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Membrane Biology:
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Mutagenic Analysis of the Intracellular Portals of the Human 5-HT₃A Receptor*

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Background: 5-HT₃R cytoplasmic arginine residues, within a putative amphipathic α-helix (MA helix), influence unitary conductance (γ); how other residues within this motif affect γ is unknown.

Results: Alanine-, arginine-, and cysteine-scanning mutagenesis reveal that γ is affected by seven additional residues.

Conclusion: The charge of 10 MA helix residues influences γ.

Significance: Our findings support a proposed structure of ion-conducting cytoplasmic portals.

Structural models of Cys-loop receptors based on homology with the Torpedo marmorata nicotinic acetylcholine receptor infer the existence of cytoplasmic portals within the conduction pathway framed by helical amphipathic regions (termed membrane-associated (MA) helices) of adjacent intracellular M3-M4 loops. Consistent with these models, two arginine residues (Arg436 and Arg440) within the MA helix of 5-hydroxytryptamine type 3 (5-HT₃) receptors act singularly as rate-limiting determinants of single-channel conductance (γ). However, there is little conservation in primary amino acid sequences across the cytoplasmic loops of Cys-loop receptors, limiting confidence in the fidelity of this particular aspect of the 5-HT₃A receptor model. We probed the majority of residues within the MA helix of the human 5-HT₃A subunit using alanine- and arginine-scanning mutagenesis and the substituted cysteine accessibility method to determine their relative influences upon γ. Numerous residues, prominently those at the 435, 436, 439, and 440 positions, were found to markedly influence γ. This approach yielded a functional map of the 5-HT₃A receptor portals, which agrees well with the homology model.

The delicate balance between neuronal excitation and inhibition in the central nervous system is crucial to maintaining normal physiological function. The members of the pentameric ligand-gated ion channel (pLGIC) superfamily are key players in this balance. In eukaryotic organisms, these comprise the excitatory, cation-selective, nicotinic acetylcholine (nACh) (1, 2) and 5-hydroxytryptamine type 3 (5-HT₃) receptors (3, 4) and the inhibitory, anion-selective, γ-aminobutyric acid type A (GABAₐ) (5, 6) and glycine receptors (7, 8) that are collectively termed the “Cys-loop receptors.” The prokaryotic homologues are pLGICs from Gloeobacter violaceus (GLIC) and Erwinia chrysanthemi (ELIC) for which, importantly, crystal structures are available (9) complementing that of a eukaryotic glutamate-gated chloride channel (GLC-1, also known as GluCl) (10, 11). The Cys-loop receptors are gated by the binding of their cognate neurotransmitters to permit the transmembrane conduction of selected ions, eliciting either synaptic excitation or inhibition (12, 13).

Cys-loop receptors assemble pseudosymmetrically as either identical, but more commonly homologous, protein subunits that surround a central ion channel pore (13, 14). Each subunit consists of four α-helical transmembrane domains, termed M1 to M4, with the second transmembrane (M2) domain lining the majority of the transmembrane pore. The N and C termini of the subunits are located extracellularly, with the large N-terminal domain harboring the ligand-binding site formed at subunit interfaces, whereas a large intracellular loop extends between the M3 and M4 domains (15).

By comparison with the extracellular and membrane spanning domains, the intracellular M3-M4 loop displays the lowest degree of sequence homology across different subunits of the same receptor family or between receptor families and is predicted to be largely unstructured (16–18). Notably, the large intracellular loop is virtually absent from prokaryotic pLGIC subunits (9), and almost complete deletion of this region, among others, was necessary to optimize crystallization of GLC-1, once more suggesting it to be largely unstructured (19). This region of Cys-loop receptors is an established target for phosphorylation (19, 20), and it exerts important influences on, for example, the assembly, maturation, targeting, and gating kinetics of the nACh receptor (18, 21–26). The M3-M4 loop has also been shown to be a critical determinant of single-channel conductance (γ) (27). Cryo-electron microscopic studies of the nACh receptor of Torpedo marmorata have revealed the presence of a helical amphipathic stretch, referred to as the mem-

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brane-associated (MA) helix, at the C-terminal end of the loop (15) (see Fig. 1). Five such helices extend below the ion channel pore, forming a vestibule that is perforated by five narrow openings, or portals (see Fig. 1). Structure-function studies (28–30) demonstrate that specific residues within human 5-HT₃A and rat α₄β₂ nACh receptors positioned within these portals, as inferred by structural models based on homology with the *T. marmorata* nACh receptor, exert a strong influence on γ that is additional to that of the extensively characterized M2 domain and flanking sequences (31, 32). Furthermore, equivalent residues within MA helices of human α₁ glycine receptors also influence γ (33). The same region in the 5-HT₃A receptor additionally impacts upon divalent versus monovalent cation permeability, channel gating, and the kinetics of desensitization (34–36). However, ion size selectivity for monovalent cations appears to depend upon the M2 domain (37), and the M3–M4 loop is not essential for receptor function (38).

Structure-function studies of Cys-loop receptors, including the 5-HT₃A receptor, have extensively probed the M2 domain and flanking sequences for their impact upon ion conduction and selectivity (13, 35–39). The amino acid sequences of the membrane-spanning regions are relatively well conserved across Cys-loop receptors, and systematic mutagenesis essentially confirms the validity of homology models of the 5-HT₃A receptor M2 domain based on the medium resolution structure (3.6 Å) of the *Torpedo* nACh receptor (see Fig. 1A). We previously demonstrated (27, 28) a collective influence on ion conduction of three conserved arginine residues within the MA helix of the 5-HT₃A receptor (see Fig. 1, B and C). In particular, the replacement of the three arginine residues by their human 5-HT₃B subunit counterparts (QDA, respectively, generating a receptor construct coined 5-HT₃A(QDA)) increased γ 29-fold (27, 28). This influence is interpretable in the context of the portal-like structures observed in cryo-EM studies of the *Torpedo* nACh receptor (see Fig. 1). However, no high resolution structural data are available for the cytoplasmic residues of any of the mammalian Cys-loop receptors, weakening confidence in homology models such as ours (29), based on the *Torpedo* structure.

In this study, we broadened the analysis to include most 5-HT₃A receptor amino acids within the MA helix lining the putative intrasubunit portals evident in the 5-HT₃ receptor model (see Fig. 1). There is little conservation of amino acid identity in this region between the 45 human Cys-loop receptor subunits. In the 5-HT₃A subunit, there are several positively charged residues within the MA helix. By contrast, in nACh receptors, the preponderance of charge is negative (3).

To gain insights into the effect of charge throughout the MA helix, we first established the value for γ of the 5-HT₃A(QDA) receptor when the uncharged amino acid alanine was present at each position. With these values as a reference, we determined γ when each residue was arginine to examine the influence of positive charge. However, arginine and alanine also differ with regard to their size and hydrophobicity. Therefore, we also introduced a cysteine at each position and attempted methanethiosulfonate (MTS) modification using positively and negatively charged 2-aminoethyl-methanethiosulfonate (MTSEA) and 2-sulfonatoethyl-methanethiosulfonate (MTSES), respectively. This yields additional structural information both providing evidence of the accessibility of each MA helix residue and allowing comparison between the influence of positive and negative charges of equivalent bulk. The alternative approach of scanning the portals with negatively charged amino acids for comparison with the effect of arginine is confounded by the substantially larger volume of the latter. We have previously demonstrated that the volume of residue 436 in the 5-HT₃A subunit influences γ (29). The influence of multiple residues within the MA helix upon γ strongly supports the existence of cytoplasmic ion conducting portals within the 5-HT₃A receptor.

**EXPERIMENTAL PROCEDURES**

**5-HT₃A Receptor Constructs and Transfection of Subunit cDNAs—**cDNAs encoding human 5-HT₃A(QDA) and mutant subunit constructs thereof were cloned into plk5. Point mutations were introduced into the 5-HT₃A construct using standard molecular biological techniques, and all constructs were fully sequenced to confirm fidelity. Wild-type and mutant receptor subunit cDNAs were co-transfected into tsA-201 human embryonic kidney cells with a cDNA encoding green fluorescent protein to identify transfected cells. Transfection was performed by electroporation (400 V, 125 microfarads, infinite resistance) using a Bio-Rad Gene Electroporator II (Bio-Rad). Cells were subcultured twice weekly and incubated in a medium composed of Dulbecco’s modified Eagle’s medium and 10% (v/v) calf serum, supplemented with 100 μg ml⁻¹ streptomycin and 100 units ml⁻¹ penicillin. Cells were maintained at 37 °C in an atmosphere of 5% CO₂ (100% relative humidity). Cell culture reagents were purchased from Life Technologies.

**Electrophysiology—**The outside-out patch configuration was used to record single-channel currents from patches excised from transfected tsA-201 cells. The bath solution contained (in mM): 140 NaCl, 2.8 KCl, 2.0 MgCl₂, 1.0 CaCl₂, 10 glucose, and 10 HEPES (pH 7.2 adjusted with NaOH). Patch electrodes were filled with a solution comprising (in mM): 130 potassium gluconate, 5 NaCl, 2 MgCl₂, 5 EGTA, and 10 HEPES (pH 7.2 adjusted with KOH) and had resistances within the range 4–10 megohms. 5-HT (10 μM) was dissolved in the bath solution and applied locally by pressure ejection to outside-out patches held at −74 mV (holding potential includes correction for liquid junction potential). Stock solutions of MTS reagents (200 mM) obtained from Toronto Research Chemicals (Ontario, Canada) were stored at −20 °C. Prior to each experiment, MTS reagents were diluted in the electrode solution for outside-out patch experiments to yield a final concentration of 200 μM. Single-channel currents were recorded using an Axopatch 200A amplifier (Axon Instruments) and low pass-filtered at a cut-off frequency of 1 kHz. Data were digitized (Digitdata 1322A, Axon Instruments) at 10 kHz onto the hard drive of a PC for subsequent offline analysis. Sections of data recorded from outside-out patches, in which unitary events predominated, were selected for analysis. Multiple Gaussian distributions were fitted (least squares minimization) to all-points amplitude histograms using the Strathclyde Electrophysiology Software developed and provided by J. Dempster (Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, UK). Single-channel conductance (γ) values are reported as the chord conductance determined from the rela-
Analysis of the 5-HT\textsubscript{3}A Receptor MA Stretch

The single-channel conductance ($\gamma$) of human and mouse homomeric 5-HT\textsubscript{3}A receptors is below the limit of direct resolution by single-channel recording and has been inferred by fluctuation analysis to be within the range 0.3–1.3 pS, dependent, at least in part, upon the concentration of divalent cations within the extracellular medium (27, 28, 34, 45–49). We have previously demonstrated that the replacement of three arginine residues (Arg\textsuperscript{432}, Arg\textsuperscript{436}, Arg\textsuperscript{440}) within the MA stretch of the human 5-HT\textsubscript{3}A subunit by their human 5-HT\textsubscript{3}B subunit counterparts (QDA, respectively) increases $\gamma$ by 29-fold, as inferred by fluctuation analysis (27) and by 40-fold as revealed directly by single-channel recording (28). To facilitate single-channel analysis of the mutants produced in this study, mutations were introduced into the 5-HT\textsubscript{3}A(R432Q, R436D, R440A) receptor background, henceforth referred to as 5-HT\textsubscript{3}A(QDA). Fig. 1 presents an alignment of residues within the MA stretch of human 5-HT\textsubscript{3}A, 5-HT\textsubscript{3}B, and 5-HT\textsubscript{3}A(QDA) subunits.

**Alanine Scan of the Human 5-HT\textsubscript{3}A(QDA) MA Helix**—Residues within the human 5-HT\textsubscript{3}A(QDA) MA stretch vary with respect to their physicochemical properties; specifically, 10 are charged (5 positive, 5 negative), 6 are nonpolar, and only 1 is polar (Fig. 1). At the outset of this study, we mutated each residue, from Arg\textsuperscript{426} to Trp\textsuperscript{442}, individually to alanine, which presents a simple methyl side chain, thus negating the potential influences of charge and large volume upon $\gamma$. Such mutant receptors also provided reference values for $\gamma$ to which comparisons were made in subsequent amino acid substitution experiments involving the introduction of arginine and cysteine residues and modification of the latter by MTS reagents.

Within the MA stretch of the human 5-HT\textsubscript{3}A(QDA) subunit construct, alanine occurs naturally at position 439 and replaces the normally resident arginine by mutagenesis at the 440 locus. Expression of the remaining alanine-substituted subunits produced functional receptors, although introduction of the W442A mutation resulted in a very low level of channel activity. It is notable that this tryptophan residue, unlike any of the other amino acids examined in this study, is absolutely conserved across all subunits of cation-selective Cys-loop receptors. In addition, mutation of this residue to alanine in nicotinic a7 receptors severely compromises their cell surface, but not total, expression level (50). The introduction of alanine residues typically resulted in a decrease in the $\gamma$ of the expressed receptors (Fig. 2, Table 1). With the exceptions of the R426A, Q427A, D432A, and W442A mutations, this change in $\gamma$ was significant (as indicated in Fig. 2 and Table 1) following the neutralization of charged residues. Thus, for mutations neutralizing negative charge, the percentage of reduction in $\gamma$ was: E430A, 23%; D433A, 22%; E434A, 28%; D436A, 64%; E437A, 23%; and D441A, 19%. The K431A mutation, removing positive charge, increased $\gamma$ by 19%. Only two substitutions of polar or nonpolar uncharged residues caused a significant change in $\gamma$, those being L429A, which was associated with a 25% reduction, and V438A, which caused a 15% reduction. Of all the exchanges examined, the D436A substitution had the most profound effect upon $\gamma$.

**Arginine Scan of the Human 5-HT\textsubscript{3}A(QDA) MA Helix**—Positively charged arginine residues at positions 436 and 440, but not 426 and 432, of the MA helix singularly have a significant impact on the $\gamma$ of the human 5-HT\textsubscript{3}A receptor (27, 28). In this study, we employed an arginine scan of the 5-HT\textsubscript{3}A(QDA) MA stretch of the 5-HT\textsubscript{3}A receptor was created with the Deepview Swiss-Pdb Viewer using the T. marmorata 2BG9 structure, as described previously (28). Images were rendered using PyMOL.

**Structural Modeling**—The homology model of the 5-HT\textsubscript{3}A receptor was created at the homology model of the 5-HT\textsubscript{3}A receptor based on the Torpedo nACh receptor structure. The pentameric protein is surface-rendered with foreground subunits made transparent. Arrows highlight the putative conduction pathway, one pointing to the outer vestibule, the others pointing out three of the five cytoplasmic portals. B, five 5-HT\textsubscript{3}A(QDA) receptor MA helices viewed from above (top panel) and from the cytoplasm (bottom panel). These structures are depicted with both transparent surface rendering and ribbons. The residues substituted by mutagenesis in the current study are indicated in color. Differing colors were used to distinguish each of the five subunits. C, the amino acid numbering is that of the human 5-HT\textsubscript{3}A subunit (h5-HT\textsubscript{3}A). The arginine residues that are collectively responsible for the sub-picosielectrical conductance of unitary events between cation. However, the relationship between voltage and the conductance of unitary events predominated. 5-HT\textsubscript{3}A receptors exhibit inward rectification. However, the relationship between voltage and the conductance of unitary events predominated. 5-HT\textsubscript{3}A receptors exhibit inward rectification.
helix to probe for additional locations in which the presence of an arginine residue influences γ. An arginine residue is naturally present at the 426 position of the human 5-HT₃_A(QDA) MA helix. Of the 16 mutant 5-HT₃_A(QDA) receptors produced (from 427 to 442), the W442R mutation resulted in a nonfunctional receptor (Fig. 3, Table 1).

It should be noted that comparisons of γ are between alanine- and arginine-containing receptor constructs. Thus,

![Graph showing the influence of alanine substitutions upon the single-channel conductance (γ) of the 5-HT₃_A(QDA) receptor.](image)

**FIGURE 2.** The influence of alanine substitutions upon the single-channel conductance (γ) of the 5-HT₃_A(QDA) receptor. The bar graph summarizes the effect of individual replacement of residues 426–438 and 441 and 442 of the 5-HT₃_A(QDA) subunit by alanine. Note that alanine is already present at the 438 and 439 loci of the 5-HT₃_A(QDA) subunit. Data are reported as the mean percentage change in γ caused by each mutation, with the 5-HT₃_A(QDA) receptor acting as control. The mean reference 5-HT₃_A(QDA) receptor γ value was 35.9 pS (Table 1). Statistical analysis was performed by comparing the raw values of γ for the 5-HT₃_A(QDA) and mutant receptor constructs. The inset traces are single-channel events recorded from excised outside-out patches expressing either (top) 5-HT₃_A(QDA) receptors (control) or (bottom) 5-HT₃_A(QAA) receptors (named D436A in the graph).

![Graph showing the influence of arginine substitutions upon the single-channel conductance (γ) of the 5-HT₃_A(QDA) receptor.](image)

**FIGURE 3.** The influence of arginine substitutions upon the single-channel conductance (γ) of the 5-HT₃_A(QDA) receptor. The bar graph summarizes the effect of individual replacement of residues 427–441 of the 5-HT₃_A(QDA) subunit by arginine. Note that arginine is already present at the 426 locus of the 5-HT₃_A(QDA) subunit. Data are reported as the mean percentage change in γ caused by each mutation, with the appropriate alanine-substituted 5-HT₃_A(QDA) receptor construct acting as the reference. Statistical analysis was performed by comparing the raw values of γ for the arginine- and alanine-substituted 5-HT₃_A(QDA) receptor constructs. The inset traces are single-channel events recorded from excised outside-out patches expressing either (top) 5-HT₃_A(QDA) receptors (control) or (bottom) 5-HT₃_A(QRA) receptors (named D436R in the graph).

**TABLE 1**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Alanine substitute</th>
<th>Arginine substitute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg⁴²⁶</td>
<td>32.8 ± 1.4 (7) ⋆</td>
<td>35.9 ± 2.0 (5) ⋆</td>
</tr>
<tr>
<td>Glu⁴²⁷</td>
<td>29.9 ± 0.6 (5)</td>
<td>34.8 ± 1.2 (5)</td>
</tr>
<tr>
<td>Phe⁴³⁵</td>
<td>35.9 ± 1.4 (7)</td>
<td>39.1 ± 1.2 (5)</td>
</tr>
<tr>
<td>Leu⁴³⁹</td>
<td>27.0 ± 0.2 (5) ⋆</td>
<td>32.6 ± 1.3 (10) ⋆</td>
</tr>
<tr>
<td>Glu⁴⁴⁰</td>
<td>27.7 ± 0.9 (8) ⋆</td>
<td>30.8 ± 1.6 (7)</td>
</tr>
<tr>
<td>Gln⁴⁴¹</td>
<td>42.6 ± 0.6 (7) ⋆</td>
<td>36.6 ± 0.6 (5) ⋆</td>
</tr>
<tr>
<td>Glu⁴⁴²</td>
<td>31.6 ± 0.7 (6)</td>
<td>24.2 ± 0.7 (7) ⋆</td>
</tr>
<tr>
<td>Asp⁴⁴³</td>
<td>28.1 ± 0.7 (5) ⋆</td>
<td>22.7 ± 0.9 (6) ⋆</td>
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<tr>
<td>Glu⁴⁴⁴</td>
<td>26.0 ± 0.4 (7)</td>
<td>19.8 ± 0.7 (6) ⋆</td>
</tr>
<tr>
<td>Ile⁴⁴⁵</td>
<td>35.8 ± 0.8 (5)</td>
<td>21.3 ± 0.4 (6) ⋆</td>
</tr>
<tr>
<td>Asp⁴⁴⁶</td>
<td>13.1 ± 0.8 (9) ⋆</td>
<td>6.4 ± 0.2 (3) ⋆</td>
</tr>
<tr>
<td>Glu⁴⁴⁷</td>
<td>27.7 ± 0.7 (5)</td>
<td>21.6 ± 0.6 (5) ⋆</td>
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<tr>
<td>Val⁴⁴⁸</td>
<td>30.4 ± 1.0 (7) ⋆</td>
<td>22.7 ± 0.7 (5) ⋆</td>
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<tr>
<td>Ala⁴⁴⁹</td>
<td>35.9 ± 2.0 (5) ⋆</td>
<td>22.1 ± 1.0 (7) ⋆</td>
</tr>
<tr>
<td>Ala⁴⁵⁰</td>
<td>35.9 ± 2.0 (5) ⋆</td>
<td>18.4 ± 0.6 (9) ⋆</td>
</tr>
<tr>
<td>Asp⁴⁴¹</td>
<td>29.0 ± 0.5 (5)</td>
<td>27.8 ± 1.2 (3)</td>
</tr>
</tbody>
</table>

* Value for the 5-HT₃_A(QDA) construct lacking additional amino acid substitution.

*p < 0.05 when compared with alanine mutant.

*p < 0.001 when compared with untreated cysteine mutant.

*p < 0.001 when compared with 5-HT₃_A(QDA) subunit residue.

*p < 0.01 when compared with alanine mutant.

*p < 0.05 when compared with 5-HT₃_A(QDA) subunit residue.

*p < 0.01 when compared with untreated cysteine mutant.

*p < 0.05 when compared with untreated cysteine mutant.

*p < 0.01 when compared with 5-HT₃A(QDA) subunit residue.
although we denote mutations as they were actually constructed (e.g. L429R), the changes in \( \gamma \) reported are, for example, between Ala\(^429\) and Arg\(^429\), not Leu\(^429\). The sequential introduction of arginine residues from position Lys\(^431\) to Asp\(^440\) inclusive resulted in a significant decrease in \( \gamma \), when compared with alanine controls, at all locations (Fig. 3, Table 1). Inspection of Fig. 3 reveals that the most pronounced decreases in \( \gamma \) occur at the adjacent positions 435 (41%)/436 (51%) and 439 (38%)/440 (49%). Against this overall trend of depression, the Q432C and L429R mutations were associated with a significant increase in \( \gamma \) of 16 and 21%, respectively. Arginine substitution at positions 428, 430, and 441 had no significant effect upon \( \gamma \) (Fig. 4, Table 1). However, such a comparison includes the effect upon \( \gamma \) of the cysteine substitution itself, which in three instances (see above) was significant. Thus, we also analyzed the effect of MTSEA application employing the \( \gamma \) of unmodified cysteine constructs as a reference to isolate the change in \( \gamma \) specifically due to the reagent (Fig. 5B). Using this criterion, MTSEA caused significant reductions in \( \gamma \) at positions 434 through 440 inclusive, in addition to 428 and 431. Notably, the introduction of arginine residues at positions 431 through 440 also caused significant reductions in \( \gamma \) in comparison with alanine controls (see above). At all other loci, MTSEA treatment was associated with a trend toward a reduction in \( \gamma \). Moreover, the effects upon \( \gamma \) of arginine substitution and challenge with MTSEA at positions 431 through 441 were in excellent quantitative agreement (\( r^2 = 0.89 \)), the data being fitted by a line of regression with a slope (1.13) close to unity (Fig. 6A). However, although arginine substitution at residues 426 through 430 tended to increase \( \gamma \) in comparison with the alanine controls, MTSEA tended to produce the opposite effect. Inclusion of the data obtained for these residues resulted in a much poorer correlation between the effect of arginine substitution or treatment with MTSEA upon \( \gamma \) (\( r^2 = 0.58 \); Fig. 6B).
The application of MTSES to cysteine mutant receptors to potentially donate the negatively charged ethylsulfonate moiety tended to increase \( \gamma \) when compared with the appropriate alanine controls at most loci examined. Despite the substantially increased side chain volume of the covalently modified cysteine residues versus alanine, MTSES never caused a significant decrease in \( \gamma \). Indeed, significant increases in \( \gamma \) in comparison with alanine controls occurred at mutants E430C, D433C, E434C, I435C, D436C, E437C, V438C, and D441C (Fig. 5A, Table 1). When the data were analyzed employing the \( \gamma \) of unmodified cysteine constructs as a reference, significant increases in \( \gamma \) were found at fewer loci (i.e. 430, 433, 436, 437, 438, and 440; Fig. 5B, Table 1). In this regard, it should be recalled that the introduction of cysteine itself at positions 435 and 436 significantly increased \( \gamma \) in comparison with alanine controls and tended toward an increase at several other positions (Fig. 4). We therefore examined whether there is a relationship between the \( \gamma \) of the cysteine mutants before and following the application of MTSES, suspecting that the maximal observable effect of residue modification might be limited by a barrier to permeation elsewhere within the conduction pathway. Fig. 6C indicates that a correlation \( (r^2 = 0.68) \) exists between \( \gamma \) prior to and after MTSES application such that the largest percentage increases tend to be registered for mutants with a lower \( \gamma \) prior to MTSES treatment. By contrast, there was no correlation between the percentage decrease in \( \gamma \) caused by MTSEA and the \( \gamma \) of the cysteine mutant receptors (data not shown).

It may not be coincidental that the maximum value for \( \gamma \) found in this study (43.7 pS) is remarkably close to that of the human 5-HT\(_3\)A receptor in which the entire intracellular loop was replaced by the heptapeptide linking the M3 and M4 domains of the pLGIC GLIC from the prokaryote Gloeobacter violaceus (i.e. 43.5 pS (38)).

**DISCUSSION**

In this study, by using a combination of alanine- and arginine-scanning mutagenesis, SCAM, and single-channel recording from outside-out membrane patches, we have identified residues within the intracellular MA helix of the human 5-HT\(_3\)A receptor that influence ion permeation. SCAM and
site-directed mutagenesis have previously been applied to the mouse 5-HT$_3$A receptor to infer or identify the residues within the M2 domain and flanking sequences that line the ion channel and influence ion selectivity (41–43). The present study utilizes a similar strategy but with the considerable advantage that changes in single-channel, rather than macroscopic, current amplitude were determined, obviating ambiguities in interpretation that might arise from perturbations in receptor kinetics (e.g. Ref. 41). We have previously demonstrated the dynamic modification of a cysteine residue introduced at the 436 locus by MTS reagents (29), and in this study, a further 15 loci were probed, representing the first comprehensive evaluation of the influence of the MA stretch as a whole upon $\gamma$.

The following criteria may be applied to infer that a particular residue within the MA stretch is a component of the conduction pathway: (i) replacement of a negatively or positively charged residue by alanine within the 5-HT$_3$A(QDA) receptor construct decreases or increases $\gamma$, respectively; (ii) the introduction of an arginine residue decreases $\gamma$ when compared with the appropriate alanine-substituted control; (iii) reaction of an engineered cysteine residue with MTSEA or MTSES decreases or increases $\gamma$, respectively, when compared with alanine- or cysteine-substituted controls; and (iv) the introduction of an arginine residue and the reaction of an engineered cysteine residue with MTSEA produce a qualitatively similar effect upon $\gamma$. Criteria iii and iv can only be satisfied if the engineered cysteine residue resides within an aqueous environment and is thus accessible for covalent modification by hydrophilic MTS reagents (51). Moreover, if the introduction of an arginine residue at a particular locus has no significant effect upon $\gamma$ when compared with the appropriate alanine control, a lack of an observable effect of MTSEA (or MTSES) upon the $\gamma$ of an engineered cysteine mutant clearly does not imply that residue to be inaccessible to the MTS reagent; instead such an outcome is consistent with the residue lying outside the conduction pathway.

On the basis of the 4-Å model of the intracellular vestibule of the Torpedo nACh receptor (15, 52) and the homology model of the human 5-HT$_3$A receptor derived from that structure (29) (Fig. 7), we anticipated that a potentially large number of residues within the MA stretch might impact upon $\gamma$, in addition to the 436 and 440 loci identified in previous studies (27–29). Such a suggestion arises from the fact that numerous residues of each MA stretch helix are predicted to face into the centrally located cytoplasmic vestibule or the lateral windows that are framed by adjacent helices (Fig. 7). It should be noted that adjacent helices are rotated (by 72° in a perfectly symmetrical pentamer) with respect to each other, thus placing different residues from each of the two MA helical frames within the lateral window. In addition, the MA stretch may be a mobile structure, as suggested by changes in the conformation of the large intracellular loop of the mouse 5-HT$_3$A receptor subsequent to agonist binding (53).

Confining initial consideration to those loci at which arginine substitution caused a significant decrease in $\gamma$ when compared with the alanine matched controls (i.e. residues 431–440 inclusive, rendered in blue in Fig. 7A), MTSEA also caused a significant decrement in the $\gamma$ of receptors engineered to express a cysteine residue at 7 of these 10 positions (431, 434, 435, 436, 438, 439, and 440, rendered in blue or yellow in Fig. 7B) when also compared with alanine controls. Essentially the same pattern emerged when the comparison was made with the unmodified substituted cysteine serving as control, with the exception that residue 437 additionally displayed a significant decrease in $\gamma$. For those substituted cysteine residues that did not yield a significant reduction in $\gamma$ following treatment with MTSEA (i.e. the 432 and 433 loci), the trend was nonetheless toward decreased conduction. In addition, constructs in which the negatively charged residues within the 432–440 sequence of the 5-HT$_3$A(QDA) receptor were mutated to alanine dis
played (with the exception of Asp432) a significant reduction in $\gamma$, whereas the mutation of the solitary positive charge in this region (i.e. K431A) resulted in augmented $\gamma$. Thus, the effects of introducing positive charge (via either arginine substitution or covalent modification by MTSEA) or neutralization of negative charge (by mutation to alanine) are remarkably consistent across the 431–440 region.

Additional evidence for the involvement of the residues 433–438 inclusive in ion conduction was provided by a significant potentiation, when compared with alanine controls, of the $\gamma$ values of cysteine mutant constructs as a result of challenge with MTSES. When compared with the unmodified substituted cysteine as control, MTSES treatment was associated with a significant increase in $\gamma$ at the 433, 436, 437, 438, and 440, but not the 434 and 435 loci. We directly compared the $\gamma$ of the cysteine engineered constructs that had been challenged with MTSEA or MTSES. As is clear from inspection of Fig. 5, the two treatments generally had opposite influences upon $\gamma$ over the loci 432–441 (rendered in yellow in Fig. 7B), and at all of these, the difference in $\gamma$ was significant. However, in specific instances, MTSEA significantly reduced $\gamma$, whereas MTSES did not affect this parameter in comparison with the cysteine control (i.e. at the 431, 434, 435, and 439 loci, rendered in blue in Fig. 7B). As mentioned above, a lack of effect of MTSES does not necessarily preclude the contribution of a residue to the permeation pathway because other components of the ion pore may exert a rate-limiting influence upon $\gamma$, or the increase in side chain volume may negate any influence of introducing negative charge. Conversely, MTSES elevated $\gamma$ at residues 430 and 433, but MTSEA did not cause a significant reduction (rendered in red in Fig. 7B). It is possible that the environment local to residues 430 and 443 favors deprotonation of the primary amino

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**FIGURE 7.** Residues that determine single-channel conductance ($\gamma$) mapped onto a model of a 5-HT$_3$A receptor cytoplasmic portal. A, surface renderings of an homology model of a 5-HT$_3$A receptor portal formed by adjacent MA helices, viewed from the cytoplasm (left panel) or from within the inner vestibule (right panel). When arginine residues were present at the positions rendered in blue, 5-HT$_3$A receptors exhibited significantly reduced $\gamma$ values when compared with their alanine equivalents (Fig. 3). When arginine residues were present at the positions rendered in black, the $\gamma$ values of 5-HT$_3$A receptors were either unchanged or slightly elevated when compared with their alanine equivalents. B, the same surface renderings of MA stretches represented in A, colored according to the effect of the MTS reagents, MTSEA and MTSES, on 5-HT$_3$A receptors into which cysteine residues were substituted at the positions indicated. Cysteine substituents rendered in yellow were associated with significantly decreased and increased $\gamma$ upon MTSEA and MTSES treatment, respectively, when compared with their alanine equivalents (Fig. 5A). Those indicated in blue or red only exhibited a decrease by MTSEA or an increase by MTSES, respectively, when compared with their alanine equivalents. Cysteine substituents rendered in black were unaffected by both MTSEA and MTSES when compared with their alanine equivalents. The 5-HT$_3$A receptor was modeled on the T. marmorata structure (see “Experimental Procedures”).
group of MTSEA (pK_a = 8.5 (51)), rendering the compound neutral. Alternatively, anionic MTSES may have greater access to the residues 430 and 433 than cationic/neutral MTSEA. Irrespective of such complications, arginine introduced at loci 431–440 inclusive decreased γ and cysteine present at all of these positions, except 432, reacted with either MTSEA or MTSES. We thus conclude that all of the residues, with the possible exception of position 432, are in an aqueous environment and influence γ, albeit to a variable extent.

The effects of arginine substitution or covalent modification of engineered cysteine residues by MTS reagents are most profound at the 435, 436, 439, and 440 loci (Fig. 7). In an α-helical structure, residues 435 and 440 would subtend an angle of 140°, and we tentatively suggest that it is this arc of the helix that most closely impinges upon the permeation pathway.

At positions 426–430 inclusive, the introduction of an arginine residue either had no significant effect upon or caused an increase in γ when compared with the 5-HT_3A(QDA) receptor construct. Neither effect is compatible with these residues contributing to the permeation pathway, consistent with the structural model in which these residues are below the bottom of the portal (rendered in gray in Fig. 7A). However, such an interpretation is complicated by the observation that replacement of Leu_429 and Glu_430 by alanine caused a significant reduction in γ and that the mutation Q427C caused a small, but significant, increase. In addition, MTSEA decreased the γ of receptors containing a cysteine residue and position 428, whereas MTSES increased the γ of receptors containing a cysteine at 430. However, MTSES and MTSEA did not have opposing effects on either of these receptor constructs. The general failure of residues within the 426–431 positions to exhibit consistent changes in γ with modification of charge, either through mutagenesis or SCAM, suggests that these residues lie outside the sphere of influence of the portal. Collectively, these findings suggest that position 430 is below the bottom of the portal, in agreement with the structural model.

The sphere of influence of portal residue charge on γ extends toward the upper aspect of the portal structure as far as position 440. Residue 441 responded inconsistently to charge modification, suggesting that it lies at the upper limit of the permeation pathway derived from the Torpedo model.

An aspect of the 5-HT_3A receptor portal structure that remains largely untested are the positions of the residues spanning its “ceiling.” The model predicts that these residues are contributed by the beginning of the M3-M4 loop and the cytoplasmic M1-M2 loop. Additional systematic mutagenesis will be required in future studies to probe this aspect of the homology model. Nonetheless, our results indicated that the residue conventionally denoted -4’ (i.e., the cytoplasmic ring (31)) when mutated from asparagine (in the human 5-HT_3A receptor) to aspartate (in the murine 5-HT_3A receptor) increases γ by ∼10 pS in the context of the human 5-HT_3A(QDA) receptor construct.  

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Analysis of the 5-HT$_3$A Receptor MA Stretch

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