Tryptophan scanning of the acetylcholine receptor’s βM4 transmembrane domain
Decoding allosteric linkage at the lipid-protein interface with ion-channel gating

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Abbreviations: 
ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; AChR, acetylcholine receptor; [125I]-α-BgTx, [125I] α-bungarotoxin; TrpScanM, tryptophan-scanning mutagenesis; TD, transmembrane domain; WT, wild-type; FT, Fourier transform; TrpPPs, tryptophan-periodicity profiles; [125I]TID, 3-trifluoromethyl-3-(m[125I]iodophenyl)diazirine; [3H]DAF, 2-[3H]diazofluorene

Key words: acetylcholine receptor, tryptophan-scanning mutagenesis, fourier transform, transmembrane domain, ligand-gated ion channel

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel protein that mediates fast excitatory synaptic transmission in the peripheral and central nervous systems. Changes in the structure and function of the AChR can lead to serious impairment of physiological processes. In this study, we combined site-directed mutagenesis, radioligand binding assays, electrophysiological recordings and Fourier analyses to characterize the functional role and structural aspects of the βM4 transmembrane domain of the Torpedo AChR. We performed tryptophan replacements, from residues L438 through F455, along the βM4 transmembrane domain. Expression levels of mutants F439W-G450W and F452W-I454W produced peak currents similar to or lower than those in wild-type (WT). Tryptophan substitutions at positions L438 and T451 led to a deficiency in either subunit expression or receptor assembly. Mutations L440W, V442W, C447W and S453W produced a gain-of-function response. Mutation F455W produced a loss of ion channel function. The periodicity profile of the normalized expression level (closed state) and EC50 (open state) revealed a minor conformational change of 0.4 residues/turn of the βM4 domain. These findings suggest that a minor movement of the βM4 domain occurs during channel activation.

Introduction

The nicotinic acetylcholine receptor (nAChR) is an integral membrane protein that belongs to the superfamily of ligand-gated ion channels.1-4 The nAChR from Torpedo californica is an allosteric and integral membrane protein comprised of five protein subunits arranged pseudo-symmetrically in a stoichiometry of α2βγδ2.5,6 Each subunit contains a large hydrophilic N-terminus, four transmembrane domains (TD) of 20–30 amino acids regions named M1, M2, M3 and M4, a variable intracellular loop between M3 and M4, and a short extracellular C-terminus.7,8 The M2 domain, which sits closest to the pore lumen, forms the pore lining in the closed state conformation, whereas both M1 and M2 domains contribute to the formation of the ion channel pore in the open state conformation.9-11 The M3 and M4 domains are in contact with the lipid membrane12,13 away from the ion channel pore and the ligand binding site.14

A high resolution structure from a crystallized AChR remains to be elucidated; however, structural information has been reported by a model of the Torpedo marmorata nAChR at 4 Å resolution.14 This model provided detailed information on the secondary structure and global arrangement of the TDs and is the best approximation to the AChR structure. Fourier transform infrared spectroscopy,15-17 photoaffinity labeling,12,13 and two-dimensional 1H-NMR spectroscopy,18 are other techniques utilized to probe both AChR structure and function. Recently, using some of these techniques, the crystal structure of the extracellular domain of nAChR α3 subunit has been reported at 1.94 Å resolution. This article provided detailed information about the cavity of the extracellular domain of nAChR α3 but no information about the TDs of the α3 subunit.19

Some methods such as X-ray crystallography and NMR spectroscopy are not standard techniques used with all proteins. Many proteins do not form crystals, and many are too large to solve by NMR.20 Although tryptophan-scanning mutagenesis (TrpScanM) is not a high resolution method, it is a good procedure for determining the structure of intractable proteins. For example, TrpScanM has been used successfully to characterize transmembrane domains of proteins such as inward rectifier potassium channels,21,22 voltage-activated potassium channels,23-28 nAChR channels,29-33 glutamate receptor channels,34 GABA receptors,35 and P2X receptors.36 TrpScanM has been used to collect valuable data about the structure...
Table 1 Biophysical parameters of the wild-type and βM4-mutant AChR

| AChR type | Expression level (fmol) | EC50 (µM) | Hill coefficient | Imax (-nA/fmol) | Imax (-nA) |
|-----------|------------------------|----------|-----------------|-----------------|-------------|
| 2αβγ (Wild type) | 9.2 ± 1.7            | 22.7 ± 3.7 | 1.35 ± 0.059     | 1674 ± 230      | 7047 ± 718  |
| 2αβγ(L438W)βγ | 0.22 ± 0.08#          | N.D.     | N.D.            | N.D.            | N.D.        |
| 2αβγ(F439W)βγ | 2.2 ± 0.14#           | 7.6 ± 1.80* | 1.20 ± 0.13     | 1480 ± 205      | 3100 ± 381* |
| 2αβγ(L440W)βγ | 6.2 ± 0.70*           | 12.2 ± 0.99* | 1.37 ± 0.088     | 1832 ± 236      | 10850 ± 657* |
| 2αβγ(F441W)βγ | 2.4 ± 0.96#           | 99.4 ± 38#  | 1.21 ± 0.17     | 814 ± 516*      | 2493 ± 2255 |
| 2αβγ(V442W)βγ | 2.2 ± 0.20#           | 11.8 ± 0.56* | 1.42 ± 0.065     | 3683 ± 620*     | 7810 ± 1219 |
| 2αβγ(F443W)βγ | 5.5 ± 0.70*           | 5.2 ± 0.14#  | 1.31 ± 0.087     | 1316 ± 203      | 6843 ± 253  |
| 2αβγ(F444W)βγ | 1.3 ± 0.40#           | 49.3 ± 5.30* | 1.64 ± 0.11     | 3357 ± 605*     | 3515 ± 663+ |
| 2αβγ(V445W)βγ | 2.6 ± 0.50#           | 18.6 ± 3.04* | 1.55 ± 0.067     | 1373 ± 268*     | 3196 ± 313* |
| 2αβγ(L446W)βγ | 2.9 ± 0.64#           | 23.4 ± 3.40  | 1.31 ± 0.087     | 2366 ± 669*     | 6027 ± 688  |
| 2αβγ(C447W)βγ | 3.5 ± 0.62*           | 5.8 ± 1.13#  | 1.11 ± 0.24     | 3283 ± 448*     | 13650 ± 432* |
| 2αβγ(S448W)βγ | 7.90 ± 1.52           | 21.2 ± 5.83  | 1.31 ± 0.12     | 568 ± 141*      | 3479 ± 370* |
| 2αβγ(L449W)βγ | 0.57 ± 0.09#          | 128.1 ± 20.33# | 1.61 ± 0.072   | 5400 ± 2155*    | 2700 ± 600# |
| 2αβγ(G450W)βγ | 2.7 ± 0.26#           | 39.8 ± 3.83#  | 1.34 ± 0.059     | 804 ± 115*      | 2167 ± 355# |
| 2αβγ(T451W)βγ | 0.32 ± 0.14#          | N.D.     | N.D.            | N.D.            | N.D.        |
| 2αβγ(F452W)βγ | 5.03 ± 1.03*          | 28.0 ± 4.12  | 1.38 ± 0.052     | 1211 ± 232      | 5471 ± 590  |
| 2αβγ(F453W)βγ | 4.2 ± 0.40*           | 16.82 ± 1.5* | 1.30 ± 0.088     | 2985 ± 350*     | 11179 ± 642* |
| 2αβγ(L454W)βγ | 6.8 ± 1.14            | 24.8 ± 4.03  | 1.52 ± 0.087     | 984 ± 139*      | 5857 ± 594  |
| 2αβγ(F455W)βγ | 1.16 ± 0.25#          | N.D.     | N.D.            | N.D.            | N.D.        |

Values are given as the mean ± the standard error of the mean for each parameter and were calculated using 5–16 oocytes. The normalized response (nA/fmol) of each oocyte was assessed as the ratio of the macroscopic peak current (nA) generated at 300 µM ACh to the superficial [125I]-α-BgTx binding (fmol). # means p < 0.001, * means 0.001 ≤ p < 0.01 and + means 0.01 ≤ p < 0.05. N.D., means no detectable current.

and function of various transmembrane domains of the AChR. For example, the pioneer studies of mutagenesis in the lipid-exposed position replacement of αCys418 and αCys421 by tryptophan resulted in a substantial increase in channel open time and demonstrated the important role of the lipid-exposed transmembrane domains to gating machinery of the AChR. Our group has extended the TrpScanM to αTM4,32 γTM4,32 γTM3,37 αTM3,30,33 and βTM3,31 to assess the structure and function of the AChR. These earlier studies identified residues at which a tryptophan replacement results in a robust gain-of-function, including, for example, αCys418,38 αVal425,29 αThr422.40 Moreover, prior analyses of the changes in AChR function and expression as a function of the mutation position or periodicity provided valuable information used to predict the secondary structure of various TDs of the AChR. The periodicity for αTM3-TM4, γTM4 and βTM3 are consistent with a helical structure for these TDs. In the present study, we extended the tryptophan-scanning approach to monitor the conformational changes of the Torpedo AChR βM4 domain. The TrpScanM approach reported allowed us to construct structural models of the Torpedo AChR βM4 domain in both the closed- and open-channel states. The interpretations of these structural models suggest an overall helical secondary structure for the βM4 domain. However, in contrast to others domains, the βM4 domain displays either very discrete or localized conformational changes.

Results

Functional characterization of the M4 transmembrane domain mutations: expression of βM4 mutants. Eighteen residues, from L438 to F455, within the M4 TD of the β subunit of T. californica AChR were individually replaced with tryptophan. Transcripts of mRNA were synthesized and injected into Xenopus laevis oocytes, and the level of cell-surface expression of mutant AChRs was assessed by [125I]-α-bungarotoxin ([125I]-α-BgTx) binding analysis, as described in Experimental Procedures. All the βM4-mutants showed different nAChR expression levels (Table 1). Twelve of the βM4-mutants (L438W, F439W, Y441W, V442W, F444W, V445W, I446W, C447W, I449W, G450W, T451W and F455W) displayed a significant reduction in nAChR expression levels as compared to wild-type (WT). According to previous photolabeling affinity studies, L438, F439, V442, V445, I449, G450, T451 and I454 are believed to be oriented toward the interior of the nAChR, so substitution of tryptophan at these given positions could cause a steric hindrance that could affect the stability of the TD and the expression levels of the AChR, consistent with the present results. Mutations at L438W, T451W and F455W were also incapable of eliciting acetylcholine (ACh)-induced currents (Table 1). The three non-functional mutants L438W (0.22 fmols), T451W (0.32 fmols), and F455W (1.16 fmols) gave expression levels lower than WT (9.2 fmols) and equivalent to other functional mutants in different AChR subunits. For example, in previous TrpScanM, the mutant βL284W31 gave a functional mutant that had an expression level of 0.30 fmols, similar to the mutant T451W. The mutant βV295S31 also gave a functional receptor with an expression level of 0.4 fmols. It is noteworthy that other mutations with similar or lower expression levels (F444W [1.3 fmols] and I449W [0.57 fmols]), respectively, in our scanning produced significant functional response. According to our data base of more than 300 lipid-exposed mutations, a decreased AChR expression does not necessarily mean that the receptor is
not functional. For example, the αF280W mutation displayed an expression level of 0.12 fmol and a functional receptor similar to WT. One possible explanation for the non-functional mutants found in the present study is a deficiency in channel assembly and/or oligomerization given that similar expression levels generate functional and non-functional mutants. The remaining six mutants (L440W, F443W, S448W, F452W, S453W and I454W) exhibited similar nAChR expression levels as WT.

**Effect on macroscopic current response.** The purpose of these studies is to determine the role of βM4 TD on channel function and to assess the role that lipid-protein interactions may have on the AChR function. We used the two-electrode voltage clamp technique and ACh concentrations ranging from 1 to 300 μM to generate the dose response curve for each mutation. Two mutants (L440W and S453W) enhanced the maximum macroscopic response by almost 1.5–2-fold compared to WT. Ten mutants (F439W, Y441W, F443W, F444W, V445W, I446W, S448W, I449W, G450W and F452W) significantly reduced the maximum macroscopic response; C447W, V442W and I454W displayed a similar response as WT. The I_{max} values were normalized to AChR expression levels (fmol) in each oocyte. The normalized macroscopic response (I_{max}/fmol) for six mutants (Y441W, V442W, F444W, C447W, I449W and S453W) was increased by 1.5–2-fold as compared with WT. Four mutants (V445W, S448W, G450W and I454W) displayed a decrease of I_{max}/fmol values, whereas the mutants F439W, L440W, F443W, I446W and F452W were similar to WT.

All the mutations that produced functional AChRs showed typical sigmoidal ACh dose-response curve profiles (Fig. 1B). The βY441W and βY449W mutants showed shallower curves with superior horizontal asymptotes >1 (I/I_{max}; Fig. 1B). This could explain the absence of a clear saturation plateau. The potency to acetylcholine (EC_{50}) was reduced in five mutants (F439W, L440W, V445W, F444W and C447W) by 2–4 fold compared to WT. The reduction in EC_{50} values could be due to changes in affinity and/or efficacy of ACh. On the other hand, five mutants (V445W, I446W, S448W, S453W and I454W) exhibited potency similar to WT. EC_{50} values increased for five mutants (Y441W, F444W, I449W, G450W and F452W) 2–4-fold compared to WT values. Even though the mutant I449W displays an EC_{50} value of 128 μM, the dose-response curve does not reflect this difference due to the small ionic current and small expression levels of the mutant. Most of the mutants display ionic current traces similar to those of WT. In comparison to WT, only three mutants (I440W, C447W and S453W) display distinct peak currents (Fig. 1C). Those mutations have a peak current over 7,000 nA larger than WT. A previous single-channel study demonstrated that the βC447W mutation increases the open channel probability, a kinetic pattern that could be shared with the other two mutants (I440W and S453W). The mutant F439W displays a macroscopic ionic current trace with a slow decay phase compared to WT, but the amount of ionic current is too small (<3,500 nA) and a single-channel analysis might be tedious. The small ionic currents displayed by the mutant βI449W (<2700 nA) does not allow for an accurate estimation of desensitization kinetics. Although a single-channel kinetics analysis would permit the study of the desensitization kinetics of the mutant βI449W, such an analysis does not seem feasible due to the low amount of macroscopic ionic current per oocyte.

**Structural differences between closed and open states.** The periodicity of nAChR expression (fmol/Å^3) revealed structural information of the βM4 TD in its closed state, given that the toxin binding assays were performed in the absence of the agonist (Experimental Procedures). Oocytes are incubated in 20 nM [^{125}I]-α-BgTx, 5 mg/mL BSA, and MOR-2 without EGTA at room temperature for 2 hours. A typical expression level of AChR in the oocyte surface is 3–10 fmols; therefore, toxin excess is in the order of ~2 x 10^7 fold to assure that all AChRs in the oocyte surface are completely blocked by the toxin. After [^{125}I]-α-BgTx incubation, oocytes do not display ACh-induced currents at any concentration of the agonist; thus, this is a clear indication that under these conditions all AChRs on the oocyte surface are bound with [^{125}I]-α-BgTx. A recent study from the crystal structure of the extracellular domain of α1 nAChR demonstrates that [^{125}I]-α-BgTx binds to the extracellular domain of the nAChR via protein-sugar interactions. We would like to emphasize that under these conditions, AChRs expressed in the oocyte surface have never been exposed to agonist; therefore, we basically assure that all receptors are “locked” in the closed conformation without a previous activation by agonist. In contrast, the periodicity profile estimated from EC_{50} values revealed structural information of the βM4 TD in an open-channel state, given that the EC_{50} value estimates the functional state of the nAChR. The periodicity profile of the closed-channel state showed an ordered oscillation along all positions, whereas the periodicity profile of the open-channel state displayed an ordered oscillation along all positions but a small distortion at the middle of the βM4 TD between positions C447 and T451. The ordered oscillatory pattern observed along all closed- and open-state conformations is consistent with a helical motif (Fig. 2A and C).

The periodicity profiles of the βM4 TD in the closed and open states illustrated patterns of 2.43 ± 0.71 and 2.14 ± 0.39 amino acids per helical turn, respectively, revealing nearly similar helical structures for the closed and open states. In addition, the helical net diagrams built with the periodicity profile data of the open- and closed-channel state localize the βM4 non-functional mutants in the same face of the “open- and closed-channel state model” (Fig. 3). Together, these data indicate that the βM4 TD preserves a helical secondary structure in the open- and closed-channel state. The fact that all the non-functional mutants are oriented toward the same face of the helix suggests a higher degree of packing within this region of the helix, presumably toward the interior of the protein (Fig. 3). Consequently, in the helical net diagrams, the last helical turn of the maximum oscillatory peaks diagram and the first helical turn of the minimum oscillatory peaks diagram do not represent an accurate location of the non-functional mutants. This observation reveals the limitation of the helical net diagram analysis at the extremes of the helix where it is very difficult to extrapolate a maximum and/or minimum oscillatory peak in the tryptophan-periodicity profiles.

Generally, the discrete Fourier transform (FT) analysis is used for converting a sequence of values into a frequency spectrum, and thus, it may be used to detect periodic variations in the sequences. Therefore, we coupled FT to TrypScanM to estimate by another method the mean periodicities (residues per turn) and peak ratios of the sequences of AChR expression (fmol/Å^3) and ACh EC_{50} (μM) values (see Table 2). With these mean periodicities we were able to estimate the overall periodicity change undergone by the helical
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open-channel states, respectively. From these results we could determine that the helical structure of the βM4 domain underwent an overall conformational change of ~0.4 residues/turn during conformational transition states from closed- and open-channel states of the AChR. In addition, the strength of these peaks to predict helical secondary structures was evaluated by the peak ratio equation as the ratio of \( P(\omega) \) in the peak range (peak - 15° ≤ peak ≤ peak + 15°) relative to \( P(\omega) \) over the whole spectrum. The peak ratio values were 1.43 and 1.37 for closed- and open-channel states, respectively, resulting in peak abundances of 24–25% from whole spectra, and

Figure 1. Sequence alignments of the βM4 domain and functional response of wild-type and βM4-mutant AChRs. (A) LFLYVFVICSIGTGTFSIF (blue font) were examined in the current study. ***YV*FVICSI**FS** (highlighted in yellow and position number in green) are non-conserved residues among AChR species. Y*(441), F(443), F(444), C*(447) and S*(448) were labeled as lipid-exposed positions using photolabeling affinity \[^{125}\text{I}]\text{TID for the underline residues and [\(^3\text{H}\)]DAF for the asterisks residues}. The numbers at the bottom indicate the position in the Torpedo β1-subunit. (B) displays dose-response curves that were standardized to \[^{125}\text{I}]\text{-bungarotoxin (fmol) bound to AChR expressed on the oocyte surface membrane. The insert in each curve shows the dose-response curves normalized to maximum current. For the sake of clarity, we split the mutants in two groups (L438W to L446W and C447W to F455W). (C) shows representative families of macroscopic ionic current traces evoked by 1–300 µM ACh and recorded through two-electrode voltage clamp.
thus predicting reliable helical secondary structures for the βM4 domain. Taken together, FT-TrpScanM data suggest that the helical structure of the βM4 domain undergoes a conformational change of 0.4 residues/turn during channel activation of the AChR, and validate the results of periodicity change from TrpPPPs data.

**Discussion**

The highly hydrophobic M4 TD of the nAChR is the least evolutionarily conserved of the four domains composing the AChR (Fig. 1A). Previous studies demonstrate that all the membrane spanning domains M1, M2, M3 and M4 adopt an α-helical conformation.12,13,17,19,42 In the present study, we used TrpScanM to examine the functional and structural role of 18 residues (from L438 to F455) of the βM4 TD in AChR.

**Functional Interpretation vs. spatial orientation.** Tryptophan substitution at position L438W, T551W and F455W resulted in non-functional AChRs (Table 1). These mutants displayed very low AChR expression levels and no detectable ionic currents. Previous photoaffinity labeling studies have suggested that these residues are located at the interior of the protein.13,42 These results contrast with a previous study of the lipid-exposed γM4,32 and αM4,29 TD in which all the γM4 tryptophan replacements (from γC451W to γG462W), including residues located at the interior of the domain, gave functional AChRs, and αM4 tryptophan replacements value than that found for WT, suggesting an impaired ion channel function of this mutant. The C447W displayed significantly lower expression levels than did WT and also showed a lower EC50 and a higher macroscopic response than did WT. These results suggest an enhancement in the modulation and/or ion channel properties of the AChR that could categorize the C447W mutant as a gain-of-function mutation.

L440W displays an increase in the macroscopic response. L440W like C447W displays a reduction in the EC50 value for ACh and a higher macroscopic current response compared to WT. Y441W, F444W, I449W, G450W, T451W and F455W of the eleven mutations are residues that could be oriented toward the interior of the AChR due to undetected labeling of previous 3-trifluoromethyl-3-[(125I)diphenyl-diazirine ([125I]TID) photolabeling studies in the *T. californica* AChR.13 However, according to the photolabeling studies, mutations Y441W, F444W and C447W are presumably oriented toward the lipid interface. Both Y441W and F444W mutants also have a lower macroscopic response and a higher EC50 (from αC412W to αV425W) gave only one non-functional mutant (αI417W). The aforementioned studies clearly demonstrate that γM4 and αM4 can tolerate the bulky tryptophan side chain compared to the βM4. These results suggest that the βM4 domain is more tightly-packed than the γM4 and αM4 TDs. In addition, γM4 and αM4 TDs report a movement of approximately 3.3 and 3.6 residues per helical turn, respectively, compared to the βM4 TD that reports only a movement of 2.4 residues per helical turn.29,33 It is noteworthy to mention that the beta subunit does not contribute to the structural domains of the agonist binding site of the AChR; therefore, the present data is consistent with a more rigid and tightly-packed domain than that of γM4 and αM4 TDs.

We found that 11 mutations (L438W, F439W, Y441W, V442W, F444W, V445W, C447W, I449W, G450W, T451W and F455W) produced a significant reduction in the AChR expression levels, suggesting a reduced efficiency of assembly and/or oligomerization of the AChR. Eight (L438W, F439W, V442W, V445W, I449W, G450W, T451W and F455W) of the eleven mutations are residues that could be oriented toward the interior of the AChR due to undetected labeling of previous 3-trifluoromethyl-3-[(125I)diphenyl-diazirine ([125I]TID) photolabeling studies in the *T. californica* AChR.13,42 In the present study, we used FT-T rpScanM to examine the functional and structural role of 18 residues (from L438 to F455) of the AChR that could categorize the C447W mutant as a gain-of-function mutation.

L440W displays an increase in the macroscopic response. L440W like C447W displays a reduction in the EC50 value for ACh and a higher macroscopic current response compared to WT. Y441W, F444W, I449W, G450W and F452W displayed a lower macroscopic response, suggesting an impaired ion channel function. These mutants also displayed higher EC50 values. The other mutants displayed expression and macroscopic levels lower or similar to those of WT.

Mutations that produced a gain-of-function response (i.e., L440W, V442W, C447W and S423W) are clustered at opposite sides of the helix. Positions C447 and L440 are facing the lipid-protein interface (Fig. 4A and B); thus, the observed gain-of-function of these mutants is consistent with those of previously reported positions such as those of the αC418W. In contrast, positions V442 and S453 are facing the interior of the protein (Fig. 4B and C). According to the nAChR structure, both positions are oriented to a crevice facing the M1 domain (Fig. 4C). The size of the crevice allows for the
accommodation of a bulky side chain and this could explain the significant expression of these mutants. The observed gain-of-functions of the V442W and S453W mutations could be due to interactions of these positions with the M1 domain during channel gating. One possibility is that the hydrophobic interactions with the M1, which contribute to the open pore structure, could delay the closing of the channel.

The novel αC418W mutation located in the M4 domain has been shown to produce an increase in macroscopic response, a lower EC₅₀, and a lower expression level, all of which are similar to the characteristics of βC447W. The functional expression of this novel αC418W mutation was shown to be a cholesterol-sensitive mutation. Single-channel electrophysiology of αC418W showed an increase in the channel open probability, due to a decrease in the apparent channel closing rate and a probable increase in channel open probability, due to a decrease in the trophysiology of the effective opening rate, again similar to the response of αC418W and βC447W share an additional characteristic: as a consequence of the mutation, this lipid-exposed mutant favors the introduction of a caveolin-binding motif (CBM). Caveolae are specialized lipid rafts rich in cholesterol, sphingolipids and caveolins. A consensus CBM sequence has been established with the following sequences: ΦXXXΦXXXΦ and ΦXXXΦXXXΦ; in which Φ is an aromatic residue (W, F or Y) and X is any amino acid. In the αC418W mutant we identified a sequence (WIIIGTYSVEA, residues 418–426) with a putative CBM, which is formed when cysteine in position 418 is replaced by tryptophan. In the βC447W we also identified a similar sequence (EVIWTSIGTE, residues 444–452) when cysteine in position 447 is replaced by tryptophan. The formation of putative CBM by the incorporation of tryptophan at lipid exposed domains may lower the amount of receptors available in the membrane and thus could account for the reduced expression levels previously reported for αC418W and now for βC447W. The αC418W is the first lipid-exposed mutation that has been shown to produce congenital myasthenic syndrome in humans.

Closed- versus open-channel states. In the present study, the tryptophan periodicity profiles of the βM4 TD displayed a small structural difference between the open- and closed-channel state (Fig. 2A and C). The overall helix movement during channel activation estimated for the βM4 TD predicts a value of 0.4 residues-per-turn; therefore, this domain displays reduced movement when compared to the αM3 TD for which a 1.0 residue-per-turn was recently reported. The small difference between the two states could be the result of one of two types of movement during channel activation: (i) a global subtle movement of the entire βM4 TD or (ii) a discrete and localized movement, both during channel activation (Figs. 2 and 3). The localized movement could occur between residues βCys447 and βThr451. This small region contains polar residues that are characterized as forming very stable and strong hydrogen bonds that could stabilize the helix during channel activation.

One interesting observation is that the ACh-binding sites lie at the α-γ and α-δ subunit interface, giving the β-subunit a more supporting role during channel activation. Another characteristic of the β-subunit is that, in contrast to the other subunit, the βM4 domain revealed very small subunit movement between channel activation. Taking into consideration the structural characteristics of the βM4 TD, one type of movement that could occur during channel activation is the one proposed in the global movement theory. Previous experiments on the structural mechanisms of gating used electron microscopy on Torpedo postsynaptic membranes and rapid spray-freezing techniques to trip the AChR in the open-channel form. Those experiments showed that binding of ACh initiates two interconnected events in the ligand-binding domain. One is a local disturbance in the region of ACh-binding sites, and the other is a larger-scale conformational change that propagates along the transmembrane domains. In some, the rate-equilibrium free energy relationship analysis of some domains in the AChR suggest that the closed- vs. open-gating conformational change occurs as
Table 2  Secondary structure parameters of βM4 domain determined by tryptophan-periodicity profiles and Fourier transform power spectra

| Conformational states of the AChR | Tryptophan-periodicity profiles | Fourier transform power spectra |
|-----------------------------------|---------------------------------|--------------------------------|
|                                   | Periodicity residues/turn       | Rotation angle degree          | Expected mean periodicity residues/turn | Peak ratio |
| Closed-channel                    | 2.14 ± 0.39                    | 168.22                         | 165.39                                   | 2.18       | 1.4255 |
| Open-channel                      | 2.43 ± 0.71                    | 148.15                         | 139.4                                    | 2.58       | 1.3652 |

The given values correspond to analysis performed on the entire sequences of the AChR expression and ACh EC₅₀ values. The periodicities of the tryptophan-periodicity profiles were given as mean ± SD. The expected rotation angles of the tryptophan-periodicity profiles and the expected mean periodicities of the peak that corresponds to mean oscillations of the tryptophan-periodicity profiles were calculated using periodicity (residues/turn) = 360° / rotation angle.

Figure 4. Spatial orientation of the AChR βM4 transmembrane domain. (A) display the βM4 domain along the helix. (B) shows the top view of the βM4 domain. (C) Illustrate the βM1–M4 domains. The amino acids in cyan color represent the gain-of-function positions. Asterisks (*) indicates the lipid-exposed positions using photolabeling affinity [¹²⁵I]TID (Y441, F443, I444, C447) and [³H]DAF (Y441, C447, S448). The 3D model representation is from the Unwin AChR 4 Å resolution structure interpretation using Pymol viewer (PDB code 2GB9).

Experimental Procedures

General experimental procedures. X. laevis oocytes were microinjected with the T. californica AChR complementary RNAs. Mutations were engineered with the QuikChange® site-directed mutagenesis kit (Stratagene) and were confirmed by automated DNA sequencing. All mutagenic primers were designed with the tryptophan codon (TGG) instead of the WT codon at the desired position. Muscle-type AChR cRNA transcripts were synthesized with the mMESSAGE
mMACHINE® kit (Ambion). Oocytes were incubated for three days with fresh liquid medium at 19°C.

**Voltage clamp.** Macroscopic ACh-induced currents were recorded with a whole-cell two-electrode voltage clamp configuration using the Gene Clamp 500B amplifier (Axon Instruments) at room temperature. Electrodes were filled with 3 M KCl, resistances <2 MΩ. Impaled oocytes were automatically perfused with MOR-2 buffer [82.5 mM NaCl, 2.5 mM KCl, 5 mM MgCl₂, 1 mM Na₂HPO₄, 0.2 mM CaCl₂, 5 mM HEPES, and 0.5 mM EGTA (pH 7.4)] at a rate of 0.43 μL/s using a Perfusion Valve Controller VC-8 (Warner Instruments). Membrane potential was held at -70 mV. Membrane currents were filtered at 100 Hz and digitized at 1 kHz using a DigiData 1322A interface (Axon Instruments). Data acquisition was conducted through the Clampex 9.2 program (Axon Instruments). Dose-response curves were generated from macroscopic peak currents (I) obtained from six different ACh concentrations (1, 3, 10, 30, 100 and 300 μM ACh). Dose-response curves were fitted through the sigmoidal dose-response equation with variable slope using the GraphPad Prism 4 program (GraphPad):

\[
I (\text{nA}) = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + 10^{\log EC_{50} - \log [\text{ACh}]}x_{\text{Hill \ Slope}}}
\]

where \(I\) is the macroscopic peak ionic current at a given ACh concentration; \(I_{\text{min}}\) and \(I_{\text{max}}\) are the smallest and the largest currents observed, respectively; \(EC_{50}\) is the concentration of acetylcholine that provokes a response halfway between \(I_{\text{min}}\) and \(I_{\text{max}}\); and the Hill Slope is the steepness of the dose-response curve.

**[125I]-α-bungarotoxin binding assays.** \([125I]-\alpha\)-BgTx (PerkinElmer) binding assays were performed immediately after the voltage clamp measurement on the same intact oocytes. Oocytes were incubated in 20 nM \([125I]-\alpha\)-BgTx, 5 mg/mL BSA, MOR-2 without EGTA and in the absence of agonist at room temperature for 2 hours. Non-injected oocytes were incubated under the same conditions to measure non-specific binding. The excess of toxin was removed by washing each oocyte with 25 mL of MOR-2 without EGTA. Calibration curves were plotted with radioactivity (counts per minute) as a function of concentration \([125I]-\alpha\)-BgTx (fmol). Calibration curves were used to determine AChR expression levels as the ratio of the gamma counter (Beckman Coulter).

**Calibration curves were used to determine AChR expression levels (fmol/Å³). Standardized expression (\(X_{\text{Standardized}}\)) was calculated as:**

\[
X_{\text{Standardized}} (\text{fmol/Å³}) = \frac{X_{\text{Mutant}}}{V_{\text{Trp}} - V_{\text{WT}}}
\]

where \(X_{\text{Mutant}}\) is the expression of the βM4-mutant AChRs; \(V_{\text{Trp}}\) and \(V_{\text{WT}}\) are the volumes of the tryptophan and original residues, respectively. Amino acid volume values \(V_{\text{WT}}\) are from crystallographic studies. Periodicity profiles were plotted with ACh EC₅₀ or AChR expression as a function of their tryptophan substitution position along the βM4 domain. Periodicity curves were created through the cubic spline method with 10,000 segments using the GraphPad Prism 4 program. The number of residues per helical turn of the periodicity profiles was estimated as the amount of amino acids between the adjacent maximum and minimum peaks.

**Fourier transform.** The periodicities of AChR expression (fmol/Å³) and ACh EC₅₀ (µM) periodicity profiles were also determined by Fourier transform power spectra. The Fourier transform power spectra from data of AChR expression and ACh EC₅₀ periodicity profiles were generated using a least-squares Fourier transform equation:

\[
P(\omega) = \left[ \sum_{j=1}^{N} |V_j - \langle V_j \rangle| \cos (j\omega) \right]^2 + \left[ \sum_{j=1}^{N} |V_j - \langle V_j \rangle| \sin (j\omega) \right]^2
\]

where \(P(\omega)\) is the Fourier transform power spectrum as a function of angular frequency \(\omega\) (rotation angle between residue around a helical axis), \(V_j\) is the value of AChR expression or ACh EC₅₀ at a given position \(j\), \(\langle V_j \rangle\) is the mean value of \(V_j\) in the sliding window, and \(N\) is the number of values in the sliding window. For example, the Fourier transform analysis to a sequence of values from an ideal α-helical pattern (3.6 residues per turn) should give in the Fourier transform power spectrum a prominent peak at -100°, given that the \(\omega\) parameter is related to the number of residues per turn (n) by the expression \(\omega \text{ (degree)} = 360°/n\).

The mean periodicities of the sequences of AChR expression and ACh EC₅₀ values were estimated from the peak in the Fourier transform power spectrum localized in the vicinity of the rotation angle as indicated by the mean periodicities of their tryptophan-periodicity profiles. These rotation angles from mean periodicities of the tryptophan-periodicity profiles were calculated using equation 5.

The relative abundance of the peak that corresponds to mean oscillations of the tryptophan-periodicity profiles was calculated by the peak ratio equation using MATLAB® 7.4 program:

\[
\text{Peak ratio} = \left[ \frac{1/30 \int P(\omega)\, d\omega + 15°}{1/180 \int P(\omega)\, d\omega} \right]^{1/2}
\]

where \(\text{Peak ratio} \rangle > 2\), which occupies at least 33% of the whole power spectrum, has been considered indicative of a very good indicator of a helical secondary structure. The algorithm templates developed to plot the power spectrums and calculate the peak ratios are available upon request.
Helical net diagrams. Helical net diagrams were built using the amount of residues between the adjacent maximums and minimums peaks of the periodicity profile for the open- and closed-channel state. Rotation angle (Ω) per residue for each helical turn was calculated as:

\[
\Omega \text{ (degree)} = \frac{360'}{n}
\]

where \( n \) is the number of residues per helical turn. Helical rise per residue (\( d \)) for each helical turn was determined using:

\[
d(\AA) = \frac{p \times \Delta + b}{n}
\]

where \( \Delta \) is the amount of amino acids between the positions of the residue and anterior peak, \( b \) is the magnitude of the maximum rise in the anterior helical turn (thus the rise at 360°), \( p \) is the helical pitch per helical turn estimated through \( p (\AA) = -1 \times n + 9 \), and \( n \) is the amount of residues per helical turn.

Molecular modeling. The structural models of the closed- and open-channel states were built with the periodicity profile data using the Deep Viewer/Swiss-PdbViewer 3.7 program (http://www.expasy.org/spdbv/). The backbone of each helical structure was adjusted to the number of residues per helical turn determined by periodicity profiles using:

\[
3 \cos \Omega = 1 - 4 \cos^2 \left( \frac{\phi + \psi}{2} \right)
\]

where \( \Omega \) is the angle per residue and \( \phi \) and \( \psi \) are backbone dihedral angles. The helical structures were energetically minimized. The backbones of the helical structures were superimposed to compare their structure. The quality of the superimposition between two helical structures was evaluated by calculating the root mean squared deviation. The mobility of the backbone atoms between helical structures was displayed using B-factor in the superposed models. The length (\( b \)), helical pitch (\( p \)), and helical rise per amino acid (\( d \)) of the helical structure were calculated with the Deep Viewer/Swiss-PdbViewer 3.7 program. The radius (\( r \)) of the helical structure was estimated using \( r^2 = (p/2)^2 + d^2 \), considering the helical structure as a cylinder. The volume (\( V \)) of the helical structure was assessed using \( V = \pi r^2 h \).

Molecular graphics generated in the Swiss-PdbViewer program were exported to the PyMOLTM Molecular Graphics System program (DeLano Scientific LLC) to produce images of the highest quality.

Interpretation of normalized macroscopic response. The data that we have gathered thus far indicate that mutations within TM3 and TM4 do not affect agonist binding nor do they produce global changes in the AChR ion channel pore, as they have no effects on single-channel conductance, reversal potentials, or calcium permeability.38,39,41,59,60 Furthermore, a recent structural study on changes in the AChR ion channel pore, as they have no effects on and TM4 do not affect agonist binding nor do they produce global that we have gathered thus far indicate that mutations within TM3 (DeLano Scientific LLC) to produce images of the highest quality.

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