Chloroethylclonidine and 2-Aminoethyl Methanethiosulfonate Recognize Two Different Conformations of the Human $\alpha_{2A}$-Adrenergic Receptor*

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The substituted cysteine-accessibility method and two sulphydryl-specific reagents, the methane-thiosulfonate derivative 2-aminoethyl methanethiosulfonate (MTSEA) and the $\alpha_2\beta_2$-adrenergic receptor (H2A) agonist chloroethylclonidine (CEC), were used to determine the relative accessibility of engineered cysteines in the fifth transmembrane domain of the human $\alpha_{2A}$-AR (H2A). The second-order rate constants for the reaction of the receptor with MTSEA and CEC were determined with the wild type H2A (cysteine at position 201) and receptor mutants containing accessible cysteines at other positions within the binding-site crevice (positions 197, 200, and 204). The rate of reaction of CEC was similar to that of MTSEA at residues Cys-197, Cys-201, and Cys-204. The rate of reaction of CEC with Cys-200, however, was more than 5 times that of MTSEA, suggesting that these compounds may interact with two different receptor conformations. MTSEA, having no recognition specificity for the receptor, likely reacts with the predominant inactive receptor conformation (R), whereas the agonist CEC may stabilize and react preferentially with the active receptor conformation (R*). This hypothesis was consistent with three-dimensional receptor-ligand models, which further suggest that $\alpha_{2A}$-AR activation may involve the clockwise rotation of transmembrane domain 5.

$\alpha_2$-Adrenergic receptors ($\alpha_2$-ARs) belong to the family of G-protein-coupled receptors and are integral cell membrane proteins with seven transmembrane (TM) domains (1). The TM regions are predicted to be $\alpha$-helical and to form a water-accessible crevice containing the binding site for receptor ligands (2). Some of the amino acid residues forming the surface of this crevice directly interact with $\alpha_2$-AR agonists and/or antagonists, whereas some others in the TM domains may affect ligand binding indirectly (3, 4). G-protein-coupled receptors are thought to exist in an equilibrium between two (or more) conformations or allosteric states, R and R* (5). In the absence of ligand, the inactive state R predominates. Agonist binding stabilizes the receptor protein in its active state R*, promoting its coupling with G-proteins. The resulting G-protein activation initiates a cascade of intracellular events leading to physiological responses.

Recently, a technique utilizing charged methanethiosulfonate derivatives to probe the accessibility of substituted cysteine residues has been used to study the structure of the dopamine D2 and the $\beta_2$-adrenergic receptors (4, 6–8). This method identifies residues that form the surface of the binding-site crevice by replacing consecutive amino acid residues in the membrane-spanning segments with cysteine, one at a time. The sulphydryl side chain of a cysteine can face the water-accessible binding-site crevice, the interior of the protein, or the lipid bilayer. Sulphydryls facing the binding-site crevice react rapidly with sulphydryl-specific methanethiosulfonate derivatives such as 2-aminoethyl methanethiosulfonate (MTSEA), with formation of a covalent bond with the free sulphydryl group of an accessible cysteine residue (4, 8). MTSEA has no preference for receptor conformations, and it reacts, therefore, with the predominant (inactive) form of the receptor (R). In this method, agonists cannot be used to stabilize and thereby to study the structure of the active receptor species, because the presence of an agonist in the binding pocket would interfere with the access of MTSEA to the engineered cysteines. The activated state of the receptor (R*) can, however, be detected with MTSEA by using a constitutively active receptor mutant (7). This approach, combined with molecular modeling, has suggested rotation and/or tilting of the TM6 segment of the $\beta_2$-AR as a consequence of receptor activation (7).

We recently used the agonist chloroethylclonidine (CEC) to map the structure of TM5 of the human $\alpha_{2A}$-AR with a modified substituted cysteine-accessibility method (9). The reactive aziridinium ion derivative of CEC forms a covalent bond with the sulphydryl side chain of a cysteine exposed in the binding-site crevice and accessible to CEC. This method has the advantage of introducing recognition specificity by using a thiol-reactive group incorporated into an affinity ligand of the target receptor. In functional studies, CEC irreversibly activates $\alpha_{2A}$-AR, both prejunctionally in the rat vas deferens and postjunctively in the dog saphenous vein (10, 11), suggesting that the compound is covalently tethered to an endogenous cysteine near the binding site.

In our previous study, TM5 of H2A was shown to be consistent with an $\alpha$-helix with residues Val-197, Cys-201, and Ser-204, pointing into the binding-site crevice, residues Ile-198, Met-200, and Ser-204.
Ser-199, Ile-202, and Gly-203, facing the lipid bilayer, and Ser-200, pointing partly toward TM4 (9). Surprisingly, the pattern of accessibility to MTSEA in TM5 of the dopamine D2 receptor was not consistent with a fixed α-helical structure for TM5 (8). A possible explanation for these results is that this region changes conformation. Because this region is known to be directly involved in agonist binding and because the intracellular end of TM5 interacts with G-proteins, conformational changes in TM5 may be critical to the activation mechanisms of these receptors.

To explore such a conformational change in the H2A receptor, we have compared the rates of reaction of the agonist CEC and of MTSEA with accessible cysteines at positions 197, 200, 201, and 204 in TM5 of H2A. Nonaccessible cysteines at positions 202 and 203 and a H2A Ser201 mutant (containing no substituted cysteines) were used as controls. MTSEA reacted with accessible cysteines with the following rank order of reactivity: Cys-200 < Cys-201 (wt) < Cys-197 = Cys-204. In contrast, for CEC, the cysteine at position 200 was found to be the most reactive (rank order of the rate constants: Cys-204 = Cys-201 (wt) = Cys-197 < Cys-200). Our findings suggest that the agonist CEC and MTSEA may recognize two different receptor conformations, with MTSEA reacting with the predominant inactive form and CEC reacting with the agonist-dependent active conformation of TM5 of H2A. Such a hypothesis is consistent with three-dimensional models of the receptor-MTSEA and receptor-CEC complexes.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) was obtained from Amersham Pharmacia Biotech (specific activity, 56 Ci/mmol). Phenolamine, UK 14,304 (5-bromo-dihydro-1H-imidazol-2-yl)-6-quinolinamine), and CEC were supplied by Research Biochemicals (Natick, MA). MTSEA was purchased from Toronto Research Chemicals Inc. (North York, Canada). Cell culture reagents were supplied by Life Technologies, Inc. (Gaithersburg, MD). Other chemicals were of analytical grade and were purchased from commercial suppliers.

**Mutagenesis and Expression Vectors**—Site-directed mutagenesis was performed utilizing the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI) as described previously (9). The wild type H2A and the mutated receptor cDNAs were subcloned into the KpnI/BamHI sites of the expression vector pREP4 (In Vitrogen, NV Leek, The Netherlands).

**Cell Culture and Transfections**—Adherent CHO cells (American Type Culture Collection, Manassas, VA) were cultured as reported previously (9). Hygromycin B (Roche Molecular Biochemicals)-resistant (550 μg/ml) cell cultures were examined for their ability to bind the α2A-AR antagonist [3H]RX821002. The transfected cells chosen for further experiments were subsequently maintained in 200 μg/ml hygromycin B.

**Ligand Binding**—Ligand and binding assays were performed in 50 mM K+-phosphate buffer (pH 7.4 at 21 °C) as described previously (9, 12).

**Results**—The introduction of secondary mutations was verified by dideoxy sequencing of double-stranded DNA. Mutated and wild type receptors were expressed in CHO cells. Hygromycin B-resistant cell cultures were examined for their ability to bind the α2A-AR antagonist [3H]RX821002. The transfected cells chosen for further experiments were subsequently maintained in 200 μg/ml hygromycin B.

**Ligand Binding Assays**—Saturation isotherms of [3H]RX821002 binding, and LIGAND (18)-derived \( K_r \) (receptor affinity), and \( S_r \) (receptor density) values were determined in three separate experiments performed in triplicate for each cell line. The expression levels of two point-mutated receptors, H2A Ser201Cys202 and H2A Ser201Cys203, were 451 ± 10 and 2922 ± 91 fmol/mg protein, respectively. The affinities of these two mutations for the α2A-AR antagonist [3H]RX821002 were comparable with the H2Awt receptor (\( K_r \) values for H2Awt, H2A Ser201Cys202, and H2A Ser201Cys203 were 0.60 ± 0.02, 0.25 ± 0.02, and 0.68 ± 0.02 nm, respectively)
that H compounds were determined as described under "Experimental to CEC, the second-order rate constants for reaction with these other membrane-spanning segments. As G-202 contained no substituted cysteines, suggesting that residues 202 positions produces such a large inhibition of binding that the 

CHO-H CHO-H CHO-H 

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CHO-H CHO-H CHO-H (18) (although this calculated apparent K results from a combination of reversible and irreversible inhibition and is not a true K, as typically determined for reversible binding, it nevertheless supports the argument that CEC has substantial affinity for all the mutants tested). The characterization of some of these cell lines has been reported elsewhere (9).

Table I is an approximation of the rapid rate of reaction with CEC in these receptors was not due to a lack of binding affinity but rather to the absence of accessible cysteine residues on the surface of the binding-site crevice (apparent K = 260 ± 32, 345 ± 38, and 808 ± 37 nm, respectively) (Table I).

A similar characterization of CHO cells expressing wt and mutant receptors, the addition of CEC to competition binding assays inhibited specific binding of 2.5 nM [H]RX21002 with steep monophasic competition curves (9). The concentration of CEC that inhibited specific [H]RX21002 binding by 50% (IC50) was used to calculate apparent K values (inhibition constant) according to the Cheng-Prusoff equation (19) (although this calculated apparent K results from a combination of reversible and irreversible inhibition and is not a true K, as typically determined for reversible binding, it nevertheless supports the argument that CEC has substantial affinity for all the mutants tested). The characterization of some of these cell lines has been reported elsewhere (9).

Reactions with CEC—Fig. 1A illustrates the inhibition of antagonist binding produced by CEC. Binding to the mutants Cys-197, Cys-200, and Cys-204 is very sensitive to CEC, whereas the mutants Ser-201, Cys-202, and Cys-203 are relatively resistant to CEC, and the wild type receptor Cys-201 shows intermediate sensitivity. The rate and extent of receptor inactivation by CEC with cysteine substitutions for Ile-202 and Gly-203 was comparable with that of H2A Ser201, which contained no substituted cysteines, suggesting that residues 202 and 203 face the lipid bilayer or are closely packed against other membrane-spanning segments.

To quantitate the susceptibility of the engineered cysteines to CEC, the second-order rate constants for reaction with these compounds were determined as described under "Experimental Procedures" (Table II). It is apparent from these data (Fig. 1) that H2A Ser201 is not resistant to the effects of CEC and that it contains, therefore, one or more relatively slowly reacting cysteines. For all of the mutants except H2A wt(Cys-201), however, the simple single exponential decay function gave a reasonable fit of the CEC data, especially at low concentrations of CEC (Fig. 1A). This is likely the case because Cys-197, Cys-200, and Cys-204 are so reactive and because reaction at these positions produces such a large inhibition of binding that the relatively slowly reacting endogenous cysteines are nearly irrelevant to the resulting pattern of inhibition. For the H2 wt(Cys201) receptor, however, the plateau determined by this fit was substantially greater than the experimentally determined value, even though the fit is consistent with the inhibition seen at low concentrations of CEC. If the plateau was fixed to 0, however, a single exponential fit provided a gross underestimate of the rate of reaction (data not shown), a conclusion based on the significant inhibition of binding to this receptor observed at even very low concentrations of CEC. The data for the wild type receptor are better fit by a 2-site exponential decay function, but the variation in the rates obtained for the very reactive component for this receptor (and for the other mutants) was too great to make this a reliable method for analyzing the data (data not shown). Thus the value shown in Table II is an approximation of the rapid rate of reaction with CEC and MTSEA. The Cys-200 mutant is somewhat less sensitive to MTSEA than the wild type receptor. The lines are the single exponential decay fits (see "Experimental Procedures"), except in the case of and Ser-201, where no curve fits are shown (see "Results"). The data points represent means ± S.E. of 3–6 separate experiments, each performed in triplicate.

Reactions with MTSEA—MTSEA inhibited binding to all of the mutants (Fig. 1B), with Cys-202 and Cys-203 being the most resistant, consistent with the relative inaccessibility of these cysteines. The results for the Ser-201 mutant were not accurately described by an exponential decay function; this receptor was relatively resistant to MTSEA up to a concentration of 125 µM but showed quite significant inhibition at higher concentrations.
MTSEA concentrations. Consequently, no estimate for the second-order rate constant of inactivation of this receptor with MTSEA could be computed. The reasons for this unexpected inhibitory pattern remain unclear but may reflect the reactivity of other cysteines present in the Ser-201 background. In Fig. 1B the MTSEA results for the Ser-201, Cys-202, and Cys-203 mutants are obtained without curve-fitting. Reasonable single exponential decay fits were obtained for Cys-197, Cys-201, and Cys-204. For Cys-200, the data also show a “lag” at low concentrations, similar but less pronounced than that seen with Ser-201 (see above), and thus the fit is less satisfactory than that seen with the other three reactive residues. This results in an overestimation of the rate of reaction of Cys-200 with MTSEA, although it is impossible to determine the exact rate of reaction of this substituted cysteine in the presence of the Ser-201 background.

Receptor-mediated Changes in Extracellular Acidification Rate—CEC increased the extracellular acidification rate in CHO cells expressing Ho2Awt in a concentration-dependent and irreversible manner (Fig. 2). After CEC treatment followed by extensive washing, the reversible α2A-AR agonist UK 14,304 was unable to further increase the acidification rate, consistent with the complete reaction of the entire receptor population with the covalently tethered agonist CEC and persistent activation. Exposure of CHO cells expressing Ho2Awt and Ho2Aser201Cys200 receptors to UK 14,304 resulted in reversible increases in the rate of extracellular acidification (Fig. 3). These results confirmed the efficacy of the tethered agonist and the functionality of the mutant receptor. The functionality of this mutant receptor was also shown in a [35S]GTPγS binding assay.2

Receptor Modeling—Our current receptor model is based on an α-carbon template of the transmembrane helices in the rhodopsin family of G-protein-coupled receptors (15). Mutant receptor models were built by replacing the corresponding residues. Because only two residues at a time were replaced, and the mutations were quite conservative, such as Cys-201 to Ser and Ser-200 or Ser-204 to Cys or Val-197 to Cys and because radioligand antagonist binding to the mutants was similar to that of wt receptor, it is reasonable to assume that these mutations did not have major effects on the receptor structure; therefore, these residues were simply replaced in the wt model. CEC was docked manually into the binding-site crevice of the wild type receptor and the mutants in a position that allows the cysteine at position 201 to make a covalent bond between a reactive carbon atom and the sulfhydryl group of the cysteine residue in TM5 of the receptor. The models were then energy-minimized (Fig. 4B). In the Ho2Awt receptor, in the Ho2Aser201Cys197 mutant, and in the Ho2Aser201Cys204 mutant, cysteine residues are readily accessible in the binding-site crevice, and an ideal covalent bond can be formed. In the mutant Ho2Aser201Cys200, the cysteine residue is less accessible, and the predicted covalent bonding distance is longer than in the other cases, suggesting that a conformational change is necessary to shorten this distance and thereby to boost the rate of reaction between CEC and this cysteine. When the minimization is made without constraints, the cysteine residue can move. This allows the orientation of the TM5 helix to change, and makes the cysteine at position 200 more accessible for covalent bonding with CEC (Fig. 4C).

The starting position of MTSEA was similar to that of CEC. Fig. 4A shows the energy-minimized models of the receptor-MTSEA complexes. MTSEA forms ideal disulfide bonds with sulfhydryl residues in the Ho2Awt receptor, the mutant Ho2Aser201Cys197, and the mutant Ho2Aser201Cys204. However, the disulfide bond in the mutant Ho2Aser201Cys200 was slightly longer, and the cysteine residue was less accessible in the binding-site crevice than it is in the Ho2Aser201Cys197, Ho2Aser201Cys204, or Ho2Awt receptor models. As MTSEA has no other points of attachment in the binding-site crevice, energy minimization of the Ho2Aser201Cys200 mutant without constraints did not make the Cys-200 residue more accessible, as was the case with CEC.

DISCUSSION

Using the substituted cysteine-accessibility method and irreversible binding of the agonist CEC, we have previously mapped the residues accessible in the binding-site crevice in TM5 of human α2A-AR (9). Surprisingly, this pattern of accessibility was quite different from that observed in TM5 of the homologous dopamine D2 receptor (8). We postulated that the differences in accessibility might relate to the use of the agonist CEC. CEC has been used to discriminate between α1-AR subtypes in functional assays (20, 21), and it has also been shown to irreversibly bind to Ho2A and Ho2C but not Ho2B (9, 22). In functional studies with tissue preparations, CEC has been shown to behave as an α2-AR agonist, both at prejunctional and postjunctional α2-ARs (10, 11, 23, 24). However, the agonist action of CEC has previously not been shown in recombinant cell lines expressing only one defined receptor subtype. In this work, the agonist effects of CEC in recombinant CHO cells expressing Ho2Awt were confirmed with Cytosensor® microphysiometry. These results suggest that CEC is covalently tethered to the side chain of Cys-201 in such a manner that it produces irreversible receptor activation.

In the current study, the rate of reaction of engineered cysteines in TM5 of human α2A-AR was determined using two different sulfhydryl-specific agents, CEC and MTSEA. The rates of reaction of CEC and MTSEA were similar at the wt receptor (Cys-201). The rates of reaction of the two reagents were likewise similar, with cysteine substituted for residues 197 and 204. In contrast, the rate of reaction with a cysteine at

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2 J. M. Peltonen, unpublished observations.
CHO cells expressing human α2A-ARs. Cells were exposed to CEC for 15 min at the concentrations indicated, and a 30-min wash was employed between CEC exposures. CEC irreversibly increased the extracellular acidification rate in CHO-H2Awt cells. After CEC treatment and a further 30-min wash, the α2A-AR agonist UK 14,304 (1 μM) did not further increase the acidification rate. Each point represents the measured rate of acidification for one of the six chambers of cells (3–6 × 10^5 cells/chamber). CEC additions did not have any effect in non-transfected CHO cells (control). Each division of the abscissa represents a 45-min treatment cycle. Numerically, 1 μV s^-1 is close to 1 × 10^-5 pH units per minute (see Ref. 13).

The concept that G-protein-coupled receptors may possess some degree of spontaneous or constitutive activity and thus activate their cognate G-proteins in the absence of receptor agonist has recently become widely accepted (25, 26). G-protein-coupled receptors are thought to exist in an equilibrium between two (or more) interconvertible allosteric states, active (R*) and inactive (R) conformations. Binding of a receptor agonist causes an increase in the ratio of R* to R (5, 25). It has also been suggested that the R state can isomerize, not to a single active conformation, but to multiple different ligand-specific active conformations (27, 28). This multistate model suggests that the biological efficacy of an agonist may be a consequence of the qualitative and quantitative preference of the ligand for conformational variant forms of the receptor rather than just affecting the equilibrium between only two alternative states (28). MTSEA has no significant affinity for the receptor and, thus, reacts with cysteines accessible in the predominant resting receptor form (R). The activated state of a receptor can only be investigated with this reagent by using constitutively active mutant receptors (7). When the agonist CEC binds to TM5 of H2A, however, it likely stabilizes one or several of the active conformations (R*) and reacts with engineered cysteines accessible in the active conformation. Thus, the difference in the reactivity of CEC and MTSEA toward the substituted cysteine in position 200 in the H2A wt and H2A-Ser201Cys200 mutant may result from a difference in the accessibility of this residue in the active and the inactive forms of the receptor.

There are several other factors that might also contribute to a difference in the rate of reaction of a cysteine with CEC and with MTSEA. Local steric factors might favor the reaction of MTSEA with cysteines accessible in the active conformation. The true difference in rate of reaction of this residue with MTSEA is even greater than 5-fold, because the fit obtained for the inhibition of Cys-200 by MTSEA, as discussed above, significantly overestimates the rate of reaction of this residue with MTSEA. At particular positions including the reaction of Cys-200 with MTSEA, a simple single-exponential decay function is clearly inadequate to deal with the complex molecular interactions underlying the experimental results. There must be other sites in the receptor protein in addition to the investigated cysteines that are susceptible to inactivation by CEC and MTSEA, and these sites may also interact with each other. In addition, residual CEC and MTSEA present in the ligand binding assays may further complicate the situation, although experiments in which the reagents were removed by extensive washing gave similar results (data not shown). Nonetheless, both the qualitative results and the derived second-order rate constants support a similar reactivity of Cys-197, Cys-201, and Cys-204 to both CEC and MTSEA, and this contrasts with the substantially greater rate of reaction of Cys-200 with CEC than with MTSEA.

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FIG. 2. Effect of CEC on the extracellular acidification rate in CHO cells expressing human α2A-ARs. Cells were exposed to CEC for 15 min at the concentrations indicated, and a 30-min wash was employed between CEC exposures. CEC irreversibly increased the extracellular acidification rate in CHO-H2Awt cells. After CEC treatment and a further 30-min wash, the α2A-AR agonist UK 14,304 (1 μM) did not further increase the acidification rate. Each point represents the measured rate of acidification for one of the six chambers of cells (3–6 × 10^5 cells/chamber). CEC additions did not have any effect in non-transfected CHO cells (control). Each division of the abscissa represents a 45-min treatment cycle. Numerically, 1 μV s^-1 is close to 1 × 10^-5 pH units per minute (see Ref. 13).

FIG. 3. Effect of α2A-AR agonist UK 14,304 on the extracellular acidification rate in CHO-H2Awt (A) and CHO-H2ASer201Cys200 (B) cells. Exposure of CHO-H2Awt and CHO-H2ASer201Cys200 cells to the α2A-AR agonist UK 14,304 resulted in reversible increases in rates of extracellular acidification. Cells were exposed to UK 14,304 for 15 min at the concentrations indicated, and a 30-min wash was employed between UK 14,304 exposures. Each point represents the measured rate of acidification for one of the six chambers of cells (3–6 × 10^5 cells/chamber). UK 14,304 additions did not have any effect in non-transfected CHO cells (control). Transfected CHO cells with no UK 14,304 treatment showed stable acidification rates (basal). Each division of the abscissa represents a 45-min treatment cycle. Numerically, 1 μV s^-1 is close to 1 × 10^-5 pH units per minute (see Ref. 13).
FIG. 4. Energy-minimized hypothetical model of the H2A binding-site crevice with MTSEA and CEC. MTSEA (A) forms a covalent disulfide bond with a cysteine residue in TM5 of H2A at position 200 blue, the wild type 201 (red), and 204 (green). CEC (B) forms a covalent carbon-sulfur bond with a cysteine residue in TM5 of H2A at position 200 (blue), 201 (red), and 204 (green). An aspartate residue (Asp-113) in TM3 of H2A, the wt, and mutants participates in recognition of CEC; this is shown in blue, red, and green, respectively. C, energy-minimized model of the H2ASer201Cys200 mutant with CEC without constraints. Note that Cys-200 rotates to point into the binding-site crevice. is also consistent with our current revised receptor model based on the α-carbon template for the TM helices in the rhodopsin family of G-protein-coupled receptors (15). When CEC was docked into the receptor model based on the bacteriorhodopsin structure, residues 200, 201, and 204 were equally accessible to form the covalent carbon-sulfur bond with CEC or with MTSEA. In our current receptor model, however, the TM3 helix is tilted into the binding-site crevice, thereby limiting the size of the binding site and the access of ligands to the residue at position 200. Energy minimizations without constraints indicated that the presence of CEC may influence the orientation of position 200, thereby optimizing the interactions between the receptor and CEC, i.e. both hydrogen bonding of CEC with Asp-113 in TM3 and covalent bonding between CEC and Cys-200 in TM5 are simultaneously favored. Our results with MTSEA support the prediction that Cys-200 in the H2ASer201Cys200 mutant is not quite as readily accessible as Cys-201 in H2Awt, Cys-197 in the H2ASer201Cys197 mutant, and Cys-204 in the H2ASer201Cys204 mutant. Removing constraints during the energy minimization does not cause any conformational changes in TM5 in the H2ASer201Cys200-MTSEA complex.

These results suggest that CEC and MTSEA may recognize different conformations of the receptor, with CEC accessing the cysteine at position 200 more readily than MTSEA. This may be because of agonist-dependent clockwise rotation of TM5 of the activated human α2A AR. The current receptor model thus appears to more accurately predict the three-dimensional receptor structure than the previous bacteriorhodopsin-based receptor model.

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