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Molecular mechanisms of ligand–receptor interactions in transmembrane domain V of the α_{2A}-adrenoceptor

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1 The structural determinants of catechol hydroxyl interactions with adrenergic receptors were examined using 12 α_2-adrenergic agonists and a panel of mutated human α_{2A}-adrenoceptors. The α_{2A}Ser201 mutant had a Cys→Ser201 (position 5.43) amino-acid substitution, and α_{2A}Ser201Cys200 and α_{2A}Ser201Cys204 had Ser→Cys200 (5.42) and Ser→Cys204 (5.46) substitutions, respectively, in addition to the Cys→Ser201 substitution.

2 Automated docking methods were used to predict the receptor interactions of the ligands. Radioligand-binding assays and functional [^{35}S]GTPγS-binding assays were performed using transfected Chinese hamster ovary cells to experimentally corroborate the predicted binding modes.

3 The hydroxyl groups of phenethylamines were found to have different effects on ligand affinity towards the activated and resting forms of the wild-type α_{2A}-adrenoceptor. Substitution of Ser200 or Ser204 with cysteine caused a deterioration in the capability of catecholamines to activate the α_{2A}-adrenoceptor. The findings indicate that (i) Cys201 plays a significant role in the binding of catecholamine ligands and UK14,304 (for the latter, by a hydrophobic interaction), but Cys201 is not essential for receptor activation; (ii) Ser200 interacts with the *meta*-hydroxyl group of phenethylamine ligands, affecting both catecholamine binding and receptor activation; while (iii) substituting Ser204 with a cysteine interferes both with the binding of catecholamine ligands and with receptor activation, due to an interaction between Ser204 and the *para*-hydroxyl group of the catecholic ring.
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Abbreviations: α_{2A}-AR, human α_{2A}-adrenergic receptor; CHO, Chinese hamster ovary; GPCR, G-protein-coupled receptor; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); TM, transmembrane helix; wt, wild type; UK14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine; RX821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline

Introduction

Three amino-acid residues in the fifth transmembrane domain (TM5) of adrenergic receptors (ARs) (positions 5.42, 5.43 and 5.46 according to the indexing system of Ballesteros & Weinstein (1995) have been shown to be important for agonist interactions involving catecholamine ligands. The α_{1B}- and α_{1D}-AR, and all the three β-AR subtypes as well as the α_{2B}-AR have serine residues at these three positions. The human α_{2A}- and α_{2C}-ARs have a cysteine, and the human α_{1A}-AR an alanine at position 5.43 (Figure 1). The α_{2A}-AR of rodents (previously called α_{2D}) has three serine residues at these three positions; this is reflected in some differences in the ligand-binding affinities between the rodent and human receptors (Blaxall *et al*., 1993). The OH and thiol groups of these conserved amino-acid residues are thought to be exposed in the ligand-binding crevices of the receptors, and to form hydrogen bonds with the *meta*- and *para*-hydroxyl (OH) groups of catecholamine ligands (Strader *et al*., 1989). Mutagenesis studies on β_2-AR have indicated that both Ser204 (position 5.43, corresponding to Cys201 of the human α_{2A}-AR) and Ser207 (position 5.46, corresponding to Ser204 of α_{2A}-AR) are important for binding catecholamine ligands (Kikkawa *et al*., 1997). Ser204 (5.43) of β_2-AR has been suggested to interact with the *meta*-OH group of catecholamine agonists, and Ser207 (5.46) with the *para*-OH group (Strader *et al*., 1989). A recent study indicated that the *meta*-OH group of catecholamines also interacts with Ser203 of β_2-AR (position 5.42, corresponding to Ser200 of α_{2A}-AR) (Sato *et al*., 1999). In α_{1A}-AR, the catecholic *meta*-OH group binds to Ser188 (position 5.42) and the *para*-OH group interacts with Ser192 (position 5.46) (Hwa *et al*., 1996; Wetzel *et al*., 1996).

A mutagenesis study on the human α_{2A}-AR indicated that only Ser204 (5.46) interacts with the *para*-OH group of the catecholamine phenyl ring, and that Ser200 (5.42) does not

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			42	43			46			
α_{1A}	187	F	**S**	**A**	L	G	**S**	F	Y	194
α_{1B}	206	F	**S**	**S**	L	G	**S**	F	Y	213
α_{1D}	257	F	**S**	**S**	V	C	**S**	F	Y	264
α_{2A}	199	S	**S**	**C**	I	G	**S**	F	F	206
α_{2B}	175	A	**S**	**S**	I	G	**S**	F	F	182
α_{2C}	213	S	**S**	**C**	I	G	**S**	F	F	220
β_1	227	A	**S**	**S**	V	V	**S**	F	Y	234
β_2	202	S	**S**	**S**	I	V	**S**	F	Y	209
β_3	207	L	**S**	**S**	S	V	**S**	F	Y	214

Figure 1 Alignment of amino-acid sequences from TM5 of the human AR subtypes.

directly participate in receptor–agonist interactions (Wang *et al*., 1991). However, in a more recent study with Ser→Ala200 and Ser→Ala204 mutants, it was suggested that Ser200 exerts an inhibitory influence on the ability of the catecholic *para*-OH group to interact with the receptor. Ser204 had a similar effect on the function of the *meta*-OH group of the ligand (Rudling *et al*., 1999). In addition, position 5.43 appears to be important for interaction with the *meta*-OH group of the catechol ring (Strader *et al*., 1989).

We have constructed models of the human α_{2A}-AR (Salminen *et al*., 1999; Nyrönen *et al*., 2001), whose functionality has been verified through experimental studies that include site-directed mutagenesis (Marjamäki *et al*., 1999; Salminen *et al*., 1999) and ligand-binding assays with modified catecholamine derivatives (Nyrönen *et al*., 2001). Our α_{2A}-AR model has now been updated using the recently determined structure of bovine rhodopsin (Palczewski *et al*., 2000). In the new model, most of the same residues are located within the binding cavity, as seen for the previous model that was based on the α-carbon template of frog rhodopsin (Baldwin *et al*., 1997; Nyrönen *et al*., 2001).

In our recent study on the wild-type human α_{2A}-AR (12), we utilised computer modelling, radioligand binding and functional assays to determine the amino-acid residues important for catecholamine binding and receptor activation. In addition, we suggested a molecular-level model for receptor activation. The charged amine and the β-OH group of catecholamine ligands were suggested to interact with Asp113 ($D_{3.32}$) in TM3. The *meta*- and *para*-OH groups of the phenyl ring appeared to be important for catecholamine binding and receptor activation, and were in close contact with Ser200, Cys201 and Ser204 of TM5. Docking studies did not firmly distinguish between the two possible orientations of the catechol ring, that is, whether the *para*- or the *meta*-OH groups interact, respectively, with Ser200 or Ser204, or *vice versa*. It was concluded on the basis of previous mutagenesis results (Wang *et al*., 1991; Rudling *et al*., 1999) that the *meta*-OH points towards Ser200 (and Cys 201), and that the *para*-OH forms a hydrogen bond with Ser204 (Nyrönen *et al*., 2001).

The current study examines the structural determinants of catechol hydroxyl interactions with ARs, using a panel of mutated human α_{2A}-ARs with Cys→Ser201, Ser→Cys200 and Ser→Cys204 amino-acid substitutions and 12 α_2-adrenergic ligands: nine phenethylamines and three imidazoles. Automated docking methods were used to predict the receptor interactions of these ligands. Radioligand-binding assays and functional G-protein activation assays were performed using transfected Chinese hamster ovary (CHO) cells, to test the predicted binding modes. Both active and inactive receptor conformations were probed. From an evolutionary point of view, it is interesting to compare the effects of Ser→Cys and Cys→Ser substitutions (the α_{2A}- and α_{2C}-AR are the only human ARs with a cysteine in position 5.43). In a previous study with β_2-AR, Ser→Ala204 and Ser→Ala207 substitutions had similar effects as Ser→Cys204 and Ser→Cys207 substitutions (Ambrosio *et al*., 2000); therefore, we limited this study to Ser/Cys substitutions.

Experimental procedures

Materials

2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline ([^{3}H]RX 821002) was obtained from Amersham (Buckinghamshire, U.K.; specific activity 52 Ci mmol^{-1}). Clonidine, dopamine, *R*-noradrenaline (bitartrate), 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK14,304) and *p*-aminoclonidine were supplied by Research Biochemicals (Natick, MA, U.S.A.). *R*-2-amino-1-phenylethanol and *S*-noradrenaline (hydrogen L-tartrate) were purchased from Fluka Sigma-Aldrich (Buchs, Switzerland). (*R*)-adrenaline was from Sigma (St Louis, MO, U.S.A.). (*R*,*S*)-norphenephrine and (*R*,*S*)-octopamine were obtained from Aldrich (Milwaukee, WI, U.S.A.).The enantiomers of norphenephrine and octopamine were prepared using *Pseudomonas cepacia* lipase-catalysed 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-catalysed ethanolysis and treatment with piperidine (5% (v v^{-1})) in tetrahydrofuran provided the free *R*- and *S*-octopamine enantiomers (enantiomeric excess >98 and 91%, respectively). [^{3}H]UK14,304 (62.5 Ci mmol^{-1}) and [^{35}S]GTPγS (1225 Ci mmol^{-1}) were purchased from NEN (Boston, MA, U.S.A.). Cell culture reagents were supplied by Gibco (Gaithersburg, MD, U.S.A.). Other chemicals were of analytical or reagent grade, and were purchased from commercial suppliers.

Cell culture and transfections

Adherent CHO cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured as reported previously (Pohjanoksa *et al*., 1997). Cells were transfected with a pMAMneo-based expression construct encoding the human

wild-type α_{2A}-AR (Kobilka *et al*., 1987). The pREP4-based mutant expression constructs were transfected into cells using the Lipofectin reagent kit (Life Technologies Inc., Grand Island, NY, U.S.A.). Hygromycin B (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.)-resistant (550 μg ml^{-1}) cell cultures were examined for their ability to bind the α_2-AR antagonist [^{3}H]RX821002. The transfected cells chosen for further experiments were subsequently maintained in 200 μg ml^{-1} hygromycin B.

Ligand-binding assays

Saturation- and competition-binding experiments were carried out with [^{3}H]RX821002 in K^+-phosphate buffer (Halme *et al*., 1995). Competition-binding assays were performed using an [^{3}H]RX821002 concentration close to its affinity constant (K_d) for each receptor variant, and 13–15 concentrations of the competitor ligands. In some experiments, conditions that favour the low-affinity receptor conformation were used (150 mM NaCl, 10 μM GDP, no magnesium). Additional radioligand-binding experiments were performed with the agonist [^{3}H]UK14,304 (buffer: 20 mM Tris, pH 7.4, 1 mM EDTA and 5 mM $MgCl_2$). These assays were carried out with similar membrane preparations, as used for the [^{35}S]GTPγS assay (see below) in Tris-Mg^{2+} buffer (Paris *et al*., 1989).

[^{35}S]GTPγS-binding assay

Agonist-induced stimulation of [^{35}S]GTPγS binding was measured essentially as described previously (McKenzie, 1992; Tian *et al*., 1994; Peltonen *et al*., 1998). The [^{35}S]GTPγS-binding assay was carried out using a Beckman Biomek 2000 Laboratory Automation Workstation (Beckman Instruments Inc., Palo Alto, CA, U.S.A.) and 96-well plates. 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, 1 μM GDP, pH 7.4). The reaction was started by adding an aliquot of membrane suspension (5 μg of membrane protein per well) to microwells containing the reaction buffer, 0.08–0.15 nM [^{35}S]GTPγS and agonist in a total volume of 300 μl. The samples were incubated for 25 min at room temperature. The incubation was terminated by rapid filtration through glass fibre filters, using a Tomtec Harvester 96 Mach II (Tomtec Inc., Hamden, CT, U.S.A.). The filters were washed with 3 × 4 ml of ice-cold wash buffer (20 mM Tris-HCl, 5 mM $MgCl_2$ 1 mM EDTA, pH 7.4). The bound radioactivity was determined in a Wallac 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

Model building

Receptor and ligand models The α_{2A}-AR receptor model was created with the program Modeller 4 (Sali & Blundell, 1993). The model is based on a sequence alignment of α_{2A}-AR with bovine rhodopsin, and uses the crystal structure of bovine rhodopsin as the template structure (Palczewski *et al*., 2000). Models of the receptor mutants α_{2A}Ser201, α_{2A}Ser201Cys200 and α_{2A}Ser201Cys204 were made using Sybyl (Tripos Inc., St Louis, MO, U.S.A.). Ligands (Table 1) were built using the program Hyperchem (Hypercube Inc., Gainesville, FL, U.S.A.). Special attention was given to the protonation state of the ionisable amine and imine groups in the ligands. For both the receptor models and ligands, Gasteiger–Marsili charges (required by Autodock, see below) were assigned using Sybyl. All further modifications of the ligands and receptor models were done using Sybyl.

Receptor mapping GRID version 16 (Goodford, 1985) was used to investigate essential interactions in the binding site of the α_{2A}-AR models. The chemical probes used in this study mimic charged and neutral amine groups, (phenolic) OH groups, methyl groups, aromatic carbons and hydrophobic groups (Figure 2). Probes were placed at positions throughout a 30 Å × 30 Å × 30 Å cube (3 points Å^{-1}, 27 points Å^{-3}) centred at the α_{2A}-AR ligand-binding site, and the interaction energies were calculated at each point. GRID maps were visualised using the program gOpenMol (Bergman *et al*., 1997).

The program Superstar (Verdonk *et al*., 1999) was used to map interactions between the receptor models and probes representing OH oxygens, OH hydrogens, charged and neutral amine nitrogens and amine hydrogens and aromatic carbons. Superstar predictions were made within a 15 Å radius centred

Table 1 Competition-binding affinities of 12 tested agonists obtained with [^{3}H]RX821002 to the wild-type human α_{2A}-adrenoceptor and receptor mutants expressed in CHO cells

	α_{2A}-wt		*α_{2A}Ser201*		*α_{2A}Ser201Cys200*		*α_{2A}Ser201Cys204*	
	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H
R-adrenaline	361±38	0.76	15,400±900	0.75	14,300±900	0.67	29,600±3300	0.50
R-noradrenaline	1650±330	0.15	6520±80	1.31	75,100±4500	1.20	18,600±1500	1.14
S-noradrenaline	7100±80	0.70	141,000±11,000	1.24	45,400±11,900	1.18	36,200±1400	1.05
R-norphenephrine	8600±400	2.11	113,000±14,000	1.20	91,700±20,000	1.18	30,800±3000	1.15
S-norphenephrine	11000±400	0.75	169,000±7000	0.95	85,200±7200	2.50	26,000±1900	0.93
R-octopamine	11300±800	0.95	64,100±7500	1.15	40,000±4200	0.92	58,000±10,200	1.12
S-octopamine	15,000±1000	1.15	323,000±250,000	1.21	104,000±15,000	1.03	71,300±48,500	1.15
Dopamine	4780±720	1.03	133,000±51,000	1.34	53,500±17,000	2.70	12,500±3400	1.80
R-2-amino-1-phenylethanol	2720±90	1.14	28,700±2000	1.20	8900±2800	0.91	4260±280	1.95
UK14,304	13.0±0.6	0.57	2430±30	1.06	197±2	0.98	29.2±0.7	1.48
p-Aminoclonidine	20.7±0.7	0.70	34.3±0.4	1.29	104±27	0.83	34.0±1.0	0.82
Clonidine	31.5±1.5	0.89	33.2±0.4	1.20	410±23	1.42	42.8±7.0	1.01

The apparent K_i (nM) is the inhibition constant for a one-site model (mean±s.e.m.). The results are representative of 3–4 similar experiments, each performed in triplicate. The pseudo-Hill slope (n_H) was obtained from a variable Hill slope model.

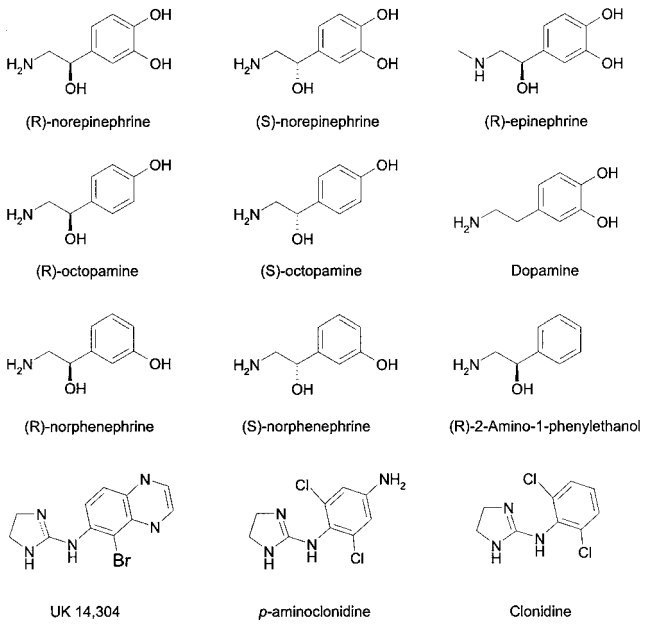

Figure 2 Chemical structures of the α_{2A}-AR ligands examined in this study.

at the OD2 atom of the side chain of Asp113 (3.32) in each of the receptor models.

Ligand docking The programs Gold 1.1 (Jones *et al*., 1997) and Autodock 2.4 (Morris *et al*., 1996) were used to dock each ligand to all receptor models. In Gold, 10 independent genetic algorithm (GA) runs were made for each ligand. The binding site in all receptor models was restricted within a 15 Å radius centred at the side-chain oxygen OD2 of Asp113 (3.32). The default docking parameters were used in Gold. To speed up the calculations, GA-based docking was stopped if the three best scoring solutions were within a 1.5 Å root-mean-squared deviation (r.m.s.d.) of each other.

With Autodock, 500–800 separate docking simulations were performed for each ligand. Each simulation consisted of 100 constant temperature cycles with 8000 steps accepted or rejected. The flexibility of the ligand was considered by allowing ligand torsion angles to rotate. The docked structures were clustered into similar groups that were within 1 Å r.m.s.d. of each other.

Binding mode selection In creating the representative binding modes for each of the ligands, we (i) selected the optimal docked conformations to the receptor (i.e., binding modes) found by Gold and Autodock; (ii) visualised on a graphics station the binding modes superimposed with the GRID and Superstar maps, choosing the binding mode that correlated best with these maps; and (iii) considered how well our model complexes correlated with the existing α_{2A}-AR ligand-binding and receptor activation data. Binding modes chosen using this protocol were subjected to energy minimisation. In all, 300 minimisation cycles were calculated using the MMFF94 force field and MMFF94 charges in Sybyl, allowing all ligand atoms and all atoms in the amino-acid side chains in the receptor-binding site within 6 Å from the ligand to relax during the minimisation procedure.

Results

Radioligand-binding assays

We established four CHO cell lines expressing wild-type and mutated α_{2A}-ARs with amino-acid substitutions at residues Cys201, Ser200 or Ser204 (positions 5.43, 5.42 and 5.46). The α_{2A}Ser201 mutant had a cysteine-to-serine substitution (null mutation with respect to the presence of a cysteine residue in the investigated region). α_{2A}Ser201Cys200 and α_{2A}Ser201 Cys204 had Ser→Cys200 and Ser→Cys204 substitutions, respectively, in addition to the Cys→Ser201 substitution.

The receptor densities (B_{max}) were 1300 ± 200 (s.e.m.) fmol mg^{-1} of protein for CHO-α_{2A}wt, 4740 ± 230 fmol mg^{-1} for CHO-α_{2A}Ser201, 2150 ± 310 fmol mg^{-1} for CHO-α_{2A}Ser201 Cys200 and 4010 ± 120 fmol mg^{-1} for CHO-α_{2A}Ser201Cys204. The equilibrium dissociation constants (K_D) of the radioligand [^{3}H]RX821002 were 0.89 ± 0.27, 0.42 ± 0.08, 3.34 ± 0.51 and 2.71 ± 0.12 nM, respectively. Binding affinity constants (K_i-values) were determined for a panel of α_2-adrenergic agonists at the wild-type α_{2A}-AR and the three α_{2A}-AR mutants in competition-binding assays with whole-cell homogenates and [^{3}H]RX821002 as the radioligand (Table 1). The overall trend was that the affinities of the agonist ligands to the receptor were decreased by the amino-acid substitutions. As the *R*-enantiomers of catecholamines are more potent activators of α_2-ARs than the *S*-enantiomers (Hoffman & Lefkowitz, 1995; Airriess *et al*., 1997), we will concentrate on the results of the *R*-enantiomers.

The affinities of the nonphenethylamine reference compounds UK14,304, *p*-aminoclonidine and clonidine were not affected to the same extent by the mutations as those of the phenethylamines (Tables 1,2). An exception to this trend was UK14,304, whose affinity was clearly lower with the α_{2A}Ser201 mutant than with the other mutants or the wild-type receptor. The affinities of UK14,304, clonidine and *p*-aminoclonidine were also 10–12-fold lower with the α_{2A}Ser201Cys200 mutant than with the other receptor species.

In order to examine the presence of a population of agonist high-affinity receptors in the membrane preparations used for the functional [^{35}S]GTPγS-binding assay (see below), additional radioligand-binding assays were performed with the agonist [^{3}H]UK14,304. The saturation-binding experiments with [^{3}H]UK14,304 revealed high-affinity receptor densities (B_{max}) of 1.1 pmol mg^{-1} of protein for CHO-α_{2A}wt, 7.4 pmol mg^{-1} for CHO-α_{2A}Ser201 and 5.5 pmol mg^{-1} for CHO-α_{2A}Ser201Cys204 (means of two to four independent determinations). The K_D-values were 0.4, 0.8 and 0.7 nM, respectively. In the CHO-α_{2A}Ser201Cys200 mutant, the affinity of this radioligand was too low to obtain reliable estimates of receptor density and affinity. The affinities of all phenethylamine compounds and UK14,304 were 1.5–4-fold lower with the CHO-α_{2A}Ser201 mutant compared to the wild-type receptor, but clonidine did not seem to lose its affinity. The Ser→Cys204 substitution caused a 148-fold loss in affinity for *R*-adrenaline, a 236-fold loss for *R*-noradrenaline, a 40-fold loss for dopamine and a 10-fold loss for *R*-octopamine compared to the CHO-α_{2A}Ser201 mutant. However, *R*-norphenephrine retained its affinity for this mutant.

Another set of competition-binding experiments was carried out using [^{3}H]RX821002 as the radioligand and membrane preparations used for the [^{35}S]GTPγS-binding assay, under

Table 2 Competition-binding affinities of agonists obtained with [^{3}H]UK14,304 to the wild-type human α_{2A}-adrenoceptor and receptor mutants expressed in CHO cells

Apparent K_i (nM)	α_{2A}-*wt*	α_{2A}*Ser201*	α_{2A}*Ser201Cys204*
R-adrenaline	1.4±0.2	3.8±1.1	563±263
R-noradrenaline	4.6±1.0	10.7±0.9	2530±330
S-noradrenaline	105±23	233±38	11,970±1670
R-norphenephrine	130±35	520±150	477±33
S-norphenephrine	2716±659	5670±680	5300±1100
R-octopamine	432±55	960±312	9600±2140
Dopamine	34±6	123±4.7	4970±236
R-2-amino-1-phenylethanol	675±99	1010±113	817±94
UK14.304	0.33±0.09	1.3±0.1	1.7±0.3
p-Aminoclonidine	0.9±0.1	1.1±0.2	0.5±0.1
Clonidine	1.5±0.04	1.4±0.2	0.4±0.1

The apparent K_i (nM) is the inhibition constant for a one-site model (mean±s.e.m.). The results are representative of at least three similar experiments, each performed in duplicate.

Table 3 Competition-binding affinities of seven tested agonists obtained with [^{3}H]RX821002 to the wild-type human α_{2A}-adrenoceptor and receptor mutants expressed in CHO cells, under experimental conditions that favour the low-affinity receptor conformation (150 mM NaCl, 10 μM GDP, no magnesium)

Apparent K_i (nM)	α_{2A}-*wt*	α_{2A}*Ser201*	α_{2A}*Ser201Cys200*
R-adrenaline	3240±390	57,400±10,000	59,000±2000
R-noradrenaline	13,900±1080	152,000±32,800	313,000±41,000
R-norphenephrine	6920±340	116,000±12,300	223,000±30,000
R-octopamine	34,000±2600	152,000±15,000	144,000±17,400
Dopamine	43,500±2400	310,000±35,700	419,000±91,000
R-2-amino-1-phenylethanol	5230±450	29,900±3800	45,900±950
UK14,304	224±16	1395 (two experiments)	1770±95

The apparent K_i (nM) is the inhibition constant for a one-site model (mean±s.e.m.). The results are representative of at least three similar experiments, each performed in duplicate.

experimental conditions that favour the low-affinity receptor conformation (150 mM NaCl, 10 μM GDP, no magnesium) (Table 3). The B_{max}-values determined in saturation-binding experiments were 1.3 pmol mg^{-1} of protein for CHO-α_{2A}wt, 54.0 pmol mg^{-1} for CHO-α_{2A}Ser201 and 3.3 pmol mg^{-1} for CHO-α_{2A}Ser201Cys200. The K_D-values were 0.51, 1.27 and 2.4 nM, respectively. These experiments were not carried out for the CHO-α_{2A}Ser201Cys204 mutant. All compounds tested appeared to have 4.5–18-fold lower affinity with the CHO-α_{2A}Ser201 mutant compared to the wild-type receptor. *R*-noradrenaline, *R*-norphenephrine and *R*-2-amino-1-phenylethanol had 1.5–2.1-fold lower affinities towards the CHO-α_{2A}Ser201Cys200 mutant, compared to CHO-α_{2A}Ser201. The affinities of *R*-adrenaline, dopamine and UK14,304 were not significantly changed by the Ser→Cys200 substitution, and *R*-octopamine even gained some affinity.

[^{35}S]GTPγS-binding assay

Agonist-induced binding of [^{35}S]GTPγS to isolated cell membranes was measured in the presence of 20 mM NaCl and 1 μM GDP, in order to monitor functional receptor activation. These conditions favour the discrimination between partial and full agonism. EC_{50}- and E_{max}-values were determined for 12 α_2-adrenergic ligands.

The maximal stimulation of [^{35}S]GTPγS binding was 247% over the basal level for the Ser201 mutant (*R*-noradrenaline), 102% for the Ser201Cys200 mutant (*p*-aminoclonidine and *R*-norphenephrine) and 65% for the Ser201Cys204 mutant (*p*-aminoclonidine). With the wild-type α_{2A}-AR, the maximal stimulation was 140% over the basal level (*R*-adrenaline). However, these efficacy values are not directly comparable as the receptor expression levels in the four clones were different.

The *R*-enantiomers of the phenethylamines (adrenaline, noradrenaline, octopamine and norphenephrine) appeared to be more potent and efficacious on the wild-type receptor than the *S*-enantiomers, which was expected according to previous studies (Airriess *et al*., 1997). Catecholamine derivatives with two catechol hydroxyl groups (adrenaline, noradrenaline and dopamine) lost part of their efficacy on the α_{2A}Ser201Cys200 receptor, and acted as partial agonists compared to *p*-aminoclonidine. The phenethylamine derivatives with one catechol hydroxyl group (octopamine and norphenephrine), in contrast, appeared to retain their efficacy with the α_{2A}Ser201Cys200 mutant receptor. All phenethylamine derivatives, except *R*-noradrenaline and *R*-2-amino-1-phenylethanol, completely lost their efficacy with the α_{2A}Ser201 Cys204 mutant receptor.

UK14,304 appeared to act as a full agonist with the wild-type receptor, and as a partial agonist with all of the mutants. *para*-Aminoclonidine was a partial agonist with the wild-type and α_{2A}Ser201 receptors, but emerged as the most efficacious agonist with the α_{2A}Ser201Cys200 and α_{2A}Ser201Cys204 receptors. Clonidine was a partial agonist with all receptors. As a rule, all of the tested imidazoline compounds retained their efficacy in all receptor mutants.

The potencies of the phenethylamines (expressed as EC_{50}-values) were in general highest with the wild-type receptor and α_{2A}Ser201, the potencies being approximately equal for both receptors. The potencies were clearly lower with the α_{2A}Ser201Cys200 mutant, and most compounds appeared inactive with the α_{2A}Ser201Cys204 mutant (Table 4).

The potencies of the imidazoline reference compounds UK14,304, *p*-aminoclonidine and clonidine were not affected to the same degree by the amino-acid substitutions as those of the phenethylamines, although the potency of UK14,304 was clearly lowered in the α_{2A}Ser201Cys200 mutant. The potency of *p*-aminoclonidine was 10-fold higher in the α_{2A}Ser201 and α_{2A}Ser201Cys204 mutants compared to the wild-type receptor.

Ligand docking

Amino-acid residues that form the ligand-binding cavity in our receptor models are shown in Figure 3. The charged amine and imine groups of the ligands were optimally coordinated to one side-chain oxygen of Asp113 ($D_{3.32}$) in TM3. The *β*-OH group of the *R*-isomers formed a hydrogen bond with the other side-chain oxygen of Asp113 ($D_{3.32}$). Also, Cys117 ($C_{3.36}$) and Thr118 ($T_{3.37}$) at the bottom of the ligand-binding cavity were in close proximity to the amine end of the docked ligands. The phenyl rings were packed with one ring face against the conserved aromatic residues in TM6: Phe390 ($F_{6.51}$), Phe391 ($F_{6.52}$), Tyr394 ($Y_{6.55}$) and possibly Phe205 ($F_{5.47}$) in TM5, and with Val114 ($V_{3.33}$) in TM3 and Leu160 ($L_{4.53}$) in TM4 packing against the other face of the ring (12). The α_{2A}-AR-binding site is rich in aromatic residues: the side chains of Phe205 ($F_{5.47}$) in TM5, Phe390 ($F_{6.51}$), Phe391 ($F_{6.52}$), Tyr394 ($Y_{6.55}$) and Trp387 ($W_{6.48}$) in TM6, and Phe411 ($F_{7.38}$) and Phe412 ($F_{7.39}$) in TM7 are accessible to the ligands in the binding cavity of our receptor model. As indicated by previous studies (Rudling *et al*., 1999; Salminen *et al*., 1999), Cys201, Ser200 and Ser204 in TM5 of α_{2A}-AR also have important roles both in orienting and binding ligands. Models of the docked conformations of *R*-phenethylamines and UK14,304 in the wild-type α_{2A}-AR are shown in Figure 4.

Discussion

Several previous investigations have proposed that the binding of catecholamine ligands to ARs involves the formation of hydrogen bonds between the catecholic OH groups and two or three conserved amino-acid residues in the TM5 of the receptor protein. When these findings have been applied to the α_{2A}-AR, it has generally been assumed that the *meta*-OH group would interact with Ser200 and Cys201, and the *para*-OH group with Ser204. In this study, we have (i) employed separate assays to explore the agonist high- and low-affinity conformations of the receptor, and (ii) used mutant receptors with amino-acid substitutions in TM5 to test whether cysteines and serines can subserve similar roles in TM5 in binding catecholamine ligands. Our α_{2A}Ser201 mutant receptor had a cysteine-to-serine substitution (null mutation), and the α_{2A}Ser201Cys200 and α_{2A}Ser201Cys204 mutants had Ser→Cys200 and Ser→Cys204 substitutions, respectively, in addition to the Cys→Ser201 substitution. The ligand-binding and activation properties of each mutant receptor were compared to those of the wild-type α_{2A}-AR, with a cysteine in position 201. Radioligand competition-binding and functional agonist activation assays were performed for 12 α_2-AR agonists (nine phenethylamine derivatives and three nonphenethylamine reference compounds). Computer modelling was used to create models of the wild-type α_{2A}-AR and α_{2A}Ser201, α_{2A}Ser201Cys200 and α_{2A}Ser201Cys204 mutant receptors, using the bovine rhodopsin crystal structure (Palczewski *et al*., 2000) as the template. Each ligand listed in Figure 2 was docked to all receptor models using procedures described in Materials.

OH groups preferentially interact with other OH groups rather than with SH groups. This notion is based on the analysis of nonbonded interactions in the Cambridge Structural Database (CSD) and the Isostar Database. Interactions between OH- and SH-groups are much less common than OH–OH interactions in all crystal structures present in the CSD. Theoretical *ab initio* calculations stored in the CSD indicate that SH–OH interactions have two possible favourable orientations, whereas in OH–OH interactions only one favourable nonredundant orientation can be found: the SH-

Table 4 Efficacy and potency results from [^{35}S]GTPγS-binding assays

	α_{2A}-*wt*		α_{2A}*Ser201*		α_{2A}*Ser201Cys200*		α_{2A}*Ser201Cys204*	
	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
R-adrenaline	179	140	76	210	19,500	44	n.d.	0
R-noradrenaline	211	102	126	247	105,000	52	1600	11
S-noradrenaline	4000	91	4320	174	n.d.	0	n.d.	0
R-norphenephrine	2990	29	2420	108	n.d.	≈25	n.d.	0
S-norphenephrine	n.d.	0	10,100	16	n.d.	0	n.d.	0
R-octopamine	23,300	49	7480	78	44,100	81	n.d.	0
S-octopamine	495,000	31	118,000	35	n.d.	≈20	n.d.	0
Dopamine	19,500	96	1330	210	n.d.	0	n.d.	0
R-2-amino-1-phenylethanol	802	7	1510	27	17,800	73	3180	15
UK14,304	8.2	133	10	161	643	87	31	46
p-Aminoclonidine	30	46	2.9	125	41	102	2.9	65
Clonidine	176	30	6.1	118	10	60	10.5	43

Half-maximal effective concentrations (EC_{50}, nM) of agonists are shown for the wild-type and mutant α_{2A}-ARs in transfected CHO cell lines. The E_{max} values for the different agonists indicate the maximal % change in the binding of the guanine nucleotide analogue over the basal level in the absence of agonists. Values are means from at least three separate experiments performed in duplicate. n.d., not determined.

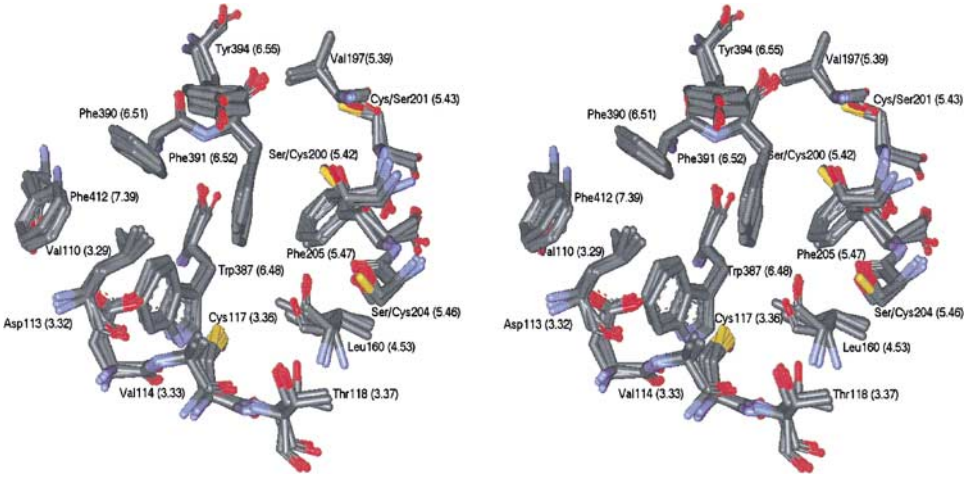

Figure 3 Stereo view of the superposition of the α_{2A}-wt, α_{2A}Ser201, α_{2A}Ser201Cys200 and α_{2A}Ser201Cys204 binding sites after flexible docking of *R*-noradrenaline. Amino-acid residues that form the binding cavity in our models are shown. Atom colour codes: grey = carbon, red = oxygen, blue = nitrogen, yellow = sulphur.

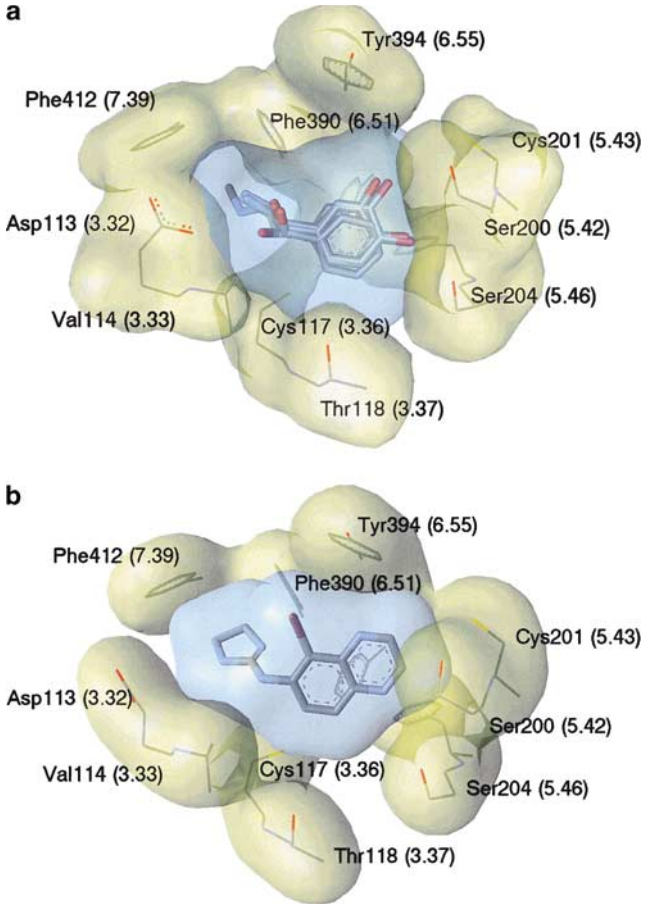

Figure 4 Binding mode models (see Experimental procedures for details) of superposed *R*-isomers of adrenaline, noradrenaline, octopamine, norphenephrine and 2-amino-1-phenylethanol (a), and UK14,304 (b). Some key amino acids that line the ligand-binding cavity model are shown. The yellow surface depicts the solvent-accessible surface of these residues, and the blue surface depicts the solvent-accessible surface of the ligand. Hydrogens are not shown for clarity, but were included in the calculation of the solvent-accessible surface. Atom colour codes: grey = carbon, red-= oxygen, blue = nitrogen, yellow = sulphur, brown = bromine (only in UK14,304).

group can act as a proton donor to the OH-group, or as a hydrogen bond acceptor from the OH-group. The orientation of the latter is indicated to be energetically more favourable ($-11.4\,kJ\,mol^{-1}$), with a longer nonbonded interaction length (2.7 Å). The interaction in which the SH-group acts as a hydrogen bond donor is slightly shorter and higher in energy ($-7.8\,kJ\,mol^{-1}$, 2.3 Å). The interaction length of the most favourable OH–OH orientation is notably shorter (1.9 Å), and the interaction energy is much lower ($-21.8\,kJ\,mol^{-1}$).

The active and resting forms of the wild-type α_{2A}-AR

In our previous report, we suggested a model for α_{2A}-AR activation Nyrönen *et al*., 2001. The new results presented in this article clarify the role of catecholamine ligand determinants that affect the affinity of the ligand towards the active and resting receptor conformations. In competition-binding experiments carried out under conditions that favour the resting receptor conformation (high sodium and GDP concentrations, no magnesium) (Table 3), the *β*-OH group appears to increase the agonist affinity somewhat (cf. dopamine vs *R*-noradrenaline). The catechol *para*-OH group decreases the affinity significantly (cf. *R*-octopamine vs *R*-2-amino-1-phenylethanol), whereas the *meta*-OH has a much smaller or no effect (cf. *R*-norphenephrine vs *R*-2-amino-1-phenylethanol). *R*-noradrenaline had higher affinity than *R*-octopamine, maybe because the decrease in the affinity caused by the *para*-OH group is moderated by the possibility to form an intramolecular hydrogen bond with the *meta*-OH group.

In order to examine the affinities of the agonists towards the active receptor conformation, additional radioligand-binding assays were performed with the agonist radioligand [^{3}H]UK14,304 (Table 2). The *β*-OH group increases the affinity somewhat (cf. dopamine vs *R*-noradrenaline), as is the case in the resting receptor conformation. *R*-2-amino-1-phenylethanol had a higher affinity towards the activated receptor compared to the resting receptor, that is, part of the affinity gain for all compounds on the high-affinity receptor appears to be built into the phenethylamine core, and is independent of the catechol hydroxyls. However, the gain in affinity for *R*-2-amino-1-phenylethanol is one to two orders of

magnitude less than what can be achieved with the aid of the catechol hydroxyls, that is, by far the largest part of the affinity gains on the activated receptor are due to the catechol hydroxyls. Interestingly, the data also indicate that the affinity gain provided by both catechol hydroxyls being present at the same time is based on a synergistic action. While the *para*-OH group (cf. *R*-octopamine vs *R*-2-amino-1-phenylethanol) slightly increases the affinity towards the active receptor conformation and the *meta*-OH group significantly increases this affinity (cf. *R*-norphenephrine vs *R*-2-amino-1-phenylethanol), the gain in affinity for *R*-noradrenaline vs *R*-2-amino-1-phenylethanol is considerably larger than the combination of the isolated *meta*- and *para*-OH effects. Overall, this synergistic effect on the affinity towards the agonist high-affinity form of the receptor clearly represents the largest effect of the catechol hydroxyls in any of the binding interactions.

The role of Cys201 in ligand-binding and receptor activation

The human α_{2A}- and α_{2C}-ARs have a cysteine at position 5.43 (201 in α_{2A}-AR), whereas the β_2- and α_{2B}-ARs have a serine at the corresponding position. It has been previously suggested that the *meta*-OH group of the catecholic ring interacts with position 5.43 (Strader *et al*., 1989). The importance of Cys201 for UK14,304 binding in the α_{2A}-AR has also been previously reported (Cockcroft *et al*., 2000). To experimentally verify the contribution of Cys201 to catecholamine binding, we tested a human receptor with an engineered Cys→Ser201 substitution, thus making this binding site resemble β_2-AR, α_{2B}-AR and the rodent α_{2A}-AR. Moreover, we wanted to test how this amino-acid replacement would affect receptor activation. The amino-acid substitution significantly reduced the binding affinities of all catecholamine ligands in competition binding with [^{3}H]RX821002 (Table 1). The affinity of *R*-norphenephrine (having only the *meta*-OH) was decreased more than that of *R*-octopamine (only *para*-OH), possibly indicating a stronger interaction between Cys201 and the *meta*-OH as opposed to the *para*-OH, which is in agreement with previous reports. In contrast to catecholamine ligands, the affinities of the imidazoline reference compounds clonidine and *p*-amino-clonidine were not affected, but the apparent affinity of UK14,304 was reduced. This reduction in affinity was restricted to the agonist low-affinity conformation of the receptor.

In [^{3}H]UK14,304 saturation-binding experiments, the affinity of this radioligand towards the α_{2A}Ser201 mutant was only to a minor extent different from the affinity observed towards the wild-type receptor (0.8 nM vs 0.4 nM). Competition-binding studies with [^{3}H]UK14,304 as radioligand were performed in order to examine the affinities of the agonists towards the agonist high-affinity form of the receptor, which supposedly corresponds to the active receptor conformation. The affinities of all phenethylamine compounds and UK14,304 were 1.5–4 fold lower in the CHO-α_{2A}Ser201 mutant compared to the wild-type receptor, while the affinities of clonidine and *p*-aminoclonidine were unaffected. In competition-binding experiments carried out under conditions that favour the resting receptor conformation (150 mM NaCl, 10 μM GDP, no magnesium), all compounds tested appeared to lose affinity compared to the wild-type receptor.

The agonist potencies did not decrease uniformly by the amino-acid substitutions in the functional GTPγS-binding assay (Table 4). The different effects of the Cys→Ser201 mutation on the binding affinities and functional potencies of agonists imply that the cysteine residue at position 201 has an important role in the binding of catecholamine ligands and UK14,304, but that a serine at the corresponding position can subserve its role in receptor activation.

Cys201 ($C_{5.43}$) probably has an important role in UK14,304 binding, because the quinoxaline ring can favourably coordinate with this residue by a hydrophobic interaction. UK14,304 is a longer molecule than the other agonists used in this study, and can more easily reach across the binding site from Asp113 ($D_{3.32}$) to the serine and cysteine residues in TM5. In the computer-modelling studies, the mutation of Cys201 ($C_{5.43}$) into a serine causes an unfavourable interaction between the serine OH group and the hydrophobic carbons in the quinoxaline in UK14,304. The two unprotonated nitrogens in the quinoxaline ring can, however, act as hydrogen bond acceptors. According to our hypothesis, Ser200 and Ser204 participate in this interaction between α_{2A}-AR and UK14,304 in the active receptor conformation. Interestingly, the functional potency of dopamine was increased by a factor of 15 by the Cys→Ser201 mutation. This may indicate the importance of this serine residue in the recognition of dopamine by the active receptor conformation. Dopamine has conformational flexibility in comparison with the other phenethylamines due to the lack of the β-OH group Nyrönen *et al*., 2001, and all human dopamine receptor subtypes have a serine at the corresponding position.

Comparison of the wild-type human α_{2A}-AR and α_{2A}Ser201Cys200

In our previous study (Nyrönen *et al*., 2001), we proposed a model for α_{2A}-AR ligand-binding and receptor activation. According to the model, structural rearrangements take place when the receptor is activated. This agonist-associated structural change could be described as a rotation of TM5 which exposes residues Ser200 and Ser204 to the binding cavity. According to previous studies, Ser200 interacts with the *meta*-OH group of the catecholamine ring, which helps to stabilise the active receptor conformation. Thus, substituting Ser200 with a cysteine might affect the affinity of this residue to the *meta*-OH group or influence the capability of agonists to stabilise the active receptor conformation.

In competition-binding studies with [^{3}H]RX821002, the Ser→Cys200 substitution (α_{2A}Ser201Cys200) clearly had different effects on the affinities of *R*-noradrenaline, *R*-norphenephrine and *R*-octopamine. When compared to the wild-type receptor, the affinity of *R*-noradrenaline was 45-fold lower, probably reflecting weakened interactions between the *meta*-OH group and the receptor presenting Cys200. This notion is supported by our docking simulations, where the *meta*-OH group is in close contact with Ser200. The affinity of *R*-octopamine (possessing only the *para*-OH group) was only slightly decreased compared to the wild-type receptor, indicating that there are no significant interactions between Ser200 and the *para*-OH group. On the contrary, the affinity of *R*-norphenephrine was 10-fold lower than in the wild-type receptor, again caused by the weaker interaction between Cys200 and the *meta*-OH group. Catecholamine interactions

with the α_{2A}Ser201Cys200 mutant receptor would, according to our model, involve interactions between catechol OH-groups and the SH-group of the cysteine. The Isostar database indicates that SH–OH interactions are not as favourable as OH–OH interactions that are formed between catechol hydroxyls and Ser200 ($S_{5.42}$) in the wild-type α_{2A}-AR, and this is supported by the statistical analysis of protein–ligand and protein–protein interactions in known crystal structures (Rantanen *et al*., 2001). This result is also reflected in the docked conformations of *R*-noradrenaline, *R*-norphenephrine and *R*-octopamine to the α_{2A}Ser201Cys200 mutant, which were not as well defined as in the wild-type receptor.

The [^{35}S]GTPγS-binding assay results for the Ser201Cys200 mutant are also in line with the proposal of residue 200 ($S_{5.42}$) interacting with the *meta*-OH of catechol ligands. Cys200 causes a dramatic rearrangement in the agonist efficacy orders compared to the wild-type and the Ser201 mutant receptor. The most efficacious agonist among the phenethylamine ligands on Ser201Cys200 is *R*-octopamine, followed closely by *R*-2-amino-1-phenylethanol, which shows a large gain in agonist efficacy over the wild-type as well as the Ser201 mutant. In contrast, clearly, the efficacies of phenethylamines possessing an OH in the *meta*-position (*R*-adrenaline, *R*-noradrenaline, *R*-norphenephrine and dopamine) have suffered. These results obviously imply an unfavourable interaction between *meta*-OHs and Cys200, relative to the interaction of *meta*-OHs and Ser200. A possible explanation for the impressive gain in agonist efficacy for *R*-2-amino-1-phenylethanol on the Ser201Cys200 mutant could be that Cys200 can form a productive hydrophobic interaction with the aromatic ring of the ligand, unencumbered by the presence of any OH substituent.

The Ser→Cys200 mutation also affected the functional [^{35}S]GTPγS binding results for the imidazolines UK14,304, clonidine and *p*-aminoclonidine. The clearest effect observed was on the agonist potency of UK14,304, which was some 65–75-fold lower than on the wild-type α_{2A}-AR or the Ser201 mutant. To some extent, UK14,304, *p*-aminoclonidine and clonidine could be regarded as analogues of catecholamines with nitrogen instead of oxygen atoms in the *meta*- and *para*-positions on the aromatic ring. However, obviously, this perspective is too simplistic, because, while it might offer an explanation for the effects of the mutation on the activity of UK14,304 in terms of the presence of a '*meta*-nitrogen', there is no comparable efficacy gain for clonidine relative to *para*-aminoclonidine, as observed for *R*-2-amino-1-phenylethanol relative to *R*-octopamine. All in all, the set of imidazoline compounds is certainly too small to allow for any definitive conclusions. However, it is worthwhile to point out that the marked reduction in the potency of UK14,304 (643 nM) compared to the wild-type receptor (8.2 nM) is in line with our inability during [^{3}H]UK14,304 saturation-binding assays to detect an agonist high-affinity binding site for this radioligand within experimentally feasible concentrations.

Comparison of the wild-type human α_{2A}-AR and α_{2A}Ser201Cys204

It has been proposed previously that Ser204 interacts with the *para*-OH group of the catechol ring in the active receptor conformation. Therefore, it would be expected that the Ser→Cys204 substitution should decrease the affinity and potency of catecholamine ligands containing a *para*-OH group. Such a preferential reduction in the binding affinities for phenethylamines was indeed observed in the agonist high-affinity binding sites probed with [^{3}H]UK14,304. Compared to the wild-type α_{2A}-AR, the affinities for the catechol compounds dopamine, *R*-adrenaline and *R*-noradrenaline on the Ser201Cys204 mutant were reduced by 145–550-fold. For the *para*-OH compound *R*-octopamine, the loss in affinity was 22-fold, while the *meta*-OH compound *R*-norphenephrine suffered a 3.7-fold reduction in affinity, and the affinity of the *meta*,*para*-unsubstituted *R*-2-amino-1 phenethylamine was essentially unchanged. While these results are largely in line with the expectation formulated above, they also hint at a minor involvement of OH substituents in the *meta* position. The three compounds with an OH in that position in addition to the *para*-OH (dopamine, adrenaline and noradrenaline) suffered greater losses than *R*-octopamine; *R*-norphenephrine also showed some loss, and only the completely unsubstituted *R*-2-amino-1-phenethylamine appeared truly unaffected. The influence of the phenethylamine hydroxyl substitution pattern was in a similar, but less distinct manner also observed in the competition-binding assay with [^{3}H]RX821002, for which the data are compiled in Table 1. The more diffuse picture in this assay is presumably due to the fact that it reflects a mixed population of agonist high-affinity and low-affinity binding sites. As has been mentioned above, the main effect of catechol hydroxyls consists in a synergistic increase in the interaction with the agonist high-affinity form of the receptor. Not surprisingly, therefore, it appears that where possible, such an effect is more easily monitored by directly measuring the interaction of ligands with the agonist high-affinity binding site.

The Ser→Cys204 substitution not only affected the affinity of most phenethylamine ligands, but also essentially obliterated the agonist responses towards catechol compounds. With the exception of some minor receptor activation by *R*-noradrenaline and *R*-2-amino-1-phenylethanol, all other compounds were inactive. This observation highlights the importance of Ser204 in the activation of the receptor by catecholamine ligands. A cysteine at this position may impair the capacity of the receptor to attain or retain an active conformation that is dependent on catecholamine agonists. In this regard, there seems to be a qualitative difference between Ser204, which appears to have a mandatory role, and Ser200, which appears to be more auxiliary or permissive in nature.

In contrast to the phenethylamines, the imidazoline ligands were still capable of forming 'productive' interactions with the Ser201Cys204 receptor mutant, albeit with possibly reduced efficacy. As a matter of fact, it is only through the agonist responses caused by the imidazoline compounds that this receptor mutant can be judged to be at least partly functional. Even though UK14,304 appeared to be less efficacious than *para*-aminoclonidine, the existence of an agonist high-affinity conformation for the Ser→Cys204 receptor variant was confirmed by the [^{3}H]UK14,304 saturation-binding assays.

High-/low-affinity ratio vs efficacy

According to multistate receptor models, the differences in the affinities of agonists towards resting and activated receptor

conformations correspond to the intrinsic activity of compounds, that is, the more a compound discriminates in favour of the activated receptor, the stronger its agonist efficacy is expected to be. This correlation is shown for the wild-type receptor in Figure 5a, and for the α_{2A}Ser201 mutant in Figure 5b. The results are in line with the above hypothesis: *R*-adrenaline, *R*-noradrenaline, dopamine and UK14,304 have 700–3000-fold higher affinities towards the activated wild-type receptor, and they are strong agonists. *R*-norphenephrine, *R*-octopamine and *R*-2-amino-1-phenylethanol have 7–80-fold higher affinities towards the activated wild-type receptor, and they are partial agonists. A similar correlation can also been seen for the α_{2A}Ser201 mutant, where the high-/low-affinity ratio ranges between 1000 and 15,000 for full agonists, and between 30 and 200 for partial agonists. The difference between *R*-norphenephrine and *R*-octopamine is small, but the rank orders of the affinity ratios and efficacies appear to be consistent for both the wild-type and the α_{2A}Ser201 mutant receptors. Another interesting point is that the somewhat larger affinity ratio for *R*-2-amino-1-phenylethanol in the α_{2A}Ser201 mutant (30 vs 7 in the wild-type receptor) also appears to translate into a higher agonist efficacy (27% over the basal level compared to 7% in the wild-type receptor). Finally, it is noteworthy to point out that the rectangular shape of the affinity ratio vs efficacy curves in Figure 5 is in line with other predictions from multistate models. The rectangular character of these curves indicates that there are upper limits of efficacy that can be achieved, and that, for these two receptors, these limits are defined by the endogenous α_2-AR agonists *R*-adrenaline and *R*-noradrenaline.

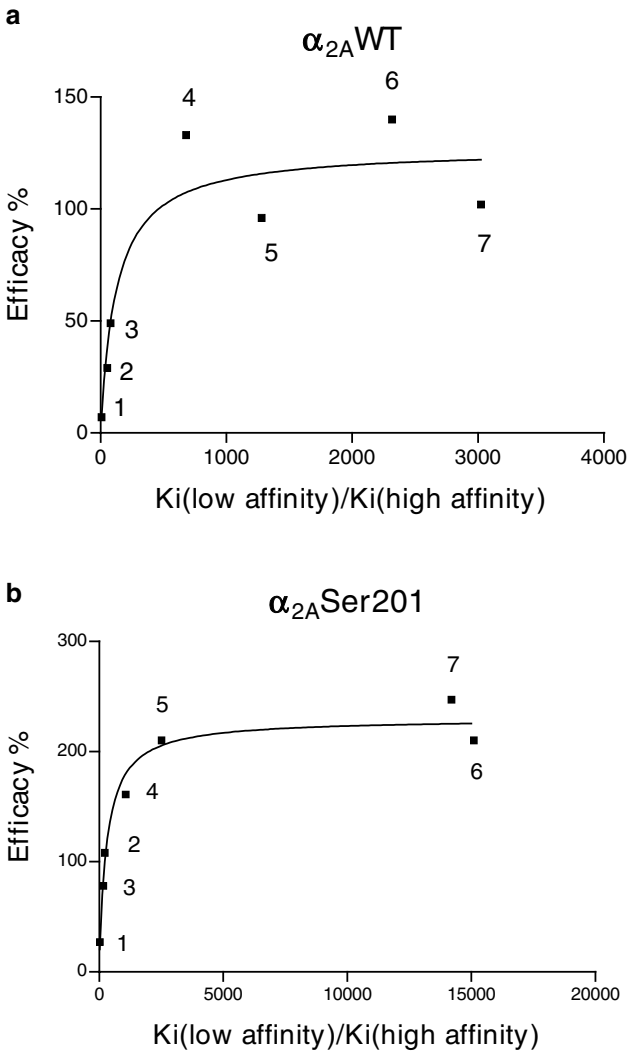

Figure 5 The correlation between the low-/high-affinity competition-binding K_i-ratio and the efficacy for seven agonists in the wild-type α_{2A}-AR (a) and the α_{2A}Ser201 mutant (b). The K_i (high affinity) values are presented in Table 2 and the K_i (low affinity) values are presented in Table 3. The agonists are numbered: 1 – *R*-2-amino-1-phenylethanol, 2 – *R*-norphenephrine, 3 – *R*-octopamine, 4 – UK14,304, 5 – dopamine, 6 – *R*-adrenaline, 7 – *R*-noradrenaline.

Conclusions

The OH groups of phenethylamine ligands seem to play different roles in the activated and resting forms of the wild-type α_{2A}-AR. The β-OH group of phenethylamines appears to increase ligand affinity towards the resting form of the wild-type α_{2A}-AR, whereas the catecholic *para*-OH group decreases the affinity significantly. In the activated receptor, the catecholic *para*-OH group slightly and the *meta*-OH group significantly increases agonist affinity.

The mutation of Ser200 ($S_{5.42}$) or Ser204 ($S_{4.46}$) into a cysteine causes dramatic deterioration in the capability of catecholamines to activate α_{2A}-AR. Our previous studies suggested that alkylating reagents of different molecular sizes capable of reacting with engineered cysteines at positions 200 and 204 recognised two different conformations of the human α_{2A}-AR (Marjamäki *et al*., 1999). This argues that a rotation of TM5 is likely to be involved in receptor activation (Salminen *et al*., 1999). Computer modelling and analysis of small molecule, protein–ligand and protein–protein crystal structures indicate that interactions of the catecholic OH groups with Ser200 and Ser204 in TM5 are favoured energetically over interactions with the corresponding cysteine mutants.

SH groups of cysteine residues can form interactions over longer distances than OH groups, but SH–OH interactions are much less frequent than OH–OH interactions. The cysteine side chain also frequently forms important hydrophobic interactions with aliphatic and aromatic groups. It has been shown that Cys201 ($C_{4.43}$) is exposed in the binding cavity (Marjamäki *et al*., 1998), and that it is important for ligand binding (Marjamäki *et al*., 1999; Salminen *et al*., 1999). Thus, it seems possible that the relative disposition of TM5, thought to be coupled to the activation of the receptor by phenethylamines, could be tuned by complex interactions that are formed between the catecholic ring, the catecholic OH-groups and Cys201, Ser200 and Ser204 of the receptor.

Cys201 of α_{2A}-AR appears to play a significant role in the binding of catecholamine ligands and UK14,304, but it is not essential for receptor activation. The present results imply that the *meta*-OH group of the phenethylamines is oriented towards Cys201 in the inactive receptor conformation. Ser200 seems to interact with the *meta*-OH group, and affects both catecholamine binding and receptor activation. Substituting Ser204 with a cysteine interferes with both the binding of catecholamine ligands and receptor activation, indicative of interactions between Ser204 and the *para*-OH group of the catecholic ring. The docking results agree with the experimental data.

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