *Nco*I and *Xho*I to generate an ~1.26-kb fragment. The *Hin*dIII/*Nco*I-generated ~130-bp fragment containing the FLAG epitope and the *Nco*I/*Xho*I-treated κ opioid receptor were ligated into *Hin*dIII and *Xho*I sites of the vector pcDNA3. A polymerase chain reaction was performed on this κ receptor construct to remove restriction enzyme sites using AGA CCC AAG CTT CAA TTC GAG C as the 5′ primer and GAG CTC GAG CTG CAG TAG TGA TCT GAG TTA as the 3′ primer. The ~1.4-kb PCR product was digested with *Hin*dIII and *Xho*I and cloned into *Hin*dIII and *Xho*I sites of the vector pcDNA3.

A silent mutation (underlined) was introduced into the human δ opioid receptor cDNA to generate a *Nco*I site around the initiation codon by polymerase chain reaction

using GCG GCC GCC ATG GAA CCG G as the 5′ primer and the SP6 promoter sequence as the 3′ primer and the human δ opioid receptor in the vector pcDNAI (*46*) as the template. The resultant product was digested with *Nco*I and *Xba*I. FLAG-tagged human δ opioid receptors were constructed by ligating the *Hin*dIII/*Nco*I-generated ~130-bp fragment containing the FLAG epitope and the *Nco*I/*Xba*I-treated δ opioid receptor into *Hin*dIII and *Xba*I sites of the vector pcDNA3. DNA sequences of the clones were determined with the method of Sanger et al. (*47*) to verify correct construction. The tag sequences did not affect binding properties of the μ, δ, and κ receptors. Epitope-tagged opioid receptors have been used in many studies (for example, see refs *48* and *49*).

Oligodeoxynucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed on the HA-tagged rat μ receptor, the FLAG-tagged human δ receptor, and the FLAG-tagged human κ receptor with the overlap PCR method described by Higuchi et al. (*50*). Mutants of HA-tagged rat μ opioid receptor and FLAG-tagged human δ and κ receptors were cloned into the vector pcDNA3, at *Hin*dIII and *Xba*I sites for μ, *Hin*dIII and *Xba*I sites for δ, and *Hin*dIII and *Xho*I sites for κ receptors, respectively. The DNA sequence was determined with the method of Sanger et al. (*47*) to confirm the presence of desired mutations and the absence of unwanted mutations.

Construction of Chimeric μ/κ Receptors. Two chimeric receptors (Figure 1), chimera I and II, were constructed previously from rat μ and κ opioid receptors (*51*). The fragments exchanged extended from the middle of the TMD4 to the middle of the third intracellular loop. Chimera I was constructed on the basic structure of the rat κ receptor with a rat μ receptor fragment (amino acid κ1−184/μ194−268/κ263−380). Chimera II was the reciprocal of chimera I and had the rat μ receptor basic structure with a fragment of the rat κ receptor (amino acid μ1−193/κ185−262/μ269−398).

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 μ, δ, or κ opioid receptor cDNA using the calcium phosphate method (*52*).

Saturation Binding. Membranes were prepared from HEK cells as described previously (*53*). K_d and B_{max} values of the wild type and mutant μ, δ, and κ receptors were determined with a series of concentrations of [^{3}H]diprenorphine (ranging from 25 pM to 2 nM). 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 (*54*).

Effect of MTS Reagents on Opioid Receptor Binding. These experiments were performed using a procedure modified from that of Javitch et al. (*15*). 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, 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 the cell suspension were incubated with freshly prepared MTS reagents at the stated concentration at room temperature for 5 min. After the reaction, cell suspensions were then diluted 10-fold, washed with buffer A, and pelleted again. The pellet were resuspended in 800 μL per dish of buffer A solution, and 200 μL aliquots were used for [^{3}H]diprenorphine and [^{3}H]bremazocine binding to intact cells as described previously (*55*). Briefly, cells were incubated with ~0.3 nM [^{3}H]diprenorphine or ~3 nM [^{3}H]bremazocine for 1 h at room temperature. 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 Dunnett's post hoc 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 μ, δ, or κ opioid receptor and MTSEA was determined to gain quantitative information on MTSEA sensitivity, according to Javitch et al. (*16*) with modifications. Each receptor was incubated with indicated concentrations of MTSEA for 5 min. The results were fit to the equations:

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

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

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 indicated concentrations of (−)-naloxone or (+)-naloxone 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 (see Figure 2B): μ, 10 mM; δ, 50 mM; κ, 3 mM. Cells were washed three times by centrifugation, then resuspended in buffer A, and assayed for [^{3}H]diprenorphine binding.

Determination of Protein Content. Protein contents of membranes were determined by the bicinchoninic acid method of Smith et al. (*56*) with bovine serum albumin as the standard.

RESULTS

Effects of MTS Reagent Pretreatment on [^{3}H]Diprenorphine Binding to the Opioid Receptors. Pretreatment of HEK293 cells transiently transfected with the μ opioid receptor with MTSEA, MTSET, and MTSES for 5 min at room temperature followed by washing inhibited [^{3}H]-diprenorphine binding to intact cells in a dose-dependent manner (Figure 2A). MTSEA is the most potent of the three MTS reagents with an IC_{50} value of 0.5 ± 0.08 mM, followed by MTSET with an IC_{50} value of 20.0 ± 1.2 mM ($n = 3$). In contrast, the negatively charged MTSES was ineffective

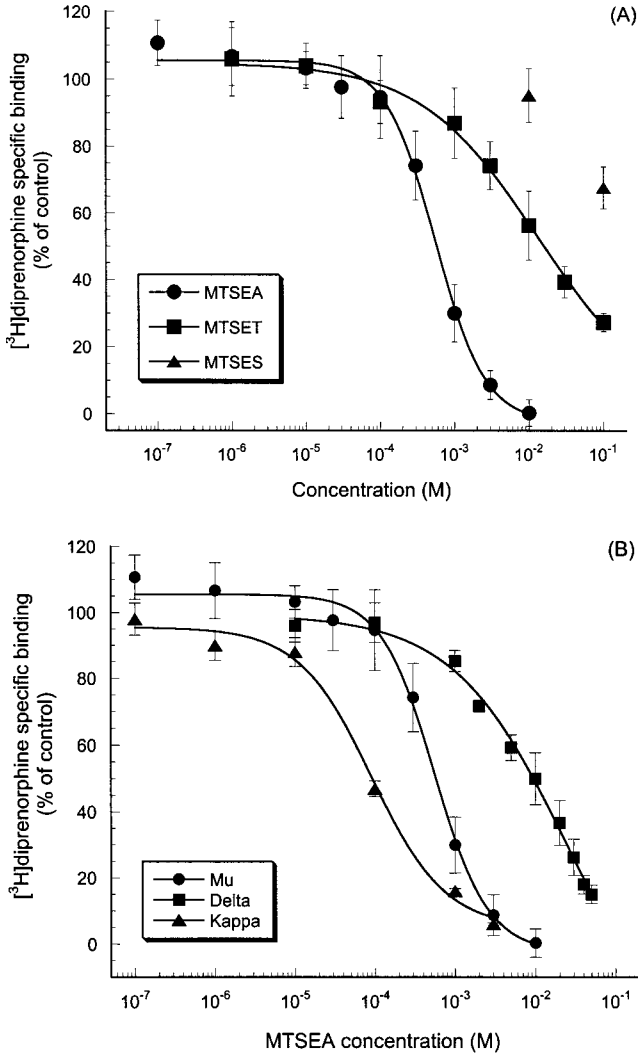


FIGURE 2: (A) Effect of pretreatment with MTS reagents on [^{3}H]-diprenorphine binding to the rat μ opioid receptor. HEK293 cells transiently transfected with the rat μ opioid receptor were treated with MTSEA, MTSET, and MTSES at the indicated concentrations for 5 min at room temperature. (B) Effect of MTSEA pretreatment on [^{3}H]diprenorphine binding to the rat μ, human δ, and human κ opioid receptors. HEK293 cells transiently transfected with the rat μ opioid receptor, the human δ receptor, or the human κ opioid receptor were treated with MTSEA at the indicated concentrations for 5 min at room temperature. [^{3}H]Diprenorphine binding was performed on whole cells after washing as described in Materials and Methods. Each point represents the mean ± sem of three independent experiments in duplicate.

at inhibiting binding at concentrations below 10 mM (Figure 2A). These results are similar to the findings of Javitch et al. (*10*) in D2 dopamine receptor. Javitch et al. (*16*) suggested that a microdomain composed of three acidic residues in TMDs 2 and 3 may account for the enhanced rate of reaction of positively charged MTSEA. We thus used MTSEA in subsequent experiments on δ and κ opioid receptors.

Pretreatment of the δ or κ opioid receptor with MTSEA followed by washing inhibited [^{3}H]diprenorphine binding in a dose-dependent manner with an IC_{50} value of 10.0 ± 2.3 mM and 0.10 ± 0.03 mM ($n = 3$), respectively (Figure 2B). The second-order rate constants of MTSEA reaction were calculated to be 3.7 ± 0.56, 0.42 ± 0.12, and 34.3 ± 2.12 M^{-1} s^{-1} for the μ, δ, and κ receptors, respectively, indicating that the order of MTSEA sensitivity is $\kappa > \mu > \delta$.

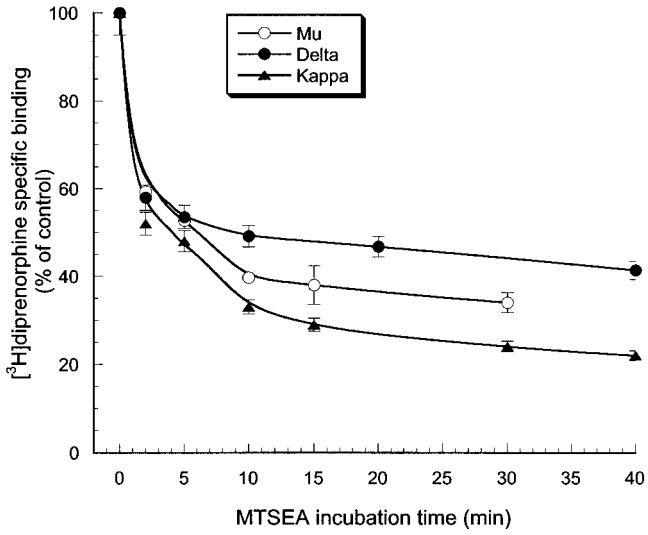


FIGURE 3: Time courses of the effect of MTSEA on [^{3}H]-diprenorphine binding to the rat μ, human δ, and human κ opioid receptors. HEK293 cells were transfected with the rat μ, the human δ, or the human κ opioid receptor and treated with 0.5, 10, or 0.1 mM MTSEA, respectively, for the indicated time periods at room temperature. [^{3}H]Diprenorphine binding was determined after washing as described in Materials and Methods. Each point represents the mean ± sem of three independent experiments in duplicate.

Time Courses of the Effects of MTSEA on [^{3}H]Diprenorphine Binding to μ, δ, and *κ Opioid Receptors.* μ, δ, and κ receptors transiently transfected into HEK293 cells were treated with 0.5, 10, and 0.1 mM MTSEA, respectively, which are IC_{50} values (5 min at room temperature) at these receptors. MTSEA pretreatment substantially reduced [^{3}H]-diprenorphine binding in 2 min and reached a plateau in about 10 min (Figure 3). At the plateau level, approximately 25−40% of [^{3}H]diprenorphine binding remained, which may in part represent the intracellular receptors. Unreacted receptors may also contribute to the binding, since the concentrations used were quite modest. A 5-min pretreatment period was chosen for all subsequent experiments since it is close to the plateau and the $t_{1/2}$ of MTSEA in aqueous media is about 12 min (*8*). In addition, a short incubation time minimizes the entry of MTSEA into cells and possible reactions with intracellular cysteines both in the receptor and in other molecules (*57*).

Protection of Opioid Receptors from MTSEA Inhibition by Naloxone. (−)-Naloxone, but not its inactive isomer (+)-naloxone, prevented the inhibitory effect of MTSEA on [^{3}H]-diprenorphine binding to the μ, δ, and κ receptors in a dose-dependent manner (Figure 4), indicating that the MTSEA reacts with a −SH group within or near the binding sites.

Effects of Pretreatment with MTSEA on [^{3}H]Diprenorphine Binding to Cys to Ser or Met Mutants of the Opioid Receptors. Each cysteine residue in the TMDs was mutated to serine one at a time. There are five cysteine residues conserved among the three opioid receptors (4.48, 5.41, 5.57, 6.47, and 7.38), one common to μ and δ receptors (1.43), and two unique to the μ receptor (3.44 and 7.47) (Table 1). Eight mutants of the rat μ receptor [C1.43(79)S, C3.44-(159)S, C4.48(190)S, C5.41(235)S, C5.57(251)S, C6.47-(292)S, C7.38(321)S, and C7.47(330)S], six of the human δ receptor [C1.43(60)S, C4.48(171)S, C5.41(216)S, C5.57-(232)S, C6.47(273)S, and C7.38(303)S], and five of the

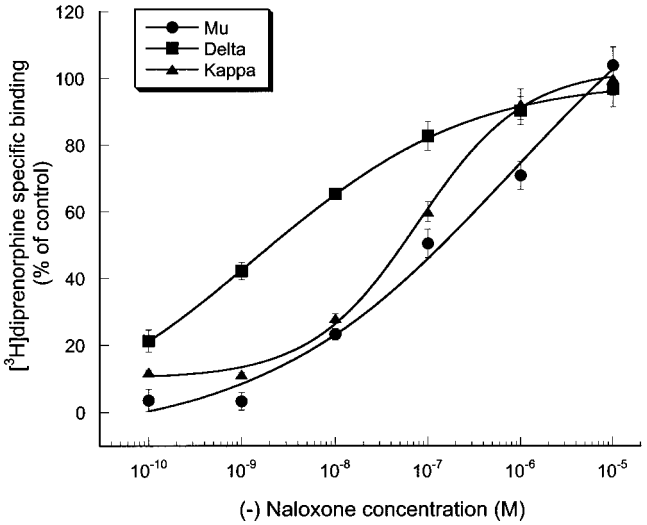


FIGURE 4: Protection by (−)-naloxone against MTSEA effects on [3H]diprenorphine binding to the rat μ, the human δ, and the human κ opioid receptors. HEK293 cells transiently transfected with the rat μ, the human δ, or the human κ opioid receptor were preincubated with the indicated concentrations of (−)naloxone for 20 min at room temperature and then reacted with MTSEA for 5 min at room temperature at a concentration that was just sufficient to cause the maximal extent of inhibition for each receptor: μ, 10 mM; δ, 50 mM; κ, 3 mM. The cells were washed three times, resuspended in buffer A, and assayed for [3H]diprenorphine binding as described in Materials and Methods. Each point represents the mean ± sem of three independent experiments in duplicate.

human κ receptor [C4.48(181)S, C5.41(229)S, C5.57(245)S, C6.47(286)S, and C7.38(315)S] were generated (Table 1). No [3H]diprenorphine binding to C7.47(330)S of the μ receptor was detected. Thus, C7.47(330)A and C7.47(330)M mutants were generated. While no [3H]diprenorphine binding to C7.47(330)A was detected, [3H]diprenorphine binding to C7.47(330)M was detectable. Each mutant bound [3H]-diprenorphine with similar affinities as the respective wild-type receptor (Table 2), indicating that none of the mutations affected the binding pockets substantially. However, each mutation reduced the expression level of the receptor to a varying extent (Table 2).

The sensitivity of each mutant to MTSEA pretreatment was examined. MTSEA concentrations used represented those producing at least 60% inhibition of the wild-type receptors: μ, 2.5 mM; δ, 15 mM; κ, 1 mM. Among all mutants, the C7.38S mutant of the μ, δ, or κ receptor became substantially less sensitive to MTSEA (Figure 5). For the μ receptor, MTSEA treatment reduced [3H]diprenorphine binding of the wild type to about 20%, whereas the C7.38S mutant still retained about 90% binding activity (Figure 5A). For the δ receptor, the wild-type binding was inhibited by 15 mM MTSEA treatment to about 35%, whereas about 65% of the [3H]diprenorphine binding to the C7.38S mutant was unaffected (Figure 5B). For the κ receptor, while 1 mM MTSEA incubation decreased the wild-type receptor binding to 20%, 80% of the [3H]diprenorphine binding to the C7.38S mutant remained (Figure 5C). These results indicate that the conserved residue Cys7.38 confers most of the sensitivity of opioid receptors to MTSEA. In contrast, C6.47S mutants of the μ and δ, but not the κ, opioid receptors appear to be more sensitive to MTSEA than the wild-type receptors (Figure 5).

Table 2: K_d and B_{max} Values of [3H]Diprenorphine Binding to the Wild Type and Cysteine to Serine or Methionine Mutants of the Rat μ, the Human δ, and the Human κ Opioid Receptors Transiently Expressed in HEK293 Cells[a]

	receptor constructs	K_d (nM)	B_{max} (pmol/mg of protein)
μ	wild type	0.14 ± 0.03	5.23 ± 0.35
	C1.43(79)S	0.38 ± 0.10	0.81 ± 0.07
	C3.44(159)S	0.15 ± 0.03	1.02 ± 0.04
	C4.48(190)S	0.35 ± 0.03	1.41 ± 0.10
	C5.41(235)S	0.12 ± 0.01	2.09 ± 0.41
	C5.57(251)S	0.14 ± 0.01	1.39 ± 0.03
	C6.47(292)S	0.13 ± 0.03	1.24 ± 0.09
	C7.38(321)S	0.11 ± 0.01	0.46 ± 0.05
	C7.47(330)M[b]	0.21 ± 0.003	2.06 ± 0.05
δ	wild type	0.29 ± 0.05	1.79 ± 0.65
	C1.43(60)S	0.77 ± 0.02	1.26 ± 0.60
	C4.48(171)S	0.24 ± 0.08	1.10 ± 0.25
	C5.41(216)S	0.24 ± 0.09	0.97 ± 0.08
	C5.57(232)S	0.26 ± 0.06	0.81 ± 0.15
	C6.47(273)S	0.20 ± 0.01	0.21 ± 0.01
	C7.38(303)S	0.30 ± 0.03	1.21 ± 0.22
κ	wild type	0.15 ± 0.02	1.56 ± 0.04
	C4.48(181)S	0.14 ± 0.02	1.44 ± 0.36
	C5.41(229)S	0.21 ± 0.03	1.94 ± 0.44
	C5.57(245)S	0.20 ± 0.03	1.16 ± 0.10
	C6.47(286)S	0.16 ± 0.03	1.73 ± 0.25
	C7.38(315)S	0.35 ± 0.03	1.43 ± 0.22

[a] Saturation binding of [3H]diprenorphine to the wild type and the mutants was performed, and K_d and B_{max} values were calculated as described in Materials and Methods. Data represent the mean ± sem of three to four independent experiments, each performed in duplicate. [b] C7.47(330)S displayed no detectable [3H]diprenorphine binding; therefore, the C7.47(330)M mutant was generated.

The findings that naloxone protected the receptors against MTSEA effect and that C7.38S appears to confer most of the sensitivity indicate that Cys7.38 residues in the opioid receptors are exposed in the binding-site crevice. It is noteworthy that Phe7.38(411) in the D2 dopamine receptor, corresponding to Cys7.38 in the opioid receptors, is accessible from the binding-site crevice and likely faces the TMD6 (*14*). The second-order reaction rate constant of F7.38C with MTSEA was determined to be 6.6 ± 1.1 M^{-1} s^{-1} (*14*), which is similar to that of the μ receptor (3.7 ± 0.56 M^{-1} s^{-1}) but higher than that of the δ receptor (0.42 ± 0.12 M^{-1} s^{-1}) and lower than that of the κ receptor (34.3 ± 2.12 M^{-1} s^{-1}).

Effects of Pretreatment with MTSEA on [3H]Bremazocine Binding to Cys to Ser or Met Mutants of the μ Opioid Receptor. We examined [3H]bremazocine binding to Cys to Ser or Met mutants of the μ opioid receptor following MTSEA pretreatment to determine whether binding of [3H]-bremazocine and [3H]diprenorphine was differentially affected. The C7.38(321)S mutant of the μ opioid receptor became significantly less sensitive to MTSEA, whereas other mutants were similarly sensitive as the wild type (data not shown). Thus, C7.38(321) contributes to the majority of MTSEA sensitivity regardless of whether binding was conducted with [3H]bremazocine or [3H]diprenorphine.

Effects of Pretreatment with MTSEA on [3H]Diprenorphine Binding to Glu6.58(297) Mutants of the κ Receptor. As shown in Figure 2B, μ, δ, and κ opioid receptors are differentially sensitive to MTSEA with the order of sensitivity being $\kappa > \mu \gg \delta$. We next examined the basis of the higher MTSEA sensitivity of the κ receptor than μ and δ receptors. According to the molecular models of opioid

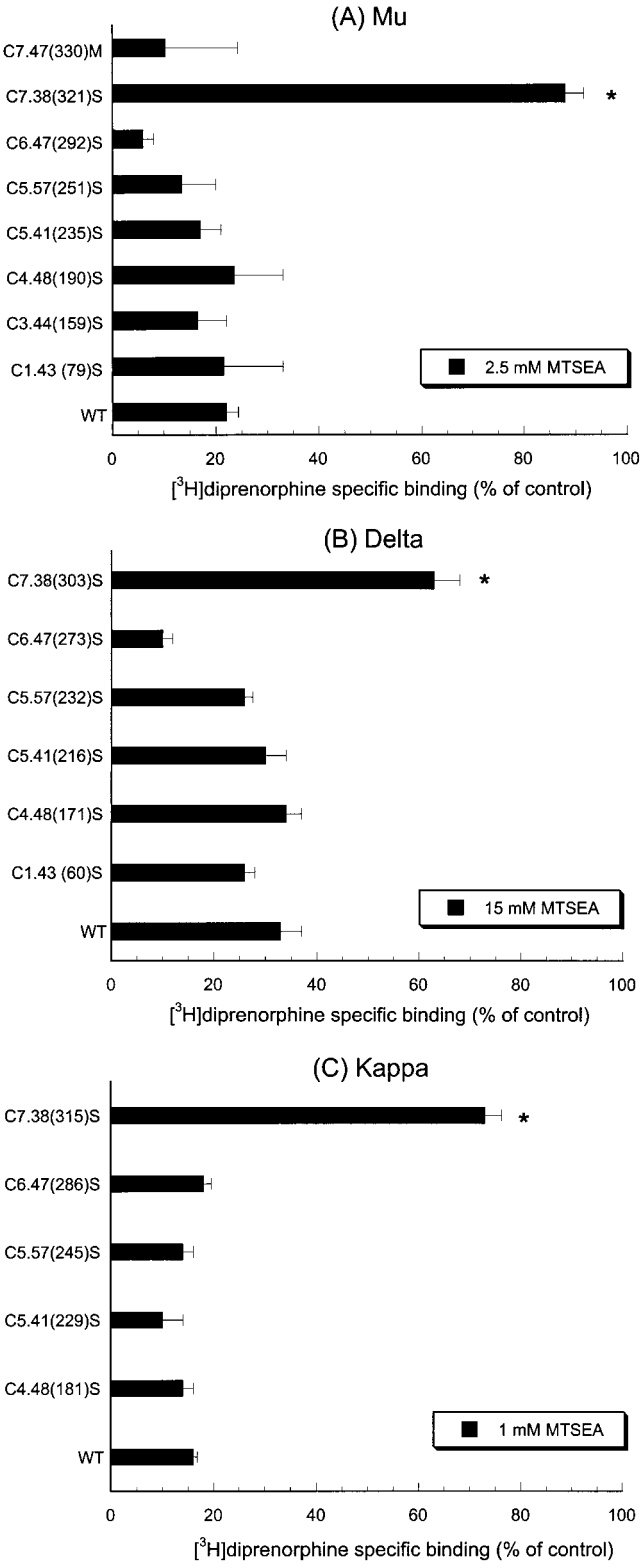


FIGURE 5: Effect of pretreatment with MTSEA on [^{3}H]diprenorphine binding to the wild type and Cys to Ser or Met mutants of (A) the rat μ opioid receptor, (B) the human δ opioid receptor, and (C) the human κ opioid receptor. HEK293 cells transiently transfected with the wild type or the mutants of each receptor were treated with MTSEA at the indicated concentrations for 5 min, and [^{3}H]diprenorphine binding was determined after washing as described in Materials and Methods. Each point represents the mean $\pm$ sem of three to eight independent experiments in duplicate. An asterisk indicates $p < 0.05$, as compared to the wild type by one-way ANOVA followed by Dunnett's post hoc test.

receptors constructed by Strahs and Weinstein (*27*), the 6.58 loci of the μ, δ, and κ receptors are in the vicinity of Cys7.38. We hypothesized that since most MTSEA molecules are positively charged at physiological pH, the negative charge of Glu6.58 in the κ receptor may attract MTSEA and facilitate its reaction with Cys7.38. To test this possibility, we generated the E6.58Q mutant. In addition, Glu6.58 was converted to Lys and Trp, the corresponding residues in the μ and δ receptors, respectively. E6.58(297)K and E6.58-(297)Q mutants had affinities for [^{3}H]diprenorphine similar to that of the wild type, with K_d values of 0.32 $\pm$ 0.04 nM ($n = 3$, mean $\pm$ sem) and 0.25 $\pm$ 0.04 nM ($n = 3$, mean $\pm$ sem), respectively. In contrast, the E6.58(297)W mutant yielded no detectable binding. The E6.58(297)K and E6.58-(297)Q mutants showed sensitivities to MTSEA similar to that of the wild-type κ receptor, with IC_{50} values of 0.10 $\pm$ 0.06 mM and 0.26 $\pm$ 0.04 mM ($n = 5-7$, mean $\pm$ sem), respectively, compared to 0.12 $\pm$ 0.03 mM for the wild-type κ receptor. The second-order rates were determined to be 41.7 $\pm$ 7.7 and 30.0 $\pm$ 4.3 M^{-1} s^{-1} for E6.58(297)K and E6.58(297)Q mutants, respectively, compared to 37.9 $\pm$ 2.9 M^{-1} s^{-1} for the wild type. These results indicate that Glu6.58-(297) does not play a significant role in the heightened sensitivity of the κ receptor to MTSEA.

Role of the Second Extracellular Loop of the κ Receptor in Its MTSEA Sensitivity. The second extracellular loop of the κ opioid receptor contains several acidic residues and was shown to be important in the high-affinity binding of dynorphin peptides, which have several Lys and Arg residues (*51*, *58*). We therefore tested the hypothesis that the second extracellular loop is important for the higher sensitivity of the κ receptor to MTSEA, compared with the μ and δ receptors. We previously constructed two chimeric μ/κ receptors (*51*). Chimera I is the rat κ opioid receptor with a rat μ opioid receptor fragment from the middle of the TMD4 to the middle of the third intracellular loop containing the second extracellular loop. Chimera II is the rat μ opioid receptor containing the corresponding fragment of the rat κ opioid receptor. Chimeras I and II were transiently transfected into HEK293 cells and examined for effects of MTSEA treatment on [^{3}H]diprenorphine binding. Chimera I displayed a low level of [^{3}H]diprenorphine binding, which did not allow us to determine its MTSEA sensitivity with confidence. Chimera II had an affinity for [^{3}H]diprenorphine similar to that of the wild-type μ and κ receptors as previously demonstrated (*51*). Pretreatment of chimera II with MTSEA reduced [^{3}H]diprenorphine binding with an IC_{50} value of 0.53 $\pm$ 0.10 mM ($n = 8$, mean $\pm$ sem) (Figure 6). The wild-type rat κ receptor had an IC_{50} value of 0.08 $\pm$ 0.004 mM ($n = 4$, mean $\pm$ sem), indicating that the rat and human κ opioid receptors are similarly sensitive to MTSEA. The IC_{50} value for the μ receptor was 1.06 $\pm$ 0.13 mM ($n = 4$, mean $\pm$ sem). Thus, the sensitivity of chimera II to MTSEA is between those of the μ and κ opioid receptors. Similarly, the second-order rate of chimera II (7.58 $\pm$ 1.45 M^{-1} s^{-1}) was higher than that of the wild-type μ receptor (2.40 $\pm$ 0.51 M^{-1} s^{-1}) but lower than that of the wild-type κ receptor (28.20 $\pm$ 1.09 M^{-1} s^{-1}). These results indicate that the second extracellular loop of the κ receptor may play a role, albeit small, in the heightened sensitivity of the κ opioid receptor to MTSEA.

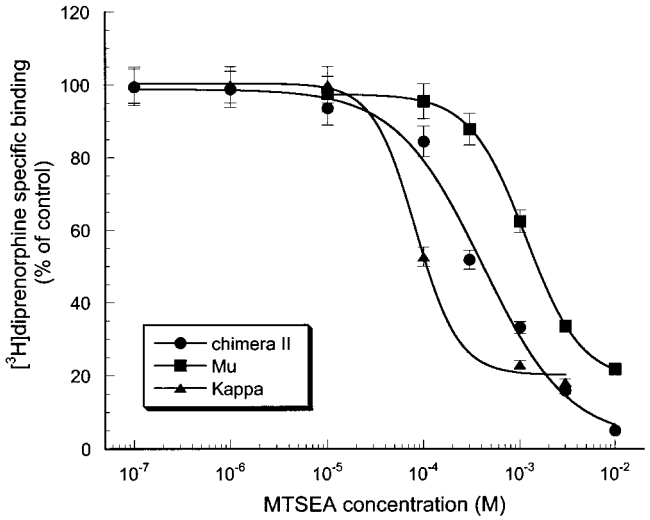


FIGURE 6: Effect of pretreatment with MTSEA on [^{3}H]diprenorphine binding to the rat μ and κ opioid receptors and the μ/κ chimera II. HEK293 cells transiently transfected with the rat μ opioid receptor (rmor), the rat κ opioid receptor (rkor), or the μ/κ chimera II were treated with MTSEA at the indicated concentrations for 5 min at room temperature, and [^{3}H]diprenorphine binding was determined after washing as described in Materials and Methods. Each point represents the mean ± sem of four to eight independent experiments in duplicate.

DISCUSSION

We have demonstrated that pretreatment with MTSEA decreases [^{3}H]diprenorphine binding to the μ, δ, and κ opioid receptors in a time- and concentration-dependent manner. The μ, δ, and κ opioid receptors have differential sensitivities to MTSEA, with the κ receptor being most sensitive and the δ receptor being least sensitive. In all three opioid receptors, the conserved residue Cys7.38 largely confers the sensitivities to MTSEA.

That preincubation with (−)-naloxone, but not (+)-naloxone, protected the receptor from the inhibitory effects of MTSEA indicates that the reactive thiol group of Cys7.38 is directly exposed in the binding site or in the vicinity of the binding site. The finding that Cys7.38 is the only Cys residue accessible in the binding-site crevices of the μ, δ, and κ opioid receptors under conditions defined in this study would be useful for medicinal chemists in designing affinity ligands reacting with cysteines. Naloxone was used in these protection experiments for three reasons. First, naloxone can be easily removed by washing. Second, an antagonist like naloxone is not likely to cause as substantial conformational changes in the receptor as an agonist, which might complicate the interpretation of results. Third, the availability of (+)-naloxone, the inactive stereoisomer of (−)-naloxone, allows us to examine whether the protective effect of naloxone is stereospecific, since the stereospecificity of naloxone binding is a hallmark of opioid receptors.

In the present study, we have used the human δ and κ opioid receptors and the rat μ opioid receptor. There are two reasons why we chose to use the rat, instead of the human, μ opioid receptor. First, in our hands, the expression level of the human μ opioid receptor was low (~0.2 pmol/mg of protein), much lower than that of the rat receptor, which may make interpretation of data ambiguous in some cases. Second, human and rat μ opioid receptors share high sequence identity, 93.5% overall (374/400) and 98.4% within the TMDs (184/187), with only one amino acid residue different in each of the TMDs 1, 4, and 5. The rat μ receptor is therefore a good model for the human receptor.

Since reaction of Cys7.38 with MTSEA appeared to be responsible for the inhibition of [^{3}H]diprenorphine binding, the differential sensitivities of μ, δ, and κ opioid receptors to MTSEA must result from different rates of reaction of Cys7.38 with MTSEA in the different receptors. Because the thiolate is vastly more reactive with MTSEA than the thiol (*7*), differences in the ionization of the Cys at 7.38 would be expected to impact on its reactivity. The microenvironment in which Cys7.38 is situated may be different among the three receptors, in terms of both the charge and the steric factors that might directly impact on the access of MTSEA to Cys7.38. According to the models of Strahs and Weinstein (1997), locus 6.58 is in the vicinity of Cys7.38, with Glu6.58(297) in the κ receptor, Trp6.58(284) in the δ receptor, and Lys6.58 (303) in the μ receptor.

The presence of nearby negatively charged residues may facilitate the interaction of MTSEA with Cys7.38 in the κ receptor, as compared to those in the μ and δ receptors. Our results that the E6.58(297)Q and E6.58(297)K mutants of the human κ receptor had sensitivities to MTSEA similar to that of the wild-type κ receptor, however, ruled out the effects of the electrostatic factor at the 6.58 locus on MTSEA sensitivity of Cys7.38.

Another possibility is that negative charges in the second extracellular loop of the κ receptor, which contains five Asp residues and three Glu residues, may facilitate the reaction of MTSEA with the κ receptor. Our results that chimera II, the μ receptor with a fragment of the κ receptor including the second extracellular loop, became twice as sensitive to MTSEA as the wild-type μ receptor suggested that the second extracellular loop may play a small role in the higher sensitivity of the κ receptor to MTSEA.

The second-order reaction rate constant of the δ receptor with MTSEA was 9- and 82-fold lower than those of μ and κ receptors, respectively. One possibility is that W6.58 of the δ receptor inhibited the reaction of MTSEA with Cys7.38 by blocking the entry of MTSEA into the binding site. Unfortunately, the low expression level of the E6.58(298)W κ receptor mutant did not allow us to test this hypothesis. However, our recent SCAM results on the TMD6 of the δ receptor that several residues on the intracellular side of the Trp6.58 were sensitive to MTSEA (Xu et al., manuscript in preparation) indicate that Trp6.58 does not block the entry of MTSEA into the binding-site crevice of the δ receptor. Thus, the most likely explanation for the low sensitivity of Cys7.38 in the δ receptor is that this conserved residue is only partially accessible from the binding-site crevice. In contrast, the rates of reaction of Cys7.38 with MTSEA in the μ and κ receptor are consistent with their being directly exposed in the binding pocket. Thus, there may be a rotation of the α-helical axis in relation to the binding pockets among the three receptors relative to each other. We are currently conducting SCAM analyses on the TMDs 7 of μ, δ, and κ opioid receptors to delineate the amino acid residues accessible in the binding-site crevices, which will provide a better understanding of the differential sensitivities of the C7.38 residues to MTSEA.

Our findings that μ, δ, and κ opioid receptors have differential sensitivity to MTSEA were different from those

of Shahrestanifar and Howells (*40*), who reported that [^{3}H]-bremazocine binding to the μ and δ receptors in membranes was equally sensitive to MTSEA. In addition, these researchers found that every cysteine to serine mutant of the μ receptor, including C7.38S, was still sensitive to MTS reagents. In contrast, Deng et al. (*41*) showed that [^{3}H]-DAMGO binding to four mutants of the human μ receptor [C3.44(161)S, C4.48(192)S, C5.41(237)S, and C7.47(332)S] exhibited lower sensitivity to MTSEA, compared with the wild type. (Note that the numbering of the human μ receptor differs from that of the rat μ receptor by 2 due to two additional residues in the N-terminal domain.) There are several possibilities that may account for the differences between these findings and our own. First, in the present study, pretreatment with MTSEA and receptor binding were conducted in a physiological buffer (buffer A), whereas Shahrestanifar and Howells (*40*) and Deng et al. (*41*) performed their studies using Tris buffer. Our buffer (buffer A) contains 140 mM NaCl, and Na^+ has been shown to induce conformational changes in opioid receptors (*33*), which may affect the accessibility of the reactive groups (*59*−*61*) and in part contribute to the observed differences. Second, while Shahrestanifar and Howells (*40*) and Deng et al. (*41*) used membrane preparations, we used intact cells, and the integrity of the cells was maintained by using a physiological buffer. In intact cell preparations, MTSEA added extracellularly reacts with water-accessible reduced cysteine residues, most likely within the extracellular domains and/or within the binding-site crevice formed by the TMDs. Although uncharged MTSEA is able to cross the membrane (*57*), the amount of MTSEA that crosses during the short incubation time (5 min) is expected to be relatively small at physiological pH, and the intracellular reducing environment would be expected to further reduce the concentration of intracellular MTSEA. Using membranes for MTSEA reaction allows MTSEA to react with reduced Cys residues both in the binding-site crevice and in intracellular domains. Thus, using intact cells vs membranes may yield different results. Third, diprenorphine, bremazocine, and DAMGO may have some different contact sites within the binding pocket, and reactions of cysteine residues with MTSEA that interfere with binding of one ligand may not affect binding of the other. Javitch et al. (*13*) found that the reaction of MTSEA with Cys118 in D2 dopamine receptor decreased the affinity of substituted benzamide antagonists, such as YM-09151-2, by 50−2800-fold, whereas the affinities of other antagonists, such as *N*-methylspiperone, were decreased ≤6-fold. However, in the present study, we found that MTSEA reaction with the Cys to Ser or Met mutants of the μ opioid receptor appears to affect [^{3}H]diprenorphine and [^{3}H]bremazocine binding similarly.

Among the three MTS reagents, the potency in inhibiting [^{3}H]diprenorphine binding to the μ opioid receptor is in the order of MTSEA > MTSET > MTSES. These findings are similar to the reports of Shahrestanifar and Howells (*40*) on the effects of MTS reagents on [^{3}H]bremazocine binding to the μ receptor. In addition, a similar rank order of potency of the three MTS compounds has also been reported for other molecules, including D2 dopamine receptor (*10*) and nicotinic acetylcholine receptor (*62*). At physiological pH, the majority of MTSEA is positively charged, and MTSET is permanently positively charged, whereas MTSES is completely negatively charged. Similar to the nicotinic acetylcholine receptor (*62*) and D2 dopamine receptor (*10*), the μ opioid receptor contains negative charges within the binding pocket, e.g., Asp residues in TMD2 and TMD3. These negative charges have been suggested to contribute to the higher reactivities of the D2 dopamine receptor to MTSEA and MTSET than to MTSES (*16*). In contrast, Gioannini et al. (*36*) reported that MTSET and MTSES treatment inhibited [^{3}H]DAMGO binding to purified bovine μ opioid receptor with equal potencies. Possible reasons for these discrepancies include the factors discussed above, such as conformational differences due to the different buffers used, different preparations (intact cells vs purified μ receptors), and the different ligands used. Gioannini et al. (*36*) found that [^{3}H]-DAMGO binding was much more sensitive to inactivation by MTS reagents than [^{3}H]bremazocine binding.

The site of MTSEA reaction determined in the present study is different from that of NEM alkylation identified by Shahrestanifar et al. (*34*), Gaibelet et al. (*32*), and Ehrlich et al. (*35*). By chimeric μ/δ opioid receptor and mutation studies, Shahrestanifar et al. (*34*) showed that H223S mutation in the second extracellular loop greatly reduced the inhibitory effect of NEM on [^{3}H]bremazocine binding (*34*). Gaibelet et al. (*32*) reported that alkylation with *N*-ethylmaleimide reduced [^{3}H]DAMGO binding affinity and that the sites of alkylation were both Cys81(1.43) in TMD 1 and Cys332(7.47) in TMD7 of the μ opioid receptor. Ehrlich et al. (*35*) reported that none of the cysteine-to-serine mutants within the TMDs of the δ opioid receptor was resistant to the inhibitory effect of NEM on binding. As discussed above, the differences between the present study and the three studies cited here may be attributed to differences in tissue preparation, ligand, and buffer used. In addition, NEM and MTSEA may have different actions. Shahrestanifar et al. (*34*, *40*) reported that although [^{3}H]bremazocine binding to the μ and δ receptors in membranes was equally sensitive to MTSEA, NEM pretreatment exerted more profound inhibition on [^{3}H]bremazocine binding to the μ receptor than the δ receptor. NEM, in addition to reacting with cysteine, can react with imidazole groups of histidines and ϵ-amino groups of lysines (*63*). In contrast, MTS reagents are specific for water-accessible reduced sulfhydryl groups (*8*). In addition, NEM was shown to react with an −SH group outside of the binding pocket, possibly within the G protein or the region of the receptor involved in G protein coupling, which resulted in diminished agonist affinity without affecting antagonist affinities or the receptor number (*37*−*39*).

Our finding that mutation of each cysteine to serine or methionine within the TMDs of the μ, δ, and κ opioid receptors did not affect binding affinity of [^{3}H]diprenorphine suggests that none of these cysteine residues are involved directly in ligand−receptor interaction, consistent with the reports of Gaibelet et al. (*32*) and Ehrlich et al. (*35*). One minor difference is that we found no detectable [^{3}H]-diprenorphine binding to the C7.47(330)S mutant but similar binding affinity for the C7.47(330)M mutant, suggesting that C7.47(330) may play an indirect role in binding by maintaining structural integrity. Thus, cysteine residues in the TMDs are not involved significantly in ligand binding in the μ, δ, and κ receptors. Reaction with NEM, MTS reagents, iodoacetamide, and *p*-hydroxymercuribenzoate has been shown to inhibit opioid receptor binding. This is most likely due to

the introduction of a bulky and/or charged group to cysteine that reduces binding by direct steric hindrance. Javitch et al. (*13*) observed that MTSEA reaction with Cys118 in the TMD3 had inhibitory effects on D2 dopamine receptor binding similar to substitution of Cys118 with Lys.

C6.47S mutants of the μ and δ, but not the κ, opioid receptors appear to be more sensitive to MTSEA than the wild-type receptors (Figure 5), suggesting that the C6.47S mutation in the μ and δ receptors may cause conformational changes leading to exposure of one or more additional −SH groups to reaction with MTSEA. This is one potential limitation of any mutagenesis study, as alterations resulting from the mutation can be due to changes in the locus mutated, global conformational changes in the receptor, and local conformation changes in or around the binding pocket. Javitch et al. (*64*) showed that C6.47, while inaccessible in the wild-type β_2-adrenergic receptor, became accessible in the binding-site crevice in a constitutively active mutant and this locus may modulate receptor conformation and affect receptor activation.

In conclusion, μ, δ, and κ opioid receptors are differentially sensitive to MTSEA, and the conserved Cys7.38 confers the MTSEA sensitivity in each receptor. 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. The negatively charged Asp and Glu residues of the second extracellular loop may facilitate the reaction of the κ receptor with MTSEA, whereas the 6.58 locus did not play a role. To the best of our knowledge, this is the first report to show that a conserved residue among highly homologous G protein-coupled receptors is differentially accessible in the binding-site crevice. In addition, despite attempts by several laboratories, this represents the first report on the successful generation of MTSEA-insensitive mutants of μ, δ, and κ opioid receptors, which allows us to proceed to determine the residues accessible in the binding-site crevices of these receptors by the substituted cysteine accessibility method (for example, ref *11*).

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