Common Determinants of Single Channel Conductance within the Large Cytoplasmic Loop of 5-Hydroxytryptamine Type 3 and \( \alpha_4\beta_2 \) Nicotinic Acetylcholine Receptors*

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Homomeric 5-hydroxytryptamine type 3A receptors (5-HT3ARs) have a single channel conductance (\( \gamma \)) below the resolution of single channel recording (966 \( \pm \) 75 fS, estimated by variance analysis). By contrast, heteromeric 5-HT3A/B and nicotinic acetylcholine receptors (nAChRs) have picosiemens range \( \gamma \) values. In this study, single channel recordings revealed that replacement of cytoplasmic membrane-associated (MA) helix arginine 432 (\( -4' \)), 436 (\( 0' \)), and 440 (\( 4' \)) residues by 5-HT3AR residues increases \( \gamma \) to 36.5 \( \pm \) 1.0 pS. The 0' residue makes the most substantial contribution to \( \gamma \) of the 5-HT3AR. Replacement of 0'Arg by aspartate, glutamate (\( \alpha_4 \) nAChR subunit MA 0'), or glutamine (\( \beta_2 \) subunit MA 0') increases \( \gamma \) to the resolvable range (>6 pS). By contrast, replacement of 0'Arg by phenylalanine (\( \alpha_4 \) subunit MA 0') reduced \( \gamma \) to 416 \( \pm \) 107 fS. In reciprocal experiments with \( \alpha_4 \),\( \beta_2 \) nAChRs (\( \gamma \) = 31.3 \( \pm \) 0.8 pS), replacement of MA 0' residues by arginine in \( \alpha_4 \beta_2 \) (Q443R) and \( \alpha_4(F588R)\beta_2 \) residues slightly. By contrast, the \( \gamma \) of double mutant \( \alpha_4(F588R)\beta_2(Q443R) \) was halved. The MA –4' and 4' residues also influenced \( \gamma \) of 5-HT3ARs. Replacement of nAChR \( \alpha_4 \) or \( \beta_2 \) MA 4' residues by arginine made current density negligible. By contrast, replacement of both –4' residues by arginine produced functional nAChRs with substantially reduced \( \gamma \) (11.4 \( \pm \) 0.5 pS). Homology models of the 5-HT3A and \( \alpha_4\beta_2 \) nAChRs against Torpedo nAChR revealed MA –4', 0', and 4' residues within five intracellular portals. This locus may be a common determinant of ion conduction throughout the Cys loop receptor family.

There are five families of vertebrate Cys loop receptors as follows: the nicotinic acetylcholine receptor (nAChR), the 5-hydroxytryptamine type 3 receptor (5-HT3R), the zinc-activated ion channel, the \( \gamma \)-aminobutyric acid type A receptor, and the strychnine-sensitive glycine receptor. In this study we investigated the influence of the 5-HT3A subunit's MA helix 432 (\( -4' \)), 436 (\( 0' \)), and 440 (\( 4' \)) residues in controlling \( \gamma \). We investigated the effect of introducing arginine into the equivalent locations within the nAChR \( \alpha_4 \) and \( \beta_2 \) subunits. Our data confirm the critical role of MA –4', 0', and 4' residues in controlling \( \gamma \) of 5-HT3AR receptors and support the hypothesis that the MA helix also forms part of the ion conduction pathway of nAChRs. Our functional data provide support for the existence of cytoplasmic portals depicted in the 4 Å structural model of the nAChR (10).

EXPERIMENTAL PROCEDURES

DNA Constructs and Transient Transfection of Subunit cDNAs—cDNAs encoding rat wild-type (WT) nAChR \( \alpha_4 \) and \( \beta_2 \) subunits (Dr. J. M. Boulter, Department of Psychiatry and Biobehavioral Sciences, UCLA), human WT 5-HT3A subunits, and mutant nAChR and 5-HT3A subunits were cloned into pGW1 (11). Point mutations were introduced using standard molecular biological techniques (7). All cDNAs were sequenced to confirm fidelity. Transfection of tsA-201, or HEK-293 cells, with subunit cDNAs, at equimolar ratios when appropriate, was performed by either the calcium phosphate precipitation method or electroporation (400 V, infinite resistance, 125 microfarads) using a Bio-Rad gene electropulser II. Transfected cells were routinely cultured at 37 °C for 24–72 h before use, although in the case of nicotinic receptor constructs incubation temperature was reduced to 29 °C overnight prior to recording in order to enhance cell surface expression (12).
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Electrophysiology—Whole-cell and outside-out patch configurations were used to record macroscopic and single channel currents, respectively, from transfected cells. The recording chamber was perfused (5 ml min⁻¹) with a solution of (in mM) KCl 140, MgCl₂ 2.0, CaCl₂ 1.0, glucose 10, and HEPES 10; pH 7.3. For macroscopic current recordings, patch-clamp electrodes contained (in mM) KCl 140, MgCl₂ 2.0, CaCl₂ 0.1, EGTA 1.1, HEPES 10; pH 7.3. Acetylcholine (1 mM) was applied by pressure ejection to whole cells to obtain estimates of peak current, which were then normalized to cell capacitance in order to determine current densities. Slowly rising and decaying whole-cell currents, suitable for fluctuation analysis, were evoked by agonists applied to voltage-clamped cells (–60 mV) via diffusion pipettes. Single channel conductances were estimated from the macroscopic current (1–1000-Hz bandwidth) response to agonist as described previously (13). Variance analysis was performed using the electrophysiology data recorder (Dr. J. Dempster, University of Strathclyde). For single channel recordings from outside-out patches, or variance analysis recorded from the latter, electrodes were filled with (in mM) potassium gluconate 130, NaCl 5, MgCl₂ 2, EGTA 5, HEPES 10; pH 7.2. Single current channels recorded from outside-out patches were either low pass filtered at 2 kHz in nACh receptor experiments or 1 kHz in 5-HT₃ receptor experiments (digitized at 10 kHz in both cases). Data were acquired as described previously (14). Agonists were applied by pressure ejection to patches voltage-clamped at either –74 mV in the case of WT and mutant 5-HT₃A constructs or –94 mV in the case of nAChR constructs (values include correction for liquid junction potential compensation). Sections of digitized data (~10-s duration), in which unitary events were predominated, were selected for analysis and were leak current-subtracted (using Clampfit) for the creation of all-points amplitude histograms using Fetchan (pCLAMP 8.0, Axon Instruments, CA). Multiple gaussians were fitted (least squares minimization) to amplitude histograms using the Simplex method within pSTAT (pCLAMP 8.0). The amplitude of the single channel current recorded from each patch was determined from the difference between the mean current amplitudes determined from the gaussians fitted to the closed and open-state currents. Single channel conductances are reported as the chord conductance derived as γ = i(Vₐ − Eₐ), where i is unitary current amplitude; Vₐ is the holding potential, and Eₐ is the reversal potential of the agonist-evoked macroscopic current response. In several outside-out patch recordings from 5-HT₃ and nACh receptors, two conductances were evident. Our analysis of single channels was restricted to the main state, which in all cases corresponded to the larger amplitude events readily detected in all-points amplitude histograms.

Statistics—Data are presented as mean ± S.E. Data sets were compared using one-way analysis of variance (ANOVA) with the post hoc Tukey’s test.

Modeling the 5-HT₃A and nAChR α₂ and β₂ Subunits onto the 4 Å Model of the Torpedo nAChR—Amino acid sequences of 5-HT₃A (WT and 5-HT₃A(R432Q,R436D,R440A) mutant) and WT α₂ and β₂ subunits were aligned against the T. marmorata α, β, and δ subunits. The 5-HT₃A template structure was down-loaded from the RCSB Protein Data Bank (code 2BG9) into the DeepView Swiss-Pdb Viewer (swissmodel.expasy.org/). Alignment of sequences of 5-HT₃A (WT and 5-HT₃A(R432Q,R436D,R440A) mutant) and WT nAChR α₂ and β₂ subunits were threaded onto the Torpedo template in DeepView. The nAChR subunits were threaded onto the template with the 2α₂β₂ stoichiometry in the ββαα arrangement. The models were then submitted to SwissModel (swissmodel.expasy.org/) for optimization. Following energy minimization (Gromos96, SwissModel), models were returned to DeepView for viewing and imaging. Excess C-terminal residues were removed from query sequences for imaging. The subunit interface illustrated (Fig. 7) represents the homology model corresponding to the Torpedo 1α-5γ interface (10).

RESULTS

Residues in the MA Helix of the 5-HT₃A Subunit Control γ—Application of 5-HT (10 μM) to outside-out membrane patches excised from HEK cells transiently transfected with cDNA encoding the human 5-HT₃A subunit activated currents without resolvable single channel events (Fig. 1A), consistent with our previous observations (5). Variance analysis of whole-cell currents activated by 5-HT (10 μM) reveals WT 5-HT₃A receptors to have a single channel conductance (γ) of 966 ± 75 fS (Table 1).

We aligned the human 5-HT₃A subunit amino acid sequence with those of the human 5-HT₃B subunit and various nAChR subunits, including the T. marmorata α, β, γ, and δ subunits. The 5-HT₃A sequence was then threaded on to the 4 Å structural model of the Torpedo nAChR (10), and the homology model was energy-minimized (see “Experimental Procedures”). The 5-HT₃A subunit contains a series of regularly spaced arginine residues located within the MA helix (Fig. 1B). Here we denote these residues MA –4′, 0′, and 4′ for ease of comparison with the homologous residues in the structurally related nAChR subunits (Fig. 1B).

By using variance analysis of whole-cell currents, we previously suggested that agonist residues at positions MA –4′ (Arg-432) and 4′ (Arg-440) participate with MA 0′ (Arg-436) in the control of γ. In agreement, single channel recordings of 5-HT-activated currents from outside-out patches containing triple mutant homomeric 5-HT₃A(R432Q,R436D,R440A) receptors (in which MA –4′, 0′, and 4′ residues were replaced by corresponding 5-HT₃B residues) revealed robust unitary current events with a γ of 36.5 ± 1.0 pS (Fig. 1, C and D, Table 1), i.e. an ~40-fold increase in γ in comparison to WT 5-HT₃A receptors. The determination of γ by variance analysis is indirect and can result in an underestimation of this parameter. Indeed, the value of γ reported here from direct observation of single channel events mediated by the 5-HT₃A(QDA) receptor is greater than that which we reported previously using variance analysis. There values recently published by others using a similar recording configuration (15). Therefore, we re-investigated the contribution of the MA –4′, 0′, and 4′-5-HT₃ receptors in the control of γ individually and in combination by single channel analysis, where resolution permitted. Fig. 2A illustrates the effect of replacing the MA –4′, 0′, and 4′ residues in 5-HT₃A with equivalent residues from the 5-HT₃B subunit. Direct measurements of γ using single channel analysis are qualitatively similar to estimates of γ that we reported previously using variance analysis. There is a good correlation between γ values determined using these two approaches (γ² = 0.98, Fig. 2B). The systematic underestimation of γ using variance analysis could be caused by the presence of sub conduc-
tance events below the resolution of single channel recording or alternatively the use of different recording solutions.

Application of 5-HT (10 μM) to outside-out membrane patches excised from cells expressing the 5-HT3A(R432Q) receptor produced small inward current responses, but individual channel events were not discernible (data not shown). Variance analysis of 5-HT-induced current responses recorded from outside-out patches suggests a sub-pS unitary conductance (Fig. 2A; Table 1), similar to that reported previously (7) using analysis of whole-cell variance for WT 5-HT3A and mutant 5-HT3A(R432Q) receptors. This demonstrates that estimates of γ using variance analysis are not influenced by the whole-cell or outside-out patch recording configuration. A role for the −4′arginine in the control of γ can be directly observed by comparing the amplitude of channels mediated by the 5-HT3A(R436D,R440A), in which the −4′arginine is preserved, to the amplitude of channels mediated by 5-HT3A(QDA) mutation in which the −4′Arg was replaced by Gln. Outside-out membrane patches expressing 5-HT3A(R436D,R440A) receptors exhibited clearly discernible 5-HT-activated single channel events of a modestly reduced conductance in comparison to 5-HT3A(QDA) receptors (Fig. 2C; Table 1). Collectively, these data reveal Arg-432 to have a limited influence on γ. In contrast to the R432Q exchange, replacement of Arg-436 of the 5-HT3A subunit by the equivalent 5-HT3B subunit residue (Asp) caused a substantial increase in γ such that single channel events were evident in outside-out patch recordings (Fig. 2C). From the all points amplitude histograms (Fig. 2D), we determined γ for channels mediated by the 5-HT3A(R436D) mutant receptor to be 9.1 ± 0.6 pS (Table 1). The importance of Arg-436 is further emphasized by the 5-HT3A(R432Q,R440A) receptor, which mediates 5-HT-activated single channels of a greatly reduced (p < 0.001) conductance compared with the 5-HT3A(QDA) receptor (Fig. 2; Table 1).
represents mutant 5-HT3A(R436D) receptors in which the excised outside-out patch containing homomeric dashed line due to the 5-HT3B subunit. The unitary events are clearly determined by variance analysis of 5-HT-activated currents recorded from resolved unitary channel events (4). The MA 0 helix (432), 0 Phe, 4 Ala, the MA 0 helix (440) residue has been replaced by aspartate (the MA 0 residue of the 5-HT3A subunit). Unitary events are clearly resolved in the expanded section of data. D, the all-points amplitude histogram of the unitary events in D was fitted with the sum of two gaussians (representing closed and open states). The single channel amplitude is 0.64 pA, corresponding to a chord conductance of 8.6 pS.

###TABLE 1
The influence of amino acid identity on the γ values for 5-HT3A and αββα nACH constructs

The γ values were determined from the amplitudes of single channels in recordings from outside-out patches activated by 5-HT (10 μM) or ACh (100 nM) unless indicated otherwise. Variance analysis was used to determine γ when resolvable channels were not observed in outside-out patch recordings.

| 5-HT3A constructs | Substituted residue(s) | γ | n |
|-------------------|------------------------|---|---|
| Wild-type 5-HT3A | WT                     | 0.97 ± 0.08<sup>b</sup> | 12<sup>c</sup> |
| R436Q             | −4′Gln                  | 0.79 ± 0.04<sup>b</sup> | 7  |
| R436D             | 0′Asp                  | 9.1 ± 0.6*** | 6  |
| R436E             | 0′Glu                  | 12.8 ± 0.8*** | 6  |
| R436Q             | 0′Gln                  | 6.3 ± 0.5*** | 7  |
| R436F             | 0′Phe                  | 0.42 ± 0.11<sup>b</sup> | 5  |
| R440A             | 4′Ala                  | 5.2 ± 0.5*** | 4  |
| R432Q,R436D       | −4′Gln, 0′Asp           | 17.5 ± 0.6*** | 5  |
| R432Q,R440A       | −4′Gln, 4′Ala           | 6.4 ± 0.2*** | 3  |
| R436D,R440A       | 0′Asp, 4′Ala            | 25.0 ± 0.3*** | 3  |

| 5-HT3A QXA constructs | Substituted residue(s) | γ | n |
|-----------------------|------------------------|---|---|
| R432Q,R436D,R440A    | −4′Gln, 0′Asp, 4′Ala   | 36.5 ± 1.0 | 17 |
| R432Q,R436E,R440A    | −4′Gln, 0′Glu, 4′Ala   | 34.8 ± 1.0 | 14 |
| R432Q,R436Q,R440A    | −4′Gln, 0′Glu, 4′Ala   | 23.1 ± 0.6*** | 9  |
| R432Q,R436E,R440A    | −4′Gln, 0′Phe, 4′Ala   | 1.9 ± 0.3*** | 7  |

| nACH αββα constructs | Substituted residue(s) | γ | n |
|-----------------------|------------------------|---|---|
| Wild-type nACH       | WT                     | 31.3 ± 0.8 | 6  |
| α5β4(ES484R)β4       | α5 WT β4, 4′Arg        | 24.8 ± 1.1*** | 6  |
| α5β4(E439R)β4        | α5 WT β4, 4′Arg        | 16.3 ± 0.9*** | 6  |
| α5β4(ES484R)β4(E439R)| α5 WT β4, 4′Arg        | 11.4 ± 0.5*** | 12 |
| α5β4(ES484R)β4       | α5, 0′Arg β4, WT       | 27.9 ± 0.5**  | 4  |
| α5β4(ES484R)β4,β4    | α5 WT β4, 0′Arg        | 26.8 ± 0.7**  | 4  |
| α5β4(ES484R)β4,β4    | α5, 0′Arg β4, 0′Arg    | 16.4 ± 0.5*** | 5  |

<sup>a</sup> Statistical significance was determined by ANOVA with post hoc Tukey’s test, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)
<sup>b</sup> Values estimated by variance analysis of 5-HT (10 μM)-activated currents recorded from either whole cells or outside-out patches.
<sup>c</sup> Includes values reported previously (7).
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exchange of the 5-HT$_{3A}$ Arg-440 residue by the equivalent 5-HT$_{3B}$ residue (Ala) produced an increase in $\gamma$ compared with the WT 5-HT$_{3A}$ receptor (Fig. 2A; Table 1). Furthermore, the 5-HT-activated $\gamma$ of the 5-HT$_{3A}$(R432Q,R436D) receptor is reduced ($p < 0.001$) relative to the 5-HT$_{3A}$(QDA) receptor (Table 1). Therefore, the data emphasize that for the 5-HT$_{3A}$ receptor the nature of the residue occupying the MA 0' position is an important determinant of $\gamma$, but Arg-440 (MA 4') and to a lesser extent Arg-432 (MA --4') are also influential in this regard.

Introduction of MA 0' Residues of nAChR into the 5-HT$_{3A}$ Subunit Influences $\gamma$—The $\gamma$ of neuronal nicotinic receptors (e.g. $\alpha_2$ and $\alpha_3\beta_2$) is much greater than that of the 5-HT$_{3A}$ receptor. Given that the much higher $\gamma$ of the heteromeric 5-HT$_{3A}$/5-HT$_{3B}$ receptor versus the homomeric 5-HT$_{3A}$ receptor is due, at least in part, to the nature of MA residues and the MA 0' residue in particular, we investigated the influence of the residues that occupy homologous MA 0' positions in the nAChR $\alpha_2$(Glu), $\beta_2$(Gln), and $\alpha_2$(Phe) subunits on the $\gamma$ of the 5-HT$_{3A}$ receptor (see Fig. 1B).

Activation of the 5-HT$_{3A}$(R436E) receptor elicited single channel events that were readily resolved in outside-out patch recordings (Fig. 3A; Table 1). Furthermore, the $\gamma$ of the 5-HT$_{3A}$(R432Q,R436E,R440A) receptor was similar to that of the 5-HT$_{3A}$(QDA) receptor, demonstrating that negatively charged Glu (the MA 0' residue in the $\alpha_2$ subunit) and Asp (the MA 0' residue in the 5-HT$_{3B}$ subunit) residues are approximately equally effective in facilitating $\gamma$ at this location (Table 1). The 5-HT$_{3A}$(R436Q) receptor also mediated discernible 5-HT-activated single channels (Fig. 3B; Table 1). The $\gamma$ of the 5-HT$_{3A}$(R432Q,R436Q,R440A) receptor was modestly reduced in comparison to the 5-HT$_{3A}$(QDA) receptor (Table 1). Hence, for the 5-HT$_{3A}$ receptor, these two constructs reveal that Gln (the MA 0' residue of the nACh $\beta_2$ subunit) facilitates $\gamma$, although it is not as effective in this regard as Asp or Glu (Table 1). No single channel events were resolvable in outside-out patch recordings of 5-HT$_{3A}$(R436F) receptors, although inward current responses were clearly present (Fig. 3C). Variance analysis of whole-cell currents confirmed that this receptor has a $\gamma$ below the resolution of single channel recording (Table 1). Similarly, the 5-HT$_{3A}$(R432Q,R436F,R440A) receptor exhibited a low $\gamma$ estimated by fluctuation analysis of the 5-HT-induced patch current (Table 1). These experiments demonstrate that the introduction into the 5-HT$_{3A}$ subunit of the MA 0' residues found in the nAChR $\alpha_2$, $\beta_2$, but not $\alpha_4$ subunits substantially increases $\gamma$.

To test for a possible involvement of poorly resolved unitary events in the effect of the 5-HT$_{3A}$ mutations on $\gamma$, we attempted to correlate the mean variance of single channel currents with $\gamma$. There was no correlation between the open channel current variance and $\gamma$ values of mutant 5-HT$_{3A}$(R432D), 5-HT$_{3A}$(R436E), and 5-HT$_{3A}$(R436Q) receptors ($r^2 = 0.29$ from linear regression; data not shown). A lack of an inverse correlation between open channel current variance and $\gamma$ values suggests that changes in $\gamma$ are not an aberration because of incompletely resolved unitary events.

Replacement of MA 4' Residues of $\alpha_2\beta_2$ nAChRs by Arginine Reduces Current Density—in contrast to the WT homomeric 5-HT$_{3A}$ receptor, single channel events mediated by both homomeric and heteromeric...
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We transfected HEK cells with triple mutant α4(E584R, E588R, E592R) and β2(E439R, Q443R, E447R) constructs (i.e. the −4′, 0′, and 4′ residues of both subunits mutated to Arg.) Interestingly, no whole-cell phosphorylation by casein kinase II (4) was obtained for the mutant or a WT subunit. Strikingly, no whole-cell response (14 cells tested) was obtained for the triple mutant or a WT subunit. This receptor provides the additional advantage that MA residues can be introduced into all, or just some, of the subunits within heteromeric nAChRs by expressing mutant α4 and β2 subunits together or in combination with WT subunits.

We transfected HEK cells with triple mutant α4(E584R, E588R, E592R) and β2(E439R, Q443R, E447R) constructs (i.e. the −4′, 0′, and 4′ residues of both subunits mutated to Arg.) The homologous residue occupying these positions in the 5-HT3A subunit). ACh (100 nM) readily activated channels recorded from patches of GFP-positive HEK cells (six patches tested). By contrast ACh (1 mM) failed to activate single channels in outside-out patches excised from transfected GFP-positive HEK cells (six patches tested). Unsurprisingly, single channels were not detected when the 4 receptors expressed. When ACh (1 mM) was also relatively ineffective on cells expressing WT α4 and triple mutant β2 or vice versa (Fig. 4). Therefore, we investigated the influence on the whole-cell current to ACh (1) of MA −4′, 0′, and 4′ mutations within one, or both, nAChR subunits. Robust responses were obtained for the −4′ and 0′ mutants, expressed either with the corresponding mutant or a WT subunit. Strikingly, no whole-cell response (14 cells tested) was obtained for the α4(E592R)β2(E447R) receptor (Fig. 4). Indeed, even when the 4 mutation was only carried by one subunit, either the α4 or the β2, the whole-cell current was dramatically reduced (Fig. 4). Unsurprisingly, single channels were not detected from outside-out patches excised from such cells. Clearly, the exchange of the negatively charged glutamate residue by the positively charged arginine residue at the 4′ position of either the α4 or the β2 subunit has a dramatic effect on the functional expression of the receptor. We are currently investigating the nature of this deficit, and we note that in both the α4 and β2 subunits the mutation disrupts a putative site for phosphorylation by casein kinase II (i.e. SXX(D/E)).

Replacement of MA −4′ or 0′ Residues of α4β2 nAChRs by Arginine Reduces γ—In contrast to the MA 4′ mutants, resolvable single channels could readily be detected for receptors carrying the −4′ or 0′ mutations. We first examined the role of the MA 0′ residue in nAChR. In comparison to WT α4β2 nAChRs, the γ of receptors assembled from α4(F588R) and β2(Q443R) subunits was approximately halved, when assessed both by fluctuation analysis of whole-cell currents (Fig. 5, A and B) and by direct observation of single channel events (Table 1; Fig. 5, D and G). However, when the α4(F588R) mutant was co-assembled with WT β2 subunits, there was only a small reduction of γ (Table 1) determined from outside-out patch recordings (Fig. 5, E and G). Similarly, when the β2(Q443R) mutant was expressed with the WT α4 subunit, γ was only modestly reduced (Table 1; Fig. 5, F and G).

Gating kinetics can influence estimates of γ. For example, rapid open channel block can cause channel "flickering" resulting in failure to resolve full openings (19). To investigate the possibility that such a mechanism could account for the apparent reduction in γ caused by mutation of MA 0′ residues in α4 and β2 subunits, we attempted to correlate the mean open channel current variance (derived from the gaussian fits to all-points amplitude histograms) to the mean conductances of WT and mutant α4β2 nAChR combinations. Current densities were determined by normalizing the peak amplitudes of ACh-activated current to cell capacitance. We calculated the mean current density mediated by WT α4β2 receptors (Fig. 4). ACh (1 mM) was also relatively ineffective on cells expressing WT α4 and triple mutant β2 or vice versa (Fig. 4). Therefore, we investigated the influence on the whole-cell current to ACh (1) of MA −4′, 0′, and 4′ mutations within one, or both, nAChR subunits. Robust responses were obtained for the −4′ and 0′ mutants, expressed either with the corresponding mutant or a WT subunit. Strikingly, no whole-cell response (14 cells tested) was obtained for the α4(E592R)β2(E447R) receptor (Fig. 4). Indeed, even when the 4 mutation was only carried by one subunit, either the α4 or the β2, the whole-cell current was dramatically reduced (Fig. 4). Unsurprisingly, single channels were not detected from outside-out patches excised from such cells. Clearly, the exchange of the negatively charged glutamate residue by the positively charged arginine residue at the 4′ position of either the α4 or the β2 subunit has a dramatic effect on the functional expression of the receptor. We are currently investigating the nature of this deficit, and we note that in both the α4 and β2 subunits the mutation disrupts a putative site for phosphorylation by casein kinase II (i.e. SXX(D/E)).

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FIGURE 5. Replacement of MA 0 residues in the nACh α4β2 receptor by arginine reduces single channel conductance. A, examples of macroscopic direct current- and alternating current-coupled whole-cell currents, recorded at a holding potential of −60 mV, evoked by nicotine (10 μM) acting at WT α4β2 and mutant α4(F588R)β2(Q443R) heteromeric nAChRs. In mutant nAChR subunits MA 0 residues were replaced by arginine (the MA 0 residue of the 5-HT3A subunit). B, plots of current variance versus mean current yield, by linear regression, γ values of 26 and 12.2 pS for WT and mutant receptors, respectively, in this example. C–F, single channel currents activated by acetylcholine (100 nM) applied to outside-out patches (clamped at −94 mV) containing WT and mutant α4β2 nAChRs. All-points amplitude histograms were fitted with the sum of two gaussians. D–F, unitary events were mediated by double mutant α4(F588R)β2(Q443R), single mutant α4(F588R)β2, and single mutant α4β2(Q443R) receptors, respectively. G, bar graph of mean chord conductance (±S.E.) for WT, single, and double mutant nAChRs. Mutant α4(F588R)β2, α4β2(Q443R), and α4(F588R)β2(Q443R) receptors exhibited chord conductances that were significantly below those of WT α4β2 receptors, determined by ANOVA with post hoc Tukey’s test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). H, plot of mean current variance versus mean γ values for WT and mutant (α4(F588R)β2(α4Mβ2)), α4β2(Q443R)(α4Mβ2), and α4(F588R)β2(Q443R)(α4Mβ2) nAChRs. Data from gaussian fits to all points amplitude histograms, including those shown in C–F. Horizontal and vertical bars represent ± S.E. for γ and variance values, respectively.
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FIGURE 6. Replacement of MA –4′ residues in the nACh α4β2 receptor by arginine reduces single channel conductance. A–C, single channel currents activated by acetylcholine (100 nM) applied to outside-out patches (clamped at −94 mV) containing mutant α4β2 nAChRs. All-points amplitude histograms were fitted with the sum of two gaussians. A–C, unitary events were mediated by double mutant α4(E584R)β2(E439R), single mutant α4(E584R)β2, and single mutant α4β2(E439R) receptors, respectively. D, a bar graph of mean chord conductance (± S.E.) for WT, single, and double mutant nAChRs. Mutant α4(E584R)β2, α4β2(E439R), and α4(E584R)β2(E439R) receptors exhibited chord conductances that were significantly below those of WT receptors, determined by ANOVA with post hoc Tukey’s test. All values were significantly different from one another at p < 0.001.

11.4 ± 0.5 pS; Fig. 6, A and D) when compared with that of WT α4β2 nAChRs (Fig. 5C). In common with the nAChR 0′ residue, when the −4′ mutation was introduced only into one of the subunits, either the α4 (Fig. 6B) or the β2 (Fig. 6C), there was a less dramatic but nevertheless significant reduction of γ. In fact, the α4β2 (E439R) receptor had the lowest γ of any of the nAChRs tested carrying a single point mutation on one of its subunits (γ = 16.3 ± 0.9 pS; Table 1).

Taken together these results demonstrate that in common with the 5-HT3A receptor, the nature of the MA 0′ residue greatly influences the γ of the α4β2 nAChR. However, for the 5-HT3A receptor, the impact of the −4′ residue on γ was modest in comparison to that of the 0′ residue, and for the α4β2 nAChR the influence of the −4′ residue is greater than that of the 0′ residue. Furthermore, for the α4β2 nAChR the −4′ or the 0′ arginine residue must be introduced on both the α4 and the β2 subunit to achieve the maximum reduction of γ.

DISCUSSION

This study demonstrates that elements of the cytoplasmic loop within the region referred to as either the MA helix (10) or helical amphipathic stretch (6) influence the single channel conductance (γ) of both the 5-HT3A and nACh receptors; a property traditionally assigned to the pore-lining M2 domains (2, 4). A mechanistic explanation is derived from ultrastructural studies of the Torpedo nAChR showing rods of density, corresponding to MA helices, projecting into the cytoplasm to form an “inverted pentagonal cone” that constitutes the inner vestibule...
of the ion channel (9, 10). Unlike conventional depictions of the vestibule, the images of the nAChR suggest that permeating ions must pass through "portals" formed between adjacent subunits. The dimensions of the portals are estimated to be comparable with those of the permeant cations and would thus be anticipated to influence the rate of ion flux (9, 10). Thus, we hypothesize that amino acids within the MA helices of the homomeric 5-HT₃A receptor line five portals (Fig. 7A). In each portal three repetitive arginine residues (MA −4′, 0′, and 4′) form a rate-limiting barrier to ion conduction (7). These residues are responsible for the femtosiemen conductance that is a unique hallmark of homomeric 5-HT₃A receptors (5). In contrast, the 5-HT₃B subunit cannot form homomeric receptors but increases the γ of heteromeric 5-HT₃A/B receptors to ~16 pS.

We produced homology models of the 5-HT₃A and α₁β₂ nACh receptors by threading their amino acid sequences onto the 4 Å resolution structure of the αβγδ Torpedo nAChR. The energy-minimized homomeric 5-HT₃A receptor model is asymmetric by virtue of the fact that the original α-carbon coordinates were derived from a heteromeric nAChR. Nevertheless, this qualitative approach suggests that MA −4′, 0′, and 4′ arginine residues are located at the cytoplasmic mouths of the 5-HT₃A portals (Fig. 7A). Introduction of 5-HT₃B subunit MA −4′Gln, 0′Asp, and 4′Ala residues into the model predicts that these more compact residues lessen a steric impediment to ion flux providing a potential explanation for the increased γ seen in recordings from the 5-HT₃A(R432Q,R436D,R440A) receptors (Fig. 7B). The triple mutant 5-HT₃A(QRA) receptor has a >36-fold higher γ compared with that of the WT 5-HT₃A receptor. It is likely that the increased density of acidic residues in 5-HT₃A(QRA) also participates in their large γ.

It is evident that there are several acidic residues lining the putative α₁β₂ nAChR portals (Fig. 7C). Instead of the basic residues at MA −4′, 0′, and 4′ locations within the 5-HT₃A subunit, α₁ and β₂ subunits have acidic (glutamate and glutamine, respectively) residues at both the MA −4′ and 4′ locations. The portals of the nAChRs also appear less cluttered by voluminous residues lining their cytoplasmic mouths compared with those of the 5-HT₃A receptor (Fig. 7). These observations are in keeping with the substantially larger γ observed for α₁β₂ nAChRs compared with 5-HT₃A receptors.

The MA 0′ residue has the largest impact on γ of any single 5-HT₃A receptor residue tested. Replacement of the MA 0′ arginine residue of the 5-HT₃A subunit by the equivalent residue in the 5-HT₃B subunit (aspartate) is sufficient to increase γ from ~900 fS (below the resolution of direct observation) to ~10 pS enabling direct observation of unitary events in outside-out patch recordings. Furthermore, introduction of MA 0′ residues of either α₁ or β₂ subunits (glutamate and glutamine, respectively) into the 5-HT₃A receptor also substantially increased γ. Substitution of the MA 0′ arginine by phenylalanine, the MA 0′ residue of the α₄ subunit, by contrast, reduced the γ of 5-HT₃A(R436F) receptors. Like arginine, phenylalanine is a voluminous residue, and its ability to maintain γ within the femtosiemen range supports the hypothesis that the volume of the MA 0′ residue is one determinant of γ.

The critical role of the 5-HT₃A MA 0′ residue in controlling γ is emphasized by replacing the 0′ arginate with arginine (5-HT₃A(QRA)) in the large γ 5-HT₃A(QRA) construct. This leads to an 83% reduction in γ (Table 1). The MA −4′ and 4′ residues neighboring 0′ in the MA helix (Fig. 7A) also make a significant contribution to γ. This can be most directly observed when either the −4′ or the 4′ arginines are returned to the 5-HT₃A(QRA) construct. Under these conditions the single channel conductances of 5-HT₃A(RDA) and 5-HT₃A(QDR) are reduced by 32 and 51%, respectively, compared with 5-HT₃A(QRA) (Table 1).

Introduction of arginine into either the MA −4′ or 0′ locations of the α₁β₂ nAChR also caused significant reductions in γ. This effect was most obvious when arginine was introduced into either the −4′ or 0′ locations of both the α₁ and β₂ subunits (i.e. α₁(E584R), β₂(E439R), and α₁(E588R) β₂(Q443R) receptors, respectively), when the γ values of the mutant receptors were reduced by 63 and 50%, respectively, from that of the WT receptor. Under these conditions all five portals of each of the mutant receptors contain basic residues at critical positions in the cytoplasmic conduction pathway. Although the introduction of arginines into the MA −4′ and 0′ locations of the α₁ and β₂ subunits substantially reduced γ, mutant nAChRs had conductances considerably larger than that of the WT 5-HT₃A receptor. It is likely that the higher density of basic residues seen in the 5-HT₃A receptor is required to achieve the femtosiemen level. To test this hypothesis, we constructed triple mutant α₁(E584R,F588R,E592R) and β₂(E439R,Q443R,E447R) constructs. Whether expressed in combination with WT α₁ and β₂ or their respective triple mutant partners, these constructs failed to produce sufficient functional receptor expression to enable quantification of γ. Furthermore, introduction of arginine into the MA 4′ positions of either the α₁ or β₂ subunits caused a near abolition of functional expression. We note that this mutation disrupts a putative casein kinase II phosphorylation site; however further experiments will be required to determine the cause of the reduced current density.

A more extensive investigation of the relationship between the physicochemical properties of MA −4′, 0′, and 4′ residues and γ of 5-HT₃ and nACh receptors is warranted to elucidate their precise roles in ion conduction. Nevertheless, this study demonstrates that the substitution of the MA −4′ and 0′ residues in both α₁ and β₂ subunits leads to a substantial reduction of γ, suggesting that cytoplasmic residues influence ion conduction through heteromeric α₁β₂ nAChRs in a manner consistent to that observed in homomeric 5-HT₃A receptors. These data support a role for cytoplasmic portals in the ion conduction pathways of Cys loop receptors.

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