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Determination of Amino Acid Residues that are Accessible from the Ligand Binding Crevice in the Seventh Transmembrane-Spanning Region of the Human A_1 Adenosine Receptor

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ABSTRACT

The substituted-cysteine accessibility method (SCAM) was applied to transmembrane span seven of the human A_1 adenosine receptor (hA_1AR) to reveal a subset of amino acids that are exposed to the ligand-binding crevice. The SCAM approach involved a systematic probe of receptor structure by individual substitutions of residues K265 (7.30) to R296 (7.61) with cysteine. In most cases, hA_1AR substituted-cysteine mutant membranes displayed antagonist dissociation binding constants that did not differ significantly from wild-type (WT). Radioligand binding assays were used to compare cell membranes that were treated with hydrophilic, sulfhydryl-specific methanethiosulfonate derivatives with control cell membranes. Position H278 was previously reported to be required for A_1AR ligand binding; however, that report did not establish that H278 represents a contact point for ligands. Cysteine-substitution at H278 yields membrane preparations with greatly decreased receptor density compared with WT membranes from cells in the same transfection experiment. However, H278C membranes retain a measurable fraction of antagonist binding. This observation allows for the investigation of binding-crevice accessibility at position 278 and suggests that H278 may not be required for binding of antagonist ligands. Our data reveal the binding-crevice accessibility of residues T270 (7.35), A273 (7.38), I274 (7.39), T277 (7.42), H278 (7.43), N284 (7.49), and Y288 (7.53) in the hA_1AR. These data are consistent with the high-resolution structure of bovine rhodopsin that features three α-helical turns in this region that are interrupted by an elongated, nonhelical structure from positions 7.43 to 7.48 in the primary amino acid sequence.

Adenosine receptors are members of the GPCR superfamily of cell surface receptor proteins. To date, four types of adenosine receptors (A_1, A_{2a}, A_{2b}, and A_3) have been cloned from a variety of species, including human (Tucker et al., 1994). Many functional studies have described the signaling roles of the different subtypes of adenosine receptor (Poulsen and Quinn, 1998). Extensive structure-activity relationships exist for ligands that were engineered to be selective for the various subtypes of the receptor (Linden, 1991). However, a clear picture of how these receptors bind their various agonist and antagonist ligands to transduce signals across the cell membrane has yet to emerge because of a lack of definitive structural evidence in the field. With the recent publication of an atomic-resolution crystal structure for bovine rhodopsin, there are promising new developments for the crystallization of GPCRs that may soon define the intricate molecular details of many members of this receptor protein class (Palczewski et al., 2000). Until these data begin to emerge, structural information may still be collected, albeit indirectly, through site-directed mutagenesis, biophysical probes, biochemical labeling, and pharmacological assays.

The recent application of SCAM to the dopamine D_2 receptor demonstrated that much information concerning the secondary structure of the TM-spanning regions of GPCRs, such as the identification of amino acid side chains that are accessible from the ligand-binding crevice, may be obtained in a

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ABBREVIATIONS: GPCR, G protein-coupled receptor; SCAM, substituted-cysteine accessibility method; NECA, 5′-*N*-ethylcarboxamidoadenosine; GppNHp, 5′-guanylylimidodiphosphate; [^{3}H]DPCPX, 8-cyclopentyl-1,3-di(2,3-^{3}H-propyl)xanthine; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTS-TEAH, [6-(triethylammonium)hexyl] methanethiosulfonate bromide; MTS-EDANS, sodium *N*-(methanethiosulfonylethylcarboxamidoethyl)-5-naphthylamine-1-sulfonic acid; WT, wild-type; HF, hexahistidine-FLAG-epitope-tagged; hA_1AR, human A_1 adenosine receptor; TM, transmembrane span; HE buffer, Na HEPES/Na_4EDTA buffer; K_D, dissociation binding constant; ANOVA, analysis of variance; MTS-X, methanethiosulfonate derivatives; K_I, inhibition binding constant; B_{max}, maximum receptor density.

systematic way (Javitch et al., 1995a,b; Fu et al., 1996; Javitch, 1998; Javitch et al., 1998, 1999). The SCAM method has also been used previously to study many other proteins, including the nicotinic acetylcholine receptor (Akabas et al., 1992, 1994a), the cystic fibrosis transmembrane conductance regulator (Akabas et al., 1994b), and the β_2-adrenergic receptor (Javitch et al., 1997).

In the present study, we have applied SCAM to the hA_1AR TM VII to reveal amino acids that are accessible to the ligand-binding crevice. This region of the receptor has previously been implicated in ligand binding through studies involving chemical modification of amino acid side chains (Klotz et al., 1988), analysis of receptor chimeras (Tucker et al., 1994), and site-directed mutagenesis (Olah et al., 1992; Townsend-Nicholson and Schofield, 1994; Tucker et al., 1994). Both chemical labeling of position H278 with the histidine-specific reagent diethylpyrocarbonate (Klotz et al., 1988) and a substitution with leucine (H278L) (Olah et al., 1992) were reported to dramatically reduce ligand binding. Data derived from site-directed mutagenesis at positions 270 and 277 indicated potential interactions with N^6-substituted agonists or C_8-substituted xanthine antagonists (270), and the nucleoside moiety of 5′-ribose-substituted agonists (277), respectively (Tucker et al., 1994). In addition, the hydroxyl moiety at position 277 in the hA_1AR was shown to be of particular importance for high-affinity 5′-substituted agonist (NECA) binding but not for the binding of antagonists (Townsend-Nicholson and Schofield, 1994).

The goal of the present study was to systematically identify the water-accessible subset of amino acids that may contribute to the hA_1AR ligand-binding crevice in the G protein-uncoupled state. We have attempted to bias the system toward the G protein-uncoupled state of the receptor by including 100 μM GppNHp, a nonhydrolyzable GTP-analog, in all assays involving agonist to target the low-affinity state of agonist binding. The A_1-selective antagonist [^{3}H]DPCPX has previously been reported to possess inverse agonist activity (Shryock et al., 1998). Although it is not a suitable radioligand for exploring the active conformation of the hA_1AR, this compound is a convenient choice for mapping binding crevice accessibility of amino acids in the G protein-uncoupled state. This initial identification of the potential ligand contact points in the uncoupled conformation of the receptor should provide important constraints for molecular modeling of the A_1AR and possibly other GPCRs.

Materials and Methods

The $HFhA_1AR$ cDNA in the pDoubleTrouble (pDT) vector was donated by Dr. Joel Linden (University of Virginia, Charlottesville, VA). Dr. Lee Limbird (Vanderbilt University, Nashville, TN) provided COS-M6 cells, developed by Edith Womack in the laboratories of Drs. Michael Brown and Joseph Goldstein (University of Texas Southwestern Medical Center, Dallas, TX). Methanethiosulfonate reagents were purchased from Toronto Research Chemicals (Ontario, Canada). The reagents utilized in this study were MTSEA, MTSET, MTSES, MTS-TEAH, and MTS-EDANS (Fig. 1). Miniprep DNA was purified on QiaPrep spin columns (Qiagen, Valencia, CA). Individual mutant cDNA constructs were amplified in DH5α and purified using Wizard MIDI and MEGA plasmid DNA prep kits (Promega, Madison, WI). Sequencing was conducted with the "BIGDYE" Terminator Cycle Sequencing Ready Reaction Kit (ABI-Prism, Warrington, Great Britian) and the reaction products were processed by the Genetic Medicine Sequencing Lab at Vanderbilt University (Nashville, TN). All DNA primers and synthetic oligonucleotides were synthesized in either the Genetic Medicine Lab or in the DNA Chemistry Core Facility at Vanderbilt University.

Tissue culture reagents were obtained from the following manufacturers: DMEM (Tissue Culture Core, Vanderbilt University, Nashville, TN); fetal bovine serum (Atlanta Biologicals, Norcross, GA); HEPES (EM Science, Gibbstown, NJ), Penicillin G/streptomycin, Na (Life Technologies, Grand Island, NY). [^{3}H]DPCPX had a specific activity of 127 and 123 Ci/mmol for respective lots that were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Theophylline, adenosine deaminase, and all other materials were purchased from Sigma (St. Louis, MO).

DNA Constructs. Substituted-cysteine mutants were generated one at a time using the N-terminal, double-epitope (hexahistidine-FLAG)-tagged (HF) WT hA_1AR cDNA as the template for mutagenesis. However, the primary amino acid sequence numbering from the epitope-free WT receptor has been used when referring to mutations at various amino acid positions. The recombinant epitope-tagged receptor displayed no appreciable differences in binding or signaling compared with natural WT receptor sources (Robeva et al., 1996). The lab, for continuity, commonly used $HFhA_1AR$ constructs to facilitate potential immunoprecipitation of the receptor protein in future experiments. This sequence was subcloned into either the pDT mammalian expression vector (Robeva et al., 1996) or into the pCMV4 mammalian expression vector (Andersson et al., 1989). The WT construct was then modified by the unique-site-elimination method (Zhu, 1996) of mutagenesis to contain a set of unique restric-

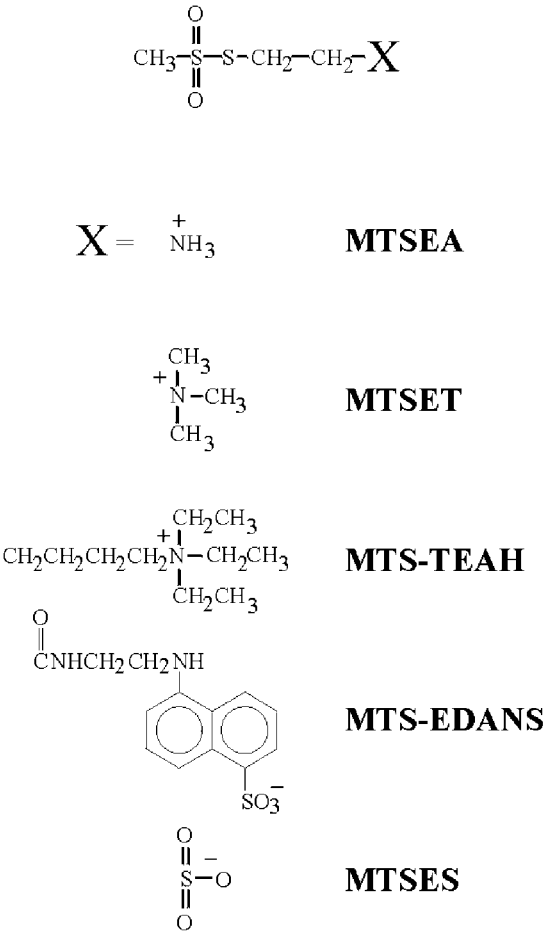

Fig. 1. Structures of MTS-X reagents. Chemical structures of MTS-X reagents contain groups of various size and charge for the purpose of probing structure in proteins that have been engineered to contain substituted-cysteine residues.

tion sites [*Pml*1 (*Bbr*P1), *Eco*RV, *Cla*I, *Bsp*E1, and *Afl*II] throughout the TMVII region of the primary cDNA sequence. These sites allowed for double-digestion of the WT construct to remove a fragment of approximately 30 to 50 base pairs in the desired region of TMVII. Various pairs of synthetic oligonucleotides containing the desired mutations, 'silent' diagnostic restriction sites, and ends that matched the corresponding digested vector backbone DNA were then ligated into the backbone fragment to generate H264C through R296C in the HFhA$_1$AR cDNA. This 'oligonucleotide replacement' strategy allowed for a more rapid generation and screening of the required substituted-cysteine mutant constructs than conventional mutagenesis kits or polymerase chain reaction approaches. Many mutants in the same region of the cDNA may be generated at the same time in parallel by incorporation of the desired synthetic oligonucleotides into the appropriately digested cDNA backbone. Restriction digests served as the primary screen for the incorporation of the desired mutations. Plasmid DNA samples from colonies that produced the expected restriction fragmentation patterns were purified and sequenced from the mid-TMV region of the cDNA sequence through the STOP codon.

Cell Culture and Harvest. Transient transfection of COS-M6 cells with substituted-cysteine mutant receptor constructs was accomplished using a modified DEAE-Dextran transfection protocol (Aruffo, 1998). Briefly, purified plasmid DNA was used in transfection experiments at concentrations of either 1.25 μg/ml or 2.50 μg/ml, depending upon the individual levels of receptor expression. COS-M6 monolayer cultures in 150-mm dishes were grown to approximately 80% confluence in sterile DMEM complete media (DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES, isotonic sodium, and 100 U/ml penicillin G/100 μg/ml streptomycin, Na). The transfection media consisted of DMEM (as above except with no serum); 0.4 mg/ml DEAE-dextran; 0.1 mM chloroquine; and the individual DNA constructs. Incubations were carried out in a humidified atmosphere at 37°C, 5% CO_2/95% air, without passage and media was changed after 48 h.

Crude cell membranes were harvested 80 to 112 h after transfection as described previously (Scholl and Wells, 2000) with the following additions. The final membrane pellets were resuspended in 1.0- to 1.5-ml cold 1 mM Na_4EDTA, 10 mM Na HEPES, pH 7.4 buffer (HE buffer) plus a 1:1000 dilution of protease inhibitors (20 mg/ml benzamidine in dH_2O; 2 mg/ml aprotinin and leupeptin in dH_2O; 2 mg/ml pepstatin A in dimethyl sulfoxide; and 17.4 mg/ml phenylmethylsulfonyl fluoride in isopropanol). Membrane protein levels were estimated from standard curves of a known amount of γ-globulin protein (0.2–0.8 mg/ml) using the method of Bradford (1976).

Radioligand Binding Assays. Standard radioligand binding assays were conducted in triplicate at concentrations near 1.2 nM, the observed K_D value for [^{3}H]DPCPX, an A_1-selective antagonist, at the WT HFhA$_1$AR. All reactions were normalized for membrane protein and contained 20 to 100 μg per tube. Membranes were diluted from thawed −80°C frozen stocks and treated with adenosine deaminase at 1 U of deaminase/80 μg of membrane protein for 20 min at 30°C. Nonspecific binding in the presence of 10 mM theophylline was subtracted from total binding to obtain the specific binding to receptors in each reaction. Individual reaction tubes contained 175 μl of HE buffer, 10 mM theophylline (nonspecific tubes only); 1.2 to 1.3 nM [^{3}H]DPCPX; and 50 μl of deaminase-treated membranes in a total volume of 250 μl. Binding reactions were processed as described previously (Scholl and Wells, 2000). Saturation binding assays were conducted at six concentrations per set of membranes that ranged from 0.1 nM to 6.0 nM [^{3}H]DPCPX. Specific cpm were converted to femtomoles or picomoles of receptor per milligam of membrane protein added and plotted versus concentration of free radioligand. Dissociation constants, K_D, and maximum receptor density, B_{max}, were determined using nonlinear regression in the one site binding model of the GraphPad Prism 2.01 software package (GraphPad Software, Inc., San Diego, CA). One-way ANOVA with Dunnett's post-test was applied to determine statistically significant differences from WT. Values of p less than 0.05 were considered significant results.

Treatments with MTS-X Reagents. Standard MTS-X (Fig. 1) treatments were performed in parallel for each substituted-cysteine mutant HFhA$_1$AR and WT HFhA$_1$AR. In each series of experiments, WT membranes served as negative controls because they displayed no significant inhibition by MTS-X. Positive control mutant membranes (A273C or I274C) were included in each assay as well to verify the activity of the reagents in the system. Deaminase-treated membrane dilutions were prepared as described above for each set of mutants. Membranes were divided into 320-μl aliquots in Eppendorf tubes corresponding to treatment and control groups for each set of WT and mutant membranes. Control groups were treated with dH_2O alone for 2 min and then placed on ice. MTS-X–treated groups received MTS-X reagents at the final concentrations of 2.5 mM MTSEA, 1.0 mM MTSET, 1.0 mM MTS-TEAH, 1.0 mM MTS-EDANS, and 10 mM MTSES, which reflect equally reactive concentrations with free thiols in solution (Stauffer and Karlin, 1994).

MTS-X reagents in powder form were stored in a desiccator at −80°C. Aliquots of stock aqueous solutions were thawed as necessary for reactions and were always kept on ice when not frozen at −80°C. Generally, hydrated MTS-X reagents were stable for several freeze/thaw cycles in this manner according to both their consistent activities in membrane assays as well as a direct spectrophotometric assay for MTS-X activity (Karlin and Akabas, 1998). Reactions were carried out at room temperature for 2 min and then placed on ice. Alternatively, reactions were diluted 60 to 100 fold in cold HE buffer in 40-ml tubes, centrifuged at 17,000 rpm in an SS-34 rotor for 25 min at 4°C, and resuspended in 400 μl of HE buffer, with similar results in most cases. Some of the more robustly inhibited mutants displayed marginally higher percentage inhibition values compared with controls without dilution, probably because of a longer exposure time to higher concentrations of MTS-X reagents.

Standard [^{3}H]DPCPX binding assays (at ~ 1.2 nM) were performed in triplicate on treated and control membranes for each group. The extent of reaction with MTS-X reagents was quantified by comparison of specific binding in treated membranes for each construct with the corresponding control membrane counts. This value was expressed as percentage inhibition and was equal to 100 × [1 − (specific cpm 'MTS-X-treated' / specific cpm 'control')]. One-way ANOVA with the Bonferroni post-test was used here to determine statistically significant differences in percentage inhibition values between each mutant tested and WT MTS-X–treated controls.

Protection from MTS-X Reagents. Protection experiments were performed in the following groups for each accessible mutant that had an inhibition signal strength greater than 14%. Group I (reaction controls) provided controls for membranes that received vehicle only and for membranes that were treated with only MTS-X reagents. Group II (washout controls) contained the control samples for a preincubation with either 500 μM theophylline or 200 μM NECA without subsequent exposure to MTS-X reagents. Group III (protection experiments) included membranes that had been pretreated with either 500 μM theophylline or 200 μM NECA and were then exposed to MTS-X reagents in the continuing presence of the protecting ligand. In each group, aliquots of 242 μl of deaminase-treated membranes were processed in parallel for each set of conditions. The final incubation volume for all experiments was 336 μl. This volume included 78 μl of HE buffer. Tubes of theophylline-treated membranes contained 500 μM theophylline. Tubes of NECA-treated membranes contained 100 μM GppNHp (to bias the receptor toward the G protein-uncoupled state) and 200 μM NECA. Before the treatment with MTS-X reagents, membranes were incubated at room temperature for 20 min in 40 ml centrifuge tubes with the protecting ligands (or buffer only). Subsequent reactions with MTS-X reagents were performed at room temperature for 10 min with the addition of either dH_2O (control) or the appropriate concentration of MTS-X followed by an approximate 100-fold dilution with 30 ml of ice-cold HE buffer. Results were expressed as percentage protection

(Table 3). The percentage protection equals 100 × (1 −[% Inhibition '+ protect ligand + MTS-X' / % Inhibition '+ MTS-X-HE Only']).

Results

Mutant HFhA$_1$AR H264C through R296C cDNAs were prepared and sequenced as described under *Materials and Methods*. These constructs were used to transiently transfect COS-M6 cells that were harvested to obtain crude cell membrane preparations containing the mutant receptor proteins. Saturation binding assays on mutant and WT membranes were performed with [^{3}H]DPCPX (see *Materials and Methods*) that yielded B_{max} values from 200 fmol of receptor/mg of protein to 2.5 pmol of receptor/mg of protein (Fig. 2). Dissociation constants for [^{3}H]DPCPX were indistinguishable from observed WT HFhA$_1$AR K_D values in most cases (Table 1). Of the 33 mutants that were tested, only three, Y271C, I274C, and T277C, had K_D values that differed statistically from the observed WT value.

The criteria used to determine the accessibility of substituted-cysteine residues from the aqueous ligand-binding crevice included the observation of a reproducible, irreversible inhibition (one that cannot be washed out with buffer) in membranes treated with MTS-X reagents versus control membranes that are not exposed to MTS-X. Additionally, incubation of mutant membranes with antagonist or agonist ligands both before and during treatment with MTS-X reagents should limit the extent of reaction of MTS-X with the exposed residues. This effect is observed as a decrease in the apparent inhibition in ligand pretreated membranes compared with membranes that are pretreated with HE buffer only. Both criteria for accessibility were satisfied for a subset of 6 to 10 of the substituted-cysteine mutants that were examined for each different MTS-X reagent. Neither of these criteria was satisfied for WT HFhA$_1$AR. Sensitivity to MTS-X reagents was reported as percentage inhibition of specific binding compared with corresponding sets of control membranes that were processed in parallel in each experiment (Fig. 3). The following substituted-cysteine mutants were significantly inhibited (Table 2): MTSEA-treatment (2.5 mM) inhibited binding to T270C, I272C, A273C, I274C, L276C, H278C, and Y288C (Fig. 3A). MTS-TEAH-treatment (1.0 mM) inhibited binding in the same mutants, except L276C, and also inhibited K265C, T277C, S281C, and N284C mutants (Fig. 3B). MTS-EDANS-treatment (1.0 mM) inhibited ligand binding of cysteine mutants at the same positions as MTS-TEAH with the exception of I272C and S281C (Fig. 3C). Percentage inhibition data involving treatments with 1.0 mM MTSET (Table 2) and 10 mM MTSES (data not shown) yielded fewer "hits" and less signal strength, respectively.

Protection experiments were attempted for all substituted-cysteine mutants that had an average inhibition of ligand

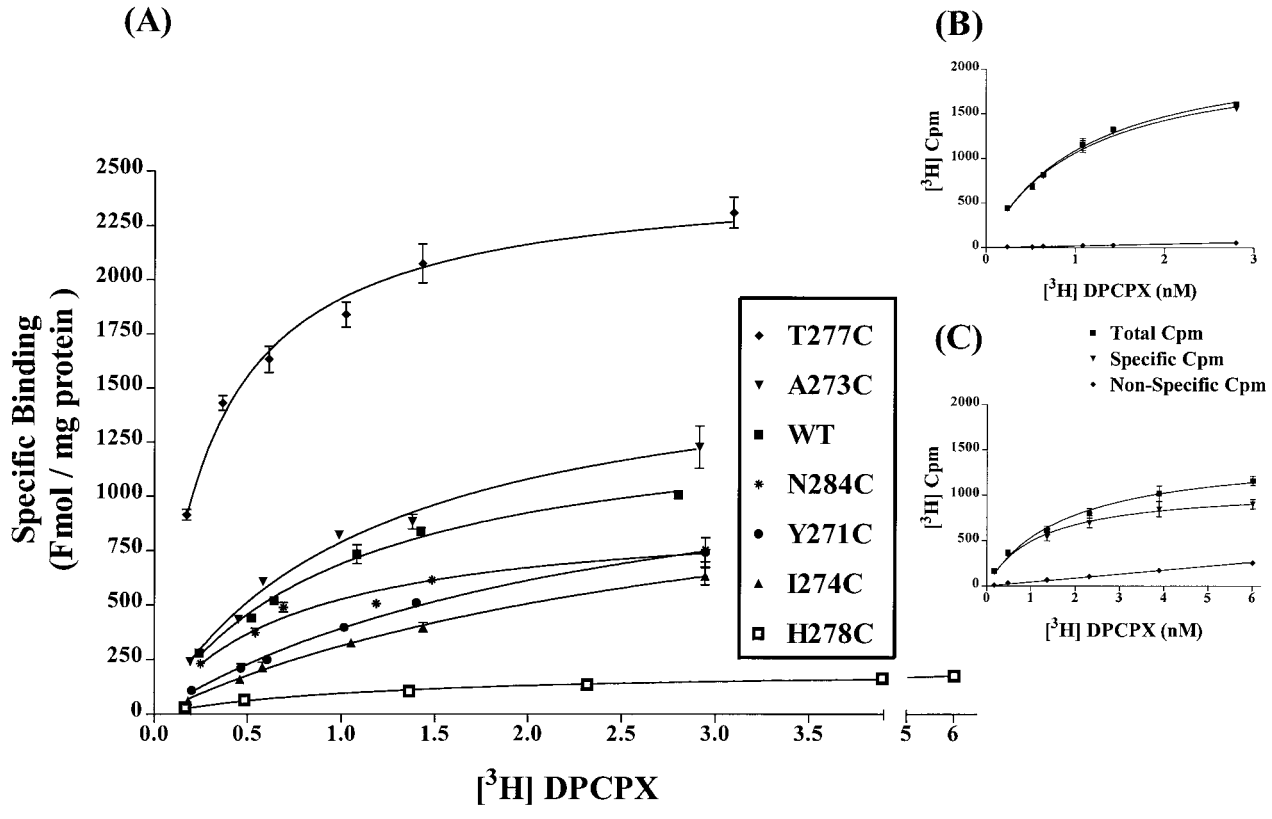

Fig. 2. [^{3}H]DPCPX saturation binding in HFhA$_1$AR TMVII substituted-cysteine mutant membranes. A, saturation binding curves for representative substituted-cysteine mutants displayed a range of B_{max} values for different groups of mutants; however, B_{max} values for different colonies of the same mutant gave similar results that varied minimally according to the day to day efficiency of transient-transfection experiments. Each saturation-binding curve was performed in triplicate and was representative of data collected for each mutant. Antagonist dissociation binding constant values for nearly all substituted-cysteine mutants were indistinguishable from WT by one-way ANOVA using Dunnett's post-test (see Table 1 and *Materials and Methods*). B, representative raw saturation data for COS-M6 cell membranes that contained WT HFhA$_1$AR are shown as total cpm, nonspecific cpm, and the resulting specific cpm curve. C, the mutant with the lowest B_{max}, H278C, is included in the inset for comparison of binding signal strength. This measurement was achieved using cells that were transfected with twice the concentration of the corresponding mutant DNA construct (compared with the amount of DNA in WT transfections) as well as membrane protein levels of 100 μg per tube in saturation binding experiments.

binding greater than 14%. Successful competition with MTS-X, an "irreversible" covalent inhibitor, by ligands that bind noncovalently and thus dissociate from the receptor, required large concentrations of protecting ligand and, often, less-than-saturating concentrations of MTS-X that still gave a repeatable, measurable signal. The initial ligand concentrations used in protection experiments were 500 μM theophylline and 200 μM NECA (approximately 200 times the K_I values for these compounds at WT HFhA$_1$AR (Scholl, 2000)). The initial MTS-X concentrations used were based upon the apparent sensitivities of each mutant to the various MTS-X reagents (Fig. 3), rather than approximate rate constants for each mutant/MTS-X combination, and were adjusted in each experiment, as necessary, to obtain stronger percentage inhibition signals or more easily detectable protection. Individual sets of membranes were treated with final concentrations ranging from 0.025 mM to 2.5 mM MTSEA, 0.5 mM to 1.0 mM MTS-TEAH, and 0.25 mM to 1.0 mM MTS-EDANS or a dH_2O control. MTSET-protection experiments included preincubation with the standard concentrations of protecting ligands and treatment with MTSET concentrations of 1.0 mM (A273C, N284C, and Y288C), 0.5 mM (T270C and H278C), and 0.05 mM (I274C) (Table 3). MTSES protection experiments included preincubation with the standard concentrations of protecting ligands and treatment with MTSES concentrations of 10 mM for A273C and 1 mM for I274C. We were unable to unambiguously demonstrate protection against 10 mM MTSES in H278C membranes because of a low percentage inhibition signal. Theophylline and NECA both caused substantial protection of A273C and I274C ligand binding (data not shown).

In most cases, both agonist and antagonist ligands substantially protected [^{3}H]DPCPX binding in the substituted-cysteine mutants from MTS-X with percentage protection values $>$ 50% (Table 3). The following exceptions were noted. Both ligands failed to substantially protect I274C from MTS-EDANS. Incubation of I274C membranes with concentrations as high as 2.0 mM theophylline or 1.5 mM NECA yielded no significant protection from 0.25 mM MTS-EDANS (Table 3). Also, the standard concentrations of protecting ligands were unable to produce a substantial protection from 0.5 mM MTS-TEAH at I274C (Table 3). Interestingly, protection of I274C from the less bulky MTSET and the even smaller MTSEA was much more robust (Table 3). NECA showed comparatively low protection levels from MTS-X of mutants T277C and beyond toward the extracellular end of the TM-span (Table 3). A 3- to 5-fold increase in percentage inhibition was observed in NECA-pretreated N284C and Y288C membranes that were exposed to 1.0 mM MTS-EDANS (Table 3). T270C membranes that were treated with 200 μM NECA displayed robust percentage protection values from MTSEA, MTSET, and MTS-TEAH (Table 3) in contrast with membranes treated with 500 μM theophylline (minimal percentage protection). Accessibility data are summarized in a helical net diagram (Fig. 4).

TABLE 1
[^{3}H]DPCPX dissociation binding constants
Dissociation binding constant averages (K_D values) were reported ± S.E.M. and were compared to WT K_D values using one-way ANOVA with Dunnett's post-test.

Construct	K_D	S.E.M.	N
	nM		
WT	1.24	0.07	6
H264C	1.36	0.03	3
K265C	0.84	0.07	3
P266C	0.97	0.02	3
S267C	1.29	0.10	3
I268C	1.70	0.15	3
L269C	0.95	0.05	3
T270C	1.24	0.12	3
Y271C**	2.61	0.52	3
I272C	1.00	0.11	3
A273C	1.09	0.19	6
I274C**	3.55	0.15	3
F275C	0.68	0.09	3
L276C	1.23	0.17	3
T277C**	0.35	0.07	3
H278C	1.26	0.20	4
G279C	1.65	0.05	3
N280C	1.21	0.21	3
S281C	1.13	0.26	3
A282C	1.08	0.05	3
M283C	0.95	0.15	3
N284C	0.70	0.06	3
P285C	1.10	0.19	3
I286C	0.91	0.05	3
V287C	1.39	0.18	3
Y288C	0.73	0.07	3
A289C	1.11	0.13	3
F290C	1.40	0.06	3
R291C	1.44	0.03	3
I292C	1.62	0.12	3
Q293C	1.20	0.06	3
K294C	1.39	0.10	3
F295C	1.42	0.16	3
R296C	1.11	0.12	3

**, significantly different from WT ($p < 0.01$).

Discussion

Recently, a study in which all endogenous hA$_1$AR cysteines were individually mutated to both serine and alanine revealed no significant differences in ligand binding compared with WT in TM-span cysteines (with the exception of C85S, which displayed rightward shifts of agonist competition binding curves) (Scholl, 2000). In our assays, MTS-X reagents did not significantly inhibit antagonist binding to WT HFhA$_1$AR. Therefore, mutant constructs H264C through R296C were generated using the WT HFhA$_1$AR cDNA as the template for mutagenesis. It is likely, given the current data, that the endogenous transmembrane cysteines are *not* exposed to the antagonist binding-crevice. Interpretation of SCAM data involved an initial pharmacological characterization of substituted-cysteine mutant receptor membranes. Receptors that bind A$_1$-selective antagonist [^{3}H]DPCPX with an approximate WT affinity should possess conformations that retain the various substituted-cysteine side chains in a WT arrangement. Because all mutants tested possessed K_D values that were within 4-fold of the WT HFhA$_1$AR binding constant, we may reasonably assume that accessible substituted-cysteine residues reported on the actual accessibility of the side chains that they replaced in the primary amino acid sequence.

Determination of the accessibility of each substituted-cysteine mutant was based on the standard SCAM criteria. Ligand binding in MTS-X-treated mutant membranes may be inhibited by the occupation or blockade of the binding site by the side chain of a reacted cysteine or, alternatively, by an alteration in the structure of the binding crevice itself in an MTS-X–labeled receptor. Because only aqueous-exposed cysteine side chains (thiolate anion form) react at an appreciable rate with charged, hydrophilic MTS-X (Karlin and Akabas,

1998), the ligand binding inhibition effect itself (regardless of mechanism) is evidence that the reaction occurred and that it occurred in an aqueous environment. Unless an aqueous compartment in addition to the ligand binding crevice exists in the largely hydrophobic transmembrane domain of the receptor and a modification at this hypothetical location causes a gross structural perturbation, inhibition of ligand binding serves as a reliable reporter for binding crevice accessibility of a substituted-cysteine residue.

A pattern of MTS-X inhibition was generally conserved across the panel of TMVII mutants that were tested. However, some variations in the inhibition patterns among the different regents were observed that were probably due in part to their differing sizes and in part to the local environment of each individual substituted-cysteine. For example, only the smallest MTS-X tested, MTSEA, caused an irreversible inhibition of I272C and L276C radioligand binding that was almost completely protected by the standard concentrations of theophylline and NECA (Table 3). This result was probably caused by the smaller size of MTSEA compared with the other reagents (Fig. 1) and its demonstrated ability at physiological pH to cross membrane interfaces and react with cysteines that the other reagents in this study cannot access (Holmgren et al., 1996). Consistent with this interpretation, a much bulkier analog of MTSEA, MTS-TEAH, showed only slight inhibitions of I272C and L276C ligand binding (Fig. 3B), one of which (I272C) was statistically significant (Table 2).

Other examples of the identification of various subsets of ligand binding crevice accessible residues included the significant inhibition of ligand binding at K265C, T277C, and N284C with only the larger MTS-X reagents (Fig. 4). Treat-

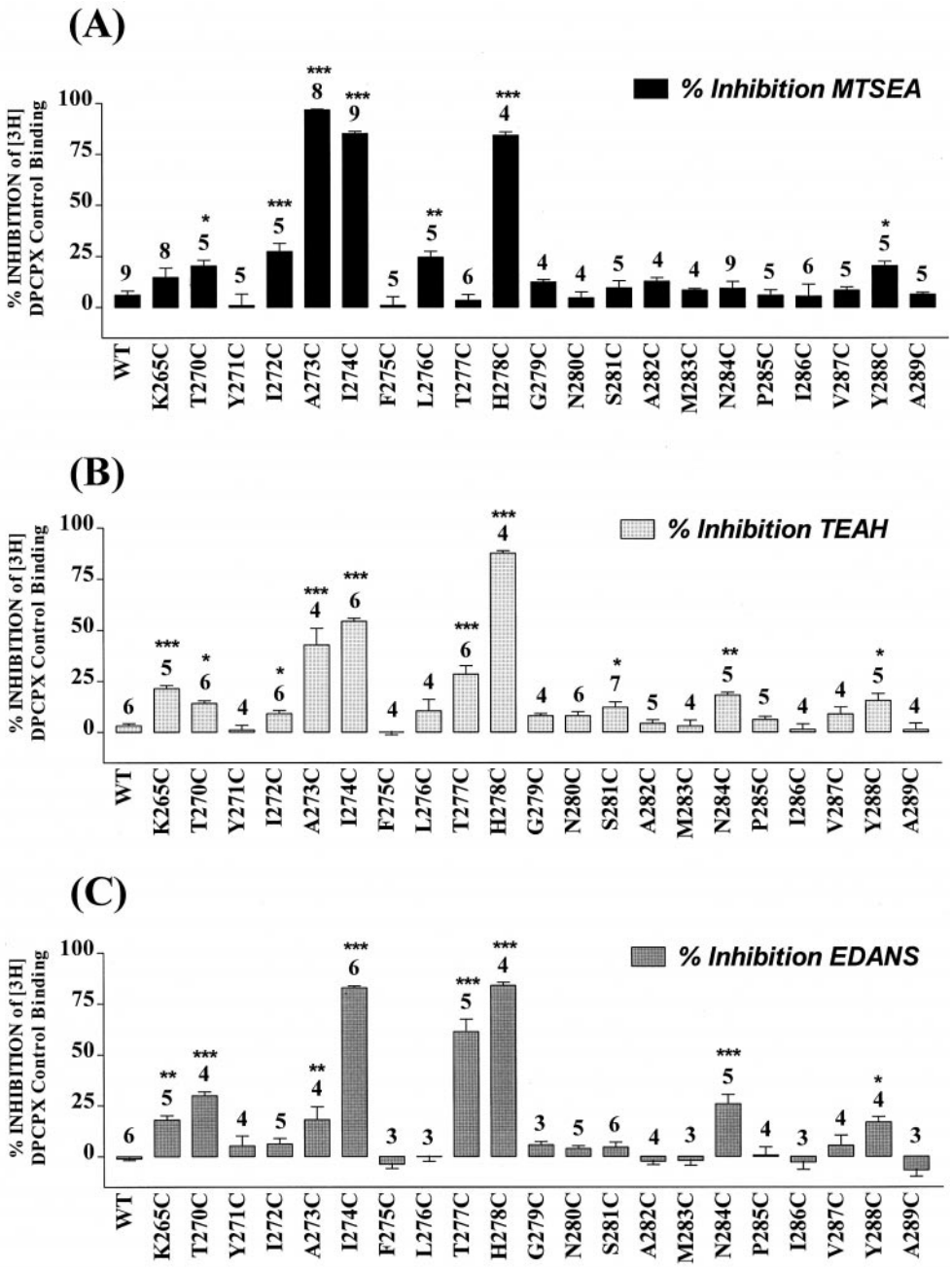

Fig. 3. Percentage inhibition of [^{3}H]DPCPX binding in TMVII substituted-cysteine mutants by treatment with MTS-X. Treatment of substituted-cysteine mutant membranes with 2.5 mM MTSEA (A), 1.0 mM MTS-TEAH (B), or 1.0 mM MTS-EDANS (C) produced inhibition of [^{3}H]DPCPX binding (see under *Materials and Methods*). Numbers of independent experiments are shown above the bars in bold. Values for $p < 0.05$ were considered statistically significant: * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$. Error bars represent the S.E.M.

ment with MTSET, MTS-TEAH, and MTS-EDANS, but NOT with MTSEA, significantly inhibited radioligand binding at N284C (Table 2). Cysteine mutants K265C and T277C had a significant irreversible inhibition of radioligand binding with only MTS-TEAH or MTS-EDANS-treatment (Table 2). The inhibition of T277C ligand binding by MTS-TEAH and MTS-EDANS was blocked by individual preincubation or 'capping' of T277C mutant membranes with 2.5 mM MTSEA, 1.0 mM MTSET, and 10.0 mM MTSES (data not shown). None of these smaller reagents yielded significant irreversible inhibition of binding in individual experiments with T277C membranes (Table 2). We infer from these data that this position was accessible to all five MTS-X. In light of the MTS-'capping' data for T277C, we also propose that a similar explanation accounts for the inhibition patterns observed at K265C and N284C. The lack of an observable effect with MTSEA, MTSET, and MTSES at these positions probably resulted from the reacted cysteine's being too small to interfere with [^{3}H]DPCPX binding, our only method of detecting a reaction with the mutant cysteine. Because larger MTS-X reagents were able to reveal accessibility, binding of larger radioligands may be disrupted by the smaller cysteine-MTS-X adducts and could offer a valid alternative approach to detect mutant residues that are accessible to smaller hydrophilic MTS-X but lie farther from the binding site.

Incubation with theophylline or NECA reduced the extent

TABLE 2

MTS-X percentage inhibition data

Data are expressed as percentage of inhibition (Y) and reflect specific binding in MTS-X-treated membranes compared with individual mutant membrane controls. Significant differences from WT are indicated by bold values. These residues were tested further with the corresponding MTS-X reagent in protection experiments. Other values had no statistical significance using the applied tests (see under *Materials and Methods*). Mutants H264C, P266C-I268C, G279C-M283C, and F290C-R296C had no significant inhibition of binding and were not shown.

Constructs	% Inhibition											
	EA			ET			TEAH			EDANS		
	Y	S.E.M.	*N*	Y	S.E.M.	*N*	Y	S.E.M.	*N*	Y	S.E.M.	*N*
WT	6.0	2.1	9	7.0	2.4	10	3.1	1.1	6	−1.1	0.8	6
K265	14.6	4.7	8	−3.6	7.1	5	**21.4**	**1.6**	**5**	**18.0**	**2.1**	**5**
T270	**20.2**	**2.9**	**5**	**24.1**	**4.2**	**5**	**14.1**	**1.4**	**6**	**29.9**	**1.9**	**4**
Y271	1.0	5.5	5	12.4	2.9	4	1.1	2.3	4	5.2	4.9	4
I272	**27.3**	**3.9**	**5**	12.0	3.7	4	**9.1**	**1.5**	**6**	6.1	2.8	5
A273	**96.8**	**0.3**	**8**	**43.1**	**7.8**	**10**	**42.8**	**8.2**	**4**	**18.2**	**6.4**	**4**
I274	**85.1**	**1.1**	**9**	**83.8**	**3.8**	**8**	**54.3**	**1.6**	**6**	**82.8**	**0.9**	**6**
F275	0.9	4.3	5	1.8	2.8	4	0.0	1.3	4	−3.8	2.2	3
L276	**24.6**	**2.8**	**5**	5.7	5.8	8	10.5	5.5	4	−0.1	2.3	3
T277	3.3	2.9	6	3.4	2.1	5	**28.6**	**3.9**	**6**	**61.3**	**6.2**	**5**
H278	**84.4**	**1.7**	**4**	**81.4**	**1.9**	**7**	**87.5**	**1.3**	**4**	**83.9**	**1.5**	**4**
N284	9.3	3.3	10	**20.4**	**3.1**	**6**	**18.1**	**1.3**	**5**	**25.8**	**4.6**	**5**
P285	5.9	2.7	5	4.8	0.8	5	6.1	1.5	5	0.7	4.0	4
I286	5.5	6.0	6	4.1	1.3	4	1.3	2.7	4	−2.7	3.7	3
V287	8.5	1.6	5	4.6	1.7	4	8.9	3.4	4	5.5	5.0	4
Y288	**20.4**	**2.2**	**5**	**16.3**	**3.1**	**4**	**15.5**	**3.6**	**5**	**16.8**	**2.7**	**4**
A289	6.4	1.0	5	2.0	2.4	4	1.2	3.2	4	−6.7	3.1	3

TABLE 3

MTS-X percentage protection data

This table logs percentage of protection (Y) for each mutant from each MTS-X reagent tested by treatment with theophylline and NECA. This value proved a convenient index to assess the relative amounts of protection that were observed across a spectrum of conditions that were tested for each mutant (see under *Materials and Methods* for experimental conditions).

	% Protection											
	EA			ET			TEAH			EDANS		
	Y	S.E.M.	*N*	Y	S.E.M.	*N*	Y	S.E.M.	*N*	Y	S.E.M.	*N*
Theophylline												
T270	37.9	5.2	3	7.0	3.7	3	13.9	13.9	3	33.0	12.5	4
I272	78.2	10.6	4									
A273	83.0	6.0	3	76.4	7.6	4	47.2	19.8	3	89.8	6.0	3
I274	53.4	6.8	4	87.9	8.1	3	11.5	8.2	4	2.7	2.0	3
L276	76.6	13.0	3									
T277							85.2	14.2	4	98.4	1.6	3
H278	49.8	3.4	3	58.5	5.5	3	66.7	10.5	3	48.7	13.4	3
N284				68.6	15.9	3	12.3	6.3	3	74.1	13.0	3
Y288	47.0	8.4	3	43.1	15.1	3	74.6	13.3	3	100.0	0.0	3
NECA												
T270	87.1	9.4	3	66.3	14.7	3	55.0	29.3	3	31.4	9.3	4
I272	97.3	2.7	4									
A273	93.0	6.1	3	73.7	10.3	4	95.2	4.8	3	100.0	0.0	3
I274	55.4	14.9	4	70.7	1.9	3	23.2	4.2	4	12.0	9.7	3
L276	91.9	8.1	3									
T277							46.8	6.1	4	32.0	5.8	3
H278	36.8	3.8	3	26.0	4.7	3	23.1	1.5	3	34.6	7.6	3
N284				40.6	10.7	3	81.8	9.1	3	Gain Inhib!		3
Y288	50.3	18.7	3	54.7	18.5	3	31.4	22.3	3	Gain Inhib!		3

of reaction of MTS-X with the receptor at standard assay concentrations for most of the mutants tested. For example, A273C was generally very well protected from MTS-X by standard concentrations of theophylline and NECA, as were I272C, L276C, and Y288C (Table 3). These results were consistent with a close proximity of each substituted-cysteine mutant tested to the ligand-binding site. However, we cannot be certain that any protected residues actually contact ligand.

Some exceptions to this protection were noted. Theophylline treatment was unable to substantially protect T270C ligand binding from MTS-X, perhaps because of the loss of an interaction site at the hydroxyl group with the theophylline (1,3-dimethylxanthine) structure. Alternatively, removal of the threonine hydroxyl group, a potential hydrogen bond donor or acceptor, from the binding crevice could destabilize, by an indirect effect, other important contact residues for theophylline in the binding site. However, ligand contact at this position is probably not essential for theophylline binding because the K_I value for theophylline in T270C membranes is shifted only 4-fold to the right compared with WT (data not shown). The T270C cysteine side chain may simply reside beyond the theophylline binding site that could be located deeper in the binding crevice.

Position 277 has been proposed as a potential contact point for NECA (Townsend-Nicholson and Schofield, 1994; Tucker et al., 1994) and position 278 was reported to be required for agonist and antagonist binding (Klotz et al., 1988; Olah et al., 1992). The present data reveal substantially greater protection from MTS-X by theophylline than by NECA at these positions (Table 3). These data are consistent with an important interaction with NECA in this region of the receptor. The replacement of threonine (277) or histidine (278) with cysteine caused an apparent loss of affinity of more than 200-fold, albeit immeasurable, for the 5′-substituted agonist NECA at each mutant in competition binding studies with [^{3}H]DPCPX (data not shown). These data accounted for the lower amounts of protection by NECA from MTS-X in T277C and H278C membranes compared with theophylline that incidentally had an unaltered K_I value in these two mutants compared with WT (data not shown).

The increase in percentage inhibition that was observed only in NECA-pretreated N284C and Y288C membranes that were exposed to the negatively-charged MTS-EDANS (Table 3) suggested a possible occurrence of a conformational change in the bottom third of TMVII. It is interesting that this effect does *not* occur in these mutants either in NECA preincubated membranes that were treated with MTS-TEAH, a bulky positively charged reagent, or in theophylline preincubated membranes that were treated with the bulky, negatively charged MTS-EDANS. Therefore, it is *not* the effect of simply a larger or a negatively charged MTS-X that produced this increased inhibition but a combination of both in the presence of a 5′-substituted agonist (and GppNHp). Future experiments will explore this result in greater detail at other TMVII residues that neighbor N284 and Y288 through the examination of binding in membranes that have been preincubated with a variety of adenosine receptor agonists and then treated with MTS-EDANS. Other future studies could examine possible shifts in accessibility of individual substituted-cysteine side chains upon G-protein coupling by using agonist preincubations in the presence of GTP and physiological Mg^{2+}. However, a truly definitive exploration of this type would include experiments with a constitutively active receptor form (Javitch et al., 1997), which at this time does not exist for the hA_1AR.

The inferred accessibility pattern for hA_1AR TMVII is in agreement with the recent X-ray crystal structure for bovine rhodopsin (Palczewski et al., 2000). If the most conserved residue in the TMVII region of GPCR structure, P285, is designated as position 7.50, the accessibility of individual amino acid residues can be compared across GPCRs. This indexing simplifies the identification of corresponding residues in comparisons and sequence alignments of different GPCRs (Fu et al., 1996). According to this nomenclature, positions 7.53, 7.49, 7.43, 7.42, 7.39, 7.38, and 7.35 are accessible to the ligand-binding crevice in both our present biochemical data and the X-ray structure for bovine rhodopsin that features three turns of α-helix interrupted by an elongated region of nonhelical primary sequence (7.43–7.48). In conclusion, we have used SCAM to identify a subset of the components of the A_1 adenosine receptor antagonist binding site (G-protein-uncoupled state) by demonstrating the binding crevice accessibility of amino acids from the TMVII region of this GPCR.

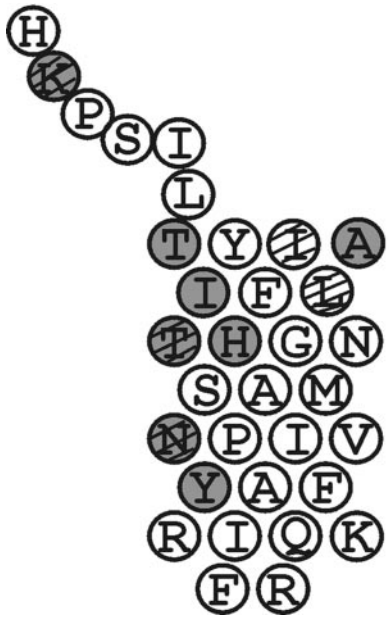

Fig. 4. hA_1AR TMVII helical net data summary. The accessibility data for MTSEA, MTSET, MTS-TEAH, and MTS-EDANS are summarized in this figure. Two-dimensional helical nets are convenient ways of quickly visualizing SCAM data. An idealized α-helix has been split down one side and stretched flat into two dimensions to generate a helical net. Because idealized helices make a turn every three to four residues, amino acids that are approximately one helical turn apart will line up down a single face in this schematic. Shaded residues were 'accessible' to all MTS-X tested; hatched residues were 'accessible' to MTSEA only; and shaded/hatched 'accessible' residues were identified by larger MTS-X only. Residues with a white background were not significantly inhibited by any MTS-X.

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