Membrane Biology:
Mutagenic Analysis of the Intracellular Portals of the Human 5-HT \(_3A\) Receptor

Jane E. Carland, Michelle A. Cooper, Matthew R. Livesey, Tim G. Hales, John A. Peters and Jeremy J. Lambert
J. Biol. Chem. 2013, 288:31592-31601.
doi: 10.1074/jbc.M113.503300 originally published online September 12, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.503300

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 21 of which can be accessed free at
http://www.jbc.org/content/288/44/31592.full.html#ref-list-1
Mutagenic Analysis of the Intracellular Portals of the Human 5-HT\textsubscript{3}A Receptor

Jane E. Carland, Michelle A. Cooper, Matthew R. Livesey, Tim G. Hales, John A. Peters, and Jeremy J. Lambert

From the Division of Neuroscience, Medical Research and Medical Education Institutes, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, United Kingdom

Background: 5-HT\textsubscript{3}R 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 (Arg\textsuperscript{436} and Arg\textsuperscript{440}) within the MA helix of 5-hydroxytryptamine type 3 (5-HT\textsubscript{3}) receptors act singularly as rate-limiting determinants of single-channel conductance. 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-HT\textsubscript{3}A receptor model. We probed the majority of residues within the MA helix of the human 5-HT\textsubscript{3}A 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-HT\textsubscript{3}A 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)\textsuperscript{5} 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-HT\textsubscript{3}) receptors (3, 4) and the inhibitory, anion-selective, γ-aminobutyric acid type A (GABA\textsubscript{A}) (5, 6) and glycine receptors (7, 8) that are collectively termed “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 (10). 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-

* The work was supported by the Wellcome Trust, Tenovus Scotland, the Anonymous Trust (to J. A. P. and J. J. L.), the Biotechnology and Biological Sciences Research Council in conjunction with Lilly (to M. R. L.), and the Science and Technology Facilities Council (to J. E. C.).

† Present address: Dept. of Pharmacology, School of Medical Sciences, Sydney Medical School, A27, Edward Ford Bldg., The University of Sydney, NSW 2006 Australia.

‡ Present address: Centre for Integrative Physiology, School of Biomedical Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh EH8 9XD, Scotland, United Kingdom.

© 2013 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
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-HT$_3$A 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-HT$_3$A 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-HT$_3$A receptor, have extensively probed the M2 domain and flanking sequences for their impact upon ion conduction and selectivity (13, 35, 39–44). 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-HT$_3$A 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-HT$_3$A receptor (see Fig. 1, B and C). In particular, the replacement of the three arginine residues by their human 5-HT$_3$B subunit counterparts (QDA, respectively, generating a receptor construct coined 5-HT$_3$A(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-HT$_3$A receptor amino acids within the MA helix lining the putative intrasubunit portals evident in the 5-HT$_3$ 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-HT$_3$A 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-HT$_3$A(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-HT$_3$A 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-HT$_3$A receptor.

**EXPERIMENTAL PROCEDURES**

5-HT$_3$A Receptor Constructs and Transfection of Subunit cDNAs—cDNAs encoding human 5-HT$_3$A(QDA) and mutant subunit constructs thereof were cloned into prk5. Point mutations were introduced into the 5-HT$_3$A 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 Electropulser 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% CO$_2$ (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 MgCl$_2$, 1.0 CaCl$_2$, 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 MgCl$_2$, 5 EGTA, and 10 HEPES (pH 7.2 adjusted with KOH) and had resistances within the range 4–10 megaohms. 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 (Digidata 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$_3$A Receptor MA Stretch

tionship $\gamma = i/(V_m - E_{\text{rev}})$, in which $i$ is the current amplitude of single-channel events, $V_m$ is the holding potential ($-74$ mV), and $E_{\text{rev}}$ is the experimentally determined reversal potential. Data were typically obtained from patches subjected to 4–10 challenges with 5-HT. Patches invariably have multiple openings evident during the initial application of 5-HT. Analysis was performed during portions of the recording when unitary events predominated. 5-HT$_3$A receptors exhibit inward rectification. However, the relationship between voltage and the conductance of unitary events between $-60$ and more negative potentials is essentially linear (35).

**Structural Modeling**—The homology model of the 5-HT$_3$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.

**Statistics**—Data are presented as the arithmetic mean ± S.E. Data sets were compared using one-way analysis of variance with a post hoc Tukey’s test. $p < 0.05$ was considered significant.

**RESULTS**

The single-channel conductance ($\gamma$) of human and mouse homomeric 5-HT$_3$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$^{432}$, Arg$^{336}$, Arg$^{440}$) within the MA stretch of the human 5-HT$_3$A subunit by their human 5-HT$_3$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$_3$A(R432Q, R436D, R440A) receptor background, henceforth referred to as 5-HT$_3$A(QDA). Fig. 1 presents an alignment of residues within the MA stretch of human 5-HT$_3$A, 5-HT$_3$B, and 5-HT$_3$A(QDA) subunits.

**Alanine Scan of the Human 5-HT$_3$A(QDA) MA Helix**—Residues within the human 5-HT$_3$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$^{432}$ to Trp$^{442}$, 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$_3$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 $\alpha_7$ 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$_3$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$_3$A receptor (27, 28). In this study, we employed an arginine scan of the 5-HT$_3$A(QDA) MA
The 438 and 439 loci of the 5-HT3A(QDA) subunit. Data are reported as the mean percentage change in γ caused by each mutation, with the 5-HT3A(QDA) receptor acting as control. The mean reference 5-HT3A(QDA) value was 35.9 pS (Table 1). Statistical analysis was performed by comparing the raw values of γ for the 5-HT3A(QDA) and mutant receptor constructs. The inset traces are single-channel events recorded from excised outside-out patches expressing either (top) 5-HT3A(QDA) receptors (control) or (bottom) 5-HT3A(QRA) receptors (named D436A in the graph).

FIGURE 3. The influence of arginine substitutions upon the single-channel conductance (γ) of the 5-HT3A(QDA) receptor. The bar graph summarizes the effect of individual replacement of residues 427–441 of the 5-HT3A(QDA) subunit by arginine. Note that arginine is already present at the 426 locus of the 5-HT3A(QDA) subunit. Data are reported as the mean percentage change in γ caused by each mutation, with the appropriate alanine substituted 5-HT3A(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-HT3A(QDA) receptor constructs. The inset traces are single-channel events recorded from excised outside-out patches expressing either (top) 5-HT3A(QDA) receptors (control) or (bottom) 5-HT3A(QRA) receptors (named D436R in the graph).

TABLE 1
Single channel conductances (γ) for 5-HT3A(QDA) receptors mutated at distinct loci to alanine, arginine, or cysteine

| Locus | Alanine substituent | Arginine substituent | Cysteine substituent |
|-------|---------------------|----------------------|----------------------|
| Arg426 | 32.8 ± 1.4 (7)       | 35.9 ± 2.0 (5)*      | 31.2 ± 1.9 (5)       |
| Glu427 | 29.9 ± 0.6 (5)       | 34.8 ± 1.2 (5)*      | 32.9 ± 1.1 (5)       |
| Phe428 | 35.9 ± 1.4 (7)       | 39.1 ± 1.2 (5)       | 35.1 ± 0.4 (5)       |
| Leu429 | 27.0 ± 0.2 (5)*      | 32.6 ± 1.3 (10)      | 27.5 ± 0.9 (8)       |
| Glu430 | 27.7 ± 0.9 (8)*      | 30.8 ± 1.6 (7)       | 30.7 ± 1.1 (5)       |
| Lys431 | 42.6 ± 0.6 (7)*      | 36.6 ± 0.6 (5)*      | 42.4 ± 1.6 (5)       |
| Glu432 | 31.6 ± 0.7 (6)       | 24.2 ± 0.7 (7)*      | 32.6 ± 1.3 (5)       |
| Asp433 | 28.1 ± 0.7 (5)*      | 22.7 ± 0.9 (6)*      | 27.2 ± 1.2 (6)       |
| Glu434 | 26.0 ± 0.4 (7)*      | 19.8 ± 0.7 (6)*      | 28.0 ± 0.9 (7)       |
| Ile435 | 35.8 ± 0.8 (5)       | 21.3 ± 0.4 (6)*      | 43.7 ± 0.5 (5)*      |
| Asp436 | 13.1 ± 0.8 (9)*      | 6.4 ± 0.2 (3)*       | 18.4 ± 0.5 (12)*     |
| Glu437 | 27.7 ± 0.7 (5)*      | 21.6 ± 0.6 (5)*      | 29.4 ± 0.9 (5)       |
| Val438 | 30.4 ± 1.0 (7)*      | 22.7 ± 0.7 (5)*      | 31.4 ± 0.8 (6)       |
| Ala439 | 35.9 ± 2.0 (5)*      | 22.1 ± 1.0 (7)*      | 36.3 ± 1.1 (6)       |
| Ala440 | 35.9 ± 2.0 (5)*      | 18.4 ± 0.6 (9)*      | 29.2 ± 0.9 (5)*      |
| Asp441 | 29.0 ± 0.5 (5)       | 27.8 ± 1.2 (3)       | 32.7 ± 1.2 (6)       |

* p < 0.05 when compared with alanine mutant.
+ p < 0.001 when compared with untreated cysteine mutant.
= p < 0.001 when compared with alanine mutant.
\( \gamma \) p < 0.001 when compared with 5-HT3A(QDA) subunit construct.
\( \gamma \) p < 0.001 when compared with 5-HT3A(QDA) subunit residue.
\( \gamma \) p < 0.001 when compared with untreated cysteine mutant. 
\( \gamma \) p < 0.001 when compared with 5-HT3A(QDA) subunit residue.
Effect of MTS Reagent Application to Human 5-HT₃A(QDA)
MA Helix Cysteine Mutant Receptors—The covalent modification of cysteine residues by MTS reagents allows for the introduction of side chains with differing physicochemical properties. We have previously shown that that extracellular and intracellular applications of various MTS reagents, including MTSEA and MTSES, to human 5-HT₃A(QDA) receptors lacking engineered cysteine residues have no effect on γ (29). We examined the effect of positively charged MTSEA and negatively charged MTSES reagents, applied to outside-out patches through the recording electrode on the γ of cysteine mutant receptors. These two reagents were chosen because of their similar molecular volumes. Therefore, a comparison of their effects should not be confounded by differences in steric hindrances caused by the modified cysteine residue (29).

For hydrophilic MTS reagents to react with a substituted cysteine residue, the latter must reside in an accessible aqueous environment (51). The positively charged aminoethyl moiety donated to a cysteine residue by MTSEA results in a side chain with a volume and charge similar to that of an arginine residue, although the latter is considerably more basic. Nevertheless, qualitative similarities between the effects of arginine substitutions and covalent modification of cysteine residues by MTSEA would be anticipated for those cysteine residues that are accessible. Application of MTSEA (200 μM) within the patch pipette typically resulted in a decreased γ when compared with the matched alanine controls. The suppression of γ was significant for receptors containing the F428C, K431C, E434C, I435C, Q432C, K431C, E434C, R439C, and A440C mutations, the greatest decrease being associated with modification of cysteine residues at positions 435 (46%), 436 (45%), and 440 (50%) (Fig. 5A, Table 1). However, such a comparison includes the effect upon γ of the cysteine substitution itself, which in three instances (see above) was significant. Thus, we also analyzed the effect of MTSEA application employing the γ of unmodified cysteine constructs as a reference to isolate the change in γ specifically due to the reagent (Fig. 5B). Using this criterion, MTSEA caused significant reductions in γ 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 γ in comparison with alanine controls (see above). At all other loci, MTSEA treatment was associated with a trend toward a reduction in γ. Moreover, the effects upon γ of arginine substitution and challenge with MTSEA at positions 431 through 441 were in excellent qualitative agreement (r² = 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 γ 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 γ (r² = 0.58; Fig. 6B).

Although we denote mutations as they were actually constructed (e.g. L429R), the changes in γ reported are, for example, between Ala⁴²⁹ and Arg⁴²⁹, not Leu⁴²⁹. The sequential introduction of arginine residues from position Lys⁴³¹ to Asp⁴⁴⁰ inclusive resulted in a significant decrease in γ when compared with alanine controls, at all locations (Fig. 3, Table 1). Inspection of Fig. 3 reveals that the most pronounced decreases in γ occur at the adjacent positions 435 (41%)/436 (51%) and 439 (38%)/440 (49%). Against this overall trend of depression, the Q427R and L429R mutations were associated with a significant increase in γ of 16 and 21%, respectively. Arginine substitution at positions 428, 430, and 441 had no significant effect upon γ (p > 0.05). In summary, there is an overall trend for arginine substitutions at positions 427 through 430 to increase γ and 431 through to 440 to decrease γ.

Cysteine Scan of the Human 5-HT₃A(QDA) MA Helix—Cysteine residues were introduced sequentially along the MA helix from Arg⁴²⁶ to Trp⁴⁴² to enable SCAM analysis. Of the 17 mutant receptors constructed, only the 5-HT₃A(QDA) W442C construct reduced channel activity to an extent that made it unfeasible to determine the effect of introducing MTS reagents. Typically, the introduction of cysteine residues had little effect on γ when compared with the alanine controls that served to “standardize” the substituted residue (Fig. 4, Table 1). However, a significant increase in γ was caused by the mutations I435C (22%) and D436C (40%), and a decrease (19%) followed the A440C exchange. Overall, there is a good correlation between the effect of alanine and cysteine substitutions upon γ with a tendency for the latter to result in a slightly higher value (Fig. 4). Such an effect may result from the modest negativity of the cysteine side chains, ~8% of which would be deprotonated at pH 7.2, assuming a pKₐ of 8.3. Alternatively, a reduction in hydrophobicity when cysteine substitutes for alanine might underlie the trend to increased γ.

FIGURE 4. The influence of cysteine substitutions upon the single-channel conductance (γ) of the 5-HT₃A(QDA) receptor. The bar graph summarizes the effect of individual replacement of residues 426–441 of the 5-HT₃A(QDA) subunit by cysteine. Data are reported as the mean percentage change in γ caused by each mutation, with the appropriate alanine substituted 5-HT₃A(QDA) receptor construct acting as control. Statistical analysis was performed by comparing the raw values of γ for the cysteine- and alanine-substituted 5-HT₃A(QDA) receptor constructs. The inset traces are single-channel events recorded from excised outside-out patches expressing either (top) 5-HT₃A(QDA) receptors (control) or (bottom) 5-HT₃A(QDA) receptors (named D436C in the graph).
The application of MTSES to cysteine mutant receptors tended to increase $\gamma$ versus 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-HT₃A 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 γ.

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-HT₃A(QDA) receptor construct decreases or increases γ, respectively; (ii) the introduction of an arginine residue decreases γ when compared with the appropriate alanine-substituted control; (iii) reaction of an engineered cysteine residue with MTSEA or MTSES decreases or increases γ, 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 γ. 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 γ when compared with the appropriate alanine control, a lack of an observable effect of MTSEA (or MTSES) upon the γ 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-HT₃A receptor derived from that structure (29) (Fig. 7), we anticipated that a potentially large number of residues within the MA stretch might impact upon γ, 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-HT₃A receptor subsequent to agonist binding (53).

Confining initial consideration to those loci at which arginine substitution caused a significant decrease in γ 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 γ 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 γ. For those substituted cysteine residues that did not yield a significant reduction in γ 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-HT₃A(QDA) receptor were mutated to alanine dis-
played (with the exception of Asp\textsuperscript{432}) a significant reduction in γ, whereas the mutation of the solitary positive charge in this region (i.e. K431A) resulted in augmented γ. 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 γ of the 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 γ at the 433, 436, 437, 438, and 440, but not the 434 and 435 loci. We directly compared the γ 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 γ over the loci 432–441 (rendered in yellow in Fig. 7B), and at all of these, the difference in γ was significant. However, in specific instances, MTSEA significantly reduced γ, 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 γ, or the increase in side chain volume may negate any influence of introducing negative charge. Conversely, MTSES elevated γ 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 433 favors deprotonation of the primary amino acid.
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/neural 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_426 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 portal structure in keeping with the model. Proceeding beyond this position with arginine or cysteine scanning to the entirely conserved tryptophan at 442 had detrimental effects most likely upon gating by analogy to the α7 nicotinic nACh receptor (50).

An aspect of the 5-HT_3 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_3 receptor) to aspartate (in the murine 5-HT_3 receptor) increases γ by ~10 pS in the context of the human 5-HT_3A(QDA) receptor construct.

More challenging will be progress toward determining the structure of the M3-M4 loop of Cys-loop receptors lying outside the Torpedo nACh receptor model. Complete and higher resolution structural models of GLIC and ELIC are of no help in this regard because these bacterial pLGICs lack the large intracellular loop that is a hallmark of Cys-loop pLGICs and the crystallization of GLIC-1 was achieved with the loop largely deleted (10). The missing link between the beginning of the M3-M4 loop and the start of the MA helix is a stretch of 85 amino acids in the human 5-HT_3A subunit for which there is no structural information. We recently probed this region by deleting stretches of amino acid residues of variable length from close to the start of the MA stretch progressing in the N-terminal direction toward M3 (30). The analysis revealed that deletions that truncated the loop length to between 90 and 75 amino acids were well tolerated using inward rectification of macroscopic current responses to 5-HT as a convenient index of the influence of the MA stretch (30). However, truncation to 70 residues abolished rectification and also increased γ. Interestingly, the M3-M4 loop of all human Cys-loop receptor subunits exceeds 70 residues, suggesting that this is the minimum length necessary to maintain unperturbed function (30).

Our developing functional map of the 5-HT_3 receptor (27, 29, 30, 35) reveals that, despite the relatively poor conservation of amino acids within MA helices, residues that obey criteria for inclusion as components within the conduction pathway lie within the portal structure derived from the Torpedo model.

Acknowledgment—We thank Dr. Tarek Deeb (Tufts University) for expert contributions to the generation of homology models.

REFERENCES
1. Millar, N. S., and Gotti, C. (2009) Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology 56, 237–246
2. Changeux, J. P. (2012) The nicotinic acetylcholine receptor: the founding father of the pentameric ligand-gated ion channel superfamily. J. Biol. Chem. 287, 40207–40215
3. Barnes, N. M., Hales, T. G., Lummis, S. C. R., and Peters, J. A. (2009) The 5HT_3 receptor – the relationship between structure and function. Neuropharmacology 56, 273–284
4. Lummis, S. C. R. (2012) 5HT_3 receptors. J. Biol. Chem. 287, 40239–40245
5. Olsen, R. W., and Sieghart, W. (2008) International Union of Pharmacology. LXV. Subtypes of γ-aminobutyric acidA receptors: classification on the basis of subclass composition, pharmacology, and function. Update. Pharmacol. Rev. 60, 243–260
6. Sigel, E., and Steinmann, M. E. (2012) Structure, function and modulation of GABA_A receptors. J. Biol. Chem. 287, 40224–40231
7. Lynch, J. W. (2009) Native glycine receptor subtypes and their physiological roles. Neuropharmacology 56, 303–309
8. Dutertre, S., Becker, C. M., and Betz, H. (2012) Inhibitory glycine receptors: an update. J. Biol. Chem. 287, 40216–40223
9. Hill, R. J., and Dutzler, R. (2009) A prokaryotic perspective on pentameric ligand-gated ion channel structure. Curr. Opin. Struct. Biol. 19, 418–424
10. Hibbs, R. E., and Gouaux, E. (2011) Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature 474, 54–60
11. Wolstenholme, A. J. (2012) Glutamate-gated chloride channels. J. Biol. Chem. 287, 40232–40238
12. Miller, P. S., and Smart, T. G. (2010) Binding, activation and modulation of Cys-loop receptors. Trends Pharmacol. Sci. 31, 161–174
13. Thompson A. J., Lester, H. A., and Lummis S. C. R. (2010) The structural basis of function in Cys-loop receptors. Q. Rev. Biophys. 43, 449–499
14. Lester, H. A., Dibas, M. I., Dahan, D. S., Leite, J. F., and Dougherty, D. A. (2004)
Cys-loop receptors: new twists and turns. Trends Neurosci. 27, 329–336
15. Miyazawa, A., Fujisho, Y., and Unwin, N. (2003) Structure and gating mechanism of the acetylcholine receptor pore. Nature 423, 494–495
16. Le Novère, N., Corriger, P. J., and Changeux, J. P. (1999) Improved secondary structure predictions for a nicotinic receptor subunit: incorporation of solvent accessibility and experimental data into a two-dimensional representation. Biophys. J. 76, 2329–2345
17. Kottwitz, D., Kukhtina, V., Dergousova, N., Alexeev, T., Utkin, Y., Tsetlin, V., and Hucho, F. (2004) Intracellular domains of the 6-subunits of Torpedo and rat acetylcholine receptors—expression, purification, and characterization. Protein Expr. Purif. 38, 237–247
18. Kukhtina, V., Kottwitz, D., Strauss, H., Heise, B., Chebotareva, N., Tsetlin, V., and Hucho, F. (2006) Intracellular domain of nicotinic acetylcholine receptor: the importance of being unfolded. J. Neurochem. 97, 63–67
19. Smart, T. G. (1997) Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. Curr. Opin. Neurobiol. 7, 358–367
20. Swope, S. L., Moss, S. J., Raymond, L. A., and Huganir, R. L. (1999) Regulation of ligand-gated ion channels by protein phosphorylation. Adv. Second Messenger Phosphoprotein Res. 33, 49–78
21. Yu, X. M., and Hall, Z. W. (1994) A sequence in the main cytoplasmic loop of the α subunit is required for assembly of mouse muscle nicotinic acetylcholine receptor. Neuron 13, 247–255
22. Williams, B. M., Temburun, M. K., Levey, M. S., Bertrand, S., Bertrand, D., and Jacob, M. H. (1998) The long intracellular loop of the α subunit targets nAChRs to subdomains within individual synapses on neurons in vivo. Nat. Neurosci. 1, 557–562
23. Valor, L. M., Mulet, J., Sala, F., Sala, S., Ballesta, J. J., and Criado, M. (2002) Role of the large cytoplasmic loop of the α7 neuronal nicotinic acetylcholine receptor subunit in receptor expression and function. Biochemistry 41, 7931–7938
24. Xu, J., Zhu, Y., and Heinemann, S. F. (2006) Identification of sequence motifs that target neuronal nicotinic receptors to dendrites and axons. J. Neurosci. 26, 9780–9793
25. Kracun, S., Harkness, P. C., Gibb, A. J., and Millar, N. S. (2008) Influence of the M3-M4 intracellular domain upon nicotinic acetylcholine receptor assembly, targeting and function. Br. J. Pharmacol. 153, 1474–1484
26. Mucklerjee, J., Kuryatov, A., Moss, S. J., Lindstrom, J. M., and Anand, R. (2009) Mutations of cytosolic loop residues impair assembly and maturation of α7 nicotinic acetylcholine receptors. J. Neurochem. 110, 1885–1894
27. Kelley, S. P., Dunlop, J. I., Kirkness, E. F., Lambert, J. J., and Peters, J. A. (2003) A cytoplasmic region determines single-channel conductance in 5HT3 receptors. Nature 424, 321–324
28. Hales, T. G., Dunlop, J. I., Deeb, T. Z., Carland, J. J., Kelley, S. P., Lambert, J. J., and Peters, J. A. (2006) Common determinants of single channel conductance within the large cytoplasmic loop of 5-hydroxytryptamine type 3 and α3β2 nicotinic acetylcholine receptors. J. Biol. Chem. 281, 8062–8071
29. Deeb, T. Z., Carland, J. E., Cooper, M. A., Livesey, M. R., Lambert, J. J., Peters, J. A., and Hales, T. G. (2007) Dynamic modification of a mutant cytoplasmic cysteine residue modulates the conductance of the human 5-HT3A receptor. J. Biol. Chem. 282, 6172–6182
30. Baptista-Hon, D. T., Deeb, T. Z., Lambert, J. J., Peters, J. A., and Hales, T. G. (2013) The minimum M3-M4 loop length of neurotransmitter-activated pentameric receptors is critical for the structural integrity of cytoplasmic portals. J. Biol. Chem. 288, 21558–21568
31. Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K., and Numa, S. (1988) Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. Nature 335, 645–648
32. Keramidou, A., Moorhouse, A. J., Schofield, P. R., and Barry, P. H. (2004) Ligand-gated ion channel mechanisms underlying ion selectivity. Prog. Biophys. Mol. Biol. 86, 161–204
33. Carland, J. E., Cooper, M. A., Sugiharto, S., Jeong, H. J., Lewis, T. M., Barry, P. H., Peters, J. A., Lambert, J. J., and Moorhouse, A. J. (2009) Characterization of the effects of charged residues in the intracellular loop on ion permeation in α1 glycine receptor channels. J. Biol. Chem. 284, 2023–2030
34. Hu, X. Q., Sun, H., Peoples, R. W., Hong, R., and Zhang, L. (2006) An interaction involving an arginine residue in the cytoplasmic domain of the 5-HT3A receptor contributes to receptor desensitization mechanism. J. Biol. Chem. 281, 21781–21788
35. Livesey, M. R., Cooper, M. A., Deeb, T. Z., Carland, J. E., Kozuska, J., Hales, T. G., Lambert, J. J., and Peters, J. A. (2008) Structural determinants of Ca2+ permeability and conduction in the human 5-hydroxytryptamine type 3A receptor. J. Biol. Chem. 283, 19301–19313
36. Mochizuki, S., Miyake, A., and Furuichi, K. (1999) Ion permeation properties of the effects of charged residues in the intracellular loop on ion permeation in a Cys-loop receptor. J. Biol. Chem. 274, 2180–2185