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Daniel T. Baptista-Hon
University of Dundee, UK

Tarek Z. Deeb
George Washington University

Jeremy J. Lambert
University of Dundee, UK

John A. Peters
University of Dundee, UK

Tim G. Hales
George Washington University

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The Minimum M3-M4 Loop Length of Neurotransmitter-activated Pentameric Receptors Is Critical for the Structural Integrity of Cytoplasmic Portals*

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Daniel T. Baptista-Honi†, Tarek Z. Deeb†, Jeremy J. Lambert‡, John A. Peters‡, and Tim G. Hales‡§†

From the †Institute of Academic Anaesthesia and the ‡Division of Neuroscience, University of Dundee, Dundee DD1 9SY, Scotland, United Kingdom and the §Department of Pharmacology and Physiology, The George Washington University, Washington, D. C. 20037

Background: Residue 436 within 5-HT3A cytoplasmic portals determines single channel conductance (γ) and rectification. Results: M3-M4 loop truncation to 75 residues spared inward rectification; truncation to 70 abolished rectification and increased γ. Human Cys-loop neurotransmitter receptor M3-M4 loops all exceed 70 residues. Conclusion: M3-M4 lengths of >70 are critical. Significance: Cytoplasmic portals may be essential for normal function of Cys-loop neurotransmitter receptors.

The 5-HT3A receptor homology model, based on the partial structure of the nicotinic acetylcholine receptor from Torpedo marmorata, reveals an asymmetric ion channel with five portals framed by adjacent helical amphipathic (HA) stretches within the 114-residue loop between the M3 and M4 membrane-spanning domains. The positive charge of Arg-436, located within the HA stretch, is a rate-limiting determinant of single channel conductance (γ). Further analysis reveals that positive charge and volume of residue 436 are determinants of 5-HT3A receptor inward rectification, exposing an additional role for portals. A structurally unresolved stretch of 85 residues constitutes the bulk of the M3-M4 loop, leaving a >45-Å gap in the model between M3 and the HA stretch. There are no additional structural data for this loop, which is vestigial in bacterial pentameric ligand-gated ion channels and was largely removed for crystallization of the Caenorhabditis elegans glutamate-activated pentameric ligand-gated ion channels. We created 5-HT3A subunit loop truncation mutants, in which sequences framing the putative portals were retained, to determine the minimum number of residues required to maintain their functional integrity. Truncation to between 90 and 75 amino acids produced 5-HT3A receptors with unaltered rectification. Truncation to 70 residues abolished rectification and increased γ. These findings reveal a critical M3-M4 loop length required for functions attributable to cytoplasmic portals. Examination of all 44 subunits of the human neurotransmitter-activated Cys-loop receptor family reveals that, despite considerable variability in their sequences and lengths, all M3-M4 loops exceed 70 residues, suggesting a fundamental requirement for portal integrity.

5-Hydroxytryptamine type 3 (5-HT3)2 receptors are members of a family of pentameric ligand-gated ion channels (pLGICs), which includes the eukaryotic Cys-loop receptors and the bacterial pLGICs. Other members of the Cys-loop receptor family include the nicotinic acetylcholine (nACh), glycine, and γ-aminobutyric acid type A (GABA_3) receptors. There are five genes that encode 5-HT3 subunits (A–E), which have features common to all Cys-loop subunits: an extracellular N-terminal domain containing the Cys-loop motif, four membrane-spanning domains (M1–M4), and a large cytoplasmic loop between M3 and M4. Homologous bacterial pLGICs lack both the N-terminal Cys-loop motif and the large M3-M4 loop (1, 2).

5-HT3A subunits combine to form functional homomeric receptors with a sub-pS single channel conductance (γ), unique among all Cys-loop receptors examined to date. Unitary microscopic currents are too small to be directly resolved using conventional electrophysiological recording techniques (3). 5-HT3A receptors also exhibit inwardly rectifying macroscopic and single channel current-voltage relationships, slow desensitization kinetics, and substantial Ca2+ permeability (3). 5-HT3B, 5-HT3C, 5-HT3D, and 5-HT3E subunits are unable to form functional homomers (4). However, incorporation of the 5-HT3B subunit into heteromeric 5-HT3AB receptors has profound effects on the biophysics of receptor function (5). Human 5-HT3AB receptors have a linear current-voltage relationship, fast desensitization, negligible Ca2+ permeability, and single channel γ of 16 pS (5).

A chimeric strategy, replacing segments of 5-HT3A subunit amino acid sequences with equivalent residues in the 5-HT3B subunit, revealed that amino acid determinants of Ca2+ permeability lie within the M2 domain (6). By contrast, a similar approach revealed that the molecular determinants for γ lie within the helical amphipathic (HA) stretch in the M3-M4

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† To whom correspondence should be addressed: the Institute of Academic Anaesthesia and Division of Neuroscience, University of Dundee, Dundee DD1 9SY, Scotland, United Kingdom. Tel.: 44-1382-383-443; E-mail: t.g.hales@dundee.ac.uk.

The abbreviations used are: 5-HT3, 5-hydroxytryptamine type 3; HA, helical amphipathic; nACh, nicotinic acetylcholine; pLGIC, pentameric ligand-gated ion channel; GluCl, glutamate-activated Cl− channel; pS, picoseconds; ANOVA, analysis of variance.
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loop. Three Arg residues at positions 432, 436, and 440, when replaced by equivalent residues (Gln, Asp, and Ala) in the 5-HT₃B subunit, produced 5-HT₃A(QDA) receptors with greatly increased γ (~40-fold). The R436D substitution had the largest individual impact (7). Methanethiosulfonate modifications of the substituted Cys residues in mutant 5-HT₃A(QCA) receptors revealed that the introduction of positive or negative charge decreased or increased γ, respectively, whereas increasing molecular volume without charge reduced γ (8). Such data provide further evidence that 5-HT₃A residue 436 is a rate-limiting determinant of conductance. In the 4-Å resolution partial structural model of the T. marmorata nACh receptor, HA stretches from adjacent subunits frame narrow intracellular portals through which ions must pass to enter or exit the cytoplasm (9). The five cytoplasmic portals enclose a vestibule that is contiguous with the transmembrane pore. The homology model of the 5-HT₃A receptor, based on the structure of the nACh receptor, indicates that Arg-436 lies at the mouth of each portal (see Fig. 2A).

Although the nACh receptor model (9) provides insight into the structure of cytoplasmic portals framed by adjacent HA stretches, the bulk of the M3-M4 loop remains structurally unresolved, probably due to a disorganized structure (10), and its role in Cys-loop receptor remains unclear. X-ray structures with stretches from adjacent subunits form narrow intracellular portals through which ions must pass to enter or exit the cytoplasm (9). The five cytoplasmic portals enclose a vestibule that is contiguous with the transmembrane pore. The homology model of the 5-HT₃A receptor, based on the structure of the nACh receptor, indicates that Arg-436 lies at the mouth of each portal (see Fig. 2A).

In this study, we demonstrate using mutagenesis that, in addition to its critical role in determining γ, residue 436 governs 5-HT₃A receptor inward rectification, a property that correlates with the volume of the amino acid. Therefore, the distinctive 5-HT₃A receptor properties of γ and inward rectification can both be used to assay the rate-limiting nature of residues located within cytoplasmic portals. Systematic truncation of the 5-HT₃A M3-M4 loop proximal to the HA stretch revealed that an M3-M4 loop length of >70 amino acids is required to maintain rate-limiting portals.

MATERIALS AND METHODS

Cell Culture and Transfection—Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg ml⁻¹ penicillin, and 100 units ml⁻¹ streptomycin at 37 °C and 5% CO₂. Cells were seeded at low density in 35-mm dishes for electrophysiological recording, and transfections were performed by calcium phosphate precipitation, using 1 μg of total cDNA per dish, as described previously (6). cDNAs encoding wild-type and mutant 5-HT₃ subunits were cloned into the pCDM8 mammalian expression vector using HindIII (5') and XhoI (3') sites. Unless stated otherwise, for heteromeric expression of 5-HT₃ subunits, a 1:1 transfection ratio was used. cDNA encoding enhanced green fluorescence protein (in pEGFP vector, 0.1 μg) was included to identify successfully transfected cells using fluorescence microscopy. Cells were washed with media 16 h after transfection and used after 48–72 h. Tissue culture reagents were obtained from Invitrogen (Paisley, UK).

Mutagenesis of 5-HT₃A Subunits—Point mutations and truncations of the M3-M4 loop were performed by overlap extension polymerase chain reaction (PCR) (12). Single point mutations and deletions were generated by chimeric PCR primers. PCR products were digested using HindIII and XhoI restriction endonucleases and ligated into pCDM8 vector. All mutagenesis reactions and ligation were verified using agarose gel electrophoresis, and constructs were sequenced to confirm fidelity prior to functional testing (Genetics Core Services, University of Dundee). All PCR and cloning reagents were from Fermentas (Thermo Fisher, Loughborough, UK).

Electrophysiology—The whole cell configuration of the patch clamp technique was used to record 5-HT-evoked currents from HEK-293 cells expressing wild-type (WT) or mutant 5-HT₃A receptors. Recording electrodes were fabricated from borosilicate glass capillaries, and when filled with intracellular solution, had resistances of 1–3 MΩ. The electrode solution contained (in mM): 140 CsCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, 10 HEPES (pH 7.4 with CsOH). The extracellular solution contained (in mM): 140 NaCl, 2.8 KCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose (pH 7.4 with NaOH). Unless otherwise stated, cells were voltage-clamped at an electrode potential of ~60 mV. Macroscopic current-voltage (I-V) relationships were determined by adjusting the electrode potential from ~60 mV to +60 mV in 20-mV increments. To determine the linearity of I-V relationships, the ratio of peak currents evoked at 60 mV (the rectification index) was used. A unitary rectification index indicates a linear I-V relationship. A unitary rectification index was used as a marker of the successful incorporation of the 5-HT₃B subunit, which causes linearization of the I-V relationship (5). Unless otherwise stated, currents were evoked by rapid application of 100 μM 5-HT, using the three-pipe Perfusion Fast Step system (Warner Instruments), as described previously (6). The outside-out patch configuration was used to record single channel currents from excised patches from transfected cells as detailed previously (5). Electrodes were coated with Sylgard (Corning, NY) to minimize capacitance and noise. Currents were evoked by transient pressure application (Picospreizer II, General Valve Corp., Fairfield, NJ) of 5-HT (3 μM). Single channel I-V relationships were determined by adjusting the electrode potential between −100 and −40 mV, in 20-mV increments.

All electrophysiological data were recorded using an Axopatch 200B amplifier. Data were low pass-filtered at 1 kHz for single channel data and 2 kHz for whole cell data, digitized at 20 kHz using a Digidata 1320A interface, and acquired using pClamp8 software (all from Molecular Devices).

Data Analysis—The peak amplitudes of 5-HT-evoked currents were measured using pClamp10 software. Desensitization kinetics were measured by fitting a double exponential function to the decaying phase of a 5-HT-evoked current. Sin-
The HA Stretch Determines 5-HT₃ Receptor Rectification—The γ of homomeric 5-HT₃₄ receptors is <1 pS, and 5-HT-evoked currents exhibit an inwardly rectifying I-V relationship (3). Incorporation of the 5-HT₃₂ subunit into heteromeric 5-HT₃₂₄ receptors increases γ to 16 pS and abolishes rectification (5). The use of a chimeric strategy, in which regions of the 5-HT₃₂ subunit were introduced into the 5-HT₃₄ subunit, isolated determinants of γ within the HA stretch of the large cytoplasmic M3-M4 loop (6–8). We adopted the same chimeric strategy here to identify the location of residues that determine rectification (Fig. 1). Consistent with previous work, WT 5-HT₃₄ receptors displayed inward rectification and the incorporation of the 5-HT₃₂ subunit increased the rectification index to near unity (5). Replacement of the entire HA stretch in the 5-HT₃₂ subunit with that of the 5-HT₃₂ subunit (chimera C4) conferred a linear IV relationship and a near unitary rectification index. In this study, we investigated the role of Arg residues within the HA stretch of the 5-HT₃₂ subunit alone or in combination with either R432Q or R440A (HA 0 and 4 positions, respectively) substitutions were also without significant effect on the rectification index when incorporated into the 5-HT₃₂ subunit either individually or in combination. However, the 5-HT₃₂(R436D) mutation (at the HA 0 position) significantly increased the rectification index close to unity when incorporated into the 5-HT₃₂ subunit. The average rectification index data for WT and mutant receptors are shown in Fig. 2B. The 5-HT₃₂A(R426S) construct, in which Arg at the HA 0 position was replaced by Ser (the HA 0 residue in 5-HT₃₂B), had a rectification index similar to that of WT 5-HT₃₂A receptors (Fig. 2B). Likewise 5-HT₃₂A R432Q and R440A (HA 0′ and HA 4′ positions, respectively) substitutions were also without significant effect on the rectification index (see “Materials and Methods”). The rectification index data for WT and mutant receptors are shown in Fig. 2B. The 5-HT₃₄A(R426S) construct, in which Arg at the HA 0′ position was replaced by Ser (the HA 0′ residue in 5-HT₃₄B) subunit, with that of the 5-HT₃₂B subunit (chimera C3 and C4 constructs). As reported previously, the 5-HT₃₂B subunit, C1, C2, and C3 required co-expression with the 5-HT₃₂ subunit for function (5, 7).

The Charge and Volume of the 0′ Residue within the HA Stretch Influences 5-HT₃ Receptor Rectification—Our previous studies demonstrate that Arg-432, Arg-436, and Arg-440, at −4′, 0′, and 4′ positions within the HA stretch, are responsible for the sub-pS single channel conductance of 5-HT₃₂A receptors, with Arg-436 having the largest individual impact on γ, giving rise to its designation as HA 0′ (13). The homology model of the 5-HT₃₂A receptor, based on the structure of the T. marmorata nACh receptor (9), indicates that these Arg residues lie near the cytoplasmic entrances of putative ion-conducting portals (Fig. 2A). In this study, we investigated the role in rectification of Arg residues within the HA stretch of the 5-HT₃₂A receptor. Residues were systematically substituted, individually and in combination, by their amino acid counterparts within the 5-HT₃₂B subunit (Fig. 2A). WT and mutant constructs were transfected into HEK-293 cells, and the amplitudes of currents, evoked by 5-HT (100 μM) at +60 mV, were expressed as a ratio of those evoked at −60 mV (1.0 to 1.0 mV), providing a rectification index (see “Materials and Methods”). The average rectification index data for WT and mutant receptors are shown in Fig. 2B. The 5-HT₃₂A(R426S) construct, in which Arg at the HA 0′ position was replaced by Ser (the HA 0′ residue in 5-HT₃₂B), had a rectification index similar to that of WT 5-HT₃₂A receptors (Fig. 2B). Likewise 5-HT₃₂A R432Q and R440A (HA 0′ and HA 4′ positions, respectively) substitutions were also without significant effect on the rectification index when incorporated into the 5-HT₃₂ subunit either individually or in combination. However, the 5-HT₃₂A(R436D) mutation (at the HA 0′ position) significantly increased the rectification index close to unity when incorporated into the 5-HT₃₂ subunit alone or in combination with either R432Q or R440A. These data demonstrate that the HA 0′ residue is the key determinant of rectification in 5-HT₃ receptors, the same HA stretch residue that plays a predominant role in determining γ (13).

The charge of the HA 0′ residue is the principal determinant of γ, with residue volume playing a more minor role through steric hindrance (8). We replaced Arg-436 by alternative amino acids with differing properties and examined the rectification...
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FIGURE 2. The HA stretch contains the determinants of inward rectification in 5-HT₃A receptors. A, ribbon diagram (left panel) based on the *T. marmorata* structure and alignment of human, mouse, and rat 5-HT₃A and 5-HT₃B subunit HA stretch residues (right panel). The three conserved arginine residues (432 at HA −4′, 436 at HA 0′, and 440 at HA 4′) in the human 5-HT₃A subunit previously shown to affect single channel conductance are highlighted, along with the corresponding residues in the 5-HT₃B subunit (13). These HA stretch Arg residues frame the cytoplasmic portals in a 5-HT₃A receptor homology model (13) based on the *T. marmorata* nACh receptor structure (9). Also highlighted in the alignment is Arg-426 (at HA 0′) in the human 5-HT₃A subunit previously shown to affect single channel conductance are highlighted, along with the corresponding residues in the 5-HT₃B subunit (13). These HA stretch Arg residues frame the cytoplasmic portals in a 5-HT₃A receptor homology model (13) based on the *T. marmorata* nACh receptor structure (9). Also highlighted in the alignment is Arg-426 (at HA 0′). B, systematic replacement of arginines within the 5-HT₃A subunit HA stretch by corresponding residues in the 5-HT₃B subunit reveals that the HA stretch contains the determinants for rectification. Data are represented as the ratio of 5-HT-evoked currents at +60 mV and −60 mV (*I_{+60mV}/I_{-60mV}*) in WT 5-HT₃A and 5-HT₃AB (black bars) and mutant 5-HT₃A receptors (open bars for single mutants and gray bars for double mutants). 5-HT₃A receptors display inward rectification as revealed by the below unitary *I_{+60mV}/I_{-60mV}*(WT 5-HT₃A). Averaged data indicate that both the charge and the volume of the residue at HA 0′ correlate with the rectification index because volume also plays a role in determining γ (8). The volume of the residue at HA 0′ correlated with the rectification index (Pearson coefficient = −0.62; *p* = 0.005; Fig. 3C). Linear regression produced an *R*² value of 0.38. Similar to the relationship between charge and the rectification index, we repeated the analysis, excluding nonpolar residues. This also improved the correlation (Pearson coefficient = −0.72; *p* = 0.02) and *R*² value (0.51; Fig. 3D). These data indicate that both the charge and the volume of the HA 0′ residue influence rectification in 5-HT₃A receptors.

*M3-M4 Loop Truncation Approach*—With the exception of the representation of the HA stretches in the *T. marmorata* nACh receptor model, there are no structural data for the large.

**index of each of the mutant receptors**, represented in Fig. 3 by the single letter abbreviation of the substituent amino acid. The change in charge of the 5-HT₃A subunit HA 0′ residue caused by the substitution of the Arg by each amino acid was plotted against the rectification index (Fig. 3A). Changing the charge at HA 0′ produced a positive correlation with the rectification index (Pearson coefficient = 0.61; *p* = 0.006; Fig. 3A). Linear regression through the data points had an *R*² value of 0.37. There is considerable heterogeneity in the rectification index for nonpolar residues. We therefore repeated the analysis with only charged (positive and negative) and polar residues at HA 0′ (Fig. 3B). Excluding the nonpolar residues from the analysis improved the correlation coefficient (Pearson coefficient = 0.82; *p* = 0.004), as well as the linear fit (*R*² = 0.68). We also examined the relationship between the volume of the residue at HA 0′ and the rectification index because volume also plays a role in determining γ (8). The volume of the residue at HA 0′ correlated with the rectification index (Pearson coefficient = −0.62; *p* = 0.005; Fig. 3C). Linear regression produced an *R*² value of 0.38. Similar to the relationship between charge and the rectification index, we repeated the analysis, excluding nonpolar residues. This also improved the correlation (Pearson coefficient = −0.72; *p* = 0.02) and *R*² value (0.51; Fig. 3D). These data indicate that both the charge and the volume of the HA 0′ residue influence rectification in 5-HT₃A receptors.
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FIGURE 3. The charge and volume of residue 436 are determinants of inward rectification. Graphical representations of the relationship between \( I_{+60mV} / I_{-60mV} \) and either the amino acid charge or the volume of the HA residue are shown. Arg-436 was replaced by amino acids with differing properties. A, graph of the relationship between the change in charge, caused by the substitution of Arg-436, and \( I_{+60mV} / I_{-60mV} \). Replacement of the positively charged Arg by an uncharged residue corresponds to a change of charge of 1, whereas its replacement by a negatively charged residue corresponds to a change of charge of 2. There was a significant correlation between the change in charge at position 436 and the rectification index (Pearson coefficient = 0.61; \( p = 0.006 \)). The straight line indicates the linear fit through the data (\( R^2 = 0.37 \)). B, relationship between the change in amino acid charge at position 436 restricted to charged and polar residues. Exclusion of the nonpolar residues improved the correlation coefficient (Pearson coefficient = 0.83; \( p = 0.004 \)) and the linear fit (\( R^2 = 0.68 \)). C, graph of the relationship between the volume of the amino acid at position 436 and \( I_{+60mV} / I_{-60mV} \). In general, larger amino acids at position 436 were associated with more inward rectification. Indeed, there was a significant correlation between the volume of the residue at position 436 and the rectification index (Pearson coefficient = 0.62; \( p = 0.005 \)). This relationship was fitted by a linear regression with an \( R^2 \) value of 0.38. D, exclusion of nonpolar residues also improved the correlation between the volume and the rectification index (Pearson coefficient = 0.72; \( p = 0.02 \)) and the linear fit (\( R^2 = 0.51 \)). Amino acid volumes were determined using Spartan \(^\text{®} \) software (see "Materials and Methods").

The structure of the M3-M4 loop, between the end of M3 and the start of the HA stretch remains unresolved in the T. marmorata ACh receptor model (9). This omission is represented by a gap of >45 Å in the model. In the case of the 5-HT₃A receptor, the gap between M3 and the HA stretch is bridged by 85 residues. We reasoned that systematic removal of amino acids within this region would reveal the minimum number required to connect the 5-HT₃A subunit M3 with the HA stretch without compromising the functional integrity of the portals, assessed by the rectification index.

The sequences of the structurally unresolved sections of the M3-M4 loop of the human 5-HT₃A subunit and truncation constructs are shown in Fig. 4A. We initially truncated the M3-M4 loop close to M3. However, when expressed in HEK-293 cells and challenged with 5-HT (100 \( \mu M \)), neither of these shortened constructs produced functional receptors (\( n = 4 \) and 3 for L-10(1) and L-10(2), respectively), suggesting that residues in the region removed are essential for functional expression of 5-HT₃A receptors. We therefore adopted an alternative strategy of truncation starting closer to the HA stretch (Fig. 4A).

Truncations of 24 (\( n = 7 \)), 34 (\( n = 7 \)), 39 (\( n = 14 \)), and 44 (\( n = 6 \)) amino acids from the M3-M4 loop (termed: L-24, L-34, L-39, and L-44) produced functional homomeric constructs (Fig. 4B). The construct truncated by 55 amino acids (L-55) did not form functional homomeric receptors (\( n = 3 \)), but combined with WT 5-HT₃A subunits to form heteromeric receptors with properties distinct from those of the WT 5-HT₃A receptor (\( n = 7 \), Fig. 4C). These truncations within the M3-M4 loop did not affect expression of functional receptors, as assessed by current density measurements. 5-HT (100 \( \mu M \))-evoked current densities (in picoamperes/picofarads) were 69 ± 22 (\( n = 10 \)) for WT, 109 ± 39 (\( n = 7 \)) for L-24, 176 ± 45 (\( n = 7 \)) for L-34, 149 ± 33 (\( n = 14 \)) for L-39, 135 ± 57 (\( n = 6 \)) for L-44, and 111 ± 52 (\( n = 7 \)) for WT/L-55. These values were not statistically different from each other (\( p > 0.4 \), one-way ANOVA).

M3-M4 Loop Truncation Influences Desensitization Kinetics—5-HT (100 \( \mu M \))-evoked currents mediated by truncated homomeric 5-HT₃A L-24, L-34, and L-44 receptors exhibited desensitization kinetics similar those of WT 5-HT₃A receptors (Fig.
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FIGURE 4. M3-M4 loop truncation alters the properties of 5-HT₃A receptors. A, alignment of wild-type 5-HT₃A subunit M3-M4 region with truncation constructs. Truncation mutants are named according to the number of residues removed from the wild-type sequence. Truncation of 10 amino acids close to M3 (L-10(1) and L-10(2)) produced nonfunctional receptors (see "Results"). B, representative whole cell currents evoked by application of 100 μM 5-HT to HEK-293 cells expressing 5-HT₃A receptors (gray) or homomeric receptors composed of truncated (L-24, L-34, L-39, and L-44) constructs (black). 5-HT did not activate currents in cells expressing L-55 alone (see "Results"). C, a representative 5-HT-evoked current (black) recorded from a cell expressing the L-55 construct expressed in combination with 5-HT₃A subunits. Currents are normalized to the amplitude of the exemplar current mediated by wild-type 5-HT₃A receptors to aid comparison of desensitization kinetics. D, the bar graphs show the fast and slow desensitization τ values (left panels) and the relative contributions of their amplitudes (right panels) for wild-type and truncated 5-HT₃A receptors. The sum of two exponentials was fitted to the decaying phase of the 5-HT-evoked currents up to the point until the end of 5-HT application (see "Materials and Methods"). The use of a one-way ANOVA with post hoc Tukey’s test revealed a significant difference in the τ values and their relative amplitudes for currents mediated by the L-39 receptors when compared with the other receptors tested (p < 0.05). E, the weighted τ for desensitization (τₜₚ) of 5-HT-evoked currents mediated by L-39 was slower than the equivalent values for the all the other homomeric receptors tested (p < 0.05, one-way ANOVA with post hoc Tukey’s test).

4, D and E). Desensitization kinetics were analyzed by fitting exponential functions to the decaying phases of 5-HT-evoked currents up to the point of agonist removal. The sum of two exponentials was required to obtain the best fit to the data (Fig. 4D), and weighted τ (τₜₚ) values (Fig. 4E) were calculated for 5-HT-evoked current desensitization as described under "Materials and Methods." The fast (τ_fast) and slow (τ_slow) time constants for desensitization and their relative contributions (Fig. 4D) did not differ (one-way ANOVA), leading to similar τₚ values for 5-HT₃A L-24 (τₚ = 204 ± 48 ms, n = 7), L-34 (τₚ = 187 ± 58 ms, n = 7), L-44 (τₚ = 156 ± 12 ms, n = 6), and WT (τₚ = 229 ± 19 ms, n = 32) receptors (Fig. 4E). By contrast, 5-HT₃A L-39 receptors exhibited significantly slower τ_fast and τ_slow values than did the other homomeric receptor constructs (p < 0.05; Fig. 4D). Furthermore, the L-39 truncation caused an increase in the relative proportion of the slow component of desensitization. Together these changes led to a substantial increase in the τₚ of desensitization for currents mediated by 5-HT₃A L-39 receptors (648 ± 57 ms, n = 14) when compared with the other homomeric receptors (Fig. 4E). Receptors in HEK-293 cells expressing both the WT 5-HT₃A subunit and the 5-HT₃A L-55 construct desensitized significantly slower (τₚ = 495 ± 52 ms, n = 7, p < 0.05 Student’s t test) than did cells transfected with WT 5-HT₃A receptors alone (Fig. 4C). Desensitization kinetics could be influenced by the phosphorylation status of the M3-M4 loop. Indeed, phosphorylation has been implicated in the slow desensitization of currents mediated by 5-HT₃A receptors (14). There are Ser and Thr residues in the sections of the M3-M4 loop removed to produce the truncation constructs (Fig. 5A). A loss of potential sites for phosphorylation could account for slow desensitization kinetics of currents mediated by 5-HT₃A L-39 receptors. The L-39 truncation removes an additional Ser and a Thr residue when compared with L-34. The L-44 truncation removes another Ser and a Thr residue. There may be combinatorial effects on desensitization kinetics of different phosphorylation sites. However, according to a phosphorylation consensus site recognition algorithm (15), several of the residues in question are unlikely to be phosphorylated. The putative consensus sites are highlighted in Fig. 5A. Interestingly, this approach reveals that removal of 39 amino acids in the L-39 construct resulted in the artificial de novo introduction of a phosphorylation consensus site into the L-39 construct at Thr-369, which is not found in the L-24, L-34, or L-44 constructs.
We examined the possible contribution of phosphorylation at Thr-369 in the 5-HT$_3$A L-39 construct by making a phosphomimetic T369A substitution. A representative recording of a 5-HT (100 $\mu$M)-evoked current mediated by the 5-HT$_3$A L-39 (T369A) construct is shown superimposed on a 5-HT$_3$A L-39-mediated current (Fig. 5A, left panel). The desensitization ($\tau_w = 428 \pm 49$ ms; $n = 11$) of L-39 (T369A) was significantly greater ($p < 0.05$, Student’s $t$ test) than that of L-39 (Fig. 5C), consistent with the idea that phosphorylation at Thr-369 in the L-39 construct contributes to the slower desensitization rate when compared with the L-24, L-34, and L-44 constructs, which all lack this putative phosphorylation consensus site. We tested this hypothesis further by introducing a phosphomimetic T369E substitution into the L-34 truncation construct to produce L-34(T369E). The desensitization kinetics of 5-HT (100 $\mu$M)-evoked currents ($\tau_w = 640 \pm 48$ ms, $n = 7$) mediated by 5-HT$_3$A L-34 (T369E) receptors (Fig. 5B, right panel) were significantly slower ($p < 0.05$, Student’s $t$ test) than those of the L-34 construct that lacked the phosphomimetic substitution ($\tau_w = 187 \pm 58$ ms, $n = 7$, Fig. 5C). The rate of desensitization of currents mediated by L-34(T369E) was similar to those mediated by L-39. Collectively, these data are suggestive that phosphorylation of Thr-369 in L-39 is responsible for the slower desensitization kinetics of this construct when compared with the L-34 truncation construct.

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

**FIGURE 5. Evidence that the slow desensitization kinetics of L-39 is caused by the introduction of a phosphorylation consensus sequence.** A, sequences of the full-length and truncated M3-M4 loops with putative phosphorylation sites highlighted. Sequences were analyzed using the Group-based Prediction (GPS 2.0) algorithm (15). Several phosphorylation consensus sequences were identified (shown in bold font). A putative Ca$^{2+}$/calmodulin-dependent protein kinase site was identified in the L-39 construct at Thr-369 that was not found in the wild-type sequence or any of the other truncation constructs. B, the left panel contains traces of exemplar 5-HT (100 $\mu$M)-evoked currents mediated by L-34 receptors (gray trace) and L-34 (T369E) receptors in which Thr-369 was replaced by Glu, a phosphomimetic amino acid (black trace). The right panel contains traces of exemplar currents mediated by L-39 receptors (gray trace) and L-39 (T369A) receptors in which Thr-369 was substituted by Ala, thereby abolishing phosphorylation at this position. C, graph of the weighted $\tau$ values ($\tau_w$) for 5-HT-evoked current desensitization. L-34 (T369E) receptors (black bar) exhibited slower desensitization kinetics (*, $p < 0.05$, Student’s $t$ test) when compared with the nonmutant L-34 receptors (gray bar). L-39 (T369A) receptors (black bar) desensitized faster than did nonmutated L-39 receptors (gray bar).
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FIGURE 6. The L-44 M3-M4 loop truncation abolishes inward rectification and increases γ, A, diagrams representing a predicted portal framed by adjacent HA stretches (top panel) and a scenario in which the portal has been disrupted due to short cytoplasmic loops connecting M3 to the bottom of the HA stretch (bottom panel). B, representative traces of 5-HT (100 μM)-evoked currents mediated by wild-type 5-HT₃A receptors and receptors formed by truncation constructs, recorded at +60 mV and −60 mV. 5-HT was applied for 2 s. The graph shows the relationship between M3-M4 loop length and the rectification indexes (Iₒ/Iₒ̅ +60 mV/−60 mV). Wild-type 5-HT₃A, L-24, L-34, and L-39 receptors all displayed inward rectification, as demonstrated by rectification indexes of ≈0.5. Larger truncations (L-44 and L-55), however, resulted in receptors that displayed more similar current amplitudes at +60 mV and −60 mV. The L-55 construct was expressed with the wild-type 5-HT3A subunit. The rectification indexes of currents mediated by L-44 and 5-HT3A + L-55 were significantly higher than those mediated by wild-type 5-HT3A receptors expressed alone (*, p < 0.05; one-way ANOVA). C, representative single channel recordings of L-44 at different holding potentials. 5-HT (10 μM) was pressure-applied to excised outside-out patches to evoke microscopic currents. Channel closed and open levels are indicated by gray dashed lines. Also shown are the all points amplitude histograms of the exemplar data with the corresponding Gaussian fits to indicate the mean closed and open levels. The plot of single channel current mediated by L-44 receptors versus voltage was fitted by a linear regression yielding a mean chord conductance of 16.3 ± 1.3 pS (n = 7).

M3-M4 Loop Length of All Neurotransmitter-activated Human pLGICs Exceeds 70 Residues—The M3-M4 loops of human pLGICs are highly heterogeneous in both amino acid composition and number. Having established that >70 residues are required to maintain functional characteristics consistent with integrity of cytoplasmic portals of 5-HT₃A receptors, we examined how this relates to the M3-M4 loop lengths of the other human Cys-loop receptor subunits. We determined the length of the large cytoplasmic loop of the 5-HT3A subunit to be 114 residues from the homology model of the 5-HT₃A receptor based on the structure of the T. marmorata nACh receptor (9, 13). The M3-M4 loop lengths of the other human Cys-loop receptors were then established by alignment with the 5-HT3A amino acid sequence. With the exception of the Zn²⁺-activated ion channel (ZAC), which has an atypically short intracellular loop (16) estimated to be 43 amino acids, all the other Cys-loop receptor subunits (i.e. subunits of the neurotransmitter-activated human pLGICs) have loop lengths of ≥73 residues (Fig. 7). The M3-M4 loops of the neurotransmitter-activated cation channels range from 80 to 262 amino acids in length (Fig. 7A). The large cytoplasmic loops of the human pentameric anion channels range from 73 to 247 (Fig. 7B).

DISCUSSION

Using a chimeric strategy followed by site-directed mutagenesis, we determined that Arg-436 (in the HA 0’ position) is
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Critical for the characteristic inward rectification of 5-HT₃A receptor-mediated macroscopic currents is the residue previously demonstrated to be the principal determinant of γ for unitary events mediated by 5-HT₃A receptors, a property that is primarily dictated by the charge of the HA 0’ residue (8). In this study, substitution of numerous alternative amino acids into the HA 0’ position revealed that both the volume of the residue and its charge correlate with inward rectification. These findings add to prior evidence for the existence of cytoplasmic portals formed by adjacent Cys-loop receptor subunits. The large cytoplasmic loop length of the human 5-HT₃A subunit exceed the loop length of the L-44 construct (70 amino acids, indicated by the dotted line).

Previous studies demonstrate that rectification of 5-HT₃ receptor-mediated macroscopic currents occurs at the level of γ (17–19). Furthermore, residues in the HA stretch of the 5-HT₃A subunit impose a rate-limiting barrier to both inward and outward currents (7, 8, 13, 18), and the present study demonstrates that macroscopic current rectification is governed by the same residues. Taken together these findings imply that 5-HT₃A receptor portals impose a greater impediment to outward current than to inward current. Membrane potential is critical for the characteristic inward rectification of 5-HT₃A receptors, a property that is characteristic of recombinant human 5-HT₃A and 5-HT₃AB receptors, respectively. However, the subunit composition of 5-HT₃ receptors in vivo remains largely unknown, particularly in humans. Even for rodents, there is little definitive information available regarding the roles of homomeric and heteromeric 5-HT₃ receptors in central and peripheral neurons (reviewed in 23). There are conflicting reports as to the prevalence of the 5-HT3B subunit in the rodent brain. The situation in rodent neuroblastoma cells is simpler. The functional properties of their 5-HT₃ receptors (strong inward rectification and sub-pS single channel conductance) are those of homomeric 5-HT₃A receptors (20). The introduction of the 5-HT3B subunit changes the properties of neuroblastoma 5-HT₃ receptors consistent with the idea that these “native” receptors are indeed 5-HT3A homomers (24).

This study demonstrates that the different amino acid sequences of 5-HT3A and 5-HT3B subunits, and in particular the presence of an HA 0’ Arg or Asp in the former and latter, account for the inward rectification and linear I-V relationships that are characteristic of recombinant human 5-HT₃A and 5-HT₃AB receptors, respectively. However, the subunit composition of 5-HT₃ receptors in vivo remains largely unknown, particularly in humans. Even for rodents, there is little definitive information available regarding the roles of homomeric and heteromeric 5-HT₃ receptors in central and peripheral neurons (reviewed in 23). There are conflicting reports as to the prevalence of the 5-HT3B subunit in the rodent brain. The situation in rodent neuroblastoma cells is simpler. The functional properties of their 5-HT₃ receptors (strong inward rectification and sub-pS single channel conductance) are those of homomeric 5-HT₃A receptors (20). The introduction of the 5-HT3B subunit changes the properties of neuroblastoma 5-HT₃ receptors consistent with the idea that these “native” receptors are indeed 5-HT3A homomers (24).

There is no structural information for the M3-M4 loop in Cys-loop receptors lying outside the HA stretch. The recently solved pLGIC structural models shed no light on the structure of the large intracellular loop of Cys-loop receptors. The GLIC and ELIC bacterial pLGICs have vestigial M3-M4 loops (8 and 11 amino acids, respectively) and add no additional insights (1, 2). Of necessity, the M3-M4 loop of the C. elegans GluCl- receptor a1 subunit was largely removed to aid protein crystalization (11). The solitary pLGIC model that provides any structural data for the M3-M4 loop of Cys-loop receptors is the cryoelectron microscopy model of the T. marmorata nACh receptor, which resolves cytoplasmic portals formed by adjacent HA stretches, but lacks resolution for the larger section of the loop between M3 and the start of the HA stretch (9). The >45-Å gap is occupied by 85 residues in the case of the human 5-HT₃A subunit, which has a predicted total M3-M4 loop length of 114 residues. The large cytoplasmic loops of human Cys-loop receptor subunits exhibit diversity in length. All the
subunits of the neurotransmitter-activated human Cys-loop receptors have a loop length \( \geq 73 \) residues. It is interesting to speculate why there is not a normal distribution of loop lengths from the minimal lengths of the bacterial pLGICs up to the considerable length (262 amino acids) of the \( \alpha 4 \) nACh subunit M3-M4 loop. Our data suggest that the minimum loop length is constrained by the requirement to connect the M3 to the HA stretch without disrupting portal-associated function.

We systematically truncated the unresolved stretch of the 5-HT3A subunit to reveal the minimum number of residues required to connect the M3 with the HA stretch without compromising the functional integrity of the cytoplasmic portals. Our initial attempts to truncate at the M3 end of the loop revealed a series of residues that were essential for the expression of functional 5-HT3A receptors in HEK-293 cells. Interestingly, the rules governing 5-HT3A receptor expression in HEK-293 cells and *Xenopus* oocytes appear to differ because replacement of the entire intracellular loop by a series of Ala residues had no effect on peak 5-HT-evoked current amplitude measured in the latter (25). Alternatively, the truncations may cause the remainder of the loop to adopt a conformation that prevents functional expression. By contrast to our initial approach, which removed residues near M3, truncations starting close to the HA stretch and progressing toward M3 were well tolerated and did not significantly impact on the density of 5-HT-evoked currents. The M3-M4 loops of Cys-loop receptors have been implicated in trafficking, either through intrinsic mechanisms or by interacting with other cytosolic proteins and, in agreement with our findings, mutagenesis or deletion can have profound effects on expression (26, 27). It is possible that a series of 5-HT3A cytoplasmic residues adjacent to the M3 interacts with trafficking proteins. The PVPPXXWR amino acid motif located within this critical region of the 5-HT3A subunit is conserved in nACh \( \alpha 9 \) and \( \alpha 10 \) subunits, potentially implicating its involvement in the trafficking of other Cys-loop receptors.

Perturbations of individual amino acids within the M3-M4 loop influence the kinetics of currents mediated by some Cys-loop receptors, whereas other receptors appear unaltered by removal of the entire loop (28, 29). Mutations associated with myasthenic syndrome that cause insertions, or substitutions, in the large cytoplasmic loops of the human muscle nACh receptor \( \varepsilon \) subunit alter gating kinetics (29). Furthermore, differences in the kinetics of nACh receptors containing \( \gamma \) or \( \varepsilon \) subunits can be accounted for by specific amino acids that differ within their M3-M4 loops. Additionally, phosphorylation of a specific residue within the M3-M4 loop of the GABA_A receptor \( \beta 1 \) subunit influences GABA-evoked current desensitization (30). There is also evidence that phosphorylation regulates 5-HT3 receptor function. Casein kinase II modulates the kinetics of 5-HT3 receptor-mediated currents recorded from NG108-15 neuroblastoma cells (14). The 5-HT3A L-39 truncation construct formed receptors that mediated 5-HT-evoked currents that desensitized slower than homomeric receptors formed by the other truncation constructs and by WT 5-HT3A receptors. This could be caused by the truncation introducing a phosphorylation consensus sequence into 5-HT3A L-39, not found in the other constructs, enabling phosphorylation of Thr-369. Consistent with this idea, substitution of Thr-369 by Ala accelerated the rate of 5-HT3A L-39 (T369A) receptor desensitization to rates similar to those of WT receptors. Furthermore, a phosphomimetic mutation in 5-HT3A L-34 (T369E) caused desensitization kinetics of the L-34 truncation construct to slow to a level similar to that of the L-39 construct.

Our findings with mutant constructs suggest that phosphorylation of the M3-M4 loop can produce changes in 5-HT3A receptor kinetics. It remains to be determined whether there is a physiological role for 5-HT3A receptor phosphorylation. Our recent unpublished observations with the casein kinase II inhibitor 4,5,6,7-tetrabromobenzotriazole support previous data implicating the kinase in slowing 5-HT3A receptor desensitization (14). 4,5,6,7-Tetrabromobenzotriazole increased the rate of desensitization of the WT and M3-M4 loop truncation constructs, suggesting that the site of action of casein kinase II may lie elsewhere, possibly in the M1-M2 loop where there is a casein kinase II consensus sequence. We will explore this possibility in future studies.

Having determined that inward rectification requires the key HA 0′ Arg located at the mouth of the putative portals, we used rectification as an assay of portal function. Removal of up to 39 residues from the loop had no effect on inward rectification, suggesting that 46 residues are sufficient to bridge the gap between the end of M3 and the start of the HA stretch. By contrast, removal of five additional residues (L-44) abolished inward rectification, suggesting that 41 residues are insufficient to bridge the gap without disrupting portal function. In keeping with this interpretation, 5-HT3A L-44 receptors exhibited clearly resolvable unitary events with 16 pS-γ substantially greater than the <1 pS-γ of wild-type 5-HT3A receptors (3, 5).

It is unlikely that the absence of the five specific (SQATK) residues accounts for the loss of portal function when the loop is truncated by more than 39 residues (i.e. L-44 or L-55). The SQATK motif is not found in any of the other human pLGIC subunits. More importantly, it is also not conserved in rat or mouse 5-HT3A subunit variants (in which the equivalent sequence is FQANK) despite a high level of overall 5-HT3A amino acid sequence identity in human, mouse, and rat, even in the M3-M4 loop. Like the human 5-HT3A receptor, rodent 5-HT3A receptors exhibit strong inward rectification and a single channel conductance that is below resolution in outside-out patch recordings (17). Therefore, it is unlikely that the SQATK sequence is required for the functional integrity of 5-HT3A portals.

5-HT3A L-39 receptors, which did not have disrupted portal function, have a total loop length of 75 residues, shorter than all the M3-M4 loops of cation-permeable Cys-loop neurotransmitter receptors and only two residues longer than the shortest anionic receptor loop (that of the GABA_A receptor \( \gamma 1 \) subunit). Therefore, a loop length of 75 residues, which supports portal integrity, is within the range of lengths tolerated by neurotransmitter-activated human pLGIC subunits. By contrast, the loop length of 70 residues, which does not support portal integrity, is below the length of all the neurotransmitter-activated pLGIC...
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subunit M3-M4 loops. Taken together these findings suggest that there is a selective advantage to the cytoplasmic loops being long enough to support the structural integrity of portals. Because there is little variability between the amino acid sequences of orthologous mammalian Cys-loop subunits, this observation can be extended beyond humans.

It is interesting that pLGICs of bacteria have vestigial M3-M4 loops, whereas neurotransmitter-activated pLGICs have M3-M4 loop lengths that all exceed 72 amino acids. It is possible that the cytoplasmic portal structure has evolved to enable interactions with other intracellular proteins, enabling modulation of trafficking and function in the complex environment of a nervous system. Additionally, portals of limited dimensions may exclude molecules within the cytoplasm from entering the intracellular aspect of the channel pore, potentially causing channel blockade. Photo-affinity labeling experiments using [³H]azietomidate suggest that cytoplasmic portals of nACh receptors also serve as drug binding sites (31). As an evidence accumulates for the importance of the M3-M4 loop, the value of resolving its structure at the atomic level becomes increasingly clear.

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