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# Molecular Mechanism for Agonist-Promoted $\alpha_{2A}$-Adrenoceptor Activation by Norepinephrine and Epinephrine

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## ABSTRACT

We present a mechanism for agonist-promoted $\alpha_{2A}$-adrenergic receptor ($\alpha_{2A}$-AR) activation based on structural, pharmacological, and theoretical evidence of the interactions between phenethylamine ligands and $\alpha_{2A}$-AR. In this study, we have: 1) isolated enantiomerically pure phenethylamines that differ both in their chirality about the $\beta$-carbon, and in the presence/absence of one or more hydroxyl groups: the $\beta$-OH and the catecholic *meta*- and *para*-OH groups; 2) used [$^3$H]UK-14,304 [5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine; agonist] and [$^3$H]RX821002 [2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; antagonist] competition binding assays to determine binding affinities of these ligands to the high- and low-affinity forms of $\alpha_{2A}$-AR; 3) tested the ability of the ligands to promote receptor activation by measuring agonist-induced stimulation of [$^{35}$S]GTP$\gamma$S binding in isolated cell membranes; and 4) used automated docking methods and our $\alpha_{2A}$-AR model to predict the binding modes of the ligands inside the $\alpha_{2A}$-AR binding site. The ligand molecules are sequentially missing different functional groups, and we have correlated the structural features of the ligands and ligand-receptor interactions with experimental ligand binding and receptor activation data. Based on the analysis, we show that structural rearrangements in transmembrane helix (TM) 5 could take place upon binding and subsequent activation of $\alpha_{2A}$-AR by phenethylamine agonists. We suggest that the following residues are important in phenethylamine interactions with $\alpha_{2A}$-AR: Asp113 ($D_{3.32}$), Val114 ($V_{3.33}$), and Thr118 ($T_{3.37}$) in TM3; Ser200 ($S_{5.42}$), Cys201 ($C_{5.43}$), and Ser204 ($S_{5.46}$) in TM5; Phe391 ($F_{6.52}$) and Tyr394 ($Y_{6.55}$) in TM6; and Phe411 ($F_{7.38}$) and Phe412 ($F_{7.39}$) in TM7.

$\alpha_2$-Adrenoceptors ($\alpha_2$-ARs) belong to the rhodopsin-like class of G-protein coupled receptors (GPCRs), characterized by seven transmembrane (TM) $\alpha$-helices with an extracellular N-terminus and a cytoplasmic C-terminus (Kobilka et al., 1987; Bikker et al., 1998). The TM helices in $\alpha_2$-ARs form a water-accessible binding site for ligands in a pocket or crevice between the helices in the interior of the receptor. Residues within this cavity directly participate in ligand binding, which stabilizes the conformation of the receptor. Agonist ligands, whose pharmacological activity is manifested as an activation of downstream signaling, shift the equilibrium between the inactive and active receptor conformations in favor of the active form (Gether and Kobilka, 1998). Through their interactions with naturally occurring and synthetic ligands, $\alpha_2$-ARs mediate a variety of physiological and pharmacological effects, and are thus key targets for pharmaceutical development. $\alpha_2$-ARs have therapeutic applications in a variety of diseases, for example, in the treatment of hypertension, pain, and depression (Ruffolo et al., 1993; MacDonald et al., 1997).

For $\alpha_{2A}$-ARs, as well as all other types of $\alpha$- and $\beta$-adrenoceptors, a conserved aspartate in the third transmembrane helix (TM3) has been established as a residue critical for phenethylamine ligand binding. In the human $\alpha_{2A}$-AR, this residue corresponds to Asp113 [$D_{3.32}$ according to the indexing system of Ballesteros and Weinstein (1995)]. The negatively charged aspartate in TM3 provides an anchoring point for ligands containing positively charged amine groups (Ruffolo, 1991; Kobilka, 1995). Other residues suggested to be involved in the binding of phenethylamine ligands in $\alpha_{2A}$-AR include Cys201 ($C_{5.43}$) in TM5 (Marjamäki et al., 1999; Marjamäki et al., 1998), Ser200 ($S_{5.42}$) and Ser204 ($S_{5.46}$) also in TM5 (Marjamäki et al., 1998, 1999; Rudling et al., 1999; Salminen et al., 1999), and aromatic residues in TM6 (Ko-

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**ABBREVIATIONS:** AR, adrenergic receptor; GPCR, G protein coupled receptor; TM, transmembrane helix.

bilka, 1995). Binding of the β-OH group of the phenethylamines to adrenergic receptors has been suggested to involve a serine ($S_{2.61}$) in TM2 (Li et al., 1995; Hieble et al., 1998), a serine ($S_{4.57}$) in TM4 (Strader et al., 1989; Trumpp-Kallmeyer et al., 1992), a serine in TM7 ($S_{7.46}$; Hieble et al., 1998), and an asparagine ($N_{6.55}$) in TM6 of the $\beta_2$-AR (Wieland et al., 1996). The latter hydrogen bonding interaction could also be possible in $\alpha_{2A}$-AR, where residue 6.55 is a tyrosine. However, Hieble and colleagues (1998) have shown that this residue has no effect on the stereospecific binding of the β-OH in phenethylamines. None of these proposals is compatible with our docking results, and we will suggest an alternative determinant for the stereospecific binding of β-OH-phenethylamines to $\alpha_{2A}$-AR.

The first successful structural explanation for phenethylamine agonist binding to adrenergic receptors was the three-point attachment hypothesis outlined by Easson and Stedman in the 1930s (see Ruffolo, 1991). The original hypothesis was formulated without any empirical information on the structure of the binding site. Although no X-ray structure of $\alpha_{2A}$-AR has so far been reported, the functional, structural, and experimental data that exist in the literature for multiple classes of GPCRs can be combined to make an atomic resolution model of a particular member of the receptor family (Bikker et al., 1998). Receptor binding and activation assays, combined with a three-dimensional model of the receptor, allow us to study the receptor in great detail, which improves understanding of the conformational changes that take place upon receptor activation. The common location of the ligand binding site for many rhodopsin-like GPCRs between TM3, TM5, and TM6, and the accumulated functional and structural evidence suggest that the activation of GPCRs is connected to the movement of these transmembrane helices with respect to each other (Kobilka, 1995; Beck-Sickinger, 1996; Gether et al., 1997; Unger et al., 1997; Gether and Kobilka, 1998). We have previously introduced cysteine substitutions along TM5 and demonstrated that two alkylating agents with different chemical structures, chloroethylclonidine and 2-aminoethyl methanethiosulfonate, most likely recognize two different conformations of the human $\alpha_{2A}$-AR (Marjamäki et al., 1999). Molecular modeling (Salminen et al., 1999) of the different receptor variants with these alkylating agents supported this assertion and suggested to us that TM5 might rotate when $\alpha_{2A}$-AR is activated.

We now have a structural model for $\alpha_{2A}$-AR (Salminen et al., 1999) whose functionality has been verified through experimental studies that include site-directed mutagenesis and ligand binding assays (Marjamäki et al., 1999; Salminen et al., 1999) and that offers a better structural explanation for small molecule ligand binding in comparison to models based on the bacteriorhodopsin X-ray structure (Marjamäki et al., 1999; Salminen et al., 1999). The model is based on an α-carbon template for the backbone of the receptor, and derives from the low-resolution electron microscopy structure of frog rhodopsin and sequence alignments of hundreds of GPCRs (Baldwin et al., 1997). We also have a new model based on the X-ray structure of bovine rhodopsin (Palczewski et al., 2000). The model based on this new structure positions most of the same residues within the binding cavity as seen in our current model. However, bovine rhodopsin is not an adrenergic receptor, and TM5, demonstrated by many groups to play an essential role in catecholamine binding, is apparently less important in photoactivation of bovine rhodopsin.

In the current study, we use two sets of ligand binding experiments, a functional receptor activation assay, and molecular modeling to predict the binding modes of 12 phenethylamine ligands, and present a model of how they could promote the activation of $\alpha_{2A}$-AR. We present an atomic resolution model for the binding mode of epinephrine, norepinephrine, and their close structural analogs based on the results of these experiments, and provide a structural explanation for the binding affinity differences of the *R*- and *S*-enantiomers of these molecules. In the model, the most critical interactions for the binding of the agonists exist between the ligands and residues in TM3, TM5, TM6, and TM7 of $\alpha_{2A}$-AR, which is consistent with many earlier reports. The structural basis for the roles of the charged amine group, the *N*-methyl group of epinephrine, the β-OH group, the aromatic ring, and the catecholic *para*- and *meta*-hydroxyls for ligand binding and receptor activation in $\alpha_{2A}$-AR are also revealed by this study.

## Materials and Methods

**Experimental Materials.** [$^3$H]RX821002 [2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline] was obtained from Amersham (Buckinghamshire, UK; specific activity 52 Ci/mmol). [$^3$H]UK-14,304 [5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine] (62.5 Ci/mmol) and [$^{35}$S]GTPγS (1,225 Ci/mmol) were obtained from NEN (Boston, MA). *p*-Aminoclonidine, dopamine, (*R*)-norepinephrine (bitartrate), and unlabeled UK-14,304 were supplied by RBI/Sigma (Natick, MA). (*R*)-2-Amino-1-phenyl-ethanol and (*S*)-norepinephrine (hydrogen L-tartrate) were purchased from Fluka Sigma-Aldrich (Buchs, Switzerland). (*R*)-Epinephrine was from Sigma Chemical (St. Louis, MO). (*R,S*)-Norphenephrine and (*R,S*)-octopamine were obtained from Aldrich Chemical (Milwaukee, WI). The enantiomers of norphenephrine and octopamine were prepared using *Pseudomonas cepacia* lipase-catalyzed resolution of the racemates (Fmoc-protected in the case of octopamine) through enantioselective acylation in toluene/tetrahydrofurane (3:1). $NH_3$ treatment provided the free (*R*)- and (*S*)-norphenephrine counterparts (enantiomeric excess > 98%). *Candida antarctica* lipase B-catalyzed ethanolysis and treatment with piperidine (5% (v/v)) in tetrahydrofurane provided the free (*R*)- and (*S*)-octopamine enantiomers (enantiomeric excess > 98% and 91%, respectively). Cell culture reagents were supplied by Life Technologies (Gaithersburg, MD). Other chemicals were of analytical or reagent grade, and were purchased from commercial suppliers.

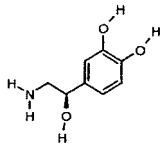
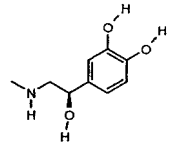
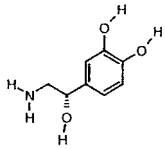
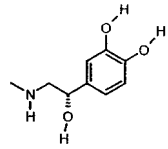
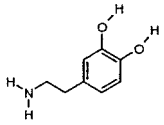
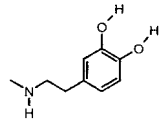
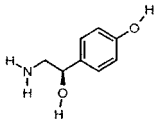
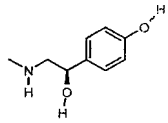
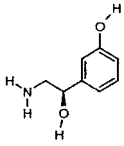
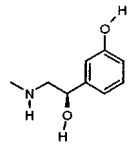
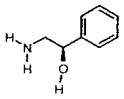
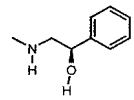
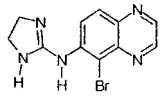
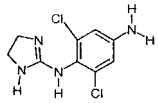
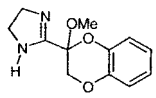
**Transfection and Cell Culture.** Adherent Chinese hamster ovary cells (American Type Culture Collection, Manassas, VA) were cultured as reported previously (Pohjanoksa et al., 1997). Cells were transfected with a pMAMneo-based expression construct encoding the human $\alpha_{2A}$-AR and standard methods (Pohjanoksa et al., 1997). Neomycin (G418; Sigma)-resistant (750 μg/ml) cell cultures were examined for their ability to bind the $\alpha_{2A}$-AR antagonist [$^3$H]RX821002. The transfected clonal cell lines were cultured in medium containing 250 μg/ml G418. The cell clone chosen for the experiments expressed $\alpha_{2A}$-AR at a density of 1.3 pmol/mg total cellular protein as determined with saturation binding experiments with [$^3$H]RX821002 (Pohjanoksa et al., 1997).

**Competition Ligand Binding Assays.** Competition binding assays with [$^3$H]RX821002 were performed as reported previously (Halme et al., 1995; Marjamäki et al., 1999), using a radioligand concentration close to its affinity constant ($K_d$) for $\alpha_{2A}$-AR and 13 to 15 concentrations of the competitive ligands. The assay buffer was 50 mM $K^+$-phosphate buffer supplemented with 10 mM $MgCl_2$. For agonist competition assays with [$^3$H]UK-14,304 as radioligand, cell membranes (about 10 μg protein/sample) and 0.6 nM [$^3$H]UK-14,304

TABLE 1

Structures and names of the ligands used in the theoretical and experimental studies involving $\alpha_{2A}$-AR

(*S*)-Octopamine, (*S*)-norphenephrine, and (*S*)-2-amino-1-phenyl-ethanol were not included in the docking study since their behavior can be predicted from the docking of the corresponding *R*-isomers and (*S*)-norepinephrine and (*S*)-epinephrine. The percentage given in parentheses is the size of the population having the conformation used in the docking studies (see *Materials and Methods* for details).

| Norepinephrine Analogs | | Epinephrine Analogs | |
|---|---|---|---|
| Structure | Name/DockingResults | Structure | Name/DockingResults |
|  | (*R*)-Norepinephrine (8%) |  | (*R*)-Epinephrine (11%) |
|  | (*S*)-Norepinephrine (19%) |  | (*S*)-Epinephrine (21%) |
|  | Dopamine (33%) |  | *N*-Methyl-dopamine (19%) |
|  | (*R*)-Octopamine (9%) |  | (*R*)-*N*-Methyl-octopamine (13%) |
|  | (*R*)-Norphenephrine (5%) |  | (*R*)-*N*-Methyl-norphenephrine (12%) |
|  | (*R*)-2-Amino-1-phenyl-ethanol (14%) |  | (*R*)-2-Methylamino-1-phenyl-ethanol (25%) |
| Reference Compounds | | | |
|  | Uk-14,304 | | |
|  | *p*-Aminoclonidine | | |
|  | RX821002 | | |

were incubated in 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, and 30 $\mu$M ascorbic acid, pH 7.4, with 12 concentrations of the test compounds covering 5.5 log units. Nonspecific binding was defined using 100 $\mu$M oxymetazoline. After 45 min at room temperature, incubations were terminated by rapid vacuum filtration through glass fiber filters. Filters were washed 3 times with 5 ml of ice-cold buffer (20 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4), dried, and counted for radioactivity in a scintillation counter. Analysis of the experiments was conducted by nonlinear least-square curve fitting with Prism software (GraphPad Software, San Diego, CA) with simultaneous analysis of three separate experiments. $IC_{50}$ values were converted to $K_i$ values by use of the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

**Functional [$^{35}$S]GTP$\gamma$S Binding Assay.** Agonist-induced stimulation of [$^{35}$S]GTP$\gamma$S binding to isolated membranes from Chinese hamster ovary cells expressing recombinant $\alpha_{2A}$-AR was measured essentially as described previously (McKenzie, 1992; Tian et al., 1994; Peltonen et al., 1998). The [$^{35}$S]GTP$\gamma$S binding assay was conducted using a Beckman Biomek 2000 Laboratory Automation Workstation (Beckman Instruments, Inc., Palo Alto, CA) and 96-well plates. Harvested cell membranes were thawed and resuspended in the reaction buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 1

mM dithiothreitol, 20 mM NaCl, and 1 $\mu$M GDP; pH 7.4 at 25°C). The reaction was started by adding an aliquot of membrane suspension (5 $\mu$g of membrane protein per well) to microwells containing reaction buffer and 0.08 to 0.15 nM [$^{35}$S]GTP$\gamma$S with agonist in a total volume of 250 $\mu$l. The microwell plates were incubated for 25 min at room temperature. The incubation was terminated by rapid filtration through glass fiber filters using a Tomtec Harvester 96 Mach II (Tomtec, Inc., Hamden, CT). The filters were washed with 3 × 4 ml of cold wash buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, and 1 mM EDTA; pH 7.4 at 4°C). The bound radioactivity was determined in a 1205 Betaplate liquid scintillation counter (Wallac Oy, Turku, Finland).

**$\alpha_{2A}$-AR Model and Ligand Models.** The previously reported model structure of $\alpha_{2A}$-AR was used in this study (Salminen et al., 1999). The model is based on an $\alpha$-carbon template derived from the low-resolution electron microscopy structure of frog rhodopsin and the alignment of a large number of GPCRs (Baldwin et al., 1997). The ligand structures (Table 1) were built with the program Hyperchem version 5.01 (Hypercube, Inc., Gainesville, FL) and optimized using a short 200 ps simulated annealing procedure described elsewhere (Salminen et al., 1999). After simulated annealing, the ligands were energy minimized in vacuo using the MM+ (extended MM2) force field.

**Automated Docking.** Norepinephrine and epinephrine and their analogs, a total of 12 different ligands, were automatically docked to the ligand binding site of the $\alpha_{2A}$-AR model. The atomic partial charges, required for the docking simulations, were assigned to atoms of both the $\alpha_{2A}$-AR model and the ligand set according to the Gasteiger method (Gasteiger and Marsili, 1980) implemented in Quanta 97 (Molecular Simulations, Inc., San Diego, CA). The computer program Autodock version 2.4 (Morris et al., 1996) was used to dock the flexible small molecule ligands in the rigid $\alpha_{2A}$-AR receptor model. Conformational searches were limited to a 25 Å$^3$ volume containing the $\alpha_{2A}$-AR ligand binding site and nearby residues. To find low-energy conformations of ligands in the receptor binding site, Autodock uses Monte Carlo simulated annealing combined with a rapid, atomic resolution, grid-based method of energy evaluation using the AMBER forcefield and a distance-dependent dielectric constant to account for the solvent effects.

**Docking Parameters.** The following scheme was used to seek low-energy ligand conformations: 1) 500 to 800 separate docking simulations were performed for each ligand; 2) for each simulation, there were 100 constant temperature cycles with 8000 steps accepted or rejected; 3) the initial simulation temperature (RT = 300 cal/mol, where R = gas constant and T = absolute temperature) was reduced by a factor of 0.97 in each cycle; and 4) flexibility of both the ligand and the orientation of the ligand in the binding site was introduced by allowing torsional rotation and molecule translation steps for the ligands of 15° and 0.2 Å, respectively, reduced by a factor of 0.97 in each cycle. In this way, over $10^7$ docked ligand-receptor conformations were evaluated for each ligand. Next, the docked structures were clustered into similar groups that differ by less than 1 Å root-mean-square deviation from each other at the binding site.

**GRID Calculations.** The computer program GRID version 16 (Goodford, 1985) was used to map essential interactions in the binding site of the $\alpha_{2A}$-AR model. GRID calculates energies of interaction between a chemical probe and the receptor. The probes used in this study mimic charged and neutral amino groups, (phenolic) hydroxyl groups, methyl groups, aromatic carbons, and hydrophobic groups. Probes were placed at positions throughout a 30 Å × 30 Å × 30 Å cube (3 points/Å, 27 points/Å$^3$) centered at the $\alpha_{2A}$-AR ligand binding site, and the interaction energies were calculated at each point. The flexibility of amino acid side chains of the $\alpha_{2A}$-AR model was considered in the evaluation of the interaction energy. The GRID maps were visualized using the program CERIUS 2 (Molecular Simulations, Inc.) and gOpenMol (Bergman et al., 1997).

**Strategy for Building Receptor-Ligand Complexes.** Initially, Autodock was used to dock epinephrine, norepinephrine, and their close analogs (Table 1) to our model structure (Salminen et al., 1999) of $\alpha_{2A}$-AR. In general, the program will lead to the identification of highly populated clusters corresponding to similar low-energy ligand-receptor complexes. Because Autodock only considers the enthalpic binding energy in its search for favorable orientations of torsionally flexible ligands, the intermolecular interaction energy function is not necessarily accurate in predicting the true binding energy. Thus, the cluster with the absolute minimum energy does not necessarily represent the best or global energy minimum of the ligand in the receptor binding-site, especially if the cluster is sparsely populated. Nonetheless, the method has been proven accurate enough to identify the possible conformations of ligand molecules at receptor binding sites (Minke et al., 1999; Rao and Olson, 1999; Salminen et al., 1999). Indeed, a highly populated cluster of similar conformations whose members are the result of many independent docking simulations suggests that the cluster is located at an energy minimum that is easily accessible. Moreover, a cluster with a higher population is more likely to represent the natural conformations of the ligand, even if a less populated cluster having a slightly lower energy is present.

Therefore, from 500 (norepinephrine and analogs with fewer degrees of freedom than epinephrine and analogs) to 800 (epinephrine and analogs) separate docking simulations were made for each ligand, and the cluster with the highest population and the lowest interaction energy was chosen as the representative conformation. According to our own experience, the most populated clusters also correlate best with other independent results, including the GRID maps and the ligand binding results. Thus, in creating the representative binding modes for each of the ligands, we: 1) selected the optimal docked conformations to the receptor (i.e., binding modes) found after cluster analysis in Autodock; 2) visualized the binding modes on a graphics station superimposed with the GRID maps, choosing the binding mode that correlated best with these maps; 3) then used the predicted binding mode to explain the ligand binding and activity data; and 4) considered how well our model complexes correlated with the existing $\alpha_{2A}$-AR ligand binding and activation data.

## Results

A total of 12 phenethylamines (Table 1), (*R*)-norepinephrine and five analogs (Fig. 1, A and B) and (*R*)-epinephrine and five analogs (Fig. 1, C and D), were docked to the structural model (Salminen et al., 1999) of $\alpha_{2A}$-AR (as indicated in Table 1, some *S*-isomers were not docked to the receptor model). $K_i$ values for competition with the antagonist radioligand [$^3$H]RX821002 to probe the lower affinity binding state and with the agonist radioligand [$^3$H]UK-14,304 to probe the higher affinity binding state and receptor activation results ($E_{max}$ and $EC_{50}$ values for [$^{35}$S]GTP$\gamma$S binding in isolated membranes) were determined experimentally for dopamine, (*R*)-norepinephrine, (*S*)-norepinephrine, (*R*)-epinephrine, (*R*)-norphenephrine, (*S*)-norphenephrine, (*R*)-octopamine, (*S*)-octopamine, and (*R*)-2-amino-1-phenyl-ethanol. Two additional agonist ligands, UK-14,304 and *p*-aminoclonidine, which are not phenethylamines but aminoimidazolines, were included for purposes of comparison. The results of these experiments are summarized in Table 2. The competition binding results obtained with the antagonist radioligand [$^3$H]RX821002 were calculated using both one-site and two-site models. Two-site models were statistically significantly superior ($p < 0.05$) to one-site models for (*R*)- and (*S*)-norepinephrine, (*R*)-epinephrine, UK-14,304, and *p*-aminoclonidine. These two-site competition binding results are shown in Table 3.

The phenethylamine analogs differ from each other by either missing one or more hydroxyl groups ($\beta$-OH, and catecholic *meta*- and *para*-OH groups) and/or in their chirality about the $\beta$-carbon atom. The docked epinephrine analogs have a methyl group attached to the amine group, which is absent in norepinephrine and its analogs. The ligand set can be divided into six chemical domains whose interactions with $\alpha_{2A}$-AR can be classified: the positively charged amine group, the hydrophobic *N*-methyl group in epinephrine, and the epinephrine analogs, the $\beta$-OH, the aromatic ring, and the

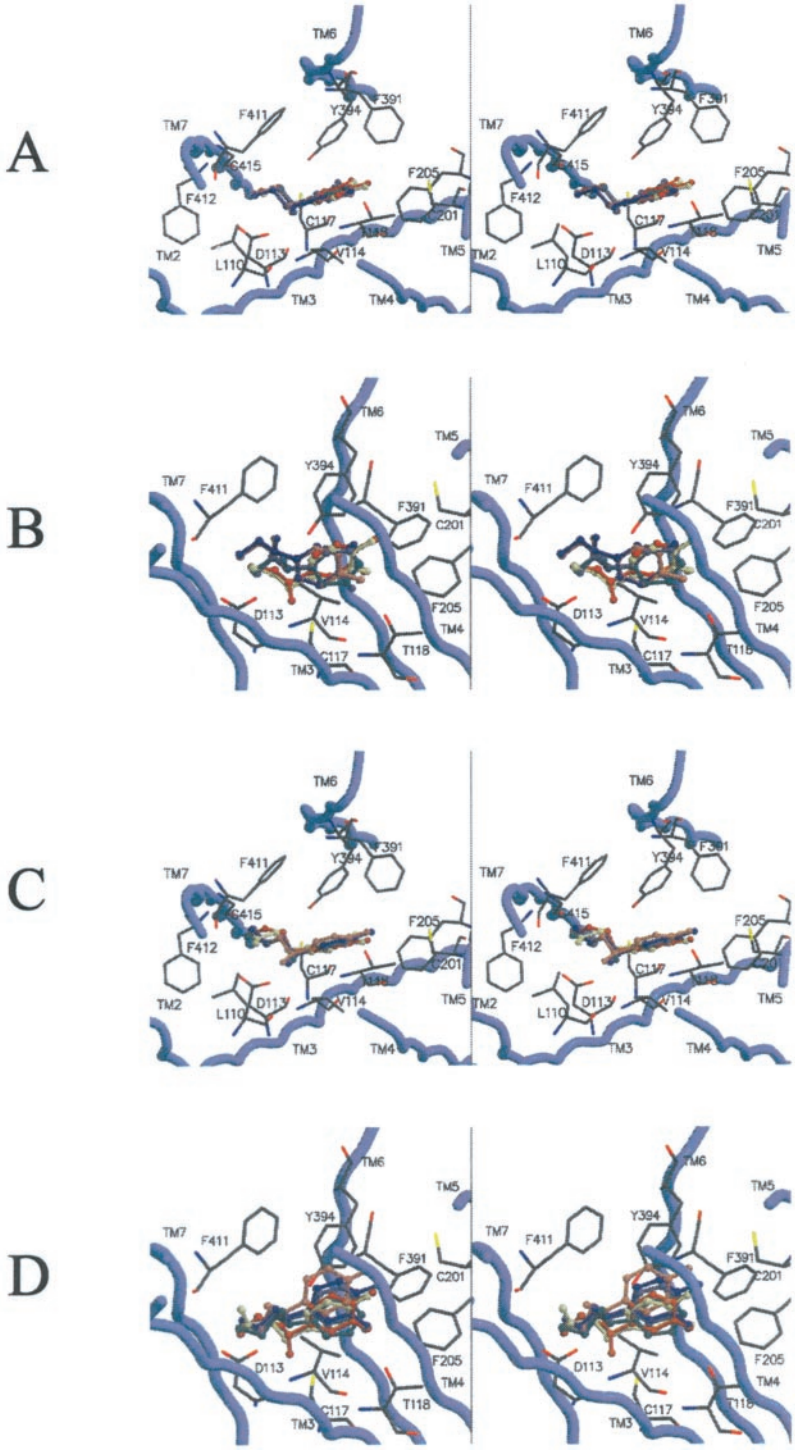



**Fig. 1.** Norepinephrine, epinephrine, and their analogs docked to the ligand binding site of the $\alpha_{2A}$-AR model structure. A and B, norepinephrine and analogs. C and D, epinephrine and analogs. In A and C, the binding site is viewed (in stereo) from the extracellular face of the receptor. In B and D, the view is perpendicular to the extracellular face and in the plane of the lipid bilayer. The $\alpha$-carbon trace of the TM helices is colored blue, and selected side chains are depicted and labeled with the single letter amino acid code (D, aspartate; F, phenylalanine; Y, tyrosine; C, cysteine; V, valine; and L, leucine). Color codes for ligands: dark blue, (*R*)-norepinephrine (A and B), (*R*)-epinephrine (C and D), *N*-methyl-dopamine (C and D); orange, (*R*)-octopamine (A and B), (*R*)-*N*-methyl-octopamine (C and D); violet, (*R*)-norphenephrine, (*R*)-*N*-methyl-norphenephrine; green, (*R*)-2-amino-1-phenyl-ethanol (A and B), (*R*)-2-methylamino-1-phenyl-ethanol (C and D).

*para*- and *meta*-catecholic OH groups. By dividing the ligands into six individual domains, we sought to understand the effect that each of these six chemical groups has on binding affinity and activation of $\alpha_{2A}$-AR.

Over $10^7$ ligand conformations were studied to produce each ligand-receptor complex model. To obtain the representative receptor-ligand complexes, we selected optimal docked poses found after cluster analysis in Autodock and visualization of the binding modes on a graphics workstation together with the GRID ligand affinity maps of the receptor binding site. The predicted binding modes of this set of ligands based on docking simulations, when taken in combination with experimental results on binding and receptor activation, makes it possible to assign functional roles to the chemical groups of the ligands, as well as to the receptor itself. Figure 2 shows the overall location of the ligand binding site on the model structure.

We have thus used a series of very similar ligand molecules that are sequentially missing different functional groups. Because we have an accurate structural model for the mode of binding between each ligand and the receptor, we can then correlate the observed structural differences of the ligands and ligand-receptor interactions with the similarities and differences in the experimental data. This then permits us to discriminate between those interactions that are important for binding affinity and those interactions that help to stabilize the activated form of the receptor.

**The Positively Charged Amine Group.** With norepinephrine and its analogs, the most important interaction is formed between the positively charged amine group (Fig. 3) of the ligands and the negatively charged side chain carboxyl group of Asp113 ($D_{3.32}$) in TM3 of the receptor (Wang et al., 1991; Kobilka, 1995). In general, the interactions between Asp113 ($D_{3.32}$) and the amine group in the ligands should be strong and anchor the amine group close to that residue, as observed in the docking simulations (Fig. 1). The GRID calculations also indicated a large volume near Asp113 ($D_{3.32}$) where charged and neutral amine groups could be placed with favorable interaction energy. The electrostatic surface potential shown in Fig. 2, B and C, clearly shows the region of negative charge about Asp113 ($D_{3.32}$) in the receptor's ligand binding site.

**The *N*-Methyl Group in Epinephrine and Analogs.** The *N*-methyl group (Fig. 3) in epinephrine and its analogs packs against Phe411 and Phe412 ($F_{7.38}$ and $F_{7.39}$) in TM7 in the docking simulations (Fig. 1, C and D). GRID maps also show that those parts of TM7 exposed to the binding cavity are favorable for hydrophobic, aromatic and methyl contacts. The presence of the *N*-methyl group in epinephrine and analogs would be predicted to improve the binding affinity by making an additional hydrophobic contact with Phe411 ($F_{7.38}$) and Phe412 ($F_{7.39}$) in TM7. Indeed, the affinity of (*R*)-epinephrine is 4 times higher than the affinity of (*R*)-norepinephrine in [$^3$H]RX821002 binding studies and 3 times

TABLE 2

Competition binding and functional assay results

The binding ($K_i$) and activation ($EC_{50}$) values are presented for molecules that were commercially available, either as pure enantiomers or as racemic mixtures that were subsequently resolved into their separate pure enantiomers. In the case of the epinephrines, only (*R*)-epinephrine was available from a commercial source. Two separate binding assays were used with competition against [$^3$H]RX821002 and [$^3$H]UK-14,304: the apparent $K_i$ is the inhibition constant for a one-site model. In the assays with [$^3$H]RX821002, two-site fits were statistically significantly superior to one-site fits only for (*R*)- and (*S*)-norepinephrine, (*R*)-epinephrine, UK-14,304 and *p*-aminoclonidine (Table 3). The functional assay is based on measurement of agonist-induced binding of [$^{35}$S]GTPγS to $\alpha_{2A}$-AR-coupled G-proteins in isolated membranes. $EC_{50}$ values of agonists are reported. $K_i$ and $EC_{50}$ ratios are the values reported for a ligand divided by that seen for (*R*)-norepinephrine. Values in parentheses are $E_{max}$ relative to (*R*)-norepinephrine. All experiments are the means ± S.E. from 3–4 separate experiments.

| Agonists | Ligand Binding Competition Assays | | | | Functional Assay | |
|---|---|---|---|---|---|---|
| | [$^3$H]RX821002 | | [$^3$H]UK 14,304 | | [$^{35}$S]GTPγS | |
| | App. $K_i$ | $K_i$ Ratio | App. $K_i$ | $K_i$ Ratio | $EC_{50}$ | $EC_{50}$ Ratio |
| | *nM* | | *nM* | | *nM* | |
| (*R*)-Epinephrine | 361 ± 38 | 0.23 | 1.4 ± 0.2 | 0.3 | 179 (137%) | 0.85 |
| (*R*)-Norepinephrine | 1,650 ± 330 | 1 | 4.6 ± 1.0 | 1 | 211 (100%) | 1 |
| (*S*)-Norepinephrine | 7,130 ± 79 | 4.3 | 105 ± 23 | 23 | 4,000 (89%) | 19 |
| Dopamine | 4,780 ± 722 | 2.9 | 34 ± 6 | 7 | 19,530 (94%) | 93 |
| (*R*)-Octopamine | 11,300 ± 834 | 6.8 | 432 ± 55 | 94 | 23,300 (48%) | 110 |
| (*S*)-Octopamine | 15,023 ± 1,042 | 9.1 | 3,622 ± 500 | 787 | 495,000 (30%) | 2,346 |
| (*R*)-Norphenephrine | 8,620 ± 400 | 5.2 | 130 ± 35 | 28 | 2,990 (29%) | 14 |
| (*S*)-Norphenephrine | 11,050 ± 405 | 6.7 | 2,716 ± 659 | 590 | N.D. (inactive) | N.D. |
| (*R*)-2-Amino-1-phenyl-ethanol | 2,722 ± 91 | 1.6 | 675 ± 99 | 147 | N.D. (<7%) | N.D. |
| UK14,304 | 13.0 ± 0.57 | 0.008 | 0.33 ± 0.09 | 0.07 | 8.2 (131%) | 0.39 |
| *p*-Aminoclonidine | 20.7 ± 0.66 | 0.013 | 0.9 ± 0.1 | 0.2 | 29.6 (45%) | 0.14 |

App., apparent. N.D., not determinable (inactive).

TABLE 3

Competition binding assay results using the antagonist radioligand and assuming two populations of binding sites

Two-site models were statistically significantly superior ($p < 0.05$), compared with one-site fits for the agonists listed in this table but not for the other agonists included in Table 2.

| Agonists | $K_i$ | $K_{iH}$ | $K_{iL}$ | % H of All Sites |
|---|---|---|---|---|
| | *nM* | *nM* | *nM* | |
| (*R*)-Epinephrine | 361 ± 38 | 27.5 ± 4.6 | 659 ± 125 | 20 ± 9 |
| (*R*)-Norepinephrine | 1,650 ± 330 | 0.44 ± 0.06 | 4,400 ± 420 | 30 ± 5 |
| (*S*)-Norepinephrine | 7,130 ± 80 | 1,000 ± 430 | 17,200 ± 500 | 31 ± 2 |
| UK-14,304 | 13.0 ± 0.57 | 0.68 ± 0.02 | 36.9 ± 1.00 | 41 ± 2 |
| *p*-Aminoclonidine | 20.7 ± 0.66 | 5.5 ± 0.23 | 118 ± 32 | 55 ± 6 |

H, high-affinity agonist binding sites; L, low-affinity agonist binding sites.

higher in the [$^3$H]UK-14,304 assay (Table 2). The rank order of affinity of (*R*)-epinephrine and (*R*)-norepinephrine is, however, the opposite for the high-affinity binding site population as revealed in a two-site analysis of the [$^3$H]RX821002 competition results (Table 3). Furthermore, the $EC_{50}$ values measured for stimulation of [$^{35}$S]GTP$\gamma$S binding are quite similar for (*R*)-epinephrine and (*R*)-norepinephrine (Table 2). Thus, the *N*-methyl group affects the binding affinity to the low-affinity receptor conformation, but is not directly coupled to the activation process.

**The β-OH Group.** With both (*R*)-epinephrine and (*R*)-norepinephrine, the β-OH group (Fig. 3) is positioned in a way that it can form a hydrogen bond with one side chain oxygen of Asp113 ($D_{3.32}$) (Fig. 1). Consistent with this view, the GRID maps calculated for $\alpha_{2A}$-AR indicate a favorable interaction for a hydroxyl group within a volume above (toward the extracellular surface) and at the level of the plane of the side chain oxygens of Asp113 ($D_{3.32}$). The interactions between $\alpha_{2A}$-AR and the β-OH are unlikely to be any further than 4 to 5 Å away from Asp113 ($D_{3.32}$) because of the restrictions imposed by the ligand geometry. If the amine group in (*R*)-norepinephrine interacts with Asp113 ($D_{3.32}$), and the catecholic end of the ligand is oriented toward residues in TM5, it is also not possible for the β-OH to interact with Ser90 ($S_{2.61}$) in TM2. The β-OH group would thus not point toward TM2, as suggested by previous mutagenesis studies (Li et al., 1995), but would be located on the opposite side of the ligand, pointing toward Asp113 ($D_{3.32}$) in TM3.

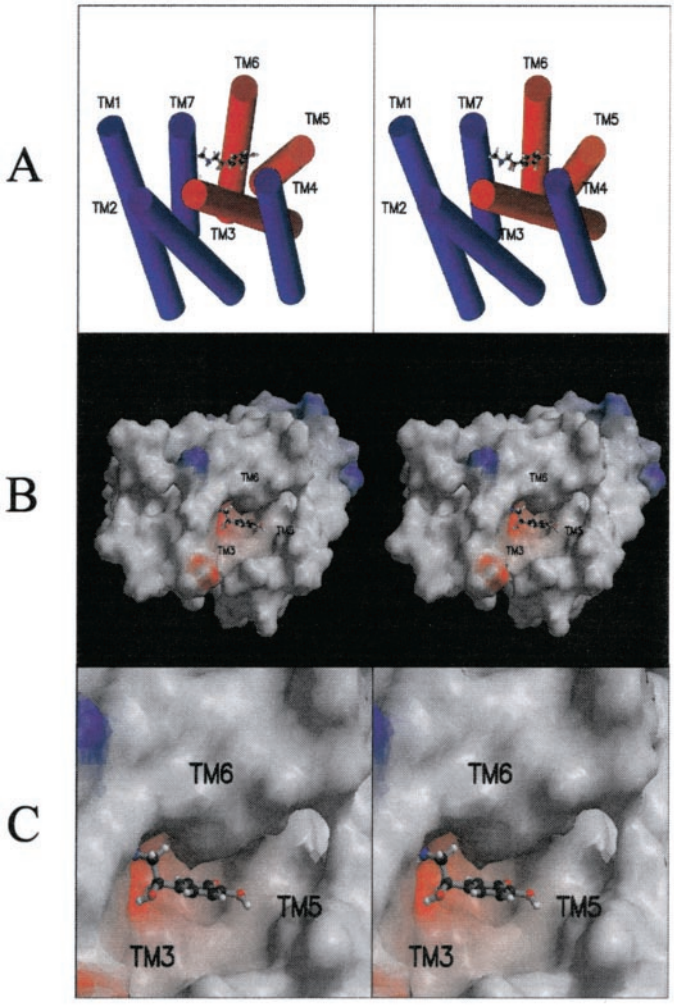



**Fig. 2.** Stereoview of the general model for (*R*)-epinephrine binding to the $\alpha_{2A}$-AR model. A, (*R*)-epinephrine docked to $\alpha_{2A}$-AR showing the relative disposition of the seven transmembrane helices (cylinders). Interactions with the ligand are mainly provided by amino acids from TM3, TM5, and TM6 (red). B, electrostatic surface of the $\alpha_{2A}$-AR model without the extracellular loops viewed from the extracellular surface; regions of negative charge are shown in red, positive charge in blue, and neutral regions are white. Note that the positively charged *N*-methyl amine group is oriented toward the negatively charged portion of the distinctive binding cavity where Asp113 ($D_{3.32}$) is located. C, close-up view of the electrostatic surface of the binding cavity. Epinephrine is shown docked in the cavity formed by TM3, TM5, and TM6. Rotation of TM5 coupled with movement toward the ligand would substantially increase the number of interactions with the ligand and could be the mechanism by which the receptor conformation is switched to the G-protein activating state. The figure was prepared using GRASP (Nicholls et al., 1991), Molscript (Kraulis, 1991), and Raster 3D (Merrit and Bacon, 1997).

In the docking simulations, the β-OH group in each of the *R*-isomers forms a hydrogen bond to Asp113 ($D_{3.32}$) (Fig. 2), whereas in the *S*-enantiomers of epinephrine and norepinephrine and their analogs, the favorable interactions with Asp113 ($D_{3.32}$) cannot take place. Consistent with this, the $K_i$ values of the *S*-isomers of norepinephrine and its analogs, derived from competitive radioligand binding assays, are larger than those of the *R*-enantiomers. The affinity of dopamine (no β-OH group) is also clearly lower than that of (*R*)-norepinephrine (Table 2). The $K_i$ values of (*S*)-norphenephrine and (*S*)-octopamine were only slightly larger than those of the *R*-isomers (1.3-fold difference in competition assays with [$^3$H]RX821002), which is a smaller difference than that seen between (*S*)-norepinephrine and (*R*)-norepinephrine (4-fold). In the [$^3$H]UK-14,304 competition binding assays, however, the affinity differences between the (*S*)- and *R*-isomers were much greater (8- to 22-fold). An even greater affinity difference was seen when the [$^3$H]RX821002 binding results were fit to a two-site competition binding model. Two-site fits were statistically significantly superior to one-site fits only for (*R*)- and (*S*)-norepinephrine, (*R*)-epinephrine, UK-14,304, and *p*-aminoclonidine (Table 3). For both (*R*)- and (*S*)-norepinephrine, the proportion of high-affinity sites was approximately 30%, but whereas the affinity difference between the stereoisomers was approximately 4-fold for the low-affinity receptor population (4,400 ± 400 vs. 17,200 ± 500 nM), the corresponding difference for the high-affinity receptor population was much greater, 2000-fold (0.5 ± 0.1 vs. 1000 ± 400 nM). Thus, the *S*-isomers do not seem to be as effective in stabilizing the high-affinity form of the receptor as the corresponding *R*-isomers. The large affinity differences observed in the [$^3$H]UK-14,304 competition assays between the *S*- and *R*-isomers may reflect the unfavorable orientation of the β-OH group in the *S*-isomers with respect to the ligand binding site of the active form of $\alpha_{2A}$-AR. This notion is supported by the results obtained for dopamine in the [$^3$H]UK-14,304 assay, because its binding affinity was intermediate to those of (*S*)-norepinephrine and (*R*)-norepinephrine.

Absence of the β-OH group, as in dopamine, removes the possibility for a hydrogen bond to contact Asp113 ($D_{3.32}$), found for the *R*-isomers of norepinephrine and epinephrine and their analogs. In the docking simulations, the β-carbon of dopamine is placed close to the bottom of the binding cavity and in the same position as the β-OH is placed in (*S*)-norepinephrine (Fig. 1, A and B). In our $\alpha_{2A}$-AR model of the inactive form of the receptor, there is free space to place a

small downward-pointing group between Asp113 ($D_{3.32}$) and Cys117 ($C_{3.36}$) in TM3. On the other hand, dopamine, without an interaction between a $\beta$-OH and Asp113 ($D_{3.32}$), is conformationally more flexible than (*S*)-norepinephrine, which may affect its ability to activate the receptor. In docking epinephrine analogs lacking the $\beta$-OH group (Fig. 1, C and D), the $\beta$-OH contact with Asp113 ($D_{3.32}$) in TM3 is removed. The *N*-methyl group bends slightly upward, the ligand packs more toward TM7, and a hydrogen bond between the amine group and Asp113 ($D_{3.32}$) side chain oxygens is optimized.

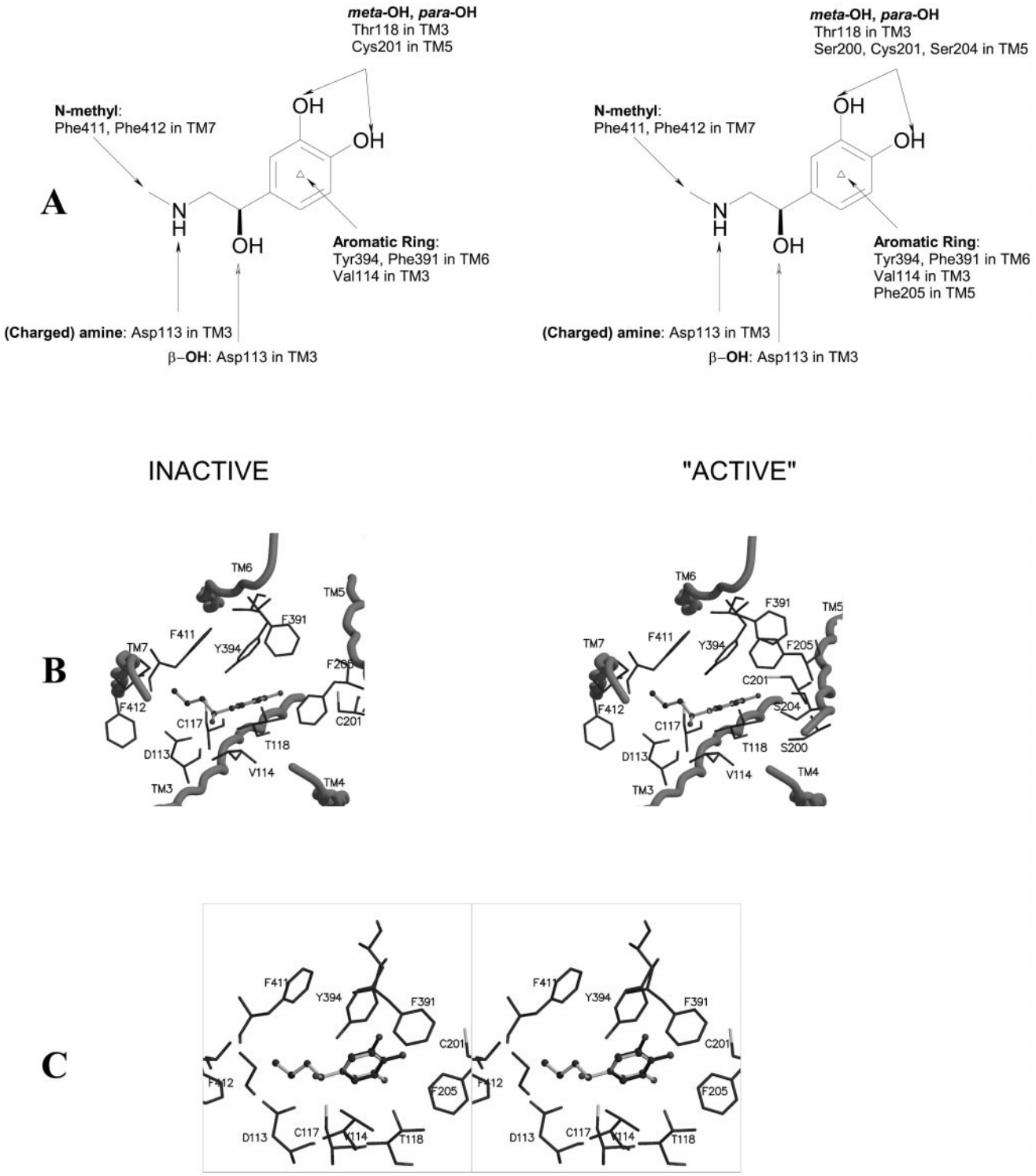



**Fig. 3.** A, key interactions of (*R*)-epinephrine with the amino acid side chains lining the $\alpha_{2A}$-AR binding site in the inactive and active forms of the receptor. B, the important amino acids that contribute to the binding of the phenethylamine ligands and possibly to the activation of the $\alpha_{2A}$-AR are mainly from TM3, TM5, and TM6. For epinephrine, the *N*-methyl group, not present in norepinephrine, has hydrophobic contacts with residues from TM7. C, the two possible orientations of the catechol ring found in this study (light color: alternative docked conformation; dark color: conformation in consensus with current literature). Experimental evidence shows (Rudling et al., 1999) that the *meta*-OH probably points "upward", toward the extracellular surface (rotamer with dark color) and thus interacts with Ser200 ($S_{5.42}$) in the active receptor conformation.

Taken together, the differences in binding affinities between the *S*- and *R*-enantiomers (Table 2; Ruffolo, 1991), and the mechanistic explanation provided by our docking simulations strongly suggest that the β-OH provides an important contribution toward the binding affinity of β-OH-phenethylamines at $\alpha_{2A}$-AR and probably also to other adrenergic receptors. The results of our analysis (Fig. 1) suggest that this contribution of the β-OH to binding affinity of the *R*-isomers occurs through coordination with the side chain of Asp113 ($D_{3.32}$).

**The Aromatic Ring.** Optimal placement of the aromatic ring, present in all of the investigated ligands, is with the ring plane packed against TM6, with additional interactions with TM3 and TM5 (Figs. 1 and 3). GRID calculations predict favorable aromatic group interactions between TM5 and TM6, induced by the aromatic side chains of Phe391 ($F_{6.52}$) and Tyr394 ($Y_{6.55}$) in TM6, both of which are conserved in all $\alpha_2$-AR subtypes, and partially by the side chains of Phe205 ($F_{5.47}$) and Cys201 ($C_{5.43}$) in TM5 (Fig. 1). On the opposite face of the docked conformation of the aromatic ring lies another conserved residue, Val114 ($V_{3.33}$) in TM3. Aromatic rings, for example the adenine ring, are often seen sandwiched between valine or another hydrophobic residue on one face of the ring and aromatic residues on the opposite face of the ring (Denessiouk and Johnson, 2000). The catecholic OH groups (see below) are very likely to be in contact with Thr118 ($T_{3.37}$) in TM3 and Cys201 ($C_{5.43}$) in TM5, and are oriented toward TM5 where Ser200 ($S_{5.42}$) and Ser204 ($S_{5.46}$) are located; interactions with these groups would affect the orientation of the aromatic ring when docked.

**The *Para*- and *Meta*-Catecholic OH Groups.** In the docking simulations, the two catecholic OH groups (Fig. 3) contact residue side chains within TM3 and TM5, and thus influence the orientation of the aromatic ring. In the docking simulations, coordination can take place between catecholic hydroxyls and the side chains of Thr118 ($T_{3.37}$) in TM3; and Ser200 ($S_{5.42}$), Ser204 ($S_{5.46}$), and Cys201 ($C_{5.43}$) in TM5. Of the latter three residues, we believe that only Cys201 is exposed to the binding cavity in the low-affinity or inactive form of the receptor (Fig. 1). In the activated or higher affinity form of the receptor, we suggest that rotation of TM5 occurs and exposes both Ser200 ($S_{5.42}$) and Ser204 ($S_{5.46}$) to the ligand-binding cavity (Marjamäki et al., 1999; Salminen et al., 1999). Because of this rotation, good contacts are formed between the *meta*-OH and *para*-OH and Ser200 ($S_{5.42}$) and Ser204 ($S_{5.46}$) (Fig. 3). Due to the inaccuracies in the docking forcefield, the distinction between the two possible orientations of the catecholic ring in $\alpha_{2A}$-AR is difficult. The calculated energy difference between two possible ligand conformations, which differ in that the catechol ring is flipped by 180°, is too small to distinguish between the two conformations. Because mutagenesis studies (Wang et al., 1991; Rudling et al., 1999) have indicated that the *meta*-OH is likely to interact with Ser200 ($S_{5.42}$) and the *para*-hydroxyl with Ser204 ($S_{5.46}$) in $\alpha_{2A}$-AR, and because this orientation also is in agreement with results obtained with other adrenergic receptors (Strader et al., 1989, Hwa and Perez, 1996), the modeled orientation of the catechol ring was adjusted to correspond to these results (Fig. 3).

The catecholic hydroxyls do not seem to be critically important for the binding affinity of the phenethylamines to the low-affinity state of $\alpha_{2A}$-AR. Differences between the $K_i$ values of (*R*)-norepinephrine and (*R*)-norphenephrine, (*R*)-octopamine, and (*R*)-amino-1-phenyl-ethanol were only 5-, 7-, and 2-fold, respectively, in the antagonist competition assays with [$^3$H]RX821002 (Table 2). (*R*)-Amino-1-phenyl-ethanol was able to bind quite well to the receptor, although it has no catecholic OH groups, but the complex was inactive in the functional assay. The loss of affinity at the high-affinity receptor conformation after removal of one or both catecholic hydroxyl groups was more dramatic when two-site fits were attempted for the [$^3$H]RX821002 competition binding results (compared with the [$^3$H]UK-14,304 competition results): the [$^3$H]RX821002 competition curves were steep and monophasic, and no high-affinity component could be modeled for the binding results of (*R*)- or (*S*)-octopamine, (*R*)- or (*S*)-norphenephrine, or (*R*)-2-amino-1-phenyl-ethanol (not shown). The other investigated $\alpha_{2A}$-AR ligands, *p*-aminoclonidine and UK-14,304, are 2–3 Å longer than the phenethylamines and can more easily form good contacts with residues from both TM3 and TM5 than can the phenethylamines. This may partly explain the high binding affinity and efficacy of these agonists.

Despite the marginal contribution of the catecholic OH groups toward the binding affinity for the low-affinity ligand-binding form of the receptor as probed by the [$^3$H]RX821002 competition ligand assays, the catecholic hydroxyls seem to be very important for receptor activation (Table 2): (*R*)-amino-1-phenyl-ethanol (no catechol-OH) is incapable of activating $\alpha_{2A}$-AR as evidenced by the [$^{35}$S]GTPγS binding results. (*R*)-Norphenephrine (missing the *para*-OH group) is a partial agonist with 14-fold lower potency and only about 30% relative efficacy in activating $\alpha_{2A}$-AR, compared with (*R*)-norepinephrine. Similarly, (*R*)-octopamine, missing the *meta*-OH group, has 110-fold lower potency and about 50% efficacy in activating $\alpha_{2A}$-AR compared with (*R*)-norepinephrine.

Dopamine, although having both catecholic OH groups, is missing the anchoring β-OH group. The β-OH group, present in either the (*R*)- or (*S*)-configuration in the other phenylethylamine ligands studied here, limits the conformational flexibility of the ligand through its interactions with the receptor. In dopamine, no such group exists and dopamine can rotate to provide a number of different conformations that would not affect the ligand's interactions with Asp113 ($D_{3.32}$) or interactions between the ligand's aromatic ring and hydrophobic amino acids lining the binding pocket. Consequently, dopamine has a higher binding affinity than (*S*)-norepinephrine in the competition binding assays (Table 2). On the other hand, the conformational freedom present in dopamine would clearly affect the positioning of the catecholic OH groups with respect to residue side chains in TM5. This is reflected in the 5-fold reduction in functional potency in comparison with (*S*)-norepinephrine, and even more dramatic loss of function compared with (*R*)-norepinephrine. This proposal is supported by the results of activation assays using a conformationally restricted dopamine analog, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphtalene: in [$^{35}$S]GTPγS assays, this analog ($EC_{50}$ = 309 nM) is 13-fold more potent in activating $\alpha_{2A}$-AR than is (*S*)-norepinephrine (S. Wurster, unpublished results). Dopamine also achieves full efficacy in comparison with (*R*)-norepinephrine in the functional [$^{35}$S]GTPγS binding assays, which is thus attributed to the presence of both catecholic OH groups.

## Discussion

**RX821002 and UK-14,304 Probe Different Activation States of the Receptor.** We have used two different ligand-binding assays in this study. [$^3$H]RX821002 is an antagonist ligand, which mainly probes the predominant inactive state of the receptor, whereas [$^3$H]UK-14,304 is an $\alpha_{2A}$-AR agonist, which probes the activated receptor. Both ligands are superficially similar, especially with regard to the imidazole end, which is likely to bind to Asp113 ($D_{3.32}$). At its opposite end, RX821002 has an unsubstituted phenyl ring, which can only participate in hydrophobic and aromatic interactions. These interactions are presumably not capable of stabilizing the activated form of the receptor. In contrast, UK-14,304 has two aromatic ring nitrogens that can participate in interactions that can help to promote or to maintain an activated conformation. This contrasts with *p*-aminoclonidine, a less potent and efficacious agonist than UK-14,304: *p*-aminoclonidine has a single amine group extending from the phenyl ring that can make one stabilizing set of polar interactions with the receptor, instead of the two possible with UK-14,304. *p*-Aminoclonidine is also shorter than UK-14,304. It is important to consider these aspects in the analysis of the data presented here.

**Ligand Binding Affinity.** The predicted location of the ligand binding site of $\alpha_{2A}$-AR and a set of representative phenethylamine binding modes are presented in Figs. 2 and 3. According to the Easson-Stedman hypothesis, an adrenoceptor should have three functional points in its binding site to accommodate the charged aliphatic nitrogen, the $\beta$-OH group, and the aromatic ring of phenethylamine ligands [see Ruffolo (1991) for a detailed discussion]. In our model (Fig. 2), these interactions are primarily contributed by residues in TM3, TM5, and TM6 (Fig. 3). In addition, we suggest that residues in TM7 should have a role in ligand binding in the case of epinephrine and analogs (Fig. 3).

The details of the attachment of phenethylamine ligands to their receptors (Fig. 3) are now suggested by us to include the following interactions for $\alpha_{2A}$-AR. First, the charged amine group would be optimally coordinated to one side chain oxygen of Asp113 ($D_{3.32}$) in TM3; the importance of this interaction has been well documented (Wang et al., 1991; Kobilka, 1995; Bikker et al., 1998; Gether and Kobilka, 1998). Additionally, $\alpha_{2A}$-AR binds (*R*)-epinephrine with a higher binding affinity in comparison to (*R*)-norepinephrine, and we attribute this to the additional contacts formed between the *N*-methyl group of epinephrine and the hydrophobic residues Phe411 ($F_{7.38}$) and Phe412 ($F_{7.39}$) in TM7. Second, with the *R*-isomers, the $\beta$-OH group would form a hydrogen bond with the other side chain oxygen of Asp113 ($D_{3.32}$). Unfortunately, this proposition cannot be directly tested with site-directed substitution mutagenesis, because the aspartate is required for receptor functionality. Receptor-ligand docking results suggest that *S*-isomerism at the $\beta$-carbon causes the $\beta$-OH group to be oriented in a direction opposite to that seen for the *R*-isomers, resulting in the loss of one Easson-Stedman contact point and reflected in poorer binding in comparison with the *R*-isomers (Table 1; Ruffolo, 1991). GRID calculations also suggest that weaker interactions occur for the *S*-isomers because the $\beta$-OH group would be placed within a volume below Asp113 ($D_{3.32}$), indicated to be slightly hydrophobic, probably due to the presence of the nearby side chains of Phe116 ($F_{3.35}$) and Cys117 ($C_{3.36}$). Cys117 ($C_{3.36}$) in TM3 is in close proximity to the amine end of the ligands and may contribute to the binding site environment, too, but its role was not clarified in the current study. Dopamine, which lacks the $\beta$-OH group, behaves in a manner similar to the *S*-isomers in that it binds poorly to $\alpha_{2A}$-AR and possibly due to conformational flexibility only activates $\alpha_{2A}$-AR with low potency (Table 2).

Third, the phenyl group of the phenethylamine ligands would pack ($\pi$-$\pi$ stacking interactions) with one ring face against conserved aromatic residues in TM6, Tyr394 ($Y_{6.55}$) and Phe391 ($F_{6.52}$), and possibly Phe205 ($F_{5.47}$) in TM5; and with Val114 ($V_{3.33}$) in TM3 packing against the other face of the ring (Fig. 3). The $\alpha_{2A}$-AR binding site is rich in aromatic residues: the side chains of Phe205 ($F_{5.47}$) in TM5, Phe391 ($F_{6.52}$) and Tyr394 ($Y_{6.55}$) in TM6, and Phe411 ($F_{7.38}$) and Phe412 ($F_{7.39}$) in TM7 are accessible to ligands in the binding cavity. In our model, a network of aromatic interactions could form if small adjustments took place in the orientations of the side chains of these residues. As indicated by our previous studies (Marjamäki et al., 1999; Salminen et al., 1999), Cys201 ($C_{5.43}$), Ser200 ($S_{5.42}$), and Ser204 ($S_{5.46}$) in TM5 of $\alpha_{2A}$-AR also have important roles both in orienting and binding ligands.

The deletion of one or both of the catecholic hydroxyl groups from (*R*)-norepinephrine results in 2- to 7-fold lower (competition assay using [$^3$H]RX821002, which mainly probes the predominant "low-affinity" inactive receptor conformation) and 28- to 146-fold lower (competition assay using [$^3$H]UK-14,304, thought to probe the "high-affinity" active conformation of the receptor) binding affinity. (*R*)-Amino-1-phenyl-ethanol has the aliphatic amine group, the $\beta$-OH group, and the phenyl ring in the correct orientation, and binds relatively well to $\alpha_{2A}$-AR; but, it lacks efficacy in activating the receptor because it has no catecholic OH groups (as reflected in [$^3$H]UK-14,304 competition assays and functional assays; Table 2). Thus, it seems that the catecholic OH groups have a more important role in receptor activation than in ligand binding. In the phenethylamines, the catecholic OH groups most probably interact with Cys201 ($C_{5.43}$) in TM5. The *para*-OH group may also coordinate to Thr118 ($T_{3.37}$) in TM3 if the catecholic ring is positioned as described previously. Furthermore, the catecholic hydroxyl groups would be able to form intimate contacts with Ser200 ($S_{5.42}$) and Ser204 ($S_{5.46}$) in TM5, if TM5 rotates clockwise (viewed from the extracellular surface) with respect to TM3 and TM6, exposing Ser200 ($S_{5.42}$) and Ser204 ($S_{5.46}$) to the ligand-binding cavity, thus enabling TM3 and TM5 to move closer toward each other (Figs. 3 and 4). This dramatic change in the structure of the receptor would certainly be reflected as structural alterations at the inner membrane surface, because intracellular loop 3, accepted to be intimately involved in G-protein activation (Jewell-Motz et al., 1998), is located between TM5 and TM6. This proposal is supported by the observed effects of alkylating agents of different sizes targeted to cysteine substitutions engineered along TM5 (Marjamäki et al., 1999), and by modeling and ligand binding studies involving these mutant receptors (Salminen et al., 1999). We also have supporting evidence for this notion from recent experiments involving the same phenylethylamine (*R*)- and *S*-enantiomers used in the present study, and the engineered TM5 serine-to-cysteine substitutions used in our

previous studies (Marjamäki et al., 1999; Salminen et al., 1999).

**Receptor Activation.** Gether and Kobilka (1998) have suggested a general model for the activation of rhodopsin-like GPCRs. We propose a similar but more detailed scheme for the activation of $\alpha_{2A}$-AR by (*R*)-phenethylamines (Figs. 3 and 4). We suggest that binding of a phenethylamine to $\alpha_{2A}$-AR is initiated by the formation of a hydrogen bond between the negatively charged carboxylate in Asp113 ($D_{3.32}$) in TM3 and the positively charged aliphatic amine group in the ligand. The *N*-methyl group of epinephrine increases the binding affinity through nonpolar interactions with hydrophobic residues in TM7. The interaction between the ligand and $\alpha_{2A}$-AR is enforced by the formation of another hydrogen bond between the $\beta$-OH of the ligand and the other oxygen in the Asp113 ($D_{3.32}$) side chain. The agonist is oriented within the binding site so that the aromatic ring is sandwiched between the aromatic interactions with Tyr394 ($Y_{6.55}$) and Phe391 ($F_{6.52}$) in TM6 and possibly Phe205 ($F_{5.47}$) in TM5, and hydrophobic interactions with Val114 ($V_{3.33}$) in TM3.

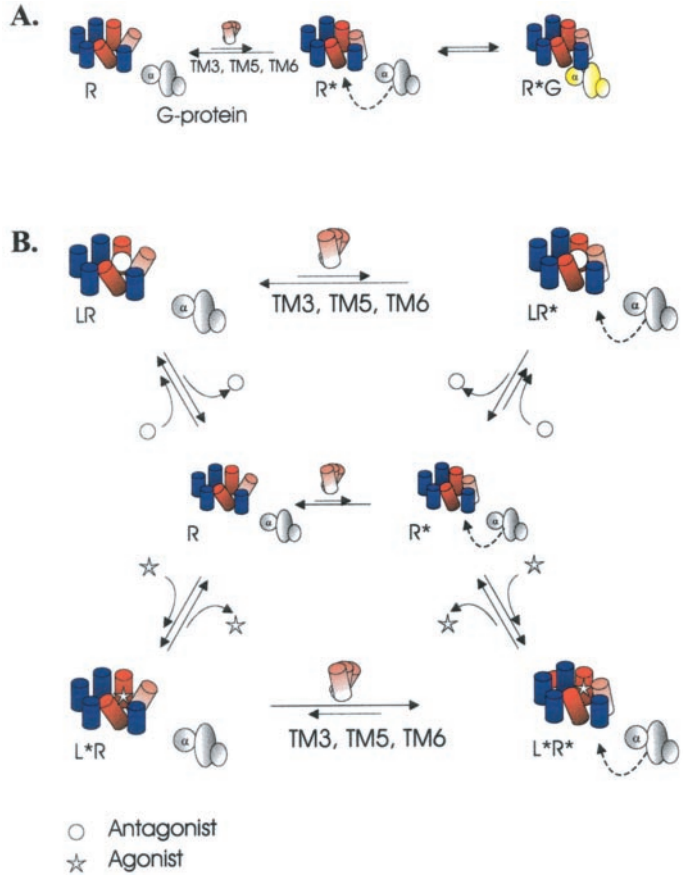



**Fig. 4.** Proposed mechanism for agonist-independent activation and agonist-induced $\alpha_{2A}$-AR activation by norepinephrine and epinephrine. A, agonist-independent activation of receptor (R) proceeds through a conformational change involving TM3, TM5, and TM6. This conformational change would be transmitted to the intracellular loops that can then bind and activate the G-protein complex ($\alpha\beta\gamma$). The energy barrier for this transition would probably be high such that the population of activated receptor (R*) capable of binding G-proteins (G) in the absence of ligand (L) would be low. This low concentration of activated receptor would, however, be able to bind tightly to agonists (stars) presented to them (B, right-hand side). In the case of agonist ligands, the equilibrium would favor the formation of the activated ligand-bound complex (L*R*), whereas antagonists (circles) with inverse agonist activity would shift the equilibrium in favor of the inactive receptor form (LR).

The orientation of the aromatic ring is locked into place by these nonpolar interactions and very probably through interactions between the catecholic OH groups and Thr118 ($T_{3.37}$) in TM3; and Ser200 ($S_{5.42}$), Cys201 ($C_{5.43}$), and Ser204 ($S_{5.46}$) in TM5.

In our model of the inactive receptor conformation, the length of the phenethylamine ligands is too short to reach easily both Asp113 ($D_{3.32}$) in TM3 and the polar residues in TM5 (Cys201 ($C_{5.43}$), Ser200 ($S_{5.42}$), and Ser204 ($S_{5.46}$)) without structural alterations. The length of the ligands does affect their ability to bind to $\alpha_{2A}$-AR (Salminen et al., 1999), because longer ligands such as UK-14,304 and *p*-aminoclonidine can more easily interact with both Asp113 ($D_{3.32}$) in TM3 and Ser200 ($S_{5.42}$), Cys201 ($C_{5.43}$), and Ser204 ($S_{5.46}$) in TM5. Aromatic nitrogens in UK-14,304 and the amino group in *p*-aminoclonidine are able to interact with Ser200 ($S_{5.42}$), Cys201 ($C_{5.43}$), and Ser204 ($S_{5.46}$) in TM5 in the active form of the receptor; with RX821002, polar interactions with TM5 probably cannot be formed. Thus, we propose that the final stage in phenethylamine ligand binding to the inactive receptor conformation is coupled to the transition of the $\alpha_{2A}$-AR inactive state to the G-protein activation state, which is then stabilized by phenethylamine ligands that have catecholic OH groups. The activation of $\alpha_{2A}$-AR can be envisioned as a rotation that exposes both TM5 serines to the ligand binding cavity and movement of TM5 toward the ligand, TM3, and TM6. This would substantially increase the number of interactions with the ligand and could be the mechanism by which structural alterations are transmitted to the third intracellular loop, and by which the equilibrium between inactive and active receptor conformations is shifted in favor of the G-protein activating state (see Figs. 3 and 4). In support of this model, our ligand binding results (Table 2) indicate that the catecholic OH groups are not so critical for binding, but are very important for receptor activation. Additionally, our previous studies strongly suggested that alkylating reagents of different sizes designed to specifically react with engineered cysteines at positions 200 and 204 recognized two different conformations of $\alpha_{2A}$-AR (Marjamäki et al., 1999) and that rotation of TM5 is likely to be involved (Salminen et al., 1999). An equilibrium model for receptor activation would suggest that a fraction of the total receptor population could, in the absence of ligands, spontaneously be in this activated conformation. A diagram describing the equilibrium between inactive and active receptor states in the presence and absence of agonist is presented in Fig. 4, along with the changes in the relative disposition of residues that are suggested to occur upon activation (Fig. 3). This model for receptor activation can also rationalize the effects of partial agonists of $\alpha_{2A}$-AR. Partial agonists may either stabilize an intermediate state, or their ability to favor the necessary conformational changes could be limited.

In summary, we have constructed a model that correlates well with the current published information on $\alpha_{2A}$-AR. The results we present offer a detailed view of the activation of $\alpha_{2A}$-AR by (*R*)-phenethylamines. This model is based both on extensive computer simulations and in vitro experiments probing both binding affinity and receptor activation using a set of structurally closely analogous $\alpha_{2A}$-AR ligands. The small differences within the ligand sets allow us to evaluate the role of each part of the ligand molecule in terms of the proposed interactions with key conserved amino acids lining

the binding cavity of the receptor itself. The model is consistent with and updates the Easson-Stedman hypothesis for catecholamine binding to the $\alpha_2$-type adrenoceptors (Ruffolo, 1991), and the activation follows a scheme as described in general for the rhodopsin-like GPCRs (Gether and Kobilka, 1998).

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