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Chloroethylclonidine Binds Irreversibly to Exposed Cysteines in the Fifth Membrane-Spanning Domain of the Human α_{2A}-Adrenergic Receptor

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ABSTRACT

The α_2-adrenergic receptors (α_2-ARs) mediate signals to intracellular second messengers via guanine nucleotide binding proteins. Three human genes encoding α_2-AR subtypes (α_{2A}, α_{2B}, α_{2C}) have been cloned. Several chemical compounds display subtype differences in their binding and/or functional activity. Site-directed mutagenesis and molecular modeling are new tools with which to investigate the subtype selectivity of ligands. In this study, we introduce a new approach to mapping of the binding site crevice of the human α_{2A}-AR. Based on a three-dimensional receptor model, we systematically mutated residues 197–201 and 204 in the fifth transmembrane domain of the human α_{2A}-AR to cysteine. Chloroethylclonidine, an alkylating derivative of the α_2-adrenergic agonist clonidine, binds irreversibly to α_{2A}-ARs by forming a covalent bond with the sulfhydryl side chain of a cysteine residue exposed in the binding cavity, leading to inactivation of the receptor. Irreversible binding of chloroethylclonidine was used as a criterion for identifying introduced cysteine residues as being exposed in the binding cavity. The results supported a receptor model in which the fifth transmembrane domain is α-helical, with residues Val197, Ser200, Cys201, and Ser204 exposed in the binding pocket. Residues Ile198, Ser199, Ile202, and Gly203 face the lipid bilayer of the plasma membrane. This approach emerges as a powerful tool for structural characterization of the α_2-ARs.

The α_2-ARs mediate diverse physiological and pharmacological effects of the neurotransmitters/hormones norepinephrine and epinephrine and related synthetic molecules. Three genes encoding human α_2-AR subtypes have been cloned, representing the pharmacologically defined subtypes α_{2A}, α_{2B}, and α_{2C} (Kobilka *et al*., 1987; Regan *et al*., 1988; Lomasney *et al*., 1990). Related α_2-AR genes also have been identified in other species, such as rat, mouse, pig, opossum, and fish (Guyer *et al*., 1990; Zeng *et al*., 1990; Lanier *et al*., 1991; Chen *et al*., 1992; Link *et al*., 1992; Svensson *et al*., 1993; Blaxall *et al*., 1994). α_2-ARs, like all other members of the GPCR family, consist of a polypeptide chain that is predicted to span the cell membrane seven times. The amino acid sequences within the seven hydrophobic TMs are highly conserved in the three α_2-AR subtypes. These TM regions are predicted to be α-helical and to form a pocket crucial for the identification and binding of ligand molecules. Binding of a receptor agonist in this binding cavity either leads to or stabilizes a conformational change in the receptor protein, promoting its coupling with G proteins. The resulting G protein activation initiates a cascade of intracellular biochemical events and physiological responses (Savarese and Fraser, 1992; Scheer *et al*., 1996).

Several α_2-AR ligands, such as oxymetazoline, chlorpromazine, prazosin, UK 14,304, and dexmedetomidine, display some degree of subtype selectivity in either their binding affinity or functional activity (Marjamäki *et al*., 1993; Jansson *et al*., 1994). A comparison of the ligand binding properties of the human α_2-AR subtypes with their species homologues also has revealed some differences. For example, Hα2A binds the antagonists yohimbine and rauwolscine with significantly higher affinity than its mouse homologue, Mα2A (Link *et al*., 1992). Analysis of the primary structures of these two receptors has identified a Cys201-to-Ser201 substitution in the TM5 of Mα2A. When Ser201 of the Mα2A was mutated to cysteine, the affinity of the mouse receptor for

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ABBREVIATIONS: AR, adrenergic receptor; CEC, chloroethylclonidine; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; SH, sulfhydryl; TM, transmembrane domain; Hα2A, human α_{2A}-adrenergic receptor; Hα2B, human α_{2B}-adrenergic receptor; Hα2C, human α_{2C}-adrenergic receptor; Mα2A, mouse α_{2A}-adrenergic receptor; WT, wild-type.

yohimbine was significantly increased. This suggested that the residue at position 201 in TM5 of α_{2A}-ARs might be exposed in the binding cavity and directly participate in ligand recognition. Site-directed mutagenesis and computer-aided modeling can be used to explore the structural determinants of receptor/ligand interactions, including species differences and subtype selectivity. Mapping of residues exposed in the binding cavity may allow the subsequent synthesis of new therapeutic agents targeted to specific ligand recognition sites.

CEC, which often has been used to discriminate between α_1-AR subtypes in functional assays (Han *et al*., 1987; Tian *et al*., 1990), has been shown to inactivate irreversibly Hα2A and Hα2C, whereas Hα2B is relatively resistant to its alkylating effect (Michel *et al*., 1993). CEC is known to undergo intramolecular cyclization to a reactive aziridinium ion before irreversible receptor inactivation (Vargas *et al*., 1993). The aziridinium ion presumably forms a covalent bond with the free SH-group of an exposed cysteine residue. The primary structure of Hα2A has a cysteine in position 201; Hα2C also has a cysteine in the corresponding position (position 215), whereas the CEC-resistant subtype Hα2B has a serine in the corresponding position (position 177) (Fig. 1). Such an amino acid substitution might explain the subtype-selective reactivity of CEC at the different human α_2-AR subtypes. To test this hypothesis, we determined the irreversible binding of CEC to the three human α_2-AR subtypes as well as the Mα2A and constructed and tested a series of mutant α_{2A}-ARs with cysteines located at different positions in this region of TM5.

Computer-aided modeling was used to predict the three-dimensional structure of the Hα2A. The TM domains of GPCRs usually are presented as fixed α-helices, with one side exposed in the binding cavity (Savarese and Fraser, 1992; Baldwin, 1993; Schwartz, 1994). With site-directed mutagenesis, however, the pattern of exposure of residues in TM5 of the dopamine D_2 receptor to a hydrophilic thiol-reactive alkylating agent was shown to be inconsistent with this prediction (Javitch *et al*., 1995). In our model of the Hα2A, TM5 was predicted to be α-helical, with residues Val197, Ser200, Cys201, and Ser204 forming part of the surface of the ligand-accessible binding site crevice and residues Ile198, Ser199, Ile202, and Gly203 facing the lipid bilayer of the plasma membrane. To map the structure and orientation of the TM5 in the Hα2A and to test this model, we mutated residues 197–201 and 204, one at a time, to a cysteine. Irreversible binding of CEC was used as a criterion for identifying a sulfhydryl side chain of an introduced cysteine as being exposed in the binding cavity and accessible to CEC. Our results confirmed the α-helical structure and predicted rotational orientation of TM5 in Hα2A.

species	subtype	TM5	201
Human	α_2A	K W Y V I S S	C I G S F F A P C L I M I L V Y V
Mouse	α_2A	K W Y V I S S	S I G S F F A P C L I M I L V Y V
Rat	α_2A	K W Y V I S S	S I G S F F A P C L I M I L V Y V
		177/177/182	
Human	α_2B	A W Y I L A S	S I G S F F A P C L I M I L V Y L
Mouse	α_2B	A W Y I L A S	S I G S F F A P C L I M I L V Y L
Rat	α_2B	A W Y I L A S	S I G S F F A P C L I M I L V Y L
		215	
Human	α_2C	T W Y I L S S	C I G S F F A P C L I M G L V Y A
Mouse	α_2C	T W Y I L S S	C I G S F F A P C L I M G L V Y A
Rat	α_2C	T W Y I L S S	C I G S F F A P C L I M G L V Y A

Fig. 1. Amino acid sequence alignment of the fifth hydrophobic TM of human and rodent α_2-AR subtypes. The sequences are aligned for maximum homology. The alignment of the entire sequences has been presented elsewhere (Pepperl and Regan, 1994). *Boxes*, amino acid residue at position 201 or corresponding position.

Experimental Procedures

Materials. [^{3}H]RX821002 [2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline] was from Amersham International (Buckinghamshire, UK; specific activity, 52 Ci/mmol). Phentolamine and CEC were from Research Biochemicals (Natick, MA). Cell culture reagents were supplied by GIBCO (Gaithersburg, MD). The 10-mer oligopeptide Tyr-Val-Ile-Ser-Ser-Cys-Ile-Gly-Ser-Phe (TM5 region of Hα2A: Tyr196 to Phe205) was supplied by the Center for Biotechnology (Turku, Finland). Other chemicals were of analytical grade and were purchased from commercial suppliers.

Reaction of CEC with oligopeptide and mass spectroscopic analysis. The 10-mer oligopeptide Tyr-Val-Ile-Ser-Ser-Cys-Ile-Gly-Ser-Phe was dissolved in 50 mM K^+-phosphate buffer, pH 7.4, at 21°, and one molar equivalent of CEC was added. The reaction mixture was incubated for 60 min at 37° and analyzed by matrix-assisted laser desorption mass spectrometry (Finnigan MAT, Hemel Hempstead, UK).

Mutagenesis and expression vectors. The cDNA encoding Hα2A (Kobilka *et al*., 1987) was inserted into the *Sma*I site of the vector pALTER-1 (Promega, Madison, WI). Site-directed mutagenesis was performed using the Altered Sites II *In Vitro* Mutagenesis System (Promega). The mutated DNA fragments were sequenced manually by dideoxy sequencing of double-stranded DNA with Sequenase (United States Biochemical, Cleveland, OH) and confirmed with an ABI377 automated sequencer (Perkin-Elmer Cetus (Norwalk, CT). The WT Hα2A and the mutated receptor cDNAs were subcloned into the *Kpn*I/*Bam*HI sites of the expression vector pREP4 (InVitrogen, NV Leek, The Netherlands), which also contains the gene for hygromycin B resistance.

The cDNAs encoding Hα2B, Hα2C, Mα2A (Regan *et al*., 1988; Lomasney *et al*., 1990; Link *et al*., 1992) and the S201C mutant of Mα2A, created and confirmed as described, were similarly subcloned into the pREP4 expression vector for receptor production.

Cell culture and transfections. Adherent CHO cells (American Type Culture Collection, Rockville, MD) were cultured in α-minimum essential medium supplemented with 2 mM glutamine, 20 mM $NaHCO_3$, 5% heat-inactivated fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were grown in 5% CO_2 at 37°. The pREP4-based expression constructs were transfected into CHO cells with use of the Lipofectin reagent kit (GIBCO, Paisley, UK). For each transfection, we used 3 μg of plasmid DNA/5 $\times$ 10^4 cells. Hygromycin B (Boehringer-Mannheim Biochemica, Mannheim, Germany)-resistant (550 μg/ml) cell cultures were examined for their ability to bind the α_2-AR antagonist [^{3}H]RX821002. The transfected cells chosen for further experiments were subsequently maintained in 200 μg/ml hygromycin B.

Receptor inactivation and ligand binding. Cells were harvested into chilled phosphate-buffered saline, pelleted, washed, suspended in ice-cold 50 mM K^+-phosphate buffer, pH 7.4, at 21°, and homogenized with an Ultra-Turrax homogenizer (model T25, Janke & Kunkel, Staufen, Germany; setting, 9500 rpm, twice for 10 sec). The homogenate was used for saturation and competition binding assays or receptor inactivation experiments.

Saturation studies were performed in K^+-phosphate buffer as described previously (Halme *et al*., 1995). Whole-cell homogenates containing 40–80 μg of protein were incubated with [^{3}H]RX821002 (0.125–8 nM). Specificity of binding was defined with 10 μM phentol-

amine. Competition studies were done as reported previously(Halme *et al.*, 1995), using [^{3}H]RX821002 concentrations close to its affinity constant (K_d) at each receptor and 13–15 concentrations of the competitor CEC.

For receptor inactivation, cell homogenates first were incubated with CEC (1 and 10 μM) in 2.5 ml of K^+-phosphate buffer for 15, 30, or 60 min at 37°. The protein content was 0.3–0.5 mg/ml during CEC treatment. Next, membranes were pelleted at 40,000 $\times$ *g* for 15 min at 4°, washed twice with 2.5 ml of ice-cold K^+-phosphate buffer, and rehomogenized with the Ultra-Turrax homogenizer. Residual α_2-AR binding was assessed by incubating the homogenate (0.1–0.2 mg/assay tube) with 2.5 nM [^{3}H]RX821002. Nonspecific binding was determined by including 10 μM phentolamine in parallel assays.

Three-dimensional modeling of Hα2A binding cavity. The molecular modeling of Hα2A and the binary complex with CEC will be described in complete detail (V. Cockcroft, A. Marjamäki, H. Frang, M. Pihlavisto, J.-M. Savola, and M. Scheinin, Ligand interaction of serine-cysteine amino acid exchanges in TM5 of α_2-adrenergic receptors, manuscript in preparation.). The structural coordinates of the high-resolution electron cryomicroscopy model of bacteriorhodopsin (Henderson *et al.*, 1990) was used as a three-dimensional template for structural mapping of GPCR sequences.

Results

Site-directed mutagenesis and transfections. To examine the structure of the TM5 domain of Hα2A, amino acid residues from Val197 to Cys201 and Ser204 were mutated to introduce or delete cysteines. The introduced mutations were confirmed and the absence of secondary mutations was verified by dideoxy sequencing of double-stranded DNA.

Mutated and WT receptors were expressed in CHO cells. Hygromycin B-resistant cell cultures were examined for their ability to bind the α_2-AR antagonist radioligand [^{3}H]RX821002. Three cell lines from each transfection expressing the expected receptor were isolated for preliminary experiments, and one cell line from each transfection was expanded for further experiments (Table 1) and subsequently maintained in 200 μg/ml hygromycin B.

Receptor inactivation studies. CEC, an alkylating derivative of clonidine, has been used previously to discriminate between α_1-AR subtypes, but it also has been shown to inactivate α_2-ARs in a subtype-selective manner. Based on our hypothesis, the reactive aziridinium ion derivative of CEC forms a covalent bond with an exposed SH-group of a cysteine residue in the receptor molecule (Fig. 2) and inactivates the receptor by steric blockade of the binding cavity. Covalent bonding of CEC to protein was confirmed by allowing it to react with a synthetic 10-mer oligopeptide corresponding to residues 196–205 of the TM5 region of the Hα2A and then undergoing mass spectroscopic analysis (Fig. 3). After 1 hr at 37°, the oligopeptide was totally alkylated in a manner consistent with our hypothesis presented in Fig. 2.

To validate our experimental conditions in the CEC inactivation assay, we first compared the effect of CEC treatment at 37° for 15, 30, or 60 min, followed by two washes, on the capacity of [^{3}H]RX821002 binding in CHO cell homogenates expressing WT Hα2A (data not shown). The incubation of cell homogenates for 30 min at 37° in the absence (control) and presence of CEC was chosen as optimal for further experiments.

First, we tested the alkylating effect of CEC on the three human α_2-AR subtypes (Hα2A, Hα2B, and Hα2C) expressed in CHO cells. CEC treatment reduced the binding capacity of Hα2A and Hα2C by 85%, whereas Hα2B was resistant to the alkylating effect of CEC (Fig. 4). This was in agreement with the involvement of a cysteine in position 201 or in a corresponding position in the alkylating effect of CEC (see amino acid sequence alignment in Fig. 1). To further characterize the interaction of TM5 cysteines and CEC, we compared the effects of CEC treatment on Hα2AWT and Mα2AWT. Instead of a cysteine, the Mα2AWT contains a serine in position 201 (Fig. 1). Incubation with CEC inactivated 75% of Hα2AWT but only 23% of Mα2A was irreversibly inactivated. When the Cys201 of Hα2A was mutated to a serine to resemble the Mα2A, it became resistant to the alkylating effect of CEC (inactivation, 15%). After the opposite mutation in Mα2A (Ser201 to cysteine), this receptor became susceptible to the irreversible effect of CEC (inactivation, 60%) (Fig. 5). This confirms our hypothesis of a structure-activity relationship between the alkylating effect of CEC and a cysteine residue in this position of TM5.

In our three-dimensional receptor model (Fig. 6), the amino acid residues Val197, Ser200, Cys201, and Ser204 were accessible and exposed in the binding cavity, whereas Ile198, Ser199, Ile202, and Gly203 were facing the lipid bilayer in an α-helical TM5 of the Hα2A. To map the surface of the ligand binding pocket, we systematically mutated residues from Val197 to Ser200 and Ser204 to cysteine. Before introducing

TABLE 1

Characterization of CHO cells expressing WT and mutated α_2-ARs.

The concentration of CEC that inhibited specific [^{3}H]RX821002 binding in competition assays by 50% (IC_{50}) was used to calculate apparent K_i values (inhibition constant) according to the Cheng-Prusoff equation.

Cell line	[^{3}H]RX821002			CEC
	B_{max}	K_d	n_H	Apparent K_i
	fmol/mg of protein	*nM*		*nM*
CHO-Hα2Awt (Cys201)	595 ± 2	0.60 ± 0.02	0.98 ± 0.02	578 ± 39
CHO-Hα2ASer201	156 ± 13	0.30 ± 0.04	0.95 ± 0.06	260 ± 32
CHO-Hα2ASer201Cys197	295 ± 22	0.53 ± 0.05	1.02 ± 0.07	95 ± 3
CHO-Hα2ASer201Cys198	3710 ± 120	0.72 ± 0.09	0.98 ± 0.02	467 ± 49
CHO-Hα2ASer201Cys199	10967 ± 260	1.90 ± 0.11	0.95 ± 0.01	2624 ± 96
CHO-Hα2ASer201Cys200	2146 ± 306	3.34 ± 0.51	0.99 ± 0.01	56 ± 6
CHO-Hα2ASer201Cys204	4009 ± 117	2.71 ± 0.12	1.00 ± 0.01	191 ± 7
CHO-Hα2Bwt	1870 ± 111	6.12 ± 0.46	1.00 ± 0.01	1016 ± 115
CHO-Hα2Cwt	1324 ± 251	1.18 ± 0.17	0.96 ± 0.04	2909 ± 4
CHO-Mα2Awt	1707 ± 164	0.89 ± 0.09	0.92 ± 0.05	539 ± 46
CHO-Mα2ACys201	1803 ± 143	1.26 ± 0.14	0.98 ± 0.01	1484 ± 223

CHO, Chinese hamster ovary.

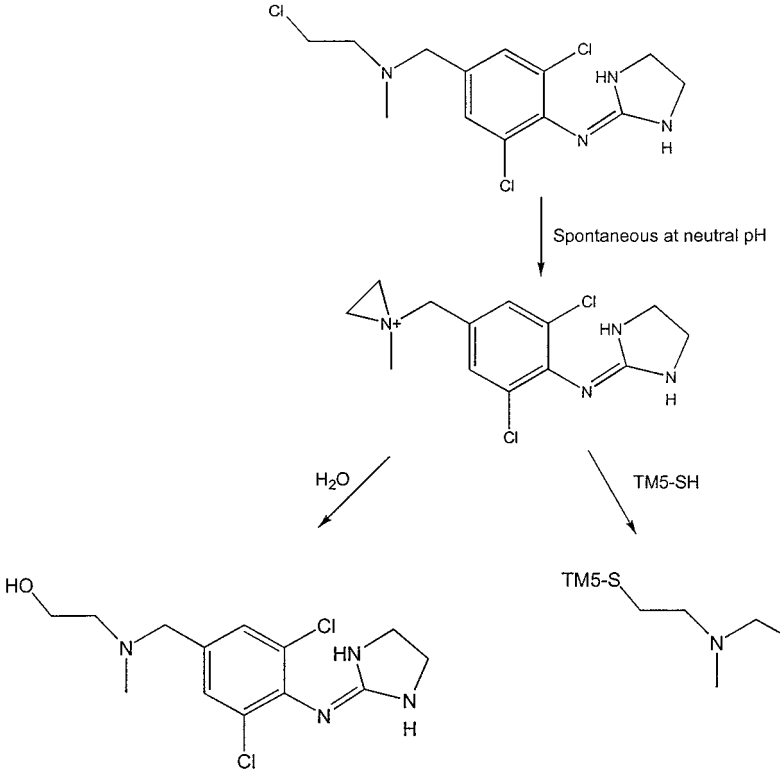


Fig. 2. CEC undergoes intramolecular cyclization to a reactive aziridinium ion as reported previously (Vargas *et al.*, 1993). The aziridinium ion then can either react with water or form a covalent adduct with a peptide containing a free SH-group (*TM5-SH*).

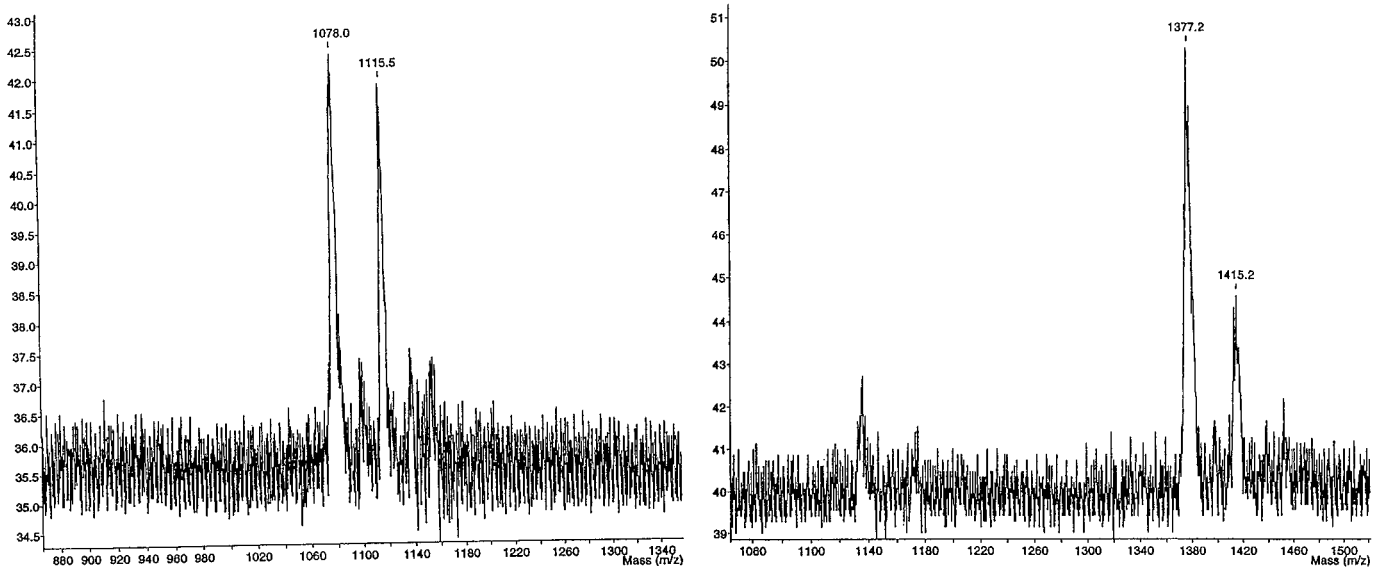


Fig. 3. Matrix-assisted laser desorption mass spectra of the oligopeptide Tyr-Val-Ile-Ser-Ser-Cys-Ile-Gly-Ser-Phe (*left*) and its covalent adduct with CEC after 1 hr at 37° (*right*). The molecular mass of the peptide increases by 299 units from 1078 to 1377, which is consistent with the reaction scheme presented in Fig. 2. Peaks with masses 38 units higher than the predicted molecular mass (1115.5 and 1415.2) are potassium adducts of peptides. After the reaction (*right spectrum*), no unreacted oligopeptide remains.

new cysteine residues to the TM5 of the Hα2A, the WT Cys201 of Hα2A was substituted with serine. This Hα2ASer201 is resistant to the alkylating effect of CEC (Fig. 5) and was used as a negative control in these experiments. We investigated the capability of CEC to inactivate WT and mutated receptors expected to contain a cysteine residue exposed in the binding crevice (Fig. 7). Relative to the WT Hα2A (inactivation, 75%), the extent of inactivation was smaller when the cysteine residue was deeper in the cavity (Ser201Cys204 mutant inactivation, 52%) and greater when the residue was closer to the extracellular surface of the plasma membrane (Ser201Cys197 mutant inactivation, 97%). This probably was due to different rates of alkylation of the receptors under our assay conditions. After a 60-min CEC treatment, the difference in the extent of receptor inactivation was minimal (Ser201Cys197, Cys201, and Ser201Cys204 inactivation, 96%, 92%, and 86% respectively), and it seems that all accessible cysteines ultimately would be alkylated, given sufficient time.

Although in our model Ser200 is pointing partly toward the

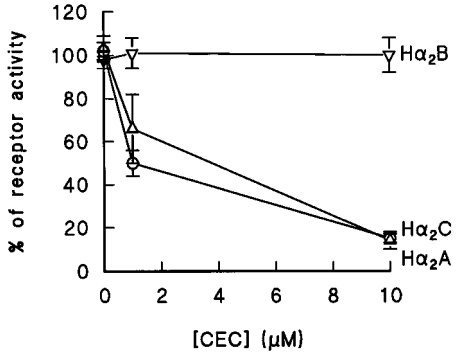


Fig. 4. Effect of CEC treatment on binding activity of human α_2-AR subtypes. CHO cells expressing Hα2A, Hα2B, and Hα2C were incubated in the absence (control) and presence of CEC (1 and 10 μM) for 30 min at 37°, followed by two washes. Residual α_2-AR binding was determined by incubation with 2.5 nM [^{3}H]RX821002. Nonspecific binding was defined by 10 μM phentolamine. For comparison purposes, binding assays for all three receptors were carried out simultaneously with the use of the same stock solutions. Results are expressed as percentage of specific [^{3}H]RX821002 binding remaining after treatment with CEC compared with control. Data represent the mean ± standard error of three separate experiments performed in duplicate.

TM4 domain, the aliphatic hydroxyl side chain of this residue can rotate toward the cavity and thus participate in ligand recognition. CEC treatment reduced the number of detectable α_2-ARs in CHO cell homogenates expressing the Hα2ASer201Cys200 mutant by 61%, indicating the SH side chain of Cys200 also is exposed in the cavity. The difference in the orientation of the residues at positions 200 and 201 also might account for the difference in the extent of receptor inactivation between Hα2AWT and Hα2ASer201Cys200 (inactivation, 75% versus 61%) (Fig. 7).

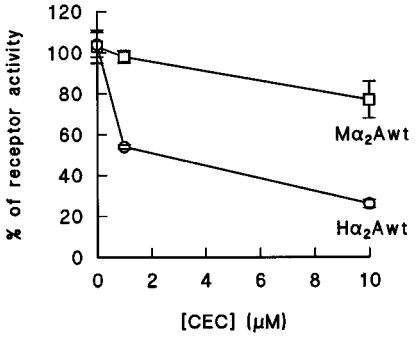
Amino acids from Val197 to Cys201 represent one full turn in the α-helical model of TM5. The residues 197–200 of Hα2ASer201 were mutated to cysteine, one at a time, and the effect of CEC treatment on the binding activity of the WT Hα2A and the mutant receptors was examined (Fig. 7). Two cysteine residues at positions 198 and 199, expected to face the lipid bilayer of the cell membrane, were relatively resistant to the alkylating effect of CEC (Ser201Cys198 and Ser201Cys199 inactivation, 25% and 24%, respectively). The results obtained with site-directed mutagenesis thus support our three-dimensional model and confirm the α-helical structure of TM5 in Hα2A.

Ligand binding assays. Saturation isotherms of [^{3}H]RX821002 binding- and LIGAND- (McPherson, 1985) derived K_d (receptor affinity) and B_{max} (receptor density) values were determined in three separate experiments for each cell line (Table 1). Three-point mutations of Hα2ASer201, Ser199, Ser200, and Ser204 to cysteine, resulted in 3–5-fold decreases in receptor affinity for the α_2-AR antagonist [^{3}H]RX821002. The expression level of the Hα2ASer201 mutant used in our experiments was only 156 ± 13 fmol/mg of total cellular protein. Similar results of receptor inactivation by CEC were, however, later obtained in experiments with another batch of Hα2ASer201 cells, expressing 4736 ± 234 fmol/mg of cell homogenate (inactivation, 7 ± 2%). This indicates that the weak alkylating effect of CEC on Hα2ASer201 presented in Fig. 5 is not dependent on the receptor expression level.

In all investigated cell lines expressing WT and mutant receptors, the addition of CEC to competition binding assays inhibited specific binding of 2.5 nM [^{3}H]RX821002 with steep monophasic competition curves. The affinity of Hα2B for [^{3}H]R821002 was relatively low (K_d = 6.12 ± 0.46 nM), and the receptor inactivation assays consequently were performed using 6 nM [^{3}H]RX821002. Similar results were obtained with both radioligand concentrations (9 ± 2% and 13 ± 2% inactivation with 2.5 and 6 nM [^{3}H]RX821002, respectively). We tested whether the lack of alkylating effects of CEC (Figs. 4, 5, and 7) would be due to low or absent binding affinity of CEC to Hα2B, Mα2A, or the Hα2A mutants Hα2ASer201, Hα2ASer201Cys198 ,and Hα2ASer201Cys199 (Table 1). The two CEC-resistant WT receptors Hα2B and Mα2A and the Hα2ASer201, Hα2ASer201Cys198, and Hα2ASer201Cys199 mutants also were capable of binding CEC (apparent K_i = 1016 ± 115, 539 ± 46, 260 ± 32, 467 ± 49, and 2624 ± 96 nM, respectively). The lack of alkylation thus is not due to lack of binding affinity but rather to the absence of an accessible cysteine residue on the surface of the binding site crevice.

Discussion

In the current study, we were able to demonstrate that an exposed cysteine residue in the binding cavity of α_2-AR is required for the alkylating effect of CEC. Although the apparent binding affinities (apparent K_i value) of CEC were comparable for the WT and mutated Hα2A-ARs, the alkylating effect of CEC treatment was dependent on the location of a reactive cysteine residue in TM5. True affinity of an irreversible ligand cannot be determined reliably in a conventional competition binding assay, and the apparent affinity of CEC determined in this way actually may represent both reversible and irreversible binding (Michel *et al.*, 1993). Simultaneous inactivation and competitive binding should, however, overestimate the apparent affinity of CEC for α_2-AR subtypes/mutants that become alkylated, indicating



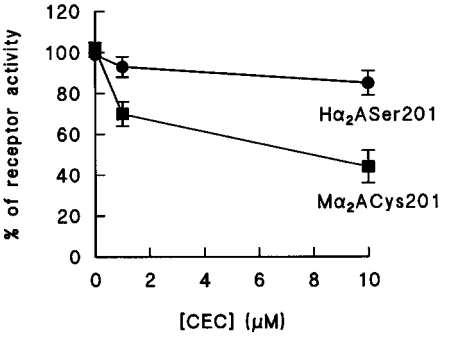


Fig. 5. Effect of CEC treatment on binding activity of Hα2AWT and Mα2AWT and Hα2ASer201 and Mα2ACys201 mutant receptors. Results were obtained as described in the legend for Fig. 4.

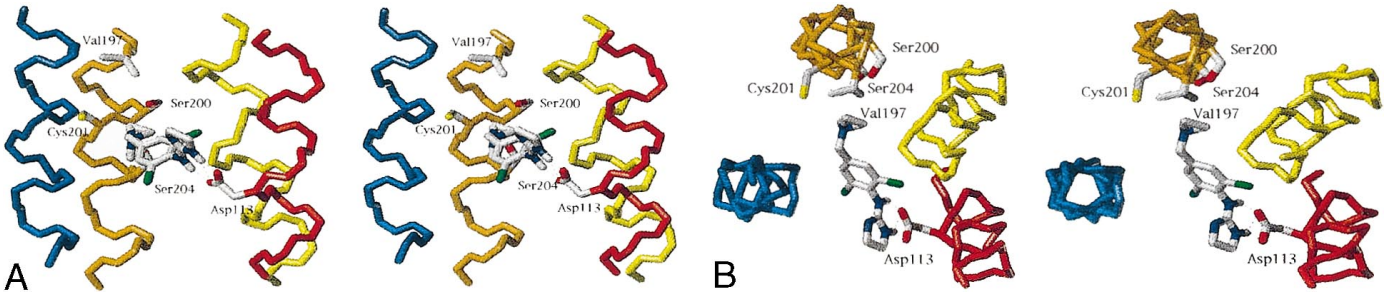


Fig. 6. Stereo view of the energy-minimized hypothetical model of the binary complex of Hα2A binding cavity with the aziridinium ion form of chloroethylclonidine. The view direction is (A) from TM1 to TM5 and (B) from above the binding cavity. Only the TM3/TM4/TM5/TM6 end of the receptor cavity is shown. The regions of the helices displayed at the level of the ligand binding site are TM3, 106–117; TM4, 157–170; TM5, 196–209; and TM6, 386–399. Carbon, *white*; oxygen, *red*; nitrogen, *blue*; chlorine, *green*; and sulfur, *yellow*. *White*, hydrogen atoms of the 2-aminoimidazoline moiety of the ligand showing hydrogen bonds with Asp113 (*dotted lines*). For main chain atoms of the transmembrane helices, TM3, *red*; TM4, *yellow*; TM5, *orange*; and TM6, *blue*. *Labels*, amino acid residue numbering of the Hα2A.

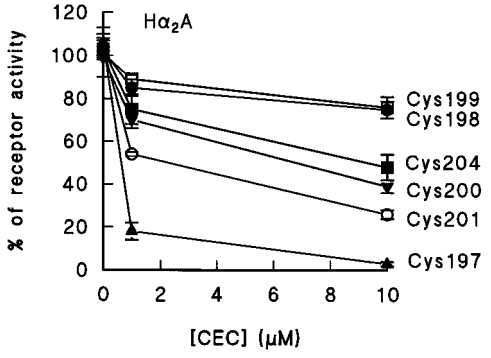


Fig. 7. Effect of CEC treatment on binding activity of Hα2ACys201 and Hα2ASer201Cys197, Hα2ASer201Cys198, Hα2ASer201-Cys199, Hα2ASer201Cys200, and Hα2ASer201Cys204 mutant receptors. Results were obtained as described in the legend for Fig. 4.

the lack of alkylating effects of CEC in our assays is not due to the lack of CEC binding.

We used a receptor model predicting the α-helical structure of TM5 in Hα2A-AR, in which the residues Val197, Ser200, Cys201, and Ser204 were pointing to the binding pocket, whereas the residues Ile198, Ser 199, Ile202, and Gly203 were facing the lipid bilayer. This model was supported by the results obtained through site-directed mutagenesis and CEC inactivation experiments. The primary structures of the TM5 regions of all α_2-AR subtypes contain a cysteine in the position corresponding to Cys209 of Hα2A (Fig. 1). This cysteine is facing the lipid bilayer of the plasma membrane in our receptor model and thus was not expected to interfere with CEC inactivation experiments. This orientation of Cys209 was supported by the results obtained with Hα2B, Mα2A, Hα2ASer201, Hα2ASer201Cys198, and Hα2ASer201Cys199 not containing cysteine residues exposed in the binding cavity and shown to be resistant to the alkylating effect of CEC (Figs. 4, 5, and 7). These results are consistent with an α-helical structure of TM5 and provide constraints for the rotational orientation of this helix in relation to the binding cavity.

The β_2-AR is one of the most extensively structurally characterized GPCRs. With site-directed mutagenesis, it has been possible to identify several amino acid residues that are critical for and probably directly involved in ligand binding. The catechol hydroxyl groups of epinephrine seem to interact with two serine residues present in TM5 of β_2-AR (Strader *et al*., 1989). The two serine residues at corresponding positions of the human α_{2A}-AR (Ser200 and Ser 204) have been shown to participate in hydrogen bond interactions with the catechol hydroxyl groups of catecholamine agonists (Wang *et al*., 1991). When Ser201 of the mouse α_{2A}-AR was mutated to cysteine, the corresponding amino acid in Hα2A, this cysteine-to-serine substitution was shown to be critical for the low affinity of the mouse receptor for yohimbine and rauwolscine (Link *et al*., 1992). Site-directed mutagenesis and analysis of the three-dimensional model of the hamster α_{1B}-AR also indicated that three serine residues, corresponding to positions 200, 201, and 204 in Hα2A, are important for agonist interactions (Cavalli *et al*., 1996). When the structural determinants of subtype-selective agonist binding of the hamster α_{1B}-AR were identified, it was shown that mutation of Ala204 to valine (corresponding to position 197 in Hα2A) in TM5 conferred onto α_{1B}-AR the binding properties of the α_{1A}-AR (Hwa *et al*., 1995). These results obtained with computer-aided modeling and site-directed mutagenesis from different members of the same receptor family support our model and the location of residues 197, 200, 201, and 204 of Hα2A in the binding site cavity.

We introduced a new useful approach to mapping of the binding site crevice of human α_2-ARs by using cysteine substitution mutagenesis and irreversible cysteine-specific covalent binding of CEC to the receptor. This method emerges as a very useful tool for structural characterization of the α_2-ARs. Using this method together with three-dimensional modeling, we were able to confirm the predicted α-helical structure of TM5 and its orientation in Hα2A. One of the goals of the current study was to assess whether a so-called molecular yardstick approach could provide distance constraints for determining improved models of GPCRs. This technique, perhaps with some modifications, also could be applicable to structural studies on TM4 and TM6 of the same receptor.

Javitch *et al*. (1995) and Fu *et al*. (1996) previously introduced a cysteine-reactive approach using as reactive agents nonspecific polar methanethiosulfonate derivatives to probe another monoamine receptor, the D_2 dopamine receptor. Our method can be seen as a simple development of this approach. We suggest our method has the advantage of introducing recognition specificity by using a thiol-reactive group incorporated into an affinity ligand of the target receptor. This allows the use of significantly lower reagent concentrations and lessens the possibility of indirectly blocking radioligand binding.

Conventional loss-of-function mutagenesis has not always produced definitive answers for the purpose of assigning amino acid residues as being inside the binding cavity. It has been shown that point mutations of positions expected to be outside the binding cavity can have a marked effect on ligand affinities, presumably through conformational control (Fong *et al.*, 1992; Sachais *et al.*, 1993). In addition, several reports exist of residue substitutions at sites at which ligand/receptor contact interaction would be expected on the basis of amino acid conservation patterns that have not shown expected disruption of ligand binding (Befort *et al.*, 1996). Although the receptor modeling presented here is still rather crude, it has been used to devise an experimental approach, gain-of-function mutagenesis, to provide, through covalent bond formation, solid information that will lead to better models of not only the receptors but also binary complexes of ligand and receptor.

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