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

Daniel T. Baptista-Honi†, Tarek Z. Deeb†, Jeremy J. Lambert‡, John A. Peters§, and Tim G. Hales††‡§1

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 ($\gamma$). Further analysis reveals that the 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 $\gamma$. 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.

Background: Residue 436 within 5-HT3A cytoplasmic portals determines single channel conductance ($\gamma$) and rectification.

Results: M3-M4 loop truncation to 75 residues spared inward rectification; truncation to 70 abolished rectification and increased $\gamma$. Human Cys-loop neurotransmitter receptor M3-M4 loops all exceed 70 residues.

5-Hydroxytryptamine type 3 (5-HT3) 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 $\gamma$-aminobutyric acid type A (GABA$_A$) 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 ($\gamma$), 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 Ca$^{2+}$ 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 Ca$^{2+}$ permeability, and single channel $\gamma$ 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 Ca$^{2+}$ permeability lie within the M2 domain (6). By contrast, a similar approach revealed that the molecular determinants for $\gamma$ lie within the helical amphipathic (HA) stretch in the M3-M4

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‡ 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, picosiemens; ANOVA, analysis of variance.
5-HT₃A receptors in
removed from GluCl to enable crystallography. The M3-M4
loop, which is vestigial in the bacterial channels and was largely
(11), have shed no further light on the structure of the M3-M4
stretches, the bulk of the M3-M4 loop remains structurally
the structure of cytoplasmic portals framed by adjacent HA
portals (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
of the homologous pLGICs of Gloeobacter violaceus (GLIC) (1)
and Erwinia chrysanthemi (ELIC) (2), as well as the glutamate-
activated Cl⁻ channel (GluCl) from Caenorhabditis elegans
(11), have shed no further light on the structure of the M3-M4
loop, which is vestigial in the bacterial channels and was largely
removed from GluCl to enable crystallography. The M3-M4
loop is not required for functional expression of recombinant
5-HT₃A receptors in Xenopus oocytes. However, its replace-
ment by the heptapeptide M3-M4 loop of GLIC resulted in
receptors with an increased single channel conductance, com-
parable with that of the 5-HT₃A(QDA) receptor (11).

In this study, we demonstrate using mutagenesis that, in
addition to its critical role in determining γ, residue 436 gov-
erns 5-HT₃A receptor inward rectification, a property that cor-
relates with the volume of the amino acid. Therefore, the dis-
tinctive 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 trunc-
ation 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 transfec-
tions 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 Xhol (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 (pEGFP vector, 0.1 μg) was included to identify suc-
cessfully 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 Invitro-
gen (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 prim-
ers. PCR products were digested using HindIII and Xhol
restriction endonucleases and ligated into pCDM8 vector. All
mutagenesis reactions and ligations were verified using agarose
gel electrophoresis, and constructs were sequenced to confirm
fidelity prior to functional testing (Genetics Core Services, Uni-
versity 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-HT₃ 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 con-
tained (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 deter-
mined 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
to ~60 mV (the rectification index) was used. A unitary rectifi-
cation index indicates a linear I-V relationship. A unitary recti-
fication index was used as a marker of the successful incorpo-
ration 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). Elec-
trodes were coated with Sylgard (Corning, NY) to minimize
capacitance and noise. Currents were evoked by transient pres-
sure application (Picosprizer II, General Valve Corp., Fairfield,
NJ) of 5-HT (3 μM). Single channel 1-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 Axo-
patch 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 cur-
rents were measured using pClamp10 software. Desensitiza-
tion kinetics were measured by fitting a double exponential
function to the decaying phase of a 5-HT-evoked current. Sin-
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The HA stretch is the determinant of 5-HT3 receptor rectification. A, diagrams representing the 5-HT3A/B chimeras, which were made by substituting different regions of the 5-HT3A (white) subunit with that of the 5-HT3B (gray) subunit. Chimeras were expressed in HEK cells, and currents were recorded at potentials between −60 and +60 mV in 20-mV increments. B, graph of the relationship between holding potential (V) in mV and current amplitude expressed relative to that of the current recorded at −60 mV. A near linear current-voltage relationship was exhibited by receptors that contained the HA stretch of the 5-HT3B subunit within the M3-M4 loop (5-HT3B and C3 and C4 constructs). As reported previously, the 5-HT3B subunit, C1, C2, and C3 required co-expression with the 5-HT3A subunit for function (5, 7).

The Charge and Volume of the 0′ Residue within the HA Stretch Influences 5-HT3A 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-HT3A 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-HT3A receptor, based on the structure of the Torpedo 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-HT3A receptor. Residues were systematically substituted, individually and in combination, by their amino acid counterparts within the 5-HT3B 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 (I(−60mV)/I(60mV)), 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-HT3A(R426S) construct, in which Arg at the HA −10′ position was replaced by Ser (the HA −10′ residue in 5-HT3B), had a rectification index similar to that of WT 5-HT3A receptors (Fig. 2B). Likewise 5-HT3A R432Q and R440A (HA −4′ and HA 4′ positions, respectively) substitutions were also without significant effect on the rectification index when introduced into the 5-HT3A subunit either individually or in combination. However, the 5-HT3A(R436D) mutation (at the HA 0′ position) significantly increased the rectification index close to unity when incorporated into the 5-HT3A 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...
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 -10'). 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₊60 mV/ I₋60 mV) 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₊60 mV/ I₋60 mV. 5-HT₃AB receptors on the other hand display a linear I-V relationship. Amino acid substitutions in the 426, 432, and 440 positions alone, or in combination, did not alter I₊60 mV/ I₋60 mV. However, R436D produced a significant increase in I₊60 mV/ I₋60 mV (p < 0.05; one-way ANOVA). R436D in combination with R432Q and/or R440A also produced receptors with a unitary I₊60 mV/ I₋60 mV (*, p < 0.05, one-way ANOVA). Taken together, these data demonstrate that residues within the HA stretch govern I-V relationship properties of 5-HT₃A receptors.

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 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
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FIGURE 3. The charge and volume of residue 436 are determinants of inward rectification. Graphical representations of the relationship between Iᵢ₊₆₀mV/Iᵢ₋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 Iᵢ₊₆₀mV/Iᵢ₋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 (R² = 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 (R² = 0.68). C, graph of the relationship between the volume of the amino acid at position 436 and Iᵢ₊₆₀mV/Iᵢ₋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 R² value of 0.38. D, exclusion of nonpolar residues also improved the correlation between the volume and the rectification index (Pearson coefficient = 0.51; p = 0.02) and the linear fit (R² = 0.51). Amino acid volumes were determined using Spartan 04 software (see “Materials and Methods”).

M3-M4 intracellular loops of any of the Cys-loop receptors (9). The structure of the M3-M4 loop between the end of M3 and the start of the HA stretch remains unresolved in the T. maromata 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 μM), 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 μM)-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—The 5-HT (100 μM)-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. 4B).
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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 (τₑ) values (Fig. 4E) were calculated for 5-HT-evoked current desensitization as described under "Materials and Methods." The fast (τₑ,fast) and slow (τₑ,slow) time constants for desensitization and their relative contributions (Fig. 4D) did not differ (one-way ANOVA), leading to similar τₑ values for 5-HT₃A L-24 (τₑ = 204 ± 48 ms, n = 7), L-34 (τₑ = 187 ± 58 ms, n = 7), L-44 (τₑ = 156 ± 12 ms, n = 6), and WT (τₑ = 229 ± 19 ms, n = 32) receptors (Fig. 4E). By contrast, 5-HT₃A L-39 receptors exhibited significantly slower τₑ,fast and τₑ,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 τₑ 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 (τₑ = 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 any known phospho sites.

**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 the M3-M4 loop truncation constructs (Fig. 5A) did not differ (one-way ANOVA), leading to similar τₑ values for 5-HT₃A 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 τₑ 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 current from the peak 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 τₑ 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 τₑ for desensitization (τₑ,w) 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).
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We examined the possible contribution of phosphorylation at Thr-369 in the 5-HT3A L-39 construct by making a phosphomimetic T369A substitution. A representative recording of a 5-HT (100 μM)-evoked current mediated by the 5-HT3A L-39(T369A) construct is shown superimposed on a 5-HT3A WT (100 μM)–evoked current (Fig. 5A, left panel). The desensitization kinetics (τw = 428 ± 49 ms; n = 11) of L-39(T369A) was significantly faster 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 μM)-evoked currents (τw = 640 ± 48 ms, n = 7) mediated by 5-HT3A 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 (τw = 187 ± 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.

Systematic M3-M4 Loop Truncation Disrupts Rectification and γ—Having determined that the 5-HT3A truncation constructs, L-24, L-34, L-39, and L-44, produce functional homomeric 5-HT3 receptors and that expression of L-55 with the wild-type 5-HT3A subunit produces functional receptors, we next explored whether inward rectification was affected by shortening the M3-M4 loop. Perhaps truncation beyond a certain critical number of amino acids would abolish rectification by disrupting the orientation of HA stretches that frame cytoplasmic portals (Fig. 6A). We measured the rectification indexes for L-24, L-34, L-39, and L-44 expressed as homomeric receptors and L-55 expressed with the WT 5-HT3A subunit. 5-HT (100 μM) was applied, and currents were recorded at −60 and +60 mV to determine the rectification index for each construct (Fig. 6B). L-24, L-34, and L-39 constructs mediated 5-HT-evoked currents that were consistently smaller in peak amplitude when recorded at +60 mV than when recorded at −60 mV. Thus, these truncation constructs, like the WT 5-HT3A receptor, exhibited inward rectification. However, the L-44 construct mediated 5-HT-evoked currents with similar amplitudes at +60 and −60 mV, indicating that there is a linear I-V relationship. The L-55 truncation mutant, when expressed together with the 5-HT3A subunit, produced receptors that also mediated 5-HT-evoked currents with similar peak amplitudes at +60 and −60 mV, again indicative of a linear I-V relationship (Fig. 6B). WT 5-HT3A receptors (rectification index = 0.41 ± 0.02, n = 4) have a total M3-M4 loop length of 114 residues. L-24 (loop length of 90), L-34 (loop length of 80), and L-39 (loop length of 75) constructs all mediated currents that were significantly smaller in amplitude at +60 mV compared with −60 mV (rectification indexes of 0.42 ± 0.06, n = 3; 0.44 ± 0.06, n = 5; and 0.53 ± 0.06, n = 6, respectively). These rectification indexes were not statistically different from those of WT 5-HT3A receptors (one-way ANOVA, Tukey’s post hoc test). L-44 (loop length of 70) and WT/L-55 (loop length of 59) truncation mutants had rectification indexes close to unity (0.82 ± 0.06, n = 4 for L-44 and 0.76 ± 0.05, n = 6 for WT/L-55). These values were statistically different from that of WT 5-HT3A receptors (p < 0.05, one-way ANOVA, post hoc Tukey’s test).

The effects of truncation suggest that a 5-HT3A subunit M3-M4 loop length of 70 residues is insufficient to support inward rectification (Fig. 6B), a property conferred by residues located within putative cytoplasmic portals (Fig. 2). The loss of inward rectification in receptors with loop lengths of ≈70 res-
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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 (I+60mV−I−60mV). 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).

The M3-M4 loop length affects the function of 5-HT₃A receptors. We performed recordings of 5-HT-evoked currents mediated by 5-HT₃A L-44 receptors in excised outside-out patches to determine whether the loss of 44 residues also affected γ, a property of 5-HT₃A receptors that is governed by residues that lie within the putative cytoplasmic portals (7, 13). 5-HT-evoked currents recorded from outside-out patches excised from HEK-293 cells expressing WT 5-HT₃A receptors resemble miniature whole cell currents with no discernible unitary events (3, 5, 7). By contrast, 5-HT-activated single channel events were observed in recordings from patches containing 5-HT₃A L-44 receptors (Fig. 6C). The chord conductance of single channels mediated by 5-HT₃A L-44 receptors was 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 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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**FIGURE 7. M3-M4 loop length of neurotransmitter-gated pentameric ion channels.** The large cytoplasmic loop length of the human 5-HT₃A subunit was established from the homology model based on the *T. marmorata* structure (13). The M3-M4 loop lengths of the other human Cys-loop receptor subunits were then estimated by aligning their sequences with that of the 5-HT₃A subunit. A, graph of the loop lengths for cationic Cys-loop receptor subunits. Included for comparison are the loop lengths of the 5-HT₃A L-39, which supported portal-mediated function, and L-44, which did not. Interestingly all of the neurotransmitter-gated pentameric ion channel subunits exceed the loop length of the L-44 construct (70 amino acids, indicated by the dotted line).

Critical for the characteristic inward rectification of 5-HT₃A receptor-mediated macroscopic currents is the residue we 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 that form rate-limiting barriers to ion conduction in some Cys-loop receptors (7–9, 13).

Previous studies demonstrate that rectification of 5-HT₃A 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 unlikely to directly influence portals, which lie outside the electrical field. It is also unlikely that charged cytosolic molecules interact with portals in a voltage-dependent manner to initiate inward rectification because this property persists in excised outside-out membrane patches (20). Instead, it is possible that rectification is caused by the asymmetric nature of the ion conduction pathway. The large extracellular vestibule contains rings of negatively charged residues, which augment single channel conductance in 5-HT₃A(QDA) mutant receptors in which portals are no longer rate-limiting (21). The negative charges likely assist the diffusion of cations into the vestibule and ion channel pore, where they will be subjected to the driving force imposed by membrane potential. By contrast, the arginine residues of the 5-HT₃A receptor portals may restrict diffusion of ions into the intracellular vestibule as well as constraining the rate at which ions can traverse in either direction through the channel. Collectively, the data suggest that the asymmetric pathway formed by cytoplasmic portals exerts a greater impediment to ion conduction in the outward direction, giving rise to inward rectification. This is, to our knowledge, the first example of intrinsic inward rectification in a cationic neurotransmitter-gated ion channel. Inward rectification of cation channels, including some nACh receptors, is often caused by voltage-dependent blockade by intracellular polyanimes (22).

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 α1 subunit was largely removed to aid protein crystallization (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 ≳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 α4 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 PVXWXR amino acid motif located within this critical region of the 5-HT3A subunit is conserved in nACh α9 and α10 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 cause insertions, or substitutions, in the large cytoplasmic loops of the human muscle nACh receptor ε subunit altering gating kinetics (29). Furthermore, differences in the kinetics of nACh receptors containing γ or ε 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 β1 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.3 We will explore this possibility in future studies.

Having determined that inward rectification requires the key HA0 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-,1 pS- substantially greater than the <1 pS- 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 γ1 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 subunits.

3 D. T. Baptista-Hon and T. G. Hales, unpublished observations.
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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]azetomidate suggest that cytoplasmic portals of nACh receptors also serve as drug binding sites (31). As 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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