Three-dimensional Models of $\alpha_{2A}$-Adrenergic Receptor Complexes Provide a Structural Explanation for Ligand Binding*

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We have compared bacteriorhodopsin-based ($\alpha_{2A}$-ARBR) and rhodopsin-based ($\alpha_{2A}$-ARr) models of the human $\alpha_{2A}$-adrenergic receptor ($\alpha_{2A}$-AR) using both docking simulations and experimental receptor alkylation studies with chloroethylcholindine and 2-aminoethyl methanethiosulfonate hydrobromide. The results indicate that the $\alpha_{2A}$-AR model provides a better explanation for ligand binding than does our $\alpha_{2A}$-ARr model. Thus, we have made an extensive analysis of ligand binding to $\alpha_{2A}$-AR and engineered mutant receptors using clonidine, para-aminoclonidine, oxytmazoline, 5-bromo-N-(4, 5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14,304), and norepinephrine as ligands. The representative docked ligand conformation was chosen using extensive docking simulations coupled with the ideation of favorable interaction sites for chemical groups in the receptor. These ligand-protein complex studies provide a rational explanation at the atomic level for the experimentally observed binding affinities of each of these ligands to the $\alpha_{2A}$-adrenergic receptor.

ExPERIMENTAL PROCEDURES

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1 The abbreviations used are: $\alpha_{2A}$-AR, human $\alpha_{2A}$-adrenergic receptor; $\alpha_{2A}$-ARBR, bacteriorhodopsin-based models of human $\alpha_{2A}$-AR; $\alpha_{2A}$-ARr, rhodopsin-based model of human $\alpha_{2A}$-AR; CEC, chloroethylcholindine; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; UK14,304, 5-bromo-N-(4, 5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; GPCR, G-protein-coupled receptor; TM, transmembrane helix/helices; WT, wild type; [3H]RX821002, [3H]2-(2-methoxy-1,4-benzo- dioxan-2-yl)-2-imidazoline.
cific activity, 56 C ü mol/mol. (±)-Norepinephrine was obtained from Merck, Celon, oxymetazoline, para-aminoclonidine, phentolamine, and UK14,304 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.

**Mutagenesis and Expression Vectors**—Site-directed mutagenesis was performed utilizing the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI) as described previously (10). The wild type α2A-AR (11) and the mutated receptor cDNAs were subcloned into the KpnI/BamHI sites of the expression vector pREP4 (InVitrogen, NV Leek, The Netherlands).

**Cell Culture and Transfections**—Adherent Chinese hamster ovary cells (American Type Culture Collection, Manassas, VA) were cultured as reported previously (10). The pREP4-based expression constructs were transfected into cells using the Lipofectin reagent kit (Life Technologies, Inc.). Hygromycin B (Roche Molecular Biochemicals)-resistant (550 µg/ml) cell cultures were examined for their ability to bind the α2A-AR antagonist [3H]HIX221002. The transfected cells chosen for further experiments were subsequently maintained in 200 µg/ml hygromycin B.

**Reactions with CEC and MTSEA**—The second-order rate constant (k) for the reaction of CEC or MTSEA with wild type α2A-AR and each investigated mutant (α2A-ARSer201, α2A-ARSer201Cys197, α2A-ARSer201Cys202, α2A-ARSer201Cys202Cys203) was determined by estimating the extent of the reaction after a fixed time, 15 min (CEC) or 2 min (MTSEA), with 7 concentrations of CEC (0.5, 1, 5, 10, 50, 100, and 500 µM) or MTSEA (5, 20, 50, 100, 150, 200, and 250 µM) as previously reported (9, 12).

**Competition Binding Assays**—Competition binding assays were performed in 50 mM K+-phosphate buffer (pH 7.4 at 21 °C) as described previously (10, 13). With the wild type α2A-AR, α2A-ARSer201 and α2A-ARSer201Cys197, the inhibition constants (Ki) for each competitor (clonidine, para-aminoclonidine, oxymetazoline, UK14,304, and norepinephrine) were analyzed with GraphPad Prism multicurve data analysis (GraphPad Software, San Diego, CA) for the three separate experiments performed in triplicate. For α2A-ARSer201Cys202 and α2A-ARSer201Cys202Cys203, only the inhibition constants (Ki) were also determined for UK14,304 and norepinephrine.

**Modeling and Comparison of the Models**—Models of α2A-AR (14) SWISS-PROT accession number P08913) were built using both the high resolution x-ray crystal structure of bacteriorhodopsin (3); Protein Data Base file code 1AP9) and a Cζ-atom template of the transmembrane helices of the rhodopsin-like GPCRs (5) as structural templates. These templates are referred to as α2A-ARWT and α2A-ARMut, respectively. The GPCR Cζ-atom template structure is based on the sequence comparison of about 500 different GPCR sequences and the cryo-electron microscopy structure of frog rhodopsin (5). Five different α2A-ARWT models and five α2A-ARMut models were made using the modeling program MODELLER (15). A representative model from each set, used for all subsequent studies, was chosen after examination of the models using the program InsightII (Molecular Simulations Inc., San Diego, CA) and by choosing the model in each set with the lowest value of the objective function, which describes the degree of fit of the model to the input structural data used in its construction, derived by the program MODELLER (15).

The models were then optimally superimposed using VERTAA. This program rapidly produces an objective comparison of two structures without any human intervention (i.e., without providing an initial alignment to seed the comparison). The program HelixTip (17) was used to define the differences in the two superimposed models, α2A-ARWT and α2A-ARMut, by specifying the vector along each helix and then calculating the angle between the vectors of the equivalent helices in the two matched structures (i.e., the difference in the helix tilt angle of the equivalent helices).

**Receptor models for each mutant were generated by replacing the corresponding residue at positions 197, 200, or 204, one at the time, with a cysteine residue and Cys-201 (the wild type sequence) with a serine residue (9).**

**GRID Maps**—The computer program GRID Version 16 (18) was used to map essential interactions in the binding site of each receptor model. GRID calculates energies of interaction between a probe and the receptor. The probes (Table I) were placed at different positions throughout the ligand binding site, and the receptor side chains were allowed to move too (using the side chain flexibility option in GRID). The GRID maps were visualized using the program CERIUS 2 (Molecular Simulations Inc., San Diego, CA).

**Docking Simulations**—CEC and MTSEA were docked manually to the binding cavity of the α2A-ARWT and the α2A-ARMut models as described in Marjamäki et al. (9).

Clonidine, para-aminoclonidine, oxymetazoline, UK14,304, and norepinephrine were initially energy minimized with the MM+ (extended MM2) forcefield in vacuum using the conjugate gradient method. The conformational space available to these small molecules was explored using simulated annealing and the MM+ forcefield implemented within the program Hyperchem 5.01 (Hypercube Inc., Gainesville, FL). Simulations were carried out in vacuum by first heating the ligands from 0 K to 100 K over 50 ps. Ligand structures were simulated at 100 K for 100 ps. The simulation temperature was then allowed to drop back to 0 K over 50 ps. A 1-fs time step was used throughout the simulation. After simulated annealing, the ligand structures were energy-minimized with the MM+ forcefield until the energy gradient was less than 0.01 kcal/mol. Atomic partial charges for the α2A-ARWT models and small molecule ligands were assigned according to the Gasteiger method (19) implemented in Quanta 97 (Molecular Simulations Inc., San Diego, CA).

The program Autodock 2.4 was used to carry out docking simulations (20–22). The small molecule ligands were flexibly docked to the rigid frog rhodopsin-based receptor models. Autodock combines Monte-Carlo simulated annealing for conformational searching with a rapid, atomic resolution, grid-based method of energy evaluation utilizing the Amber forcefield (23, 24). The overall interaction between chemical species is estimated by using Lennard-Jones atom-atom potentials and electrostatic effects summed for the individual interactions between atoms. A distance-dependent dielectric constant was used to account for the solvent-screening effects. The interaction of a probe group (corresponding to each type of atom found in the ligand) with the wild type and mutant receptor models was computed at grid positions 0.35 Å apart in a 36-Å3 box centered at the binding site using Autogrid. In the second simulation step, a 20-Å box with grid positions 0.25 Å apart was used to refine the docked structures.

The parameters used in the simulation follow the “short schedule” described by Goodsell et al. (25). To make the simulation more thorough, the following changes were made to the short schedule. 1) 100 separate docking simulations were performed for each ligand; 2) for each simulation there were 100 constant temperature cycles with 3000 steps accepted or rejected; 3) the temperature was reduced by a factor of 0.97 in each cycle; and 4) the maximal torsional rotation and translation steps used were 15° and 0.2 Å, respectively, and they were reduced by a factor of 0.97 in each cycle. In this way, over 30 million conformations were studied for each ligand-receptor complex.

After docking, cluster analysis of the docked structures was carried out. A 1-Å cut-off value of the root mean square deviation calculated over all atoms in the ligands was used to define a new cluster. Binding between the receptor molecules and the small molecules was investigated 1) using the optimal docked poses (i.e., conformation and orientation) found after this second stage of simulation and 2) visualization on a graphics station together with the GRID maps. The docked pose that best fit the GRID maps was chosen as a representative pose. Thus, the representative pose for all the docked ligands was finally chosen by combining the docking results from Autodock with the GRID maps.

**RESULTS**

**Comparison of the Bacteriorhodopsin and Frog Rhodopsin-based Models**

From the five α2A-ARWT (bacteriorhodopsin based) and the five α2A-ARMut (rhodopsin template based) models built with MODELLER (15), a single representative model was chosen for

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2 J. Lehtonen and M. S. Johnson, submitted for publication.
each with the lowest value of the MODELLER objective function: the model with the best agreement with all of the data used in the modeling process.

To evaluate the differences in the two predicted model structures, we first optimally superimposed the structures on each other and then evaluated how different the helix tilt angles were. The $\alpha_2A$-AR$_{h}$ and $\alpha_2A$-AR$_{r}$ models were superimposed using the program VERTAA, which revealed significant differences between the two models (Fig. 1, top). When all TM residues in the models were used in the comparison, 113 residues were superimposed with a root mean square deviation of 2.01 Å within the 3-Å cut-off, but none of the residues in TM4 were superimposed within this cut-off, because the closest distance between the equivalent C$_{a}$ atoms of Cys-201 and Asp-113 in the both models as well as the distance between Cys-201 in the $\alpha_2A$-AR$_{h}$ and Cys-201 in the $\alpha_2A$-AR$_{r}$ models are indicated. All figures were prepared using the program MOLSCRIPT (28) and rendered using Raster3D (16).

The differences in the relative positions of the transmembrane helices in the $\alpha_2A$-AR$_{h}$ and $\alpha_2A$-AR$_{r}$ models are larger at the ends of the helices than in the middle of them, and the helix tilt angles observed in the two models also differ. The differences between the helix tilt angles of TM3s, TM6s, and TM7s are smallest (5.9°, 5.8°, and 3.4°, respectively). The difference between the helix tilt angles is 9.9° for both TM1s and TM2s, and the difference is largest, 12.9°, between the tilt angles of TM5s. Generally, the differences in the tilt angles of the transmembrane helices in the two models can be described as a clockwise rotation of the $\alpha_2A$-AR$_{r}$ model relative to the $\alpha_2A$-AR$_{h}$ model (Fig. 1, top).

As a result of the clockwise rotation of the helices of $\alpha_2A$-AR$_{r}$ in comparison to $\alpha_2A$-AR$_{h}$ in the predicted structures, critical differences are seen in the relative positions of Asp-113 (TM3) and Cys-201 (TM5), the two key residues (10, 26, 27) implicated in ligand binding (Fig. 1B). The reactive aziridinium ion derivative of CEC forms a covalent bond with the sulphydryl side chain of Cys-201 and the carboxyl group of Asp-113 is, based on docking simulations, hydrogen bonding with the protonated imidazoline ring of CEC (Fig. 2, top and bottom) (9). In both the $\alpha_2A$-AR$_{h}$ and $\alpha_2A$-AR$_{r}$ models, Asp-113 is located at approximately the same position in the superimposed model struc-
tures, whereas Cys-201 is much less exposed to the binding cavity in the α2A-ARR model than in the α2A-ARBR model (Fig. 1, bottom). The Cα atoms of Asp-113 and Cys-201 in the α2A-ARR model, in comparison with α2A-ARBR, are closer to each other, and they are positioned at about the same distance from the membrane boundaries (Fig. 1, bottom). Because of these differences in the helix positions and tilt angles of the two models, the ligand binding site at the extracellular end of the transmembrane helices is more exposed in the α2A-ARR model than in the α2A-ARBR model.

Because large differences exist between these two models, we can probe the ligand binding site using both computational methods and experimentally using the recombinant receptor and engineered mutant receptors. Thus, comparisons made between the two approaches can provide evidence in support of one model over the other. Alternatively, it is possible that neither model adequately reflects the ligand binding environment within α2A-AR, which also should be detectable.

**CEC and MTSEA**

**Ligand Binding**—The second order rate constants for receptor alkylation with CEC and MTSEA were determined to quantitate the susceptibility of the engineered cysteines to these compounds. The consecutive amino acids extending from Val-197 to Ser-204 in TM5 were mutated to introduce or delete cysteines to examine the structure of TM5; the mutant receptors are designated α2A-ARSer201, α2A-ARSer201Cys197, α2A-ARSer201Cys200, α2A-ARSer201Cys202, α2A-ARSer201Cys203, and α2A-ARSer201Cys204. With the WT and the mutant receptors, α2A-ARSer201, α2A-ARSer201Cys197, and α2A-ARSer201Cys204, there was no significant difference in the alkylation rates obtained for CEC and MTSEA. The relative alkylation rate of the α2A-ARSer201Cys200 mutant, however, was much faster with CEC than, with MTSEA indicating that they may interact with different receptor conformations. The results obtained from reactions of the wild type and mutant receptors with CEC and MTSEA are described in complete detail elsewhere (9).

**Docking Simulations**—To simulate the binding of ligand to the two predicted models, we manually docked CEC and MTSEA to the binding cavity of α2A-ARRWT, α2A-ARRSer201Cys200, and to the mutant receptors derived from these model structures. In the wild type and mutant models based on bacteriorhodopsin, there are no differences seen in the accessibility of the reactive cysteine in TM5. Thus, Cys-200 in the α2A-ARBRSer200 model is predicted to bind equally well to both CEC and MTSEA (Fig. 2, top).

In α2A-ARRSer201Cys200, the accessibility of Cys-200 to the ligands is, however, limited. Nonetheless, when the α2A-ARRSer201Cys200-CEC complex is energy-minimized without constraints, TM5 was observed to adjust its orientation to allow proper ligand binding. As a result of this movement, covalent bonds can form between Cys-200 (TM5) and CEC, as well as hydrogen bonds between Asp-113 (TM3) and CEC (Fig. 2, bottom). In contrast, MTSEA is too short to form contacts with Asp-113 in TM3. Thus, when the α2A-ARRSer201Cys200-MTSEA complex was energy-minimized without constraints, the orientation of TM5 did not change, and the disulfide bond length...
between Cys-200 and MTSEA was longer than the disulfide bond in the MTSEA complex with $\alpha_{2A-ARR}$ or with any of the other mutant receptors (Fig. 2, bottom).

Unlike the models based on the bacteriorhodopsin structure, the predicted results obtained for the models based on the frog rhodopsin template are in agreement with the experimental ligand binding studies for CEC and MTSEA. Because of these results, we have used the $\alpha_{2A-ARR}$ model, but not the $\alpha_{2A-ARBR}$ model, in all other docking studies reported here.

### Other Ligands

**Ligand Binding**—The results from the competition of [3H]RX821002 binding to $\alpha_{2A-AR}$ WT and mutant receptors, separately expressed in CHO cells

$K_{ii}$ and $K_{ii}$ are inhibition constants for the high and low affinity sites in a statistically significant ($p < 0.05$) two-site model. Results for $\alpha_{2A-Ser201}$ mutant are statistically significantly modeled only with one-site fit. The $K_{ii}$ values are means ± S.E. from multicurve analysis of three separate experiments performed in triplicate. n.s., not significant.

### Docking Simulations

Five ligands, clonidine, para-aminoclonidine, oxytetracycline, UK14,304, and norepinephrine, which do not form covalent complexes in their binding to $\alpha_{2A-AR}$, were automatically docked to each of the wild type and mutant models. Thus, we have combined the docking results from Autodock with the GRID maps to choose the representative pose for each of these ligands. In $\alpha_{2A-AR}$ WT, the optimal docked poses of all five ligands are similar (Fig. 3). The CH$_2$ groups in the imidazoline ring of clonidine, para-aminoclonidine, and oxytetracycline pack against TM7 (Fig. 3, top and middle). The imidazoline ring of clonidine, para-aminoclonidine, oxytetracycline, and UK14,304 is placed so that the ligands can form ideal hydrogen bonds with Asp-113. In oxytetracycline, the NH group of the imidazoline ring can make one bifurcated hydrogen bond with both side-chain oxygens of Asp-113 (Fig. 3, middle). Clonidine, para-aminoclonidine, and UK14,304 can make bidentate hydrogen bonds with the side-chain oxygens of Asp-113: one with the NH group in the imidazoline ring and the other one with the NH group in the aliphatic chain (Fig. 3, top). As a result, the imidazoline ring in clonidine, para-aminoclonidine, and UK14,304 is pushed slightly toward TM7 (Fig. 3, top). GRID maps also indicate that the TM7 region is favorable for hydrophobic CH$_2$ and CH$_3$ contacts.

In norepinephrine, there is an OH group present instead of the aliphatic NH group seen in the other ligands, but it is similarly positioned. As a result, the OH group can also make a favorable hydrogen bond with a side chain oxygen of Asp-113 (Fig. 3, bottom). With an OH group probe, the GRID maps

| Ligand Binding in $\alpha_{2A-AR}$ | 23409 |
|-----------------------------------|-------|

### Table II

Competition of [3H]RX821002 binding to $\alpha_{2A-AR}$ WT and mutant receptors, separately expressed in CHO cells

$K_{ii}$ and $K_{ii}$ are inhibition constants for the high and low affinity sites in a statistically significant ($p < 0.05$) two-site model. Results for $\alpha_{2A-Ser201}$ mutant are statistically significantly modeled only with one-site fit. The $K_{ii}$ values are means ± S.E. from multicurve analysis of three separate experiments performed in triplicate. n.s., not significant.

| Ligand | $K_{ii}$ (nM) | $K_{ii}$ (nM) | $K_{ii}$ (nM) | $K_{ii}$ (nM) |
|--------|--------------|--------------|--------------|--------------|
| Norepinephrine | 1650 ± 330 | 0.44 ± 0.06 | 4400 ± 418 | 6520 ± 76 |
| UK 14,304 | 13.0 ± 0.57 | 0.68 ± 0.02 | 36.9 ± 1.00 | 2430 ± 32.1 |
| Oxytocin | 1.81 ± 0.09 | 0.10 ± 0.02 | 5.01 ± 0.20 | 41.2 ± 0.54 |
| Clonidine | 31.5 ± 1.47 | 0.07 ± 0.01 | 54.7 ± 1.83 | 33.2 ± 0.42 |
| $\mu$-Aminoclonidine | 20.7 ± 0.66 | 5.63 ± 0.23 | 119 ± 31.5 | 34.3 ± 0.40 |
| Apo $\alpha_{2A-AR}$ | 75100 ± 4500 | n.s. | n.s. | 18500 ± 1480 | 7.34 ± 0.58 | 44200 ± 1900 | n.s. | n.s. |
calculated for the $\alpha_{2A}$-AR WT, $\alpha_{2A}$-AR Ser201, and $\alpha_{2A}$-AR Ser201-Cys197 models indicate favorable interactions with the side-chain oxygens of Asp-113.

The aromatic ring, present in all of the docked ligands, is placed between TM3 and TM6, approximately parallel to TM3. GRID calculations predict favorable aromatic -CH group inter-
FIG. 4. UK14,304 docked sequentially to the wild type (Cys-201 in TM5), \( \alpha_{2A}\text{-AR} \text{Ser}201 \), \( \alpha_{2A}\text{-AR} \text{Ser}201\text{Cys197} \), \( \alpha_{2A}\text{-AR} \text{Ser201Cys200} \), and \( \alpha_{2A}\text{-AR} \text{Ser201Cys204} \) mutant receptors.
actions at this site. In clonidine and para-aminoclonidine, there are two Cl⁻ atoms present in each; one pointing toward the extracellular membrane surface (up in Fig. 3, top) and the other one pointing downward in the binding cavity (down in Fig. 3, top). In UK14,304, a Br⁻ atom is present and preferentially points up even though it could be oriented either up or down. The methyl groups of oxymetazoline are pointing in a similar way as the Cl⁻ atoms in clonidine and para-aminoclonidine. The GRID calculations made using the CH₃ and hydrophobic probes indicate that hydrophobic contacts could be formed between the bottom of the binding cavity and the methyl group pointing down in oxymetazoline (Fig. 3, middle). The largest hydrophobic zone calculated with GRID is located near Cys-201 in TM5. Thus, the longer ligands, oxymetazoline and UK14,304, can form stronger hydrophobic contacts with Cys-201 of α₂A-AR₉WT than the shorter ligands can (norepinephrine, para-aminoclonidine, and clonidine).

The cysteine residue in TM5 of the wild type receptor model seems to be important for ligand binding, since all of the docked ligands are always oriented toward that cysteine. In α₂A-AR₉Ser201, where cysteine contacts do not exist, the docked conformation of UK14,304 enforces the hydrogen bonding with Asp-113 (Fig. 4). This is true for the docking simulations of the other ligands too. Based on the GRID calculations, the perturbation caused by the replacement of Cys-201 with Ser makes the binding site environment slightly less hydrophobic. In α₂A-AR₉Ser201Cys197, the docked conformation of UK14,304 is distinctly closer to the membrane boundary than in the WT and other mutant receptors, and contacts are formed with both ends of the ligand (Fig. 4). In α₂A-AR₉Ser201Cys197, the distance from Asp-113 to Cys-197 is about 3.5 Å less than the distance from Asp-113 to Cys-201 in α₂A-AR₉WT. Thus, in α₂A-AR₉Ser201Cys197 both ends of para-aminoclonidine and clonidine can form contacts with the receptor. Our docking simulations suggest that UK14,304 binds closer to TM5 in α₂A-AR₉Ser201Cys200 than in α₂A-AR₉Ser201, because the ligand can then form contacts with Cys200 in α₂A-AR₉Ser201Cys200 (Fig. 4). In α₂A-AR₉Ser201Cys200, Cys200 is pointing slightly away from the binding cavity, and thus UK14,304 can form only one hydrogen bond with Asp-113 (Fig. 4). In α₂A-AR₉Ser201Cys204, the cysteine is one turn lower but pointing in the same direction in the binding cavity as Cys-201 in the wild type receptor. Thus, UK14,304 (Fig. 4) and the other ligands can bind to α₂A-AR₉Ser201Cys204 in a similar way as seen in the wild type receptor.

**DISCUSSION**

We have compared the bacteriorhodopsin (α₂A-ARBR) and the rhodopsin-based (α₂A-AR) models of human α₂A-AR and compared the combination with the CEC- and MTSEA-induced receptor alkylation studies using both the wild type (Cys-201 in TM5) and the mutant receptors having engineered cysteines in TM5. In the alkylation reaction, the reactive aziridinium ion derivative of CEC forms a covalent bond, and MTSEA forms a disulfide bond, with the cysteine residue accessible in the binding cavity.

Generally, the difference between the two models can be described as a clockwise rotation of the α₂A-ARBR model relative to the α₂A-AR model (Fig. 1, top). This results in a tighter and more surface-exposed ligand binding site in the α₂A-ARBR model (Fig. 1, bottom). The results with the α₂A-ARBR model further supported the rhodopsin-based model. We could not explain with the α₂A-ARBR model why the alkylation rate of α₂A-ARBRWT was higher than with CEC than with MTSEA, in both the α₂A-ARBR-CEC and α₂A-ARBR-MTSEA complexes, Cys-200 remained equally accessible to the alkylating reagent. However, the difference can be explained with the α₂A-AR model, where Cys-200 becomes more accessible because of the conformational change in the receptor, which allows CEC to have, simultaneously, a bidentate hydrogen bond to Asp-113 and a covalent bond to Cys-200. MTSEA is covalently bonded to Cys-200 and is too short to reach the vicinity of Asp-113, and thus, it does not cause any conformational change in the receptor (Fig. 2). Based on these results, we have used the α₂A-AR models in docking studies involving five other known ligands of α₂A-AR but not the bacteriorhodopsin-based models.

We have carried out extensive ligand binding simulations on α₂A-AR₉WT, α₂A-AR₉Ser201, α₂A-AR₉Ser201Cys197, α₂A-AR₉Ser201Cys200, and α₂A-AR₉Ser201Cys204 and compared the results with the experimental binding data. Cys-201 and Asp-113 have both been shown experimentally to be important for ligand binding in α₂A-AR (26, 27). Our theoretical docking studies of clonidine, para-aminoclonidine, oxymetazoline, UK14,304, and norepinephrine also indicate the importance of these residues.

In α₂A-AR₉WT, UK14,304, and oxymetazoline form interactions with both Asp-113 and Cys-201, whereas norepinephrine, clonidine, and para-aminoclonidine mainly contact Asp-113. In α₂A-AR₉Ser201Cys197, the distance between Asp-113 and Cys-197 is shorter than in the wild type receptor, and therefore, all of the ligands form strong interactions with the receptor, but the docked conformations are oriented in a very different way than in the other receptor models. Because the distance from Asp-113 to the cysteine residue in TM5 is longer in the α₂A-AR₉Ser201Cys200 and α₂A-AR₉Ser201Cys204 receptor models, the ligands are slightly too short to form interactions as strong as those seen in the wild type receptor. However, even though the distance is longer between Asp-113 and the Cys residue in TM5, the ligand interacts with the cysteine residue, not the serine, in TM5. Indeed, the docked ligands clearly prefer to interact with the cysteine residue in TM5 in each of the mutant receptors via the aromatic rings or other hydrophobic groups of the ligands. Furthermore, this is supported by the experimental binding results (Table II), which show a decrease in the binding affinity of all the ligands to α₂A-AR₉Ser201.

The binding affinity of UK14,304 to α₂A-AR₉Ser201 is nearly 200 times lower than to the wild type receptor (Table II). The favorable hydrophobic interactions between the hydrogen atoms of the aromatic ring in UK14,304 and the cysteine residue (Cys-201) have disappeared, a likely cause of this decrease in the binding affinity. In the α₂A-AR₉Ser201Cys197 and α₂A-AR₉Ser201Cys200 mutant receptors, the bidentate hydrogen bond between the ligand and Asp-113 is broken to allow the ligand to interact with both the cysteine residue in TM5 and Asp-113 in TM3 (Fig. 4). In addition, the orientation of UK14,304 changes and a hydrophobic interaction is formed between the aromatic ring of UK14,304 and the cysteine residue (Cys-201). This could explain the lower binding affinities observed for these two mutant receptors for UK14,304 (Table II). In α₂A-AR₉Ser201Cys204, the docked conformation of UK14,304 is close to the conformation seen in the wild type receptor, but the bidentate hydrogen bond to Asp-113 is broken (Fig. 4). As a result, the decrease in binding affinity is smaller compared with the two other mutant receptors (Table II).

Norepinephrine similarly forms a bidentate hydrogen bond with Asp-113 as was predicted for UK14,304. However, the bidentate interaction is formed between the oxygen atoms of Asp-113 and the hydrogen atom of the NH₂ group and the β-hydroxyl of norepinephrine (Fig. 3, bottom). Norepinephrine is smaller in size than UK14,304, and thus, norepinephrine does not ideally interact with the cysteine residues in the α₂A-AR₉Ser201Cys204 and α₂A-AR₉Ser201Cys200 mutant re-
ceptors. The binding affinities to these mutant receptors are lower than to the wild type receptor (Table II). In α2A-ARqSer201Cys197, the cysteine residue interacts with the phenyl ring of norepinephrine, but the bidentate hydrogen bond with Asp-113 is lost, and therefore, the binding affinity is lower than observed with the wild type but higher than seen with the other two mutant receptors. Because the phenolic hydroxyl group of norepinephrine interacts with Ser-201, the difference in the binding affinity of norepinephrine with α2A-ARqSer201 and with the wild type receptor is smaller than the corresponding difference for UK14,304 (Table II).

Oxymetazoline does not have the two NH groups needed to form a bidentate hydrogen bond with Asp-113. Instead, the NH group of the imidazole ring forms hydrogen bonds with both oxygen atoms of Asp-113 (Fig. 3, middle). Oxymetazoline is larger than the other ligands, having a tert-butyl group attached to the phenyl ring, which makes favorable hydrophobic interactions with the cysteine residue in TM5. In α2A-ARqSer201, the decrease in binding affinity is not as large as with UK14,304, because oxymetazoline, like norepinephrine, has a hydroxy group that interacts with Ser-201. In α2A-ARqSer201Cys197, the cysteine residue strongly interacts with the aromatic ring of oxymetazoline, and the hydrogen bond with Asp-113 is lost. Consistent with these modeling results, the observed binding affinity of oxymetazoline to α2A-ARqSer201Cys197 is lower than to the wild type receptor (Table II).

Clonidine and para-aminoclonidine do form a bidentate hydrogen bond with Asp-113 in the wild type receptor model, but they are too short to form favorable interactions with the cysteine residue in TM5 too (Fig. 3, top). Similarly, they do not favorably interact with Ser-201 in α2A-ARqSer201, and the observed change in the binding affinity compared with the wild type receptor is not large (Table II). However, in α2A-ARqSer201Cys197, the distance between Asp-113 and Cys-197 is shorter than in the wild type receptor, and this permits simultaneous interactions between ligands and the important residues. However, only a single hydrogen bond between the NH group of the imidazole ring and Asp-113 is formed instead of the bidentate hydrogen bond existing in the wild type receptor complexes. In addition, a hydrophobic interaction between the aromatic ring of both ligands and Cys-197 is formed. Consistent with these modeling results, only a slight increase in binding affinity can be seen (Table II).

In this work, we have used two programs: Autodock 2.4 (20–22) to simulate the docking of ligands to the receptor models, and GRID Version 16 (18), to probe chemically favorable interaction sites in the ligand binding site in the receptor models, and then combined these results to construct the ligand-protein complexes. Even though the binding site in the receptor is rigid in our docking simulations, the use of GRID maps allows us to examine the flexibility of the receptor binding site. In this way, the effects induced by the mutated residue in the vicinity of the binding site could be revealed, and the experimentally observed changes in binding affinity were explained at the atomic level. The combined use of molecular modeling methods and experimental data provides us with a more detailed understanding of ligand binding in the α2A-adrenergic receptor.

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