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Identification of Essential Residues Involved in the Allosteric Modulation of the Human A_3 Adenosine Receptor

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ABSTRACT

We examined the effects on allosteric modulation and ligand binding of the mutation of amino acid residues of the human A_3 adenosine receptor (A_3AR) that are hypothesized to be near one of three loci: the putative sodium binding site, the putative ligand binding site, and the DRY motif in transmembrane helical domain 3. The effects of three heterocyclic allosteric modulators [the imidazoquinoline 2-cyclopentyl-4-phenylamino-1H-imidazo[4,5-*c*]quinoline (DU124183), the pyridinylisoquinoline 4-methoxy-*N*-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide (VUF5455), and the amiloride analog 5-(*N*,*N*-hexamethylene)-amiloride] on the dissociation of the agonist radioligand, N^6-(4-amino-3-[^{125}I]iodobenzyl)-5′-*N*-methylcarboxamidoadenosine, were compared at wild-type (WT) and mutant A_3ARs. The F182A$^{5.43}$ and N274A$^{7.45}$ mutations eliminated the allosteric effects of all three modulators but had little effect on agonist binding. The N30A$^{1.50}$ and D58N$^{2.50}$ mutations abolished the allosteric effects of DU124183 and VUF5455, but not HMA, whereas the D107N$^{3.49}$ mutation abolished the effects of DU124183, but not HMA or VUF5455. The T94A$^{3.36}$, H95A$^{3.37}$, K152A^{EL2}, W243A$^{6.48}$, L244A$^{6.49}$, and S247A$^{6.52}$ mutations did not influence allosteric effects of the modulators. Sodium ions (100 mM), which modulate agonist binding at a variety of receptors, caused an ~80% inhibition of agonist binding in WT A_3ARs but did not show any effect on D58N$^{2.50}$, D107N$^{3.49}$, and F182A$^{5.43}$ mutant receptors. In contrast, NaCl induced a modest increase of agonist binding in N30A$^{1.50}$ and N274A$^{7.45}$ mutant receptors. NaCl decreased the dissociation rate of the antagonist radioligand [^{3}H]8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2.1-*i*]purin-5-one (PSB-11) at the WT A_3ARs, but not the D58N$^{2.50}$ mutant receptor. The results were interpreted using a rhodopsin-based molecular model of the A_3AR to suggest multiple binding modes of the allosteric modulators.

The G protein-coupled receptors (GPCRs) are a principal target of currently used drugs. Traditionally, the development of such drugs has been concentrated on optimizing the interaction of molecules with the binding site for endogenous agonists and competitive antagonists (orthosteric site). In addition to the orthosteric site, drugs can interact with binding sites on the receptor molecule that are distinct from the orthosteric site (i.e., allosteric sites) (Bruns and Fergus, 1990). Several lines of evidence suggest that allosteric modulators could offer advantages over orthosteric ligands (Christopoulos, 2002; Christopoulos and Kenakin, 2002), such as greater selectivity.

In the field of ion channel-coupled $GABA_A$ receptors, benzodiazepines as positive allosteric modulators have been used successfully in therapeutics. By contrast, directly acting agonists for $GABA_A$ receptors have not found therapeutic application because of their potential side effects (Macdonald and Olsen, 1994). The positive allosteric modulator for nicotinic receptors, galanthamine, has also been successfully used in the treatment of Alzheimer's disease (Olin and Schneider, 2002). In the field of GPCRs, it is encouraging that, in terms of therapeutic application, positive allosteric modulators for the calcium-sensing GPCR are now in clinical trials for the treatment of primary hyperparathyroidism (Conigrave et al., 2000).

Allosteric modulators of adenosine receptors (ARs) are of potential clinical use (Linden, 1997). Allosteric modulation of the A_1AR has been extensively investigated (Bruns and Fergus, 1990; Linden, 1997; Kourounakis et al., 2001). Allosteric modulation of the A_{2A}AR has also been reported (Gao and IJzerman, 2000). Allosteric modulators for the A_3AR, including DU124183, VUF5455, and amiloride derivatives, have recently been identified and characterized (Fig. 1) (Gao et al., 2001, 2002b, 2003). However, the identity of amino acid

ABBREVIATIONS: GPCR, G protein-coupled receptor; AR, adrenergic receptor; I-AB-MECA, N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; [^{3}H]PSB-11, 8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2.1-*i*]purin-5-one; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine; DU124183, 2-cyclopentyl-4-phenylamino-1H-imidazo[4,5-*c*]quinoline; HMA, 5-(*N*,*N*-hexamethylene-)amiloride; VUF5455, 4-methoxy-*N*-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide; TM, transmembrane helical domain; EL, extracellular loop; MD, molecular dynamics; MRS1220, 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-phenylacetamide; WT, wild-type.

residues in the A_3AR that are involved in the allosteric modulation has not previously been studied.

To identify amino acid residues that may participate in the response to allosteric modulators, we examined the roles of selected residues from transmembrane helical domains (TMs) 1, 2, 3, 5, 6 and 7 and the second extracellular loop (EL) of the human A_3AR by site-directed mutagenesis. The residues were selected based on proximity to the putative sodium binding site, the conserved DRY motif in TM3, or the putative ligand binding site (Jacobson et al., 2001).

Materials and Methods

Materials. The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). A monoclonal antibody (12CA5) against a hemagglutinin epitope and adenosine deaminase were obtained from Roche Molecular Biochemicals (Indianapolis, IN) and goat anti-mouse IgG antibody conjugated with horseradish peroxidase was from Sigma (St. Louis, MO). Oligonucleotides used were synthesized by Bioserve Biotechnologies (Laurel, MD). The vector pcDNA3 was purchased from Invitrogen (Carlsbad, CA). Human A_3AR cDNA was provided by M. Atkinson, A. Townsend-Nicholson, and P. R. Schofield (Garvan Medical Institute, Sydney, Australia) and was subcloned in pcDNA3 as pcDNA3/hA_3R. N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]I-AB-MECA; 2000 Ci/mmol) and [^{3}H]8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2.1-i]purin-5-one ([^{3}H]PSB-11) were from Amersham Biosciences (Buckinghamshire, UK); 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IB-MECA) and 5-(*N*,*N*-hexamethylene)amiloride (HMA) were from Sigma (St. Louis, MO). DU124183 and VUF5455 were from Leiden/Amsterdam Center for Drug Research (Leiden, The Netherlands). All the enzymes used in this study were obtained from New England Biolabs (Beverly, MA).

Site-Directed Mutagenesis. The protocols used were as described in the QuikChange site-directed mutagenesis kit (La Jolla, CA). Mutations were confirmed by DNA sequencing.

Numbering Scheme of GPCRs. The standardized numbering system of van Rhee and Jacobson (1996) was used to identify residues in the transmembrane domains of various receptors. Each residue is identified by two numbers: the first corresponds to the TM in which it is located, and the second indicates its position relative to the most conserved residue in that helix, arbitrarily assigned to 50. For example, His3.37 is the histidine in TM3 located 13 residues before the most conserved arginine, R3.50; Asn7.45 corresponds to Asn274.

Transient Expression of Wild-Type and Mutant Receptors in COS-7 Cells. COS-7 (African green monkey kidney) cells were grown in 100-mm cell culture dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μmol/ml glutamine. Cells were washed with phosphate-buffered saline containing calcium and then transfected with plasmid DNA (10 μg/dish) using the DEAE-dextran method (Cullen, 1987) for 1 h. The cells were then treated with 100 μM chloroquine for 3 h in culture medium and cultured for an additional 48 h at 37°C and 5% CO_2.

Membrane Preparation. After 48 h of transfection, COS-7 cells were harvested and homogenized using a Polytron homogenizer. The homogenates were centrifugated at 20,000*g* for 20 min, and the resulting pellet was re-suspended in 50 mM Tris-HCl buffer, pH 8.0, and stored at −80°C in aliquots. The protein concentration was determined by using the method of Bradford (1976).

[^{125}I]I-AB-MECA Binding Assay. For competitive binding assay, each tube contained 100 μl of membrane suspension, 50 μl of [^{125}I]I-AB-MECA (final concentration, 0.5 nM), and 50 μl of increasing concentrations of the test ligands in Tris-HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA. The mixtures were incubated at 25°C for 60 min. For dissociation kinetics, the protocols used were as we described previously (Gao et al., 2001). Briefly, [^{125}I]I-AB-MECA (0.5 nM) was preincubated with WT and mutant receptor membranes (8–20 μg protein) for 1 h at 25°C. Dissociation was started by adding 10 μM Cl-IB-MECA in the absence or presence of an allosteric modulator. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with 9 ml of ice-cold buffer. Radioactivity was determined in a γ-counter (5500B; Beckman Coulter, Fullerton, CA).

Binding of the Selective Antagonist, [^{3}H]PSB-11, to A_3ARs. Membranes (60–100 μg protein) were incubated with 8 nM [^{3}H]PSB-11 (Müller et al., 2002) at 25°C in a total assay volume of 400 μl for 60 min. Nonspecific binding was measured in the presence of 10 μM Cl-IB-MECA. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburgh, MD).

Statistical Analysis. Binding parameters were estimated using Prism software (GraphPAD, San Diego, CA). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng and Prusoff (1973) equation. Data were expressed as mean ± S.E.

Computational Methods. All calculations were performed on an SGI Octane workstation (SGI, Mountain View, CA). All ligand structures were constructed using the "Sketch Molecule" of SYBYL 6.8 (Tripos Inc., St. Louis, MO). A conformational search of allosteric modulators was performed by grid search, rotating in 60°, 180°, and −60° for all flexible bonds and 0 or 180° for the amide bond with Merck Molecular force field (MMFF94) and charge. After clustering the low-energy conformers from the result of the conformational search, the representative ones from all groups were reoptimized by semiempirical molecular orbital calculations using the PM3 method

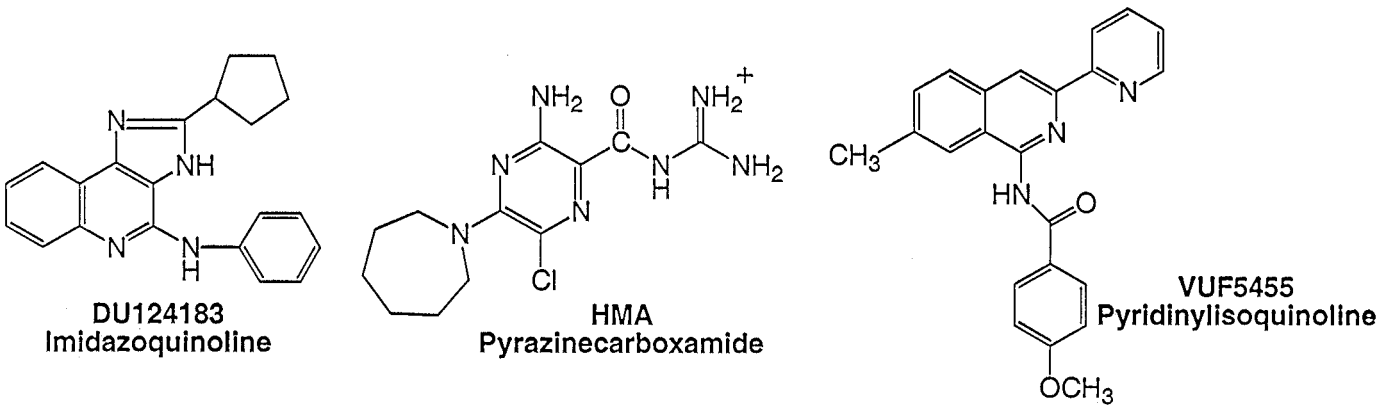

Fig. 1. Chemical structures of allosteric modulators.

in the MOPAC 6.0 package. Each of the lowest energy conformation from PM3 optimization was used for DISCO (DIStance COmparisons) computation (Martin et al., 1993), followed by default option.

A human A_3 receptor model was built using a homology modeling from the recently published X-ray structure of bovine rhodopsin (Palczewski et al., 2000) as we described previously (Gao et al., 2002a,c). The Amber all-atom force field with fixed dielectric constants as 4.0 was used for all calculations, terminating when the conjugate gradient was 0.05 kcal/mol/Å. For the conformational refinement of A_3AR, the optimized structures were then used as the starting point for subsequent 50-ps molecular dynamics (MD), during which the protein backbone atoms in the secondary structures were constrained as in the previous step. The options of MD at 300 K with 0.2-ps coupling constant were a time step of 1 fs and a nonbonded update every 25 fs. The lengths of bonds with hydrogen atoms were constrained according to the SHAKE algorithm (Ryckaert et al., 1977). The average structure from the last 10-ps trajectory of MD was reminimized with backbone constraints in the secondary structure and then without any constraints as described above.

Flexible docking was facilitated through the "FlexiDock" utility (Judson, 1997) in the Biopolymer module of SYBYL 6.8 (Tripos, St. Louis, MO). During flexible docking, the flexible bonds of allosteric modulators and the side chains of the putative binding site in the A_3AR were defined with rotatable bonds. After adding hydrogens to the receptor, atomic charges were recalculated using Kollman All-atom for the protein and Gasteiger-Hückel for the ligand. Default FlexiDock parameters were set at 3000-generation for genetic algorithms. To increase the binding interaction, the torsion angles of the side chains that directly interacted within 5 Å of the ligands according to the results of FlexiDock were manually adjusted. Finally, the complex structure was minimized using an Amber force field with fixed dielectric constant (4.0), until the conjugate gradient reached 0.1 kcal/mol/Å.

Results

Agonist and Antagonist Binding to WT and Mutant A_3ARs. Before studying the possible involvement of residues in the allosteric modulation, we first examined the agonist and antagonist binding properties of WT and mutant A_3ARs using an agonist radioligand, [^{125}I]I-AB-MECA, and an antagonist radioligand, [^{3}H]PSB-11. Several of the A_3AR mutations examined here (Table 1) were also studied previously (Gao et al., 2002a), whereas others were constructed based on predictions of molecular modeling and by homology to other GPCRs.

The potencies of the A_3 agonist Cl-IB-MECA and the A_3 antagonists MRS1220 and PSB-11 to displace [^{125}I]I-AB-MECA binding to WT and mutant receptors are summarized in Table 1. The N30A$^{1.50}$ and H95A$^{3.37}$ mutations induced a dramatic decrease in affinity for both the agonist Cl-IB-MECA and the antagonists MRS1220 and PSB-11, whereas the T94A$^{3.36}$ and F182A$^{5.43}$ mutations only induced a slight decrease of the ligand affinity. The D107N$^{3.49}$, K152A, and W243A$^{6.48}$ mutations did not significantly influence the agonist affinity but induced a 3- to 30-fold decrease of antagonist affinity. The N274A$^{7.45}$ mutation induced only a 3-fold affinity decrease of the agonist Cl-IB-MECA, whereas it induced a 500-fold affinity decrease of antagonist MRS1220. The D58N$^{2.50}$, L244A$^{6.49}$, and S247A$^{6.52}$ mutations did not cause a significant change of the ligand affinity. The M177A$^{5.38}$, V178A$^{5.39}$, S271A$^{7.42}$, and H272A$^{7.43}$ mutations led to loss of high-affinity binding of both the agonist [^{125}I]I-AB-MECA (0.5 nM) and the antagonist [^{3}H]PSB-11 (8 nM).

Effects of Mutations on the Rate of Dissociation of the Agonist Radioligand, [^{125}I]I-AB-MECA, from Human A_3ARs Expressed in COS-7 Cells in the Absence or Presence of Various Allosteric Modulators. The dissociation rates of [^{125}I]I-AB-MECA from WT and mutant human A_3ARs in the absence or presence of DU124183, HMA, and VUF5455 were examined. As shown in Fig. 2, the dissociation rate of [^{125}I]I-AB-MECA from the WT receptor was decreased by DU124183 (10 μM), HMA (100 μM), and VUF5455 (10 μM) to a similar extent, roughly by half. Surprisingly, F182A$^{5.43}$ and N274A$^{7.45}$ mutations completely abolished the allosteric effects of all three modulators. However, in the N30A$^{1.50}$ and D58N$^{2.50}$ mutant receptors, only HMA but not DU124183 or VUF5455 decreased the dissociation rate. In contrast to the D58N$^{2.50}$ mutation in TM2, the D107N$^{3.49}$ mutation in TM3 eliminated the allosteric effects of DU124183 but not HMA or VUF5455. The dissociation rates of [^{125}I]I-AB-MECA from T94A$^{3.36}$, H95A$^{3.37}$, K152A^{EL2}, W243A$^{6.48}$, L244A$^{6.49}$, and S247A$^{6.52}$ mutant receptors were reduced by the allosteric modulators by approximately half, similar to the WT receptor. The dissociation

TABLE 1

The competition of binding of a radiolabeled agonist [^{125}I]I-AB-MECA to WT and mutant human A_3 receptors by competitive ligands and allosteric modulators

Results were expressed as means ± S.E. from three independent experiments. M177A$^{5.38}$, V178A$^{5.39}$, S271A$^{7.42}$, and H272A$^{7.43}$ mutant receptors lost high-affinity binding of both agonist ([^{125}I]I-AB-MECA, 0.5 nM) and antagonist ([^{3}H]PSB-11, 8 nM).

	IC_{50}			K_i			K_d
	HMA	DU124183	VUF5455	Cl-IB-MECA	MRS1220	PSB-11	[^{125}I]I-AB-MECA
	μM			nM			nM
WT	1.8 ± 0.2	1.2 ± 0.3	2.8 ± 0.4	2.3 ± 0.62	1.6 ± 0.3	2.8 ± 0.9	1.2 ± 0.2
N30A$^{1.50}$	9.7 ± 2.2	6.2 ± 1.1	29 ± 11	226 ± 36	2761 ± 853	270 ± 68	9.8 ± 2.3
D58N$^{2.50}$	1.9 ± 0.4	1.3 ± 0.1	2.9 ± 1.1	4.6 ± 0.7	1.2 ± 0.5	2.5 ± 0.3	2.1 ± 0.3
T94A$^{3.36}$	2.2 ± 0.3	2.7 ± 0.7	6.4 ± 0.9	11.2 ± 2.5	10.1 ± 3.7	33 ± 12	4.3 ± 0.7
H95A$^{3.37}$	48 ± 23	36 ± 18	28 ± 12	60 ± 15[a]	976 ± 119[a]	1120 ± 270	5.7 ± 1.2[a]
D107N$^{3.49}$	2.0 ± 0.3	2.8 ± 0.4	9.7 ± 1.0	1.8 ± 0.4	9.2 ± 0.7	27 ± 8	0.55 ± 0.13
K152A^{EL2}	1.9 ± 0.3	3.2 ± 0.5	23 ± 71.	7 ± 0.6[a]	5.4 ± 0.7[a]	26 ± 7	3.7 ± 1.4[a]
F182A$^{5.43}$	2.4 ± 0.6	29.6 ± 7.3	2.9 ± 0.5	5.2 ± 0.8	2.7 ± 1.8	9.2 ± 2.1	3.5 ± 0.7
W243A$^{6.48}$	0.44 ± 0.07	3.6 ± 0.3	17 ± 5 2	2.9 ± 0.5[a]	45 ± 13[a]	44 ± 11	1.1 ± 0.3[a]
L244A$^{6.49}$	1.9 ± 0.3	2.1 ± 0.2	4.6 ± 0.5	2.5 ± 0.7[a]	0.63 ± 0.14[a]	2.3 ± 0.6	0.85 ± 0.11[a]
S247A$^{6.52}$	0.9 ± 0.3	1.0 ± 0.2	3.9 ± 1.2	2.2 ± 0.2[a]	3.1 ± 1.4[a]	2.9 ± 0.3	1.7 ± 0.3[a]
N274A$^{7.45}$	1.9 ± 0.3	2.4 ± 0.4	5.7 ± 1.0	7.0 ± 2.1	870 ± 220	144 ± 27	4.6 ± 1.3

[a] Data from Gao et al. (2002a).

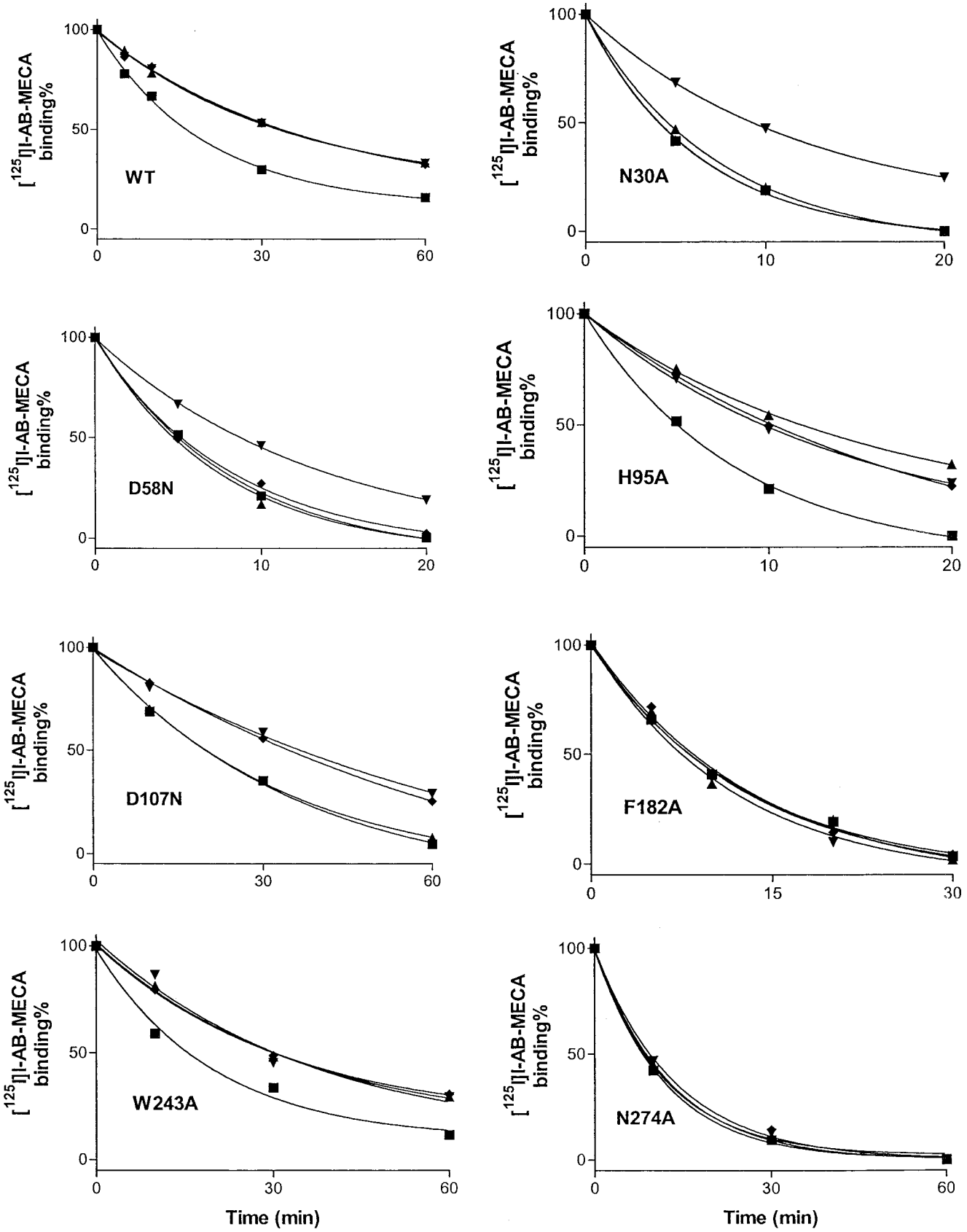

Fig. 2. The dissociation of the agonist radioligand [^{125}I]I-AB-MECA from WT and mutant ARs in the absence and presence of various allosteric modulators. (■, control; ▲, DU124183; ▼, HMA; ♦, VUF5455). The dissociation rates of [^{125}I]I-AB-MECA from N30A$^{1.50}$ and H95A$^{3.37}$ were determined at 4°C, whereas WT and other mutant receptors were measured at 25°C. The concentration of [^{125}I]I-AB-MECA was 0.5 nM. The mean k_{-1} values calculated from three independent experiments are listed in Table 2.

rates (k_{-1}) of [^{125}I]I-AB-MECA in the absence and presence of various allosteric modulators are summarized in Table 2.

Effects of Mutations on the Rate of Dissociation of the Antagonist Radioligand [^{3}H]PSB-11 from Human A_3ARs Expressed on COS-7 Cells in the Absence and Presence of Various Allosteric Modulators.[^{3}H]PSB-11 is a newly developed antagonist radioligand for A_3 receptors (Müller et al., 2002). Sodium ions (100 mM) exerted only slight effect on the equilibrium binding of [^{3}H]PSB-11 to A_3 receptors. The K_d values of [^{3}H]PSB-11 in the absence of and presence of sodium ions were 4.7 ± 0.8 and 3.8 ± 1.1 nM, respectively.

It was demonstrated in Fig. 3 that Na^+ (100 mM) decreased the dissociation rate, whereas HMA (100 μM) increased the dissociation rate of [^{3}H]PSB-11 from the WT A_3AR. The D58N$^{2.50}$ mutation abolished the effects of Na^+ (100 mM) but not HMA (100 μM). In contrast, the L244A$^{6.49}$ and S247A$^{6.52}$ mutations did not significantly influence sodium modulation. The dissociation rates (k_{-1}) of [^{3}H]PSB-11 in the absence and presence of various allosteric modulators are summarized in Table 3.

The effects of sodium ions and HMA on the dissociation of the antagonist radioligand [^{3}H]PSB-11 could not be determined for N30A$^{1.50}$, T94A$^{3.36}$, H95A$^{3.37}$, D107N$^{3.49}$, K152A^{EL2}, F182A$^{5.43}$, W243A$^{6.48}$, and N274A$^{7.45}$ mutant receptors, because of the affinity decrease of [^{3}H]PSB-11 in these mutant receptors. Hence, it was not determined whether these mutations also affected the modulatory effects by sodium ions and/or HMA on antagonist dissociation.

Effects of Sodium Ions on the Equilibrium Binding of the Agonist Radioligand [^{125}I]I-AB-MECA to WT and Mutant A_3ARs. As described above, the effects of sodium ions on the dissociation rate of the antagonist [^{3}H]PSB-11 from some mutant receptors could not be determined because of the loss of high-affinity antagonist binding. Alternatively, we examined the effect of sodium ions on the equilibrium binding of the agonist radioligand, [^{125}I]I-AB-MECA (0.5 nM), to WT and mutant receptors. As shown in Fig. 4, 100 mM NaCl induced an approximately 80% inhibition of the binding of [^{125}I]I-AB-MECA to WT receptors. The D58N$^{2.50}$, D107N$^{3.49}$, and F182A$^{5.43}$ mutant receptors were completely insensitive to 100 mM sodium ions, whereas N30A$^{1.50}$ and N274A$^{7.45}$ mutations induced a modest but significant increase of agonist binding in the presence of 100 mM NaCl. In the case of the T94A$^{3.36}$ and H95A$^{3.37}$ mutant receptors, 100 mM NaCl induced an approximately 50% inhibition of the agonist binding (Fig. 4). The effect of sodium ions in the S247A$^{6.52}$ mutant receptor was essentially the same as that in WT (Fig. 4). Similarly, the K152A^{EL2}, W243A$^{6.48}$, and L244A$^{6.49}$ mutations also did not significantly modify the modulatory effect of sodium ions. The percentage inhibition of [^{125}I]I-AB-MECA binding to these three mutant receptors by 100 mM NaCl was 73, 79, and 82%, respectively.

To distinguish between nonspecific effects of changing ionic strength and a specific effect of Na^+ (relative to K^+). The effect of equal concentration of NaCl and KCl (100 mM) on [^{125}I]I-AB-MECA binding was compared. As described above, 100 mM NaCl and 100 mM KCl induced ~80 and 20% inhibition, respectively, of the binding of [^{125}I]I-AB-MECA to WT receptors. Furthermore, 100 mM NaCl induced a significant decrease of the [^{3}H]PSB-11 dissociation rate (Table 3), but 100 mM KCl did not. The dissociation rates of [^{3}H]PSB-11 in the absence and presence of KCl were 0.23 ± 0.02 and 0.21 ± 0.04 min^{-1}, respectively, which were not significantly different.

Competition by Allosteric Modulators of the Binding of [^{125}I]I-AB-MECA to WT and Mutant Receptors. All allosteric modulators tested in this study competed for the binding of [^{125}I]I-AB-MECA to human A_3 receptors (Gao et al., 2001, 2002b, 2003). Thus, we examined the effects of mutations on this binding. The IC_{50} values are listed in Table 1. The N30A$^{1.50}$ and H95A$^{3.37}$ mutations induced a 3- to 30-fold decrease in affinity of the allosteric modulators. The D58N$^{2.50}$, T94A$^{3.36}$, D107N $^{3.49}$, L244A$^{6.49}$, S247A$^{6.52}$, and N274A$^{7.45}$ mutations induced only a slight reduction in the affinity of these allosteric modulators for ligand-unoccupied receptors. The K152A mutations did not influence the affinity of HMA but induced a 3- and 8-fold decrease of affinity of DU124183 and VUF5455, respectively. Interestingly, the W243A$^{6.48}$ mutation induced a 4-fold increase of the affinity of HMA (Fig. 5), but resulted in a 3- and 10-fold decrease of the affinity for DU124183 and VUF5455, respectively. Hence, the W243A$^{6.48}$ mutation selectively enhanced the affinity of the amiloride analog, HMA. To further confirm the unique binding properties of amiloride analogs at this mutant receptor, we further tested the effect of amiloride on WT and W243A$^{6.48}$ mutant receptors. The IC_{50} values of amiloride were 74 ± 12 and 36 ± 8 μM for WT and W243A$^{6.48}$ mutant receptors, respectively, consistent with the gain in affinity observed with the amiloride analog, HMA.

TABLE 2

Dissociation rates of the agonist radioligand [^{125}I]I-AB-MECA from WT and mutant receptors in the absence and presence of allosteric modulators

The dissociation rates of [^{125}I]I-AB-MECA from N30A$^{1.50}$ and H95A$^{3.37}$ were determined at 4°C (to slow the dissociation rate to measurable levels), whereas WT and other mutant receptors were measured at 25°C. The concentration of [^{125}I]I-AB-MECA was 0.5 nM. Results were expressed as means ± S.E. from at least three experiments

	Control	+ HMA (100 μM)	+DU124183 (10 μM)	+VUF5455 (10 μM)
WT	0.064 ± 0.007	0.028 ± 0.004	0.029 ± 0.005	0.025 ± 0.004
N30A$^{1.50}$	0.160 ± 0.036	0.083 ± 0.028	0.148 ± 0.028	0.159 ± 0.024
D58N$^{2.50}$	0.122 ± 0.017	0.064 ± 0.019	0.133 ± 0.026	0.122 ± 0.025
T94A$^{3.36}$	0.062 ± 0.008	0.038 ± 0.006	0.041 ± 0.004	0.034 ± 0.007
H95A$^{3.37}$	0.112 ± 0.011	0.070 ± 0.019	0.067 ± 0.021	0.055 ± 0.021
D107N$^{3.49}$	0.028 ± 0.006	0.014 ± 0.004	0.030 ± 0.003	0.013 ± 0.005
K152A^{EL2}	0.052 ± 0.005	0.028 ± 0.008	0.029 ± 0.009	0.027 ± 0.011
F182A$^{5.43}$	0.079 ± 0.010	0.085 ± 0.014	0.079 ± 0.012	0.074 ± 0.016
W243A$^{6.48}$	0.051 ± 0.008	0.027 ± 0.009	0.029 ± 0.002	0.031 ± 0.007
L244A$^{6.49}$	0.120 ± 0.020	0.068 ± 0.007	0.068 ± 0.008	0.062 ± 0.006
S247A$^{6.52}$	0.067 ± 0.006	0.035 ± 0.009	0.037 ± 0.008	0.035 ± 0.010
N274A$^{7.45}$	0.085 ± 0.010	0.076 ± 0.019	0.079 ± 0.015	0.083 ± 0.013

Molecular Modeling. The present mutagenesis results supported the definition of putative orthosteric binding sites for agonist and antagonist that were consistent with the previously reported A_3AR model (Gao et al., 2002a,c). Previous docking results suggested that L91$^{3.33}$, T94$^{3.36}$, H95$^{3.37}$, K152^{EL2}, Q167^{EL2}, F168^{EL2}, F182$^{5.43}$, F239$^{6.44}$, W243$^{6.48}$, L246$^{6.51}$, S247$^{6.52}$, N250$^{6.55}$, I268$^{7.39}$, S271$^{7.42}$, H272$^{7.43}$, and N274$^{7.45}$ lined the orthosteric site, being located within 5 Å of the putative binding sites for nonselective AR ligands NECA and CGS15943. An extension of the binding site delineated by the side chains of V141$^{4.56}$, M177$^{5.38}$, and V178$^{5.39}$ served as a binding region for both the N^6-substituent of Cl-IB-MECA and the benzyl group of MRS1220. The current mutational results indicated that M177$^{5.38}$, V178$^{5.39}$, N250$^{6.55}$, S271$^{7.42}$, and H272$^{7.43}$ may be critically involved in binding to A_3AR agonist and antagonist, consistent with the previous molecular model (Gao et al., 2002a,c). It should be noted that the loss of ligand binding may also be caused by the incorrect folding of the receptor, because the expression of these receptors on the cell surface was not determined. Mutation of L244$^{6.49}$ or S247$^{6.52}$ was without the effect on agonist/antagonist affinity, and these residues did not participate in direct interaction with the ligand according to the model, notwithstanding their proximity to the orthosteric site.

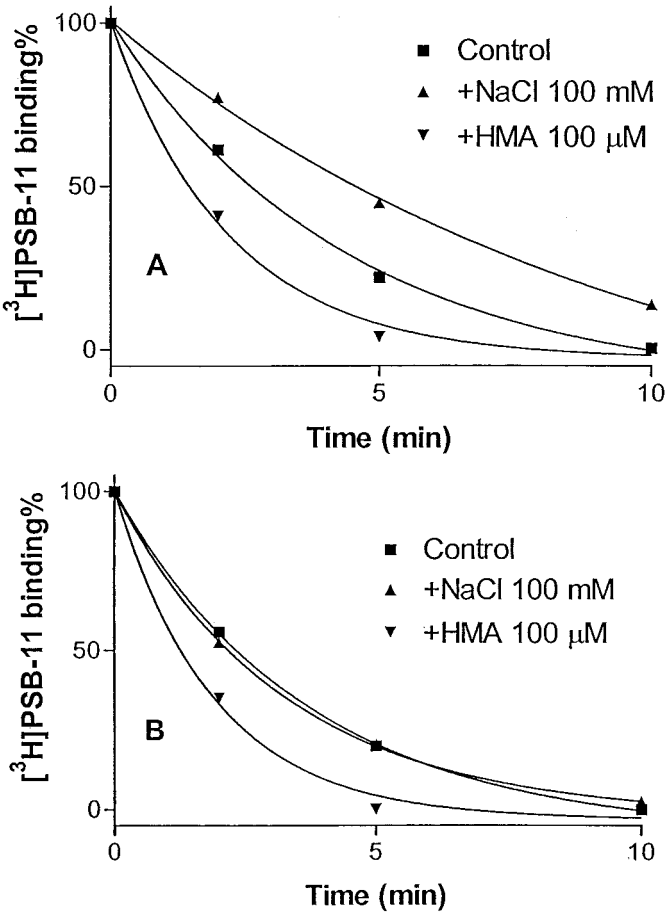

Fig. 3. The dissociation of the antagonist radioligand [^{3}H]PSB-11 from WT (A) and D58N (B) mutant ARs in the presence or absence of sodium ions and HMA. The final concentration of [^{3}H]PSB-11 used was 8 nM and the incubation temperature was 25°C. The mean k_{-1} values calculated from three independent experiments are listed in Table 3.

TABLE 3

Dissociation rates of the antagonist radioligand [^{3}H]PSB-11 from WT and mutant receptors in the absence and presence of allosteric modulators

Results were expressed as means ± S.E. from three independent experiments. The dissociation rates (k_{-1}) were determined at 25°C. The final concentration of [^{3}H]PSB-11 used in this experiment was 8 nM.

	k_{-1}		
	Control	+ HMA (100 μM)	+ Sodium Ions (100 mM)
	min^{-1}		
WT	0.23 ± 0.02	0.46 ± 0.05*	0.10 ± 0.04*
D58N$^{2.50}$	0.27 ± 0.04	0.52 ± 0.07*	0.31 ± 0.04
L244A$^{6.49}$	0.22 ± 0.04	0.45 ± 0.10*	0.11 ± 0.03*
S247A$^{6.52}$	0.24 ± 0.03	0.47 ± 0.10*	0.12 ± 0.02*

* Significantly different from control, $P < 0.05$.

The docking of allosteric modulators to the A_3AR was necessarily more complex, because multiple binding modes had to be considered. The effects of various Ala mutations on A_3AR binding affinity were generally less pronounced for the allosteric modulators than for the pure orthosteric ligands. The allosteric modulators DU124183 and VUF5455 generally showed similar effects in the inhibition of agonist binding at Ala mutant receptors, with the exception of F182A$^{5.43}$, of which mutation to Ala decreased selectively the affinity of DU124183. However, in the case of HMA, most of the mutant receptors except N30A$^{1.50}$ and H95A$^{3.37}$ displayed no significant effect on the binding affinity. In relation to DU124183 as a reference molecule, VUF5455 had more common overlap volume (130.6 Å^3) than did HMA (88.3 Å^3), as indicated by a similarity analysis using the DISCO software module, which identified matches among all active compounds based on the intermolecular distances between features. Thus, the binding sites of DU124183 and VUF5455 on the A_3AR may be more similar to each other compared with that of HMA. Therefore, we selected VUF5455 as the first allosteric modulator to be docked to the receptor at multiple potential binding sites. In the study of docking of VUF5455 to the unliganded A_3AR using the automatic docking program FlexiDock, the binding at the orthosteric site was energetically more favorable than the binding either at the presumed sodium binding site, near D58$^{2.50}$, or at the top of the TM7 region. However, in docking VUF5455 to the A_3AR/Cl-IB-MECA complex at either TM2 or TM7, the latter potential binding site at the top of the TM7 region, which was in contact with extracellular loops, was energetically more favorable (Fig. 6).

In the A_3AR model constructed from the X-ray structure of rhodopsin, several intramolecular TM networks stabilized the inactive conformation of the A_3AR through hydrophilic interactions as well as hydrophobic contacts. Similar H-bonding networks were found in rhodopsin and were also proposed to exist in the A_{2A}AR (data not shown). In the A_3AR, a group of hydrophilic amino acids (N274$^{7.45}$, S275$^{7.46}$, N278$^{7.49}$) surrounding and including D58$^{2.50}$ formed a H-bonding network (Fig. 7A). In addition, H-bonds to S97$^{3.39}$ and W243$^{6.48}$ also stabilized N274$^{7.45}$. Another hydrophilic residue, N30$^{1.50}$, which was near D58$^{2.50}$, formed a H-bond to the backbone of S275$^{7.46}$. Another H-bonding network evident in the A_3AR model involved the DRY motif at the cytosolic end of TM3 (Fig. 7B).

Discussion

We examined the effects on allosteric modulation and ligand binding of the mutation of amino acid residues of the human A_3AR that are hypothesized to be near one of three loci: the putative sodium binding site, the putative ligand binding site, and the DRY motif in TM3. Because it has been established that these three heterocyclic A_3AR allosteric modulators (DU124183, VUF5455, and HMA) also compete for radioligand binding at the orthosteric binding site, their affinities at WT and mutant receptors were examined. As for other heterocyclic antagonists (Gao et al., 2002a), the most substantial loss of affinity was observed for the H95A mutation (10- to 30-fold). Also, VUF5455 was selectively reduced in affinity at the N30A, D107N, K152A, and W243A mutant receptors. Conversely, DU124183 was selectively reduced in affinity at the F182A mutant receptor. Thus, the structurally diverse allosteric modulators have specific residues associated with the binding of each.

It was shown that certain residues contributed differently to ligand binding and to allosteric modulation either by heterocyclic derivatives or by sodium ions, suggesting a possible separation of the structural requirements for all of these processes. Most notably, the Ala substitution of Phe182$^{5.43}$ or Asn274$^{7.45}$ abolished the ability of all of the allosteric modulators, including HMA, DU124183, and VUF5455, to influence the agonist dissociation rate. Thus, although not critical for agonist binding, Phe182$^{5.43}$ and Asn274$^{7.45}$ were required in allosteric modulation.

Also, specific regions of the receptor were involved in ligand binding but seemed not to influence allosteric effects. For example, W243A$^{6.48}$ mutation caused a 4-fold increase of the affinity HMA for the unoccupied receptors, but it did not influence the effect of HMA on the dissociation of the agonist [^{125}I]I-AB-MECA. Concerning the mutations that evidently did not affect the allosterism, it must be noted that the effects of the allosteric ligands on the mutant receptors were studied at single concentrations of the compounds. Therefore, the present study can not rule out the possibility that a null mutation may in some instances be a counterbalancing effect on both parameters.

It was demonstrated that DU124183 (10 μM) inhibits [^{125}I]I-AB-MECA binding at WT by ~ 80% but only 10% at H95A mutant (Fig. 5B), yet the dissociation rates are halved for both WT and H95A mutant (Fig. 2). One explanation is that the mutation of H95 to Ala decreased the competitive binding potency but not the allosteric binding potency of

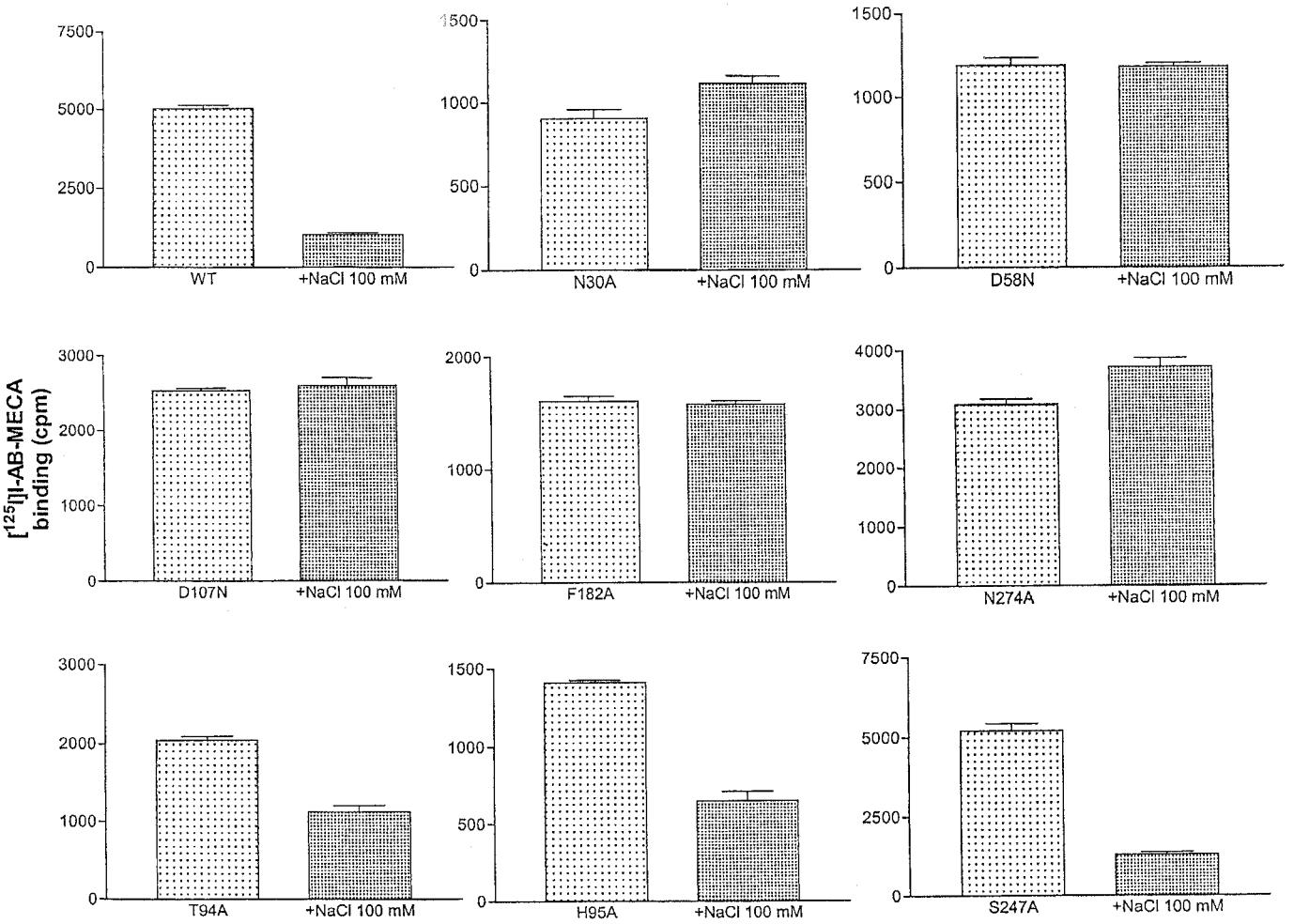

Fig. 4. Effect of sodium ions on the equilibrium binding of the agonist radioligand, [^{125}I]I-AB-MECA to WT and mutant ARs. [^{125}I]I-AB-MECA (0.5 nM) was incubated with membranes (8–20 μg of protein) from COS-7 cells expressing WT or mutant receptors in total assay volume of 200 μl of Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$ and 1 mM EDTA at 25°C for 60 min. Data were from three independent experiments performed in triplicate.

DU124183. Another possible reason is that the temperature dependence of the affinity of DU124183 in WT (measured at 25°C) and H95A mutant receptors (measured at 4°C) may be different.

As we discussed in relation to the role of the highly conserved W243$^{6.48}$ in activation of the A_3AR (Gao et al., 2002a,c), the indole side chain displayed a characteristic movement in the model exclusively upon docking of agonist, whereas the unaltered geometry of the inactive conformation in the A_3AR could accommodate the binding of antagonist. Consistent with N274$^{7.45}$ being constrained by the indole side chain of W243$^{6.48}$ in the inactive conformation and W243$^{6.48}$ participating in the hydrophobic interaction with the antagonist MRS1220 but not with the agonist Cl-IB-MECA, the N274A$^{7.45}$ mutation caused a dramatic affinity decrease for the antagonist MRS1220 but had little effect on binding of the agonist Cl-IB-MECA. Thus, mutation of those residues predicted in modeling to take part in the stabilizing the inactive conformation may also affect the binding of ligand indirectly.

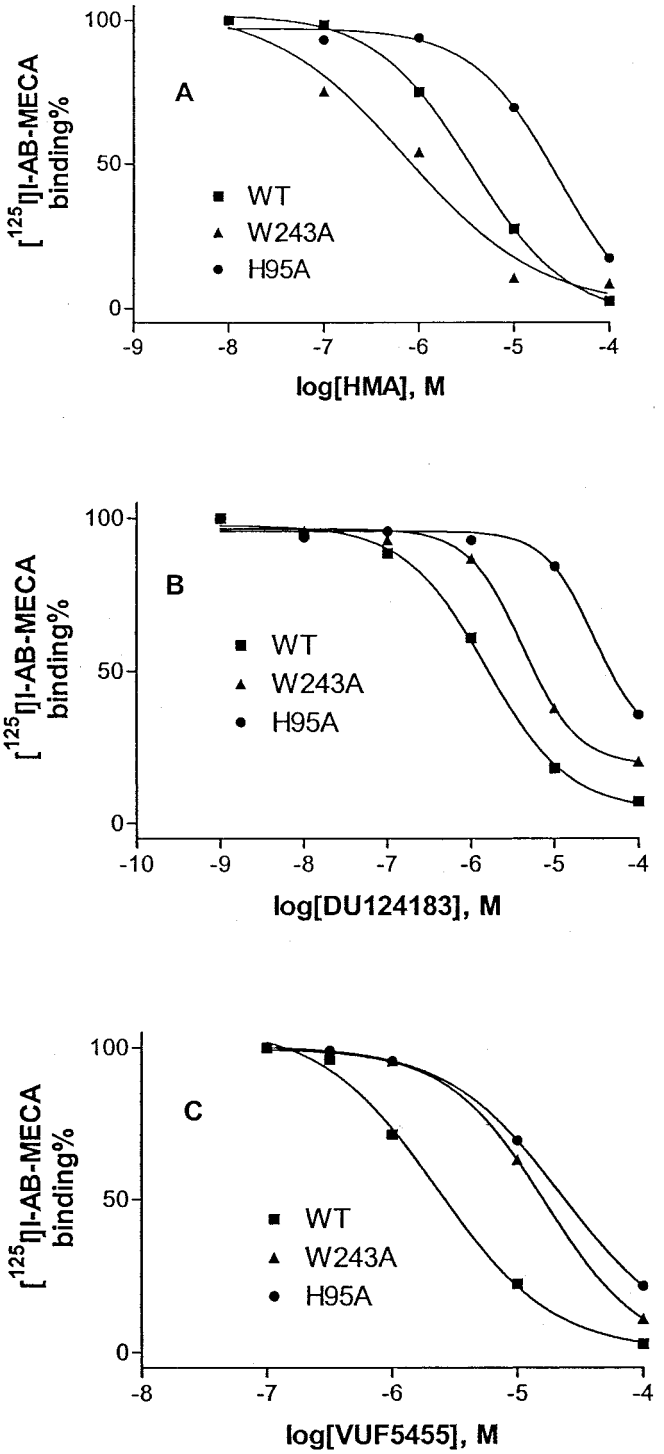

Fig. 5. Competition of allosteric modulators for the binding of [^{125}I]I-AB-MECA to WT and H95A and W243A mutant ARs. A, HMA; B, DU124183; C, VUF5455. The final concentration of [^{125}I]I-AB-MECA used was 0.5 nM. Data are from a representative experiment in duplicate. The K_i values listed in Table 1 are from three independent experiments in duplicate.

Molecular modeling and experimental results suggested that multiple binding modes of the allosteric modulators may be possible, considering their small size and low affinity compared with orthosteric ligands. Multiple binding/modulatory sites have already been proposed to be present in some other GPCRs. There seem to be two distinct allosteric sites on the M_1 receptor (Birdsall et al., 2001), and more than one allosteric site exists on the α_1-adrenergic receptor (Leppik and Birdsall, 2000). Some agonists (e.g., demox) also suggested the possibility of interaction with both orthosteric and allosteric sites of the M_2 muscarinic receptor (Angeli et al., 2002).

The possible location of the allosteric sites has been previously probed in several other GPCRs, although to a limited extent. In A_1ARs, a T277A$^{7.42}$ mutation both diminished agonist affinity and inhibited the effect of the A_1 receptor enhancer PD81,723 (Kourounakis et al., 2001). Distinct allosteric sites on the D_4 dopamine receptors have also been demonstrated by the study of sodium and zinc ions and methylisobutylamiloride (Schetz and Sibley, 2001). A single amino acid in TM7 has been found to be critical to the muscarinic receptor subtype selectivities of caracurine V de-

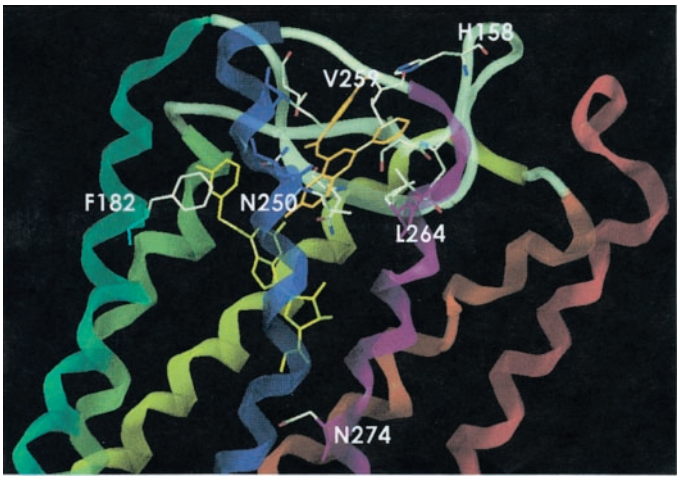

Fig. 6. Rhodopsin-based molecular model of the A_3AR, showing the previously defined docking mode of the A_3-selective agonist Cl-IB-MECA (colored according to atom type) and a putative allosteric binding site for VUF5455 (orange) on TM 7. Shown are selected residues near the allosteric modulator and residues F182 and N274, which were found to be important for the allosteric action of VUF5455. The secondary structure of each TM is displayed by ribbon using different colors, which indicate TMs 1, 2, 3, 4, 5, 6, and 7 with red, orange, yellow, green, cyan, blue, and purple color, respectively. The coordinates of this A_3AR model are available as from the Protein Data Bank ftp site under the name pdblo74.ent.Z. See http://www.rcsb.org/pdb/pdb_news2002.html#models_removal2 for more information.

rivatives and alkane-bisammonium ligands (Buller et al., 2002). In the M_1 muscarinic receptor, two residues, W101 and W400, are important for gallamine binding to the receptor, both in the unoccupied and in the antagonist (*N*-methylscopolamine)-bound state (Matsui et al., 1995). The latter residue may have a structural role, because it was important for the binding of ACh and number of antagonists. Studies of residues responsible for the m2 receptor selectivity of gallamine have used both chimeric and point-mutated receptors. Residues have been found in EL2 and EL3, especially the "EDGE" sequence in EL2 of M_2 muscarinic receptors (in M_1, the corresponding sequence is LAGQ) (Leppik et al., 1994; Gnagey et al., 1999). It has been suggested that the gallamine binding site in the M_1 muscarinic receptor is located in the region of the receptor structure close to W400 and the LAGQ sequence, and the cleft represents the access route to the binding site of ACh and other polar agonists and antagonists (Birdsall et al., 2001).

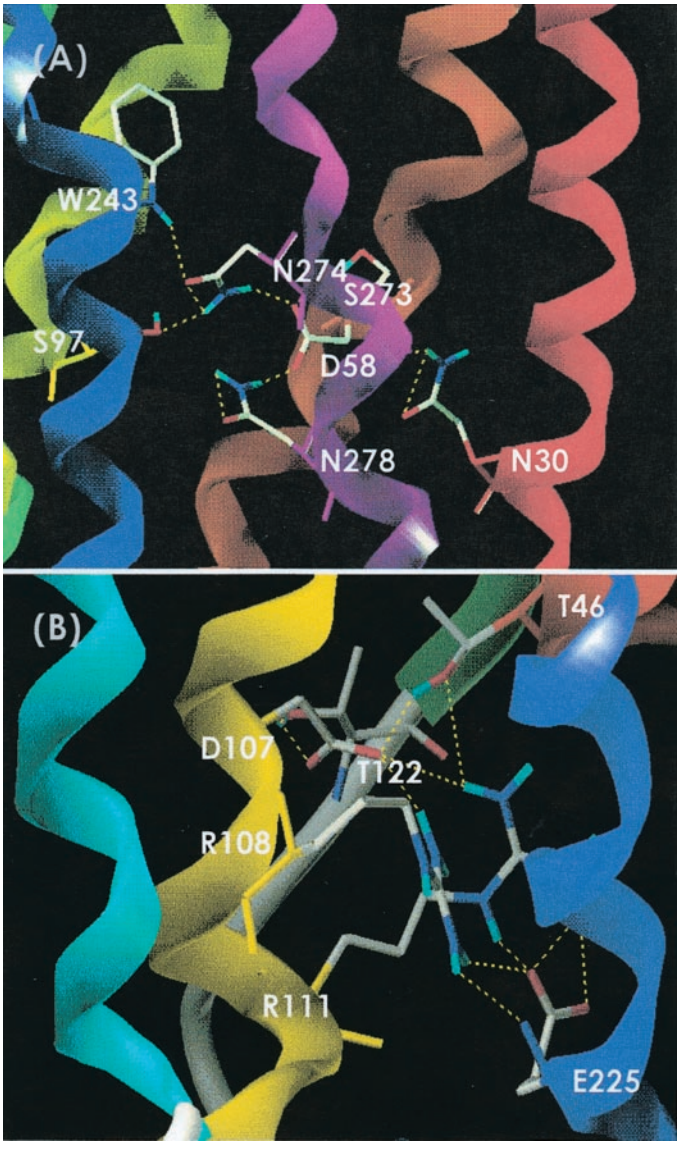

Fig. 7. Major interhelical H-bonding networks proposed for the hA_3AR based on the rhodopsin structure, in the $D58^{2.50}$ region (A) and involving the DRY motif (B). The H-bonds are represented in yellow.

Through the study of muscarinic allosteric modulators, it was suggested that the allosteric site comprises the extracellular contact points located above the orthosteric site (Christopoulos et al., 1998), which is itself postulated to be located in the upper third of the inner transmembrane pore (Wess, 1993). An allosteric binding site for various charged molecules may be near the orthosteric site of the muscarinic receptor but at a more extracellular level (Christopoulos and Kenakin, 2002). Thus, a relatively vacant region at the top of TM6 and TM7 of the A_3 adenosine receptor was searched for the possibility location of an allosteric binding site. An energetically stabilized mode of docking of VUF5455 to the A_3AR/Cl-IB-MECA complex at this region of TM7 was identified (Fig. 6).

Sodium ion binding to GPCRs is considered another form of allosteric modulation (negative) of the binding of agonists. The $Asp^{2.50}$ residue in TM2, which is conserved among GPCRs, is not generally important for ligand binding in the human A_3AR; however, it is critical in sodium modulation at this and many other GPCRs. NaCl (100 mM) caused an ~80% inhibition of agonist binding in the WT A_3AR but did not show any effect on $D58N^{2.50}$, $D107N^{3.49}$, and $F182A^{5.43}$ mutant receptors. NaCl decreased the dissociation rate of the antagonist radioligand [^{3}H]PSB-11 at the WT, but not the $D58N^{2.50}$ mutant receptor. $N30^{1.50}$ is not part of either the putative ligand binding site or the putative sodium binding site at $D58^{2.50}$, although it is in a H-bond network with the latter. Mutation of this residue caused a great decrease in affinity of both the agonist Cl-IB-MECA and the antagonist MRS1220, could play a role in stabilizing both ligand binding and sodium ion binding. Also, $D58^{2.50}$ and $N274^{7.45}$, which did not form part of the ligand binding site, seemed to be important in the H-bonding network involving $D58^{2.50}$. Furthermore, we found that $Asp^{2.50}$ is not the only residue associated with the modulatory effects of sodium ions. $Asp^{3.49}$, $Phe^{5.43}$, and $Asn^{7.45}$ are also critically involved in the allosteric effects of sodium binding at the human A_3AR. It should be noted that we have not modeled the sodium binding site, so we are unable to distinguish direct or indirect effects of mutations on sodium modulation.

The corresponding $Asp^{2.50}$ residue has been intensively studied in other GPCRs (Beukers et al., 1999). It has been demonstrated that this Asp residue is critically involved in sodium modulation in α_2-adrenergic receptors (Horstman et al., 1990), D_2 and D_4 dopamine receptors (Neve et al., 2001; Schetz and Sibley, 2001), the A_1AR (Barbhaiya et al., 1996), and neurotensin receptors (Martin et al., 1999). Here, we further demonstrated that in the A_3AR, both this residue and $Asn30^{1.50}$ are critically involved in the allosteric modulation by sodium ions but not by the amiloride analog HMA. Furthermore, $Asn30^{1.50}$ and $Asp58^{2.50}$ were also demonstrated to be involved in the modulation by DU124183 and VUF5455. Thus, the present results demonstrated that $Asn^{1.50}$, $Asp^{2.50}$, and $Asn^{7.45}$ are critically involved in the sodium modulation in the human A_3AR. Similar results were indicated for sodium effects on the D_2 dopamine receptors (Neve et al., 2001) suggesting that $Asn^{1.50}$, $Asp^{2.50}$, $Ser^{3.39}$, $Asn^{7.45}$, and $Ser^{7.46}$ might act in concert in sodium binding.

Homology modeling of the dopamine D_2 receptor has suggested the presence of a pyramidal sodium-binding pocket defined by residues $Asp80^{2.50}$, $Ser121^{3.39}$, $Asn124^{3.42}$, and

Ser420$^{7.46}$ at each vertex of the base and Asn423$^{7.49}$ at the apex (Teeter et al., 1994). N52A$^{1.50}$, N52L$^{1.50}$, and S121L$^{3.39}$ mutations profoundly altered the properties in the mutant receptors, which exhibited no detectable binding of radioligands, and S121A$^{3.19}$, S121N$^{3.39}$, S420A$^{3.46}$, and S420N$^{3.46}$ showed little or no dependence on sodium. With respect to the highly conserved (D/E)R(Y/W) motif in GPCRs, E134$^{3.49}$ in rhodopsin formed a salt-bridge with the guanidinium group of the adjacent R135$^{3.50}$ (Palczewski et al., 2000). The calculated A_3AR was stabilized by a number of interhelical H-bonds in a similar fashion: D107$^{3.49}$ H-bonded with T46^{IL1}, T122^{IL2}, R108$^{3.50}$, and R111^{IL2} (Fig. 7B). Thus, mutation of residues that are important for the intramolecular TM network may affect the stability of the inactive conformation and consequently pharmacological properties of the A_3AR. In the sodium experiment, the Ala mutant receptors of D58$^{2.50}$, D107$^{3.49}$, and F182$^{5.43}$ eliminated the modulation by sodium ions. It was suggested D58$^{2.50}$ and D107$^{3.49}$ might act as counter ions in direct sodium binding; however, F182$^{5.43}$, which is separated from the putative sodium binding pocket, might play an indirect role in stabilizing an activated conformational state or in cooperativity, interacting with the lipid membrane or in aromatic-aromatic interaction.

In the inactive conformation of the A_3AR, three amino acids, D58$^{2.50}$, D107$^{3.49}$, F182$^{5.43}$ were far from each other. Three distances between C_α atoms of D58$^{2.50}$ to D107$^{3.49}$, D58$^{2.50}$ to F182$^{5.43}$, and D107$^{3.49}$ to F182$^{5.43}$ were 22.8, 21.3, and 24.4 Å, respectively. Thus, binding of sodium could affect the overall conformation of the A_3AR. Depending on their class of allosteric modulators, DU124183 and VUF5455 binding affected the sodium binding site D58$^{2.50}$, whereas HMA affected only F182$^{5.43}$ and N274$^{7.45}$. Only DU124183 affected another anionic residue, D107$^{3.49}$, involved in sodium binding. The existence of multiple binding conformation was thus possible, depending on the allosteric modulator as suggested in ligand-selective receptor conformation.

In summary, the present study demonstrated that a number of residues in TMs are critically involved in ligand binding and/or allosteric modulation. It was demonstrated that not only Asp58$^{2.50}$, but also Asn30$^{1.50}$, Asp107$^{3.49}$, Phe182$^{5.43}$, and Asn274$^{7.45}$ are critically involved in sodium binding. Phe182$^{5.43}$ and Asn274$^{7.45}$ are not critically involved in agonist binding, but they are required for the allosteric modulation by the known A_3AR allosteric modulators.

Acknowledgments

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