Structural and Electrostatic Properties of the 5-HT₃ Receptor Pore Revealed by Substituted Cysteine Accessibility Mutagenesis*

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5-HT₃ receptors are members of the Cys loop family of ligand-gated ion channels. We used the substituted cysteine accessibility method to identify amino acid residues in the channel forming domain, M2, that face the water-accessible surface and to locate their position in the ion conduction pathway. Cysteine was substituted for each residue, one at a time, in the M2 segment (Asp⁶²⁷–Asp⁶⁸⁸). 5-Hydroxytryptamine EC₅₀ values for functional mutants revealed 11 residues to be water-accessible, with a pattern consistent with an α-helix except at Leu⁶⁸⁵ and Leu⁶⁸³. The data suggest that charge selectivity begins at a more cytoplasmic level than Val⁶⁸¹. Modification at some positions (Val⁶⁸¹, Leu⁶⁸³, Ile⁶⁸⁴, Leu⁶⁸⁷, and Ser⁶⁸⁶) resulted in channels that were locked open. Reaction rates with accessible cysteines were voltage-dependent at some residues, suggesting that access occurs via the ion channel. Overall the data observed are similar but not identical to that reported for other members of the family and confirms the high degree of structural and functional homology between receptors in the Cys loop receptor family.

The 5-HT₃ receptor is a member of the Cys loop family of ligand-gated ion channels, which includes nicotinic acetylcholine (nACh), GABA₃ and glycine receptors. These receptors are pentamers, usually formed by the co-assembly of one to four different subunits each with a large extracellular N-terminal region and four putative transmembrane domains (M1–M4). Two 5-HT₃ receptor subunits, 5-HT₃A (1) and 5-HT₃B (2), have been identified so far, and receptors can function as either homo-oligomeric (A only) or hetero-oligomeric receptors (2). Evidence suggests that the Cys loop family of receptors is modular in design, with the extracellular N-terminal domain containing the ligand binding site and the transmembrane regions containing the pore (3). There is good evidence from a variety of studies that the second transmembrane segment, M2, lines the channel (4). Studies on acetylcholine receptors, for example, have identified rings of residues that alter conductance (5) or the selectivity among monovalent (5, 6) or divalent (7) cations or channel gating (8). The high resolution structure of a protein homologous to the extracellular domain of the acetylcholine receptor was recently determined (9); however, so far details of the complete structure of any of this family of receptors are lacking.

The substituted cysteine accessibility method (SCAM) has been used to identify systematically the residues that line an ion channel. Here residues in a membrane-spanning segment are individually mutated to cysteine and each mutant receptor expressed in Xenopus oocytes. If the mutant receptors have similar properties to wild type, it can be assumed that their structure is similar to that of wild type. The accessibility of each residue can then be determined by examining the ability of small sulfydryl-specific reagents to react with the cysteine. The information gained is able to provide information on the secondary structure of channel-lining segments and the location of ion channel gates and selectivity filters and to map binding sites within the channel (10). SCAM has been used to identify pore-lining residues in a variety of ion channels, including the nACh and GABA₃ receptors. The M2 regions in these receptors gave a similar but not identical pattern of labeling and supported previous studies suggesting that this region is largely α-helical. There are, however, some discrepancies, particularly in the region surrounding the conserved central leucine residue.

The amino acid sequence of the 5-HT₃A receptor subunit displays strong sequence similarity with nACh receptor subunits, especially in the M2 region (e.g. the α1 nACh receptor subunit as illustrated in Fig. 1). We therefore wanted to confirm that the water-accessible residues in this receptor are similar to those in the nACh and GABA₃ receptors and also explore whether the use of homomeric receptors could provide additional information about the structure and function of M2. The data revealed a similar but not identical pattern of water-accessible residues in the pore to those previously observed for other members of the Cys loop ligand-gated ion channel family and also provided new information about the structure and role of certain residues located in and around the pore.

EXPERIMENTAL PROCEDURES

**Drugs and Reagents—**The sulfydryl reagents 2-[trimethylammonium](2-[trimethylammonium](methyl) methanethiosulfonate bromide (MTSET) and sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) were obtained from Biotium, Inc. (Haywood, CA). To minimize hydrolysis of these reagents, stock solutions in water were made daily, stored on ice, and diluted in buffer to the appropriate concentration just before use. All other reagents were of the highest quality.
Mutagenesis and Preparation of cRNA and Oocytes—Mutant 5-HT\textsubscript{3A} receptor subunits were developed using the eukaryotic expression vector pcDNA 3.1 (Invitrogen, Abingdon, UK) containing the complete coding sequence for the 5-HT\textsubscript{3A} subunit from NIE-115 cells as described previously (11). Mutagenesis reactions were performed using the Kunkel method (12) and confirmed by DNA sequencing. Wild type (WT) and mutant receptor subunit coding sequences were then subcloned into pGEMHE plasmid (13). This was linearized with HindIII and mutant 5-HT\textsubscript{3} receptors with 1 mM MTSET or 10 mM MTSES were prepared with the irreversible effect of the reagent. The effect of the reagent was taken as shown in the following equation.

\[ \frac{[I_{5-HT\text{MAX}} \text{after}]}{[I_{5-HT\text{MAX} \text{before}}]} - 1 \]  

(Eq. 2)

Data for each mutant were compared with wild type by one-way analysis of variance applying the Dunnett post-hoc test \((p < 0.05)\) using Prism v3.0 (Graphpad Software Inc, CA). We infer that the sulfydryl reagents have reacted with a cysteine if the subsequent 5-HT-induced currents are significantly different than the initial 5-HT-induced currents. The MTS reagents react 5 \times 10^9 times faster with ionized thiolates than with thiols (17). We assumed that only cysteines on the water-accessible surface of a protein ionize to any significant extent; thus we inferred that reactive cysteines were on the water-accessible surface of the protein. In addition, MTSET\textsuperscript{+} and MTSES\textsuperscript{−} are membrane-impermeant (18, 19), and thus when we applied them extracellularly they only had access to residues that were on the extracellular water-accessible surface of the protein.

Determination of Reaction Rate Constants—Rate constants for reaction of MTSET and MTSES with substituted cysteines were determined as described previously (20). Briefly, second order rate constants for MTSET were obtained using a voltage-step protocol as shown in Fig. 2C. Current because of leak and the effect of desensitization were subtracted from the currents recorded during application of 5-HT plus MTSET. The decrease in current was approximately linear, and the second order rate constant \((k)\) was estimated from the following equation.

\[ k = \frac{(I_2 - I_1)x}{(t_2 - t_1)} \]  

(Eq. 3)

where \(I\) is the current, \(t\) is time, \(x\) is the concentration of sulfydryl reagent, and the subscripts refer to the beginning and end of each voltage-step interval.

Reaction with MTSES caused a large and irreversible decrease in inactivation rate; therefore we used a different protocol to determine the rate of reaction with this reagent. The following sequence of reagents was applied repeatedly (see Fig. 7A): 5-HT\textsubscript{MAX} 20–30 s, CFFR 5 min, 5-HT\textsubscript{MAX} plus sulfydryl reagent 1 min, CFFR 10 min, 5-HT\textsubscript{MAX} 30 s, CFFR 5 min, 5-HT\textsubscript{50} 30 s, CFFR 5 min, 5-HT\textsubscript{MAX} 30 s, CFFR 5 min, 5-HT\textsubscript{50} 30 s, and CFFR 5 min. Average currents elicited by 5-HT\textsubscript{MAX} and 5-HT\textsubscript{50} before and after application of sulfydryl reagent were compared with the irreversible effect of the reagent. The effect of the reagent was taken as shown in the following equation.

\[ \frac{[I_{5-HT\text{MAX} \text{after}]}{[I_{5-HT\text{MAX} \text{before}}]} - 1 \]  

(Eq. 2)

The positively charged -SCH\textsubscript{2}CH\textsubscript{2}N(CH\textsubscript{3})\textsubscript{3} moeity is added to cysteine following reaction with MTSET. Following reaction with MTSES the negatively charged -SCH\textsubscript{2}CH\textsubscript{2}SO\textsubscript{3} group is added to cysteine. The reactions of wild type and mutant 5-HT\textsubscript{3} receptors with 1 mM MTSET or 10 mM MTSES were tested using alternating pulses of maximal (5-HT\textsubscript{MAX}) and EC\textsubscript{50} (5-HT\textsubscript{50}) concentrations of 5-HT before and after addition of the sulfydryl reagent. Changes in the peak current induced by the EC\textsubscript{50} 5-HT test pulses are more sensitive to effects of modification on gating kinetics, whereas changes in the peak current induced by the maximal 5-HT test pulses are more sensitive to the effects of modification on conductance (16).

Thus a typical protocol would be: 5-HT\textsubscript{MAX} 30 s, CFFR 5 min, 5-HT\textsubscript{50} 30 s, CFFR 5 min, 5-HT\textsubscript{MAX} 30 s, CFFR 5 min, 5-HT\textsubscript{50} 30 s, CFFR 5 min, 5-HT\textsubscript{MAX} plus sulfydryl reagent 1 min, CFFR 10 min, 5-HT\textsubscript{MAX} 30 s, CFFR 5 min, 5-HT\textsubscript{50} 30 s, CFFR 5 min, 5-HT\textsubscript{MAX} 30 s, CFFR 5 min, 5-HT\textsubscript{50} 30 s, and CFFR 5 min. The experiment was repeated several times.
at each holding potential. Because the reagent was in excess, we determined the second order rate constant by fitting the peaks from the test applications of 5-HT to the following equation.

\[ k_{2} = \frac{I_{f} - I_{i}}{I_{i}(1 - e^{-k_{t}t})} \]  

(Eq. 4)

where the subscripts refer to zero time and infinite time (complete reaction), respectively, and the other parameters are as above.

Calculation of Electrical Distance—The calculations were performed as described previously (20). Briefly, to determine the electrical distance \( \delta \) from the bath solution to the site of reaction, the second order rate constant was plotted as a function of the holding potential \( \phi_{0} \) and fit to the following equation.

\[ k = k_{0}e^{-(zF\phi_{0}/RT)} \]  

(Eq. 5)

where \( F \) is the Faraday constant, \( R \) is the molar gas constant, \( T \) is the absolute temperature, and \( z \) is the algebraic charge on the reagent, with all other parameters as above. MTSET is poorly permeant through the 5-HT\(_{3}\) channel, but when it does reach the residues near the cytoplasmic end of the channel, it was assumed that the rate of reagent leaving to the cytoplasm was much greater than the rate of leaving to the extracellular bath. Thus in the case of E277C the apparent \( \delta \) was \( (6 - 0.5) \) (20).

Immunofluorescent Localization of WT and Mutant 5-HT\(_{3}\) Receptors—Transfected HEK293 cells maintained as described previously (21) were washed with three changes of Tris-buffered saline (0.1 M Tris/HCl, pH 7.4, 0.9% NaCl) and fixed using ice-cold 4% paraformaldehyde in phosphate buffer (66 mM Na\(_{2}\)HPO\(_{4}\), 38 mM NaH\(_{2}\)PO\(_{4}\), pH 7.2). To label the 5-HT\(_{3}\) receptor N-terminal domain pAb120 antisemur (21) was used at 1:1000 dilution in Tris-buffered saline. Primary antibody incubation was overnight at 4 °C. Biotinylated anti-rabbit IgG (Vector) and fluorescein isothiocyanate avidin D (Vector) were used to detect secondary antibody incubation was overnight at 4°C. Coverslips were mounted in Vectashield mounting medium (Vector), and immunofluorescence was observed using a Medical Research Council Radiance confocal microscope.

RESULTS

Effect of Mutations to Cysteine—Cysteine was substituted at 25 consecutive positions (Asp\(^{274}\)–Asp\(^{298}\)) one at a time, and the function of the mutants was tested following expression in oocytes. Twenty of the mutants expressed 5-HT-activated currents in Xenopus oocytes following injection of mRNA, indicating that in general the mutations were well tolerated. Application of 5-HT to oocytes expressing WT receptors resulted in rapid inward currents averaging 806 ± 71 nA (n = 6) at a holding potential of −60 mV. Typical responses are shown in Fig. 2B. The average \( I_{\text{max}} \) for the mutants ranged from 131 nA ± 11 nA (n = 6, 1295C) to 2976 ± 327 nA (n = 6, F281C).

Dose-response curves were determined for all mutants, and the EC\(_{50}\) values are shown in Fig. 3. None of the mutants had an EC\(_{50}\) value more than 10-fold different from that of wild type receptors (1.4 ± 0.2 μM, n = 6).

Nonfunctional Mutants—The expression of G288C, Y289C, S290C, F292C, and D298C mutant receptors were examined using several batches of cRNA, but no currents were detected during application of 5-HT up to a concentration of 1 mM (~1000 × WT EC\(_{50}\)). To rule out the possibility that the lack of functional channels for these mutants was due to spontaneous formation of disulfide bonds, as was observed in the GABA\(_{A}\) receptor at 17’ and 20’ positions (22), they were perfused with 10 mM dithiothreitol for 3 min; no 5-HT induced currents were observed following this treatment. We used immunofluorescent labeling of transiently transfected HEK293 cells to determine whether these mutants, which were also shown to be nonfunctional in HEK293 cells using calcium imaging techniques (11), formed cell surface receptors. Using the 5-HT\(_{3}\) selective antiserum, pAb120, raised against the extracellular domain (21), WT, and all of the mutants had a band of fluorescence on the cell surface when examined in nonpermeabilized cells. Untransfected cells were nonfluorescent as described previously (21). Typical results for G288C are shown in Fig. 2A (inset). Thus the data suggest that the receptors were correctly synthesized, assembled and targeted to the cell surface, but the mutation resulted in loss of function.

Reaction with MTSET—The mutants were examined for their susceptibility to react with MTSET applied in the presence of 5-HT by comparing the mean of a pair of responses to...
5-HT before MTSET application to the mean of a pair of responses after MTSET application (see “Experimental Procedures” and Fig. 2). The effect of MTSET was examined by the effect on currents induced by 5-HT test pulses at EC_{50} and at maximal concentrations (Figs. 2 and 4). For wild type 5-HT_{3A} receptors, a 1-min application of 1 mM MTSET with 10 μM 5-HT had no effect on the subsequent 5-HT-induced currents. Thus we infer either that MTSET does not react with the endogenous cysteines or that reaction with them has no functional effect. For the cysteine mutants, following a 1-min application of 1 mM MTSET with 5-HT, the subsequent 5-HT EC_{50} currents were irreversibly altered with D274C, G276C, E277C, S280C, T284C, L285C, L287C, V291C, L293C, I294C, and I295C. With the majority of these residues MTSET interaction resulted in inhibition of the subsequent response. With two mutants, D274C and I295C, MTSET modification caused enhancement in inhibition of the subsequent 5-HT currents. At two other positions, L287C and S280C, reaction with MTSET in the presence of 5-HT appeared to lock the channels in the open state. Thus following washout of the MTSET and 5-HT, the currents remained elevated and did not return to their initial base-line level even after 30 min of observation. Application of 300 μM diltiazem to the MTSES locked-open channels formed by V291C reversibly blocked the current by 37% ± 9% (n = 4). Diltiazem (300 μM), however, had no effect on locked-open channels formed by L293C or I294C (data not shown).

Rate Constants for Reaction with Sulfhydryl Reagents—We determined the reaction rates with MTSET or MTSES for each of the accessible mutants. For mutants where reaction with MTSET caused inhibition of subsequent 5-HT-induced currents, the rates were measured using the procedure illustrated in Fig. 2C, and the results were analyzed using Equation 3. For some mutants the voltage dependence of the reaction rates were also examined, and these data are shown in Fig. 6 (A and B).

To determine the reaction rates of MTSET with V291C, L293C, and I294C, we used the protocol illustrated in Fig. 7A of brief sequential applications of MTSES and 5-HT followed by test pulses of 5-HT. We analyzed the results by fitting the peak currents induced by the 5-HT test pulses as a function of cumulative exposure time to MTSES with an exponential decay function as in Equation 4 to calculate the pseudo-first order rate constant (Fig. 7B). The second order rate constant was determined by dividing the pseudo-first order rate constant by the MTSES concentration (Fig. 7C).

These rate constants are slower than the reaction rates with cysteine in free solution (23) but are generally faster than the rates of reaction with cysteines found in protein clefts (e.g. in the binding site of the dopamine D2 receptor) (24). The reaction rates of MTSET with L293C and I294C, however, were similar to the rates measured with cysteines in protein clefts (24). We also examined these rate constants at a variety of different membrane potentials, and the data show that the reactions of MTSET with E277C, S280C, T284C, V291C, L293C, and I294C were voltage-dependent (Fig. 6, A and B) and that the reactions of V291C, L293C, and I294C with MTSES were voltage-dependent (Fig. 7C).

Electrical Distance—Because the sulfhydryl reagents are charged and their reactions with cysteine are assumed to be fast, the rate-limiting step of their reaction with the thiolate is their movement into the ion channel; this should be voltage-dependent. The magnitude of the voltage dependence depends on the fraction of the electrical field through which the reagent moves to reach the cysteine (20). Based on the voltage dependence of the reaction rates, we calculated the electrical distance (β) from the bulk solution to the target cysteine residues using Equation 5, for those reactions whose rates were sufficiently high to use for this analysis. The results are shown in Fig. 6C. The data reveal that the electrical distance is greatest toward the cytoplasmic end and decreases toward the extracellular end.

DISCUSSION

We identified the water-accessible residues in and flanking the M2 segment of the 5-HT_{3A} receptor subunit using the substituted cysteine accessibility method. Twenty of the twenty five cysteine substitution mutants were functional, and the EC_{50} values of these mutant receptors did not vary from wild type by more than 10-fold. We infer that their structures were similar to the structure of wild type. Thus insertion of five cysteine residues (one for each subunit) was tolerated in these mutants. For 11 of the cysteine substitution mutants, application of MTSET in the presence of 5-HT irreversibly altered the subsequent 5-HT-induced currents, and we infer that the engineered cysteine, in at least one of the five subunits, covalently reacted with MTSET. We cannot determine whether reaction occurred with more than one of the five engineered cysteines.
that is present in each functional receptor. Because the rate of reaction of these sulfydryl reagents with a thiolate anion (RS⁻/H₂S⁻) is $5 \times 10^9$ faster than the rate with the uncharged thiol (RSH) (17) and the extent of reaction of a sulfydryl reagent with an engineered cysteine directly correlates with the surface accessibility of the corresponding wild type (25), we assume that the 5-HT₃A cysteine mutants that react with MTSET and MTSES are on the water-accessible surface of the receptor. Thus in the presence of 5-HT, the residues Asp274, Gly276, Glu277, Ser280, Thr284, Leu285, Val291, Leu293, Ile294, and Ile295 in the M2 transmembrane region are on the water-accessible surface of the protein at least part of the time. The voltage dependence of the MTSET reaction rates with many of these residues implies that the access pathway to them is via the ion channel. This supports our inference that at least some of these residues form the channel lining. In the presence of 5-HT the channels undergo transitions between the open, desensitized, and closed states. We cannot distinguish in which state reaction is occurring. We did not determine MTSET reactivity in the absence of 5-HT because without data on the spontaneous open probability of each mutant it is difficult to interpret closed state reactivity data with sulfydryl reagents.

Five of the M2 segment cysteine substitution mutants did not form functional receptors when expressed in Xenopus oocytes. The nonfunctional mutants G288C, Y289C, S290C, F292C, and D298C appeared to be targeted to the cell surface, based on the immunofluorescent antibody labeling experiments in HEK 293 cells. This implies that they were correctly assembled. Thus for these five the mutations probably do not cause a gross change in receptor structure; rather the cysteine residues at these positions appeared to prevent channel opening.
The data are the means was as in Fig. 2.C accessible cysteine substitution mutants with MTSET. The protocol from the exponential fit in from the bath solution to the reactive residue were calculated (see text) tors.

A

![Graph](http://www.jbc.org/)

**Fig. 6. Rates of reaction of MTSET with mutant 5-HT₃A receptors.** A and B show the second order rate constants for reaction of accessible cysteine substitution mutants with MTSET. The protocol was as in Fig. 2C. The rates are plotted against the holding potential. The data are the means ± S.E. (n ≥ 3). The data were fitted by an exponential function (Equation 5). C, values for electrical distance (δ) from the bath solution to the reactive residue were calculated (see text) from the exponential fit in A and B.

At most reactive positions, MTSET inhibited the subsequent 5-HT-induced currents. We do not know whether the inhibition of macroscopic currents occurred because of a change in gating kinetics or to a change in single channel conductance or both. Single channel studies of each mutant would be necessary to determine this. In two of the mutants, D274C and I293C, MTSET modification potentiated the subsequent currents. This is most likely due to a change in gating kinetics because placing a positive charge on the wall of a cation conducting channel is unlikely to increase single channel conductance. An alternative explanation suggested by preliminary data from another group who have also performed SCAM on the 5-HT₃ receptor suggests that the potentiation at D274C may be due to relief of inhibition by extracellular magnesium ions (26).

Charge Selectivity—MTSET, which is positively charged, reacted with 11 cysteine mutants along the entire length of the M2 segment, whereas the negatively charged MTSES only reacted at three positions near the extracellular end of M2. The most cytoplasmic of these is the 13’ position, V291C. This implies that both anions and cations can enter the extracellular end of the 5-HT₃ channel. At V291C the ratio of the reaction rate with MTSET to the rate with MTSES is ~10 (Figs. 6 and 7), similar to the ratio of the reaction rates with 2-mercaptoethanol in free solution (23). This implies that the access pathway to this residue is not charge-selective. Thus the residues lining the extracellular vestibule and the extracellular ring of charge (5) do not serve a major role in the charge selectivity process. The fact that an anionic reagent reacted with M2 segment cysteine mutants to the 13’ level implies that the discrimination between cations and anions occurs at a more cytoplasmic position than V291C. This is consistent with results of similar experiments in the nACh receptor (27), although in the anion-selective GABA_A receptor cationic reagents were able to react with engineered cysteines as far down as the α₃ Thr2⁶¹, the 6’ level (28). In the cationic members of the gene superfamily, mutation of the 13’ residue and of two others in the M1–M2 loop changes the charge selectivity and vice versa (4, 29–31). This suggests that the major determinants of charge selectivity are near the cytoplasmic end of the channel.

The Structure of M2—The residues that react with MTSET form a pattern that is consistent with a significant proportion of M2 being in an α-helical conformation (Fig. 8, A and B). This is similar, although not identical, to SCAM data previously reported for nACh and GABA_A receptor subunits (Fig. 8C). It is also consistent with other studies that indicate that the M2 segments in all ligand-gated ion channel superfamilies are predominantly α-helical (for review see Ref. 4).

There are, however, some discrepancies that reveal interesting features of the structure. Reaction at L285C (7’) is inconsistent with a straight α-helical secondary structure in the middle of M2. Based on the accessibility of the aligned residue or a neighboring residue in SCAM studies of the GABA_A and nACh receptors (Fig. 8C), it was hypothesized that there may be a kink in the middle of M2 (27, 28). Furthermore, in the 9 Å resolution cryo-electron microscopy structure of the Torpedo nACh receptor the channel-lining segments appeared to have a bend in the middle with the ends angling away from the channel axis (29). It was hypothesized that the 9’ leucine that is conserved in almost all ligand-gated ion channel subunits is located at the bend and that it forms the channel gate (29). However, other data are not consistent with this hypothesis. In the nACh receptor Wilson and Karlin (34) using SCAM experiments inferred that the channel gate is located between Gly2⁴⁰ and Thr2³⁴⁴ (–2’ and 2’; see Fig. 1). Also, mutation of the 9’ leucine to smaller more polar residues (e.g. replacement with serine in the nACh receptor; Ref. 34) that might be expected to prevent gate formation has been shown to produce functional receptors. In addition, substitutions for the 9’ leucine significantly modify channel gating in nACh, GABA_A, and 5-HT₃ receptors and can result in stabilization of the open state relative to the resting state (35–37). Our data are also consistent with a critical role of this leucine residue; reaction of MTSET with the L285C (9’) mutants results in a receptor that appears to be locked in the open state. Thus a non-α-helical structure in this region would allow access to L285C.

Near the extracellular end, assuming that M2 is α-helical, L293C is not then predicted to be on the channel-lining face of M2, but it reacted with MTSET (Fig. 8). There are several possible explanations for its accessibility. First, this part of M2 may extend above the level of the hydrophobic interior of the bilayer into the aqueous phase either continuously or tran-
siently. This would allow the MTS reagents to gain access to non-channel-lining engineered cysteines. Alternatively, these residues may be on the back side of the M2 segment facing a water-filled crevice that extends into the interior of the membrane-spanning domain. Such a crevice was inferred to exist based on SCAM studies of the GABAA receptor (38). Furthermore, L293 is aligned with a residue in the GABAA receptor that when mutated reduces the efficacy of general anesthetics and may form part of the GABA_A receptor anesthetic binding site (39). It is interesting to speculate this same protein region may be involved in general anesthetic interactions with 5-HT_3 receptors, although there is currently no evidence for this. Consistent with L293C not being in the channel, the reaction rates with MTSET and MTSES were slow and not significantly voltage-dependent. In fact the rates were similar to rates that have been measured with engineered cysteines in the dopamine D2 receptor binding crevice (24).

MTSES modification locked the V291C, L293C, and I294C mutants in the open state. There are several possible mechanisms by which this may occur. Based on disulfide trapping experiments (22) it was hypothesized that channel opening

FIG. 7. Rates of reaction of MTSES with mutant 5-HT_3 receptors. A, a typical experiment to determine the rate of reaction of MTSES with V291C. The traces are corrected for leak current and therefore show the increase in the number of locked-open receptors after each application of MTSES. At the end of the experiment, diltiazem (300 μM) reversibly blocks current through the open channels. B, peak currents from the 5-HT test applications in A plotted against cumulative reaction time with MTSES. The data are fit with the exponential function (Equation 4) to determine the second order rate constant. C, the second order rate constants for reaction of MTSES with cysteine substitution mutants (determined as in A and B) plotted against the holding potential. The data were fit by Equation 5. The slopes are in the opposite direction to those in Fig. 6 as expected for a negatively charged reagent. The data are the means ± 32 S.E. (n ≥ 3).

FIG. 8. Helical wheel representation of water-accessible residues. A, M2 residues of 5-HT_3A subunits plotted on a helical net; B, the same residues plotted on a helical wheel. Residues at which reaction with MTSET or MTSES caused a significant irreversible change in peak current are indicated by filled squares. Residues where no change in peak current was detected are indicated by open circles. Mutants for which no current could be recorded are underlined. C, alignment of residues in the α subunit of the nACh and GABA_A receptor and the 5-HT_3A receptor subunit. Asterisks denote residues that reacted with sulfydryl reagents and therefore are assumed to be water-accessible. D, model of the 5-HT_3A receptor subunit M2 domain based on our data. The ion path is indicated above those residues that reacted with sulfydryl reagents and were therefore deemed water-accessible. The non-α-helical region in the center of M2 is also shown.
involves a rotation of the M2 segments. Modification of engineered cysteine residues in multiple subunits in their open state position may prevent the M2 segments from rotating back to their closed state conformation, thereby locking the channels open. Lock open was not seen in SCAM studies of nACh and GABA<sub>A</sub> receptor (16, 27, 28), but in these experiments there were only one or two engineered cysteines in each receptor, whereas in the homomeric 5-HT<sub>3</sub> receptor there are five. Of note, in the nACh receptor, MTSEA modification of β2V66C, which aligns with Val<sup>291</sup> in the 5-HT<sub>3</sub> receptor, caused a marked increase in single channel open time (16). The fact that MTSET caused almost complete inhibition of macroscopic currents at V291C might seem to contradict this hypothesis. It should be remembered, however, that placement of multiple positive charges on the channel wall by MTSET reaction with multiple channel-lining cysteines might lock the channels in the open state but prevent flux of cations through the channel. MTSES places multiple negative charges on the channel wall that will not block cation conduction.

To further characterize the locked-open state that resulted from MTSES modification of V291C, L293C, and I294C, we examined the ability of diltiazem, an open channel blocker of 5-HT<sub>3</sub> receptors (40), to block the locked-open current. Diltiazem partially inhibited the locked-open current in MTSES-modified V291C channels, but it did not inhibit the currents in L293C or I294C MTSES-modified channels. There are two possible explanations for this; either Leu<sup>293</sup> and Ile<sup>294</sup> are part of the diltiazem binding site and MTSES modification prevents binding, or these residues are inaccessible to the reagent, perhaps in a protein cleft.

The Electric Field in the Channel—Our data (Figs. 6, A and B, and 7C) show that the reaction of MTSET and MTSES with a number of the mutant channel residues is voltage-dependent, thus confirming that access to these residues does indeed occur via the ion channel. To further explore the location of these residues relative to the electric field in the channel, we used the information from the second order rate constants of reaction with MTSET to calculate the electrical distance from the bath solution to the reactive residues. These data are plotted in Fig. 6C and show that electrical distance is low at the extracellular end and high at the cytoplasmic end. Similar data have been calculated for the nACh receptor (20) and are as expected for these ion channels, thus providing yet more confirmatory evidence that M2 residues line the pore.

Conclusions—Thus in conclusion we have proved the accessibility and the electrostatic potential of the 5-HT<sub>3</sub> receptor ion channel using two differently charged sulfydryl derivatives. Some unexpected data have arisen that, with further experimentation, may clarify details of both the structure of M2 and the changes that occur during channel activation. Thus we observed that a number of mutant receptors were locked into the open state following reaction with the sulfydryl reagents. Mutations in M2 that result in receptors being locked open have been previously reported both in 5-HT<sub>3</sub> receptors (41) and in other ligand-gated ion channels (42–44), but this is the first report of being able to attain this state using sulfydryl reagents. Further experiments, for example using the 5-HT<sub>3B</sub> subunit, could clarify the role of these residues. We also provide data that we have interpreted as suggesting that a protein cleft is located on the non-channel-lining side of M2 toward the extracellular end. If further work confirms the existence of such a cleft there are a number of candidate molecules, such as steroids and anesthetics, for which it might form a binding site (45). Overall, however, the data suggest that M2 is mostly α-helical and that charge selectivity occurs at a more intracellular level than Val<sup>291</sup> (13). Thus the data are similar, although not identical, to those reported for nACh and GABA<sub>A</sub> receptors, further exemplifying the high degree of structural and functional homology between receptors in the Cys loop ligand-gated ion channel family.
Structural and Electrostatic Properties of the 5-HT₃ Receptor Pore Revealed by Substituted Cysteine Accessibility Mutagenesis
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