J. Membrane Biol. 170, 157–164 (1999)

The Journal of
Membrane
Biology

Alteration in Ion Channel Function of Mouse Nicotinic Acetylcholine Receptor by Mutations in the M4 Transmembrane Domain

S. Tamamizu[1,*], Y.-H. Lee[1], B. Hung[1], M.G. McNamee[1], J.A. Lasalde-Dominicci[2]

[1]Section of Molecular and Cellular Biology, University of California, Davis CA 95616, USA
[2]University of Puerto Rico, Department of Biology, PO Box 23360, San Juan, Puerto Rico 00931-3360, USA

Received: 30 December 1998/Revised: 13 April 1999

Abstract. The effect of structural alterations of the M4 transmembrane segment in the *Torpedo californica* AChR has shown that substitution of specific residues can be critical to the channel gating (Lasalde et al., 1996). In a previous study we found that phenylalanine and tryptophan substitutions at the αC418 residue in the M4 transmembrane segment of the *Torpedo californica* AChR significantly altered ion channel function (Lee et al., 1994; Ortiz-Miranda et al., 1997). Cassette mutagenesis was used to mutate the Cys residue at the corresponding C418 position in the α subunit of mouse AChR. A total of nine mutations on the mouse αC418 position were tested, including the αC418A, αC418V, αC418L, αC418S, αC418M, αC418W, αC418H, αC418E and αC418G mutants. All the mutants tested were functional except the αC418G which was not expressed on the surface of the oocyte. The data obtained from macroscopic and single channel currents demonstrate that different types of amino acids can be accommodated at this presumably lipid-exposed position without loss of ion-channel function. As with the *Torpedo* AChR, the mutation of Cys to Trp dramatically decreased the EC_{50} for acetylcholine and increased channel open time. The lack of expression of the mouse αC418G suggest that there are some differences in folding, oligomerization and perhaps transport to the surface membrane for this mutant between the *Torpedo* and the mammalian AChR.

Key words: *Torpedo californica* — Cassette mutagenesis — Single channel electrophysiology — Ion channel gating

Introduction

The nicotinic acetylcholine receptor is a member of ligand-gated ion channels including the glycine receptor, the $GABA_A$ receptor, the $5\text{-}HT_3$ receptor, and the neuronal AChR (Betz, 1990). In the vertebrate neuromuscular junction, the binding of two agonist molecules to the receptor induces a conformational change, which opens a cation-selective channel. The flow of cations initiates a membrane depolarization and causes the generation of an action potential. The muscle-type AChR is a pentamer composed of four different homologous subunits (for reviews *see* Arias, 1998; Changeux & Edelstein, 1998; Karlin & Akabas, 1995; Pradier & McNamee, 1992). Each subunit contains four hydrophobic domains of 20–30 amino acids designated as M1, M2, M3 and M4, that are proposed as membrane-spanning regions (MSRs) (Noda et al., 1982). It is known that the large N-terminal and short C-terminal domains are located on the extracellular side (DiPaola et al., 1989). The primary and secondary structure of the MSRs are crucial for the gating and permeation properties of the channel. Most of the studies that combined site-directed mutagenesis and electrophysiological analysis have focused on the M2 transmembrane segment (Giraudat et al., 1986, 1987, 1989; Hucho, Oberthur & Lottspeich, 1986; Imoto et al., 1986, 1988, 1991; Charnet et al., 1990; White & Cohen 1992; Villaroel & Sakmann, 1992). These studies provided con-

* *Present address:* The Research Division, Oriental Medicine Research Center, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642 Japan

Correspondence to: J.A. Lasalde-Dominicci

vincing evidence that M2 segments from each subunit associate about a central axis to form at least part of the aqueous ion channel. The M4 segment is the most hydrophobic and least conserved and has been shown to have the largest contact with lipid (Blanton & Cohen 1992, 1994). The first studies involving mutational analysis of M4 residues were reported on the γC451 and αC418 position of the *Torpedo* receptor (Li et al., 1990, 1992). Further analysis of the αCys418 demonstrated that tryptophan substitution at this position dramatically prolonged channel open time at low acetylcholine concentrations (Lee et al., 1994). It also has been demonstrated that γLeu440 and γMet442 located in the M4 domain of the mouse γ subunit, contribute to the increased channel open time characteristic of the embryonic receptor (Bouzat, Bren & Sine, 1994). The functional consequences of amino acid substitutions at the mouse M4 were first reported at the αCys418 position (Tamamizu, Lasalde & McNamee, 1996). Estimation of changes in free energy barrier for the channel closure state for several amino acid substitutions at the αGly421 position in the *Torpedo* receptor suggests that Van der Waal's and perhaps dipole interactions at the periphery of the AChR with the lipid interface could play a significant role in the overall mechanism of channel gating (Lasalde et al., 1996). Subsequently, the mechanistic contribution of the M4 transmembrane segment to channel gating was examined using burst-oriented analysis at several acetylcholine concentrations (Ortiz-Miranda et al., 1997). This study showed that the increase in the open channel probability of the αTrp418 was due to a decrease in the channel closing rate and possibly an increase in the effective opening rate. In a similar study it was shown that replacement of αC418 and αT422 with alanine in the mouse AChR increased the channel closing rate (Bouzat et al., 1998).

In this work we substitute this highly conserved Cys residue in the M4 domain of α subunit in the mouse muscle AChR (αC418) for a variety of amino acids including, polar, hydrophobic, negatively and positively charged side chains. A total of nine mutants generated by cassette mutagenesis were expressed on *Xenopus* oocytes and ion channel function was examined by whole-cell voltage clamping and patch clamping. The rationale for this work was to examine the structural constraints of this conserved M4 position to accommodate amino acid side chains that could induce alteration of ion-channel gating mechanism or loss of functional activity. This study provides new insight into the role of lipid-exposed residues in the AChR channel function and further supports the involvement of the αM4 transmembrane domain in the channel gating mechanism and also in the functional assembly of this relevant receptor-channel.

Materials and Methods

Materials

The mouse AChR cDNAs (pGEM1-Mα, pGEM2-Mβ, PSP65-Mγ, and pSP64T-Mδ) were a gift from Dr. H. Lester (California Institute of Technology). The cDNA coding the ε subunit was obtained from Dr. C. Gomez (University of Minnesota). pGEM-11Z(−) was purchased from Promega (Madison, WI).

Cassette Mutagenesis of the α Subunit of Mouse AChR

To utilize the commercially available M13-forward primer to carry out polymerase chain reaction (PCR), the EcoRI fragment containing the α subunit coding gene was subcloned into the EcoRI site of the pGEM-11Z(−) under the SP6 promoter. PCR mutagenesis was carried out to introduce novel restriction sites for cassette mutagenesis. The BstX I site (a native unique restriction site) is preceding, Nar I is after, and a Kpn I site is inside the mouse αM4 coding region (*see* Fig. 1). The BstX I-Kpn I cassette was employed to introduce mutations at the α418Cys position. A mutagenic primer pair were synthesized for the BstX I-Kpn I cassette mutagenesis at the α418 position. The NNS (N represents A or C or G or T, and S represents C or G) codon was used at the α418 position to generate 20 possible amino acids substitutions at this position, while eliminating two of three chances for stop codon (TAG, TGA, and TAA) and also enhancing the possibility for getting Met (ATG) and Trp (TGG) substitutions, which have only one codon choice. A novel unique restriction site, Avr II, was designed for mutants between the BstX I and Kpn I sites. Because the Avr II site does not exist in either the coding gene of the mouse α subunit, or the original vector, the mutant products from the cassette mutagenesis can be discriminated from the wild type by this Avr II restriction site. Finally, double strand DNA sequencing was performed to identify the mutation sequences.

Expression in *Xenopus laevis* Oocytes

RNA transcripts were synthesized in vitro as described by Lee et al. (1994). The RNA transcripts (10 ng/oocyte at the concentration of 0.2 μg/μl) of α, β, γ, and δ subunit at a 2:1:1:1 ratio were injected into *Xenopus* oocytes. Combinations of the RNA mixes including α, β, ε, and δ at a 2:1:1:1 ratio were injected using 0.4 μg/μl to give 20 ng/oocyte because the adult form AChRs with the mouse ε subunit ($\alpha_2\beta\varepsilon\delta$) gave less channel activity compared to the ones with the γ subunit ($\alpha_2\beta\gamma\delta$).

^{125}I-α-Bungarotoxin Binding Assay

The expression of AChR in the oocyte membrane was assayed by measuring the binding of ^{125}I-α-bungarotoxin (Amersham Life Sciences, Arlington Heights, IL) to intact oocytes according to Lee et al. (1994). Oocytes were incubated in 10 nM ^{125}I-α-bungarotoxin with 0.5 mg/ml bovine serum albumin in MOR2 at room temperature for 2 hr and then washed several times with MOR2. Nonspecific binding was determined by the amount of toxin bound to noninjected oocytes under the same conditions.

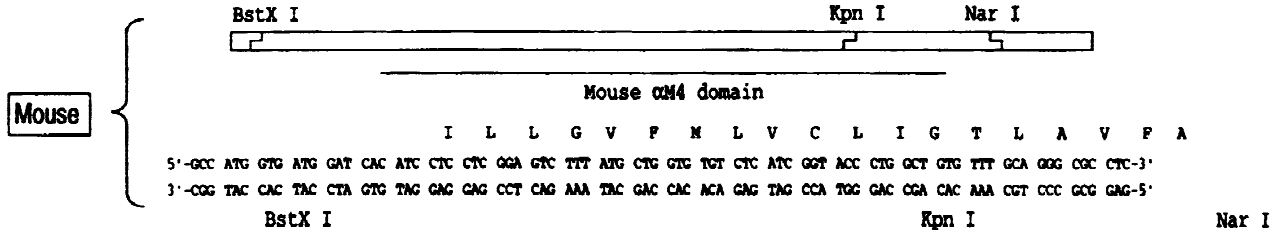

Fig. 1. Mouse αM4 cassette. Th BstXI site is a native unique restriction site. Nar I is after and Kpn I is inside the αM4 coding region. The restriction sites are unique inside the coding gene of the mouse AChR α subunit.

Voltage Clamping

ACh-induced currents were recorded with a two-electrode voltage clamp 3–5 days after mRNA injection with the Axoclamp-2A amplifier (Axon Instruments, Foster City, CA). Electrodes were filled with 3M KCl and had resistance of less than 2 MΩ. Impaled oocytes in the recording chamber were perfused at a rate of 0.5 ml/sec with MOR2 buffer (82 mM NaCl, 2.5 mM KCl, 5.0 mM $MgCl_2$, 1 mM Na_2HPO_4, 5.0 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 0.2 mM $CaCl_2$, pH 7.4) supplemented with 2.0 μM atropine. ACh solutions were made from calcium-free MOR2 to avoid activation of an endogenous Ca^{2+}-dependent Cl^- current (Barish, 1983; Mishina et al., 1984).

Patch Clamping

The oocyte vitelline membrane was removed manually after incubation in hypertonic solution composed of 150 mM NaCl, 2.0 mM KCl, 3% sucrose and 5.0 mM HEPES (pH 7.6). The oocytes were placed in a recording chamber containing bath solution (100 mM KCl, 1.0 mM $MgCl_2$ and 10 mM HEPES, pH 7.2) at 20–22°C. The patch pipettes were made of thick-walled borosilicate glass (Sutter Instruments, Novato, CA) exhibiting resistances of about 8–12 MΩ. The pipette solution contained 100 mM KCl, 10 mM HEPES, 10 mM EGTA, pH 7.2 and 4.0 μM ACh. All experiments were performed in a cell-attached patch configuration. Single channel currents were recorded using a DAGAN 3900 amplifier (Dagan, Minneapolis, MN), filtered at 5kHz (Frequency Devices, Haverhill, MA) and stored on VHS tapes using a digital data recorder (VR-10B, Instrutech, Mineola, NY). The data traces were played back into an IBM compatible computer through DigiData 1200 interface (Axon Instruments, Foster City, CA) and digitized at 20 μsec. Single channel currents were detected with a half-amplitude crossing algorithm (IPROC) and data analysis was performed using pCLAMP (Axon Instruments, Foster City, CA).

Results

Expression of Functional Mouse Mutant AChR

The Cys residue at position 418 in the α subunit of mouse receptor is highly conserved among AChR subunits and species as shown in Fig. 2. This Cys was mutated to Gly (C418G), Ala (C418A), Val (C418V), Leu (C418L), Ser (C418S), Met (C418M), Trp (C418W), His (C418H), and Glu (C418E). Mutated α subunit RNA was coinjected with wild-type β, δ and ε subunit RNAs in *Xenopus* oocytes and the expression level of mutants were determined by the ^{125}I-α-bungarotoxin binding assay. As shown in Fig. 3, the expression level of all mutants is relatively lower than that of wild type. To examine the ion channel function of the mutants, the ACh-induced currents were measured by two electrode voltage-clamp. The ACh induced maximum currents for mutant C418G was not detected at a holding potential of −80 mV whereas other mutants exhibited the same level of current amplitudes as wild type (800–1000 nA).

The M4 Transmembrane Domain

Muscle-type AChR

Torpedo	α	ILLCVFMLI**C**IIGTVSVFA
Calf	α	ILLAVFMLV**C**IIGTLAVFA
Chicken	α	LLLVIFMLV**C**IIGTLAVFA
Human	α	ILLGVFMLV**C**IIGTLAVFA
Xenopus	α	ILLAVFMTV**C**VIGTLAVFA
Mouse	α	ILLGVFMLV**C**LIGTLAVFA
Torpedo	β	LFLYVFFVI**C**SIGTFSIFL

Neuronal AChR

Chicken	α2	IFLWMFIIV**C**LLGTVGLFA
Chicken	α3	IFLWVFILV**C**ILGTAGLFL
Chicken	α4	IFLWMFIIV**C**LLGTVGLFL
Rat	α2	IFLWLFIIV**C**FLGTIGLFL
Rat	α3	IFLWVFILV**C**ILGTAGLFL
Rat	α4	IFLWMFIIV**C**LLGTVGLFL
Rat	α7	MAFSVFTII**C**TIGILMSAP
Rat	β2	LFLWIFVFV**C**VFGTVGMFL
Rat	β4	LFLWVFVFV**C**ILGTMGLFL

Fig. 2. Aligned M4 domain sequences for subunits of the AChR. The cysteine residue at position α438 in the mouse receptor is in bold and is highly conserved in other AChR.

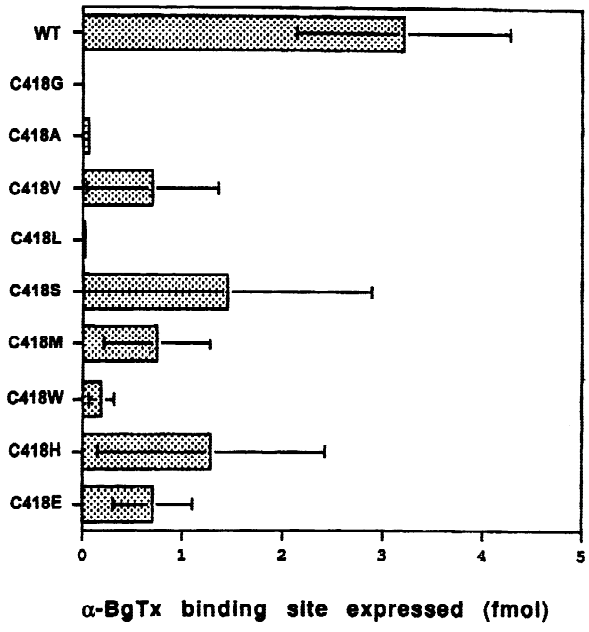

Fig. 3. Expression of surface AChRs on *Xenopus laevis* oocytes. In vitro transcribed wild type or mutant α, β, ε and δ subunit mRNAs were co-injected in *Xenopus laevis* oocytes and ^{125}I-α-bungarotoxin binding assay was performed on day 3 after injection. Values were averaged after testing 6–10 individual oocytes for each mutant.

Table 1. Functional consequences of mouse M4 mutations[a]

AChR type ($\alpha_2\beta\varepsilon\delta$)	EC_{50} (μM)	Hill coefficient[b]
Wild type (13)	28.6 ± 10.3	1.25 ± 0.22
C438G	N.E.[c]	N.E.
C438A (7)	42.3 ± 13.4	1.25 ± 0.06
C438V (6)	39.9 ± 8.72	1.13 ± 0.06
C438L (7)	32.7 ± 11.5	1.20 ± 0.07
C438S (10)	26.9 ± 10.1	1.13 ± 0.13
C438M (8)	21.3 ± 5.22	1.34 ± 0.22
C438W (9)	2.82 ± 1.17	1.46 ± 0.24
C438H (11)	13.8 ± 6.55	1.04 ± 0.06
C438E (7)	20.7 ± 9.32	1.37 ± 0.23

[a] Values are given as the mean ± SD. Numbers in parenthesis indicate the number of oocytes tested.
[b] Data points from all ACh concentrations were fitted with the Hill equation $Y = 100/[1+(K_d/A)^h]$.
[c] Not expressed.

The Mouse α418 Mutants Shifted the EC_{50}s

The EC_{50}s of the wild type and C418 mutants are listed in Table 1. Mutation C418W shifted the EC_{50} for ACh from 29 μM to 3.0 μM although the Hill coefficient from the C418W was not significantly different from the wild type. This result indicates that this mutation changes the EC_{50} for ACh without dramatic changes in cooperativity.

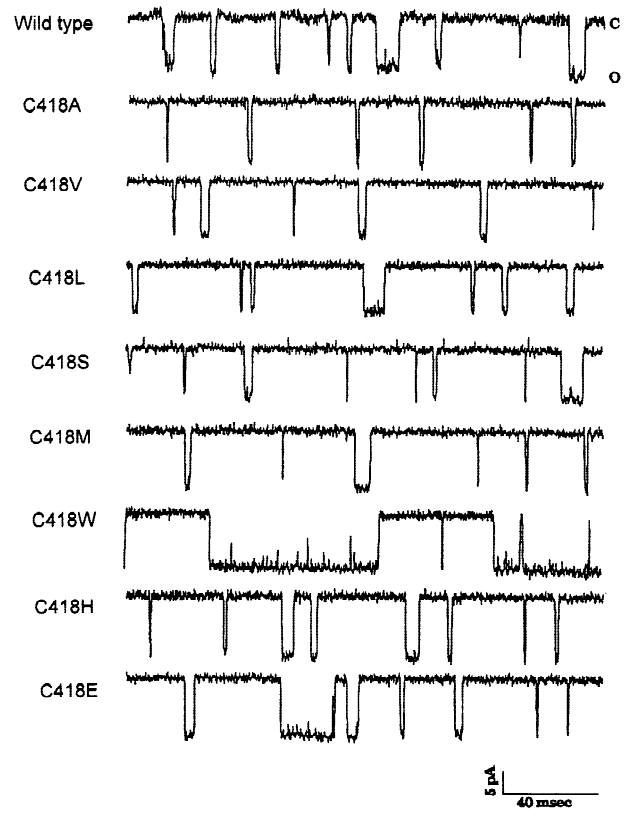

Fig. 4. Single-channel traces of wild type and mutant AChRs expressed in *Xenopus laevis* oocytes. Recordings from a cell-attached patch configuration were taken in the presence of 4.0 μM acetylcholine. The membrane potential was held at −100 mV and the chamber temperature maintained at 20 ± 1°C.

Mutations to Ala and Val increased EC_{50} for ACh and mutation to His decreased EC_{50} although the effect of mutations is not as significant as that of C418W. The EC_{50}s and the Hill coefficient of mutations, C418L, C418S, C418M, and C418E did not show significant differences compared with the wild type.

Single Channel Analysis of the C418 Mutants

Patch clamp recordings of single-channel currents for the αC418W mutant showed the most marked increase in mean channel open time compared with the wild-type channels. Figure 4 shows single channel currents recorded from cell-attached patches in oocytes expressing wild-type and the mutant AChRs. At 20 ± 1°C, wild-type channels showed a mean open time $\tau = 3.75 \pm 0.01$ msec whereas the mean open time of the αC418W was $\tau = 16.7 \pm 0.03$ msec. Mutation C418E slightly increased the mean channel open time ($t = 6.32 \pm 0.01$). As summarized in Table 2, other mutations (C418A, C418V,

Table 2. Mean open times for wild type and mutant AChR[a]

AChR type ($\alpha_2\beta\varepsilon\delta$)	Mean open time (msec)
Wild type	3.75 ± 0.01
C418A	2.99 ± 0.03
C418V	3.39 ± 0.01
C418L	3.58 ± 0.03
C418S	4.58 ± 0.01
C418M	5.44 ± 0.01
C418W	16.7 ± 0.03
C418H	4.10 ± 0.01
C418E	6.32 ± 0.00

[a] Single channel currents were recorded from cell-attached patches at −100 mV using 4.0 μM ACh.

C418L, C418S, C418M and C418H) did not show significant differences in mean channel open time compared to the wild-type channel. None of the amino acid substitutions in the αC418 showed significant change in the single channel conductance or reversal potential compared to wild-type AChR.

The C418W Mutation Increases Channel Open Time on $\alpha_2\beta\gamma\delta$ Configuration

To examine functional effects of C418W further, the subunit composition was changed from $\alpha_2\beta\varepsilon\delta$ to $\alpha_2\beta\gamma\delta$ by co-injection of mouse γ subunit RNA with α, β and δ subunit RNA instead of using the mouse ε subunit RNA. It has been shown that the $\alpha_2\beta\gamma\delta$ AChR has a longer channel open time and a relatively small conductance compared with the $\alpha_2\beta\varepsilon\delta$ AChR (Mishina et al., 1986). It was of interest to know whether 4.5-fold increase of the mean channel open time of the C418W on $\alpha_2\beta\varepsilon\delta$ configuration would be also observed on $\alpha_2\beta\gamma\delta$ configuration. As seen with ε subunit, patch clamp recordings of single-channel currents for the C418W showed marked increase in mean channel open time compared with the wild-type channels. At 20 ± 1°C, wild-type channels showed a mean open time $\tau = 4.86 \pm 0.01$ msec whereas the mean open time of the C418W was $\tau = 18.9 \pm 0.02$ msec (Table 3). This 3.9-fold increase of the mean channel open time with γ subunit is slightly smaller compared to that with ε subunit.

Table 3. Mean open times for wild type and C438W AChRs ($\alpha_2\beta\gamma\delta$)[a]

AChR type ($\alpha_2\beta\gamma\delta$)	Mean open time (msec)	Amplitude (pA)
Wild type	4.86 ± 0.01	8.1 ± 0.01
C438W	18.9 ± 0.02	6.8 ± 0.06

[a] Single channel currents were recorded from cell-attached patches at −100 mV using 4.0 μM ACh.

Discussion

The αCys418 of mouse AChR was mutated by using cassette mutagenesis to study the functional role of this highly conserved cysteine in the mammalian AChR. The Cys at position of 418 is located in the putative transmembrane segment M4 in the α subunit of the AChR. Independent labeling studies suggest this position is in contact with the lipid region of the membrane (Blanton & Cohen, 1992, 1994). This residue is also conserved among α subunits from different species and neuronal AChRs (Fig. 2). The homologous αCys418 of the *Torpedo* AChR has been investigated previously and substitution of Cys by Trp and Phe have been shown to dramatically alter channel functions, including alterations of the EC_{50} for ACh, and a significant reduction in the channel closing rate (Lee et al., 1994; Ortiz-Miranda et al., 1997). It remains unclear how a mutation at the putative lipid-protein interface of the AChR can dramatically affect the ion channel gating kinetics. To gain more insight into the structural nature as well as the functional consequences of amino acid substitution in the lipid-exposed domains of AChR, we examine the corresponding αCys position in a mammalian AChR. In this study we introduce more structural constraints in this position by replacing a cysteine with acidic and basic side chains. In addition, the αC418W mutation in the mouse AChR was examined with different wild-type combinations, the adult type ($\alpha_2\beta\varepsilon\delta$) and embryonic type ($\alpha_2\beta\gamma\delta$) AChR. This allowed us to study the mutation effects on the structure-function relationship of two type of receptors. At low ACh concentration (1.0 μM) the mouse AChR has a relatively longer open time (3.6 msec) compared to *Torpedo* AChR (0.40 msec) when expressed in *Xenopus* oocytes. Therefore, we are able to compare the consequences of amino acid substitutions at this putative lipid-exposed position when the properties of the wild-type channels are significantly different.

We found that of nine mutant AChRs tested, eight mutants (C418A, C418V, C418L, C418S, C418M, C418W, C418H and C418E) form functional AChRs in *Xenopus laevis* oocytes. This result demonstrates that various types of amino acids can be accommodated at position αC418 (except glycine) including aliphatic, aromatic, basic, and negatively charged acidic residues. If αC418 does not face the lipid bilayer, it would have contact with other amino acids from adjacent subunits and there should be a limited space in this tightly packed globular membrane protein. In this scenario, an amino acid replacement that involves a significant change in volume or physical properties should produce alteration of channel gating kinetics or loss of ion channel function.

The current results support the hypothesis that the mouse αC418 is located at the lipid-protein interface.

The C418E and C418H, which possess charged side chains, did form functional AChR channels. The hydrophobic ion theory provides a relatively simple explanation for how these charged molecules can be accommodated within a lipid bilayer (Flewelling & Hubbell, 1986). Considering the inside-positive membrane potential, the hydrophobic anion with a negative charge would be relatively more energetically favorable than the hydrophobic cation to insert into the lipid bilayer. While the C418E substitution may act as a hydrophobic anion, the C418H substitution is not so strongly charged. Previously, it was found that AChR mutated to Arg at the αC418 position did not express on the oocyte surface (Tamamizu et al., 1996). It is conceivable that the αC418H mutant may be able to insert into the membrane with its neutral form at physiological pH.

Another important observation was that a Gly substitution at the αC418 prevents the functional expression of the mouse AChR channel. Previous mutagenesis experiments at αC418 of the *Torpedo* receptor showed that glycine substitution at this position produced a decreased but measurable level of receptor expression in *Xenopus* oocytes (Lee et al., 1994; Ortiz-Miranda et al., 1997). The glycine residue at position αC418 in the mouse AChR may interfere with subunit assembly. The primary structure of the mouse and *Torpedo* αM4 has about 79% homology. There are four positions in the mouse αM4 that are different from *Torpedo*: G412, L419, L423 and A424. From these four positions only the G412 which is equivalent to the C412 in *Torpedo* has been shown to be exposed to the lipid by photoafinity labeling (Blanton & Cohen, 1992, 1994). The most significant changes between these positions occur at position G412 and L423 (C412 and S423 in *Torpedo*). In this particular case, the αC418G mutation in the mouse αM4 produces a transmembrane segment containing three glycine residues: GVFMLVGLIGTLAVFA. In the *Torpedo* receptor the same mutation only contains two glycine residues: CVFMLIGLIGTVSVFA. The disruption of secondary structure elements in the mouse αC418G mutation is probably more drastic due to the presence of three glycine residues in a ten amino acid sequence. One of the rules that allows prediction of α-helix formation with 80% accuracy is that if a sequence of six residues contains two or more "helix breakers," helix formation is unlikely (Chou & Fasman, 1974). The introduction of a glycine residue in the mouse αC418 position could well follow this rule, and this might be a possible cause of a lack of functional expression of this mutation. Although, in this particular case, the introduction of an α-helix breaker at this αM4 position is lethal to the functional assembly of the mouse AChR, it is difficult to make inferences on secondary structure elements of this lipid-exposed transmembrane segment based solely on these data.

The secondary structure of the M4 transmembrane segment of the *Torpedo* AChR remains controversial. The 9Å structure obtained by micrograph image reconstruction of frozen *Torpedo* postsynaptic membranes reported by Unwin (1993) is consistent with a β sheet structure for the M1, M3 and M4 transmembrane domains based on a lack of helix-like density profiles at the AChR periphery (Unwin, 1993). Conversely, biochemical labeling data (Blanton & Cohen, 1992, 1994) and a series of hydrogen/deuterium exchange studies using FTIR indicate an α helix structure for the four transmembrane segments (Baezinger & Méthot, 1995; Méthot & Baezinger, 1998). Another important observation is that the αC418E did produce functional AChR channel expression. Glutamic acid is considered to be the strongest β-structure breaker among all amino acids (Chou & Fassman, 1974). This β-sheet breaker does not disrupt the functional assembly of the mouse AChR receptor. The present data suggest that the functional assembly of the mouse AChR is more vulnerable to disruptions of α-helical secondary elements than to β-structure elements at the M4 trasmembrane segment.

Of nine mutants, only the αC418W shows a significant decrease in the EC_{50} for ACh, approximately 10-fold. In addition, the single channel data recorded for this mutation displays an increase in the mean channel open time of about 4.5-fold. Previously, it was found that the C418W mutation in the α subunit of *Torpedo* nAChR increased the mean channel open time from 0.4 to 16.5 msec (Lee et al., 1994). A burst oriented analysis on the *Torpedo* AChR of the same mutation showed an increase in channel open probability due to a decrease in the channel-closing rate and a modest increase in the opening rate (Ortiz-Miranda, 1997). The open time constant of the mouse αC418W, measured under the same conditions as the *Torpedo* receptor, was 16.7 msec. This value is very close to the value obtained for the αC418W *Torpedo* AChR, although, the mouse wild type has the longer open time of 3.8 msec compared to that of the *Torpedo* wild type. In addition, replacement of ε subunit by γ subunit does not affect the increased channel open time constant of the αC418W AChR significantly. These results indicate that a tryptophan substitution at this lipid-exposed position has similar effects on the channel gating mechanisms of these two different AChR species.

This mutational analysis of the mouse αC418 mutations demonstrates that the M4 transmembrane segment of the mouse AChR can support dramatic changes in the chemical properties of the substituted side

chain without loss of ion channel function. Only the glycine substitution did not produce functional AChR channel activity. In *Torpedo*, the equivalent mutation produced functional AChR channel activity with reduced level of expression compared to the wild type. The results are more consistent with the requirement for an α-helical secondary structure for the M4 domain. The large effect of the Trp substitution at the lipid-protein interface suggest that tryptophan may alter the conformation changes associated with channel closing. These findings clearly demonstrated that the primary structure of the lipid exposed αM4 transmembrane segment contribute to channel kinetics and furthermore, it can be critical to the functional assembly of the muscle-type AChR.

We thank Judy Butler, Manuel Navedo and Gisila Guzman for RNA preparation and oocyte injection. This research was supported by National Institutes of Health grants GM56371 and GM08102-27.

References

Arias, H.B. 1998. Binding sites for exogenous and endogenous noncompetitive inhibitors of the nicotinic acetylcholine receptor. *Biochimica et Biophysica Acta* **1376:**173–220

Baezinger, J.E., Méthot, N. 1995. Fourier transform infrared and hydrogen/deuterium exchange reveal an exchange-resistant core of alpha-helical peptide hydrogens in the nicotinic acetylcholine receptor. *J. Biol. Chem.* **270 (49):**29129–29137

Barish, M.E. 1983. A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol.* **342:**309–325

Betz, H. 1990. Ligand gated channels in the brain: the amino acid receptor superfamily. *Neuron* **5:**383–392

Blanton, M.P., Cohen, J.B. 1992. Mapping the lipid-exposed regions in the *Torpedo californica* nicotinic acetylcholine receptor. *Biochemistry* **31:**3738–3750

Blanton, M.P., Cohen J.B. 1994. Identifying the lipid-protein interface of the *Torpedo* nicotinic acetylcholine receptor: secondary structure implications. *Biochemistry* **33:**2859–2872

Bouzat, C., Bren, N., Sine, S.M. 1994. Structural basis of the different gating kinetics of fetal and adult acetylcholine receptors. *Neuron* **13:**1395–1402

Bouzat, C., Roccamo, A.M., Garbus, I., Barrantes, F.J. 1998. Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. *Mol. Pharmcol.* **54:**146–153

Changeux, J.P., Edelstein, S.J. 1998. Allosteric Receptors after 30 years. *Neuron* **21:**959–980

Charnet, P., Labarca, C., Leonard, R., Vogelaar, N.J., Czyzk, L., Gouin, A., Davidson, N. and Lester, H.A. 1990. An open channel blocker interacts with adjacent turns of α-helices in the nicotinic acetylcholine receptor. *Neuron* **4:**87–95

Chou, P.Y., Fasman, G.D. 1974. Prediction of protein conformation. *Biochemistry* **13 (2):**222–245

Dipaola, M., Czajkowski, C., Karlin, A. 1989. The sidedness of the COOH terminus of the acetylcholine receptor delta subunit. *J. Biol. Chem.* **264:**15457–15463

Flewelling, R.F., Hubbell, W.L. 1986. The membrane dipole potential in a total membrane potential model. Applications to hydrophobic ion interactions with membranes. *Biophys J* **49(2):**541–552

Giraudat, J., Dennis, M., Heidmann, T., Chang, J.-Y., Changeux, J.-P. 1986. Structure of the high affinity binding site for noncompetitive blockers of the acetylcholine receptor: serine-262 of the δ subunit is labeled by [^{3}H]chlorpromazine. *Proc. Natl. Acad. Sci. USA* **83:**2719–2723

Giraudat, J., Dennis, M., Heidmann, T., Hamont, P.Y., Lederer, F., Changeux, J.-P. 1987. Structure of the high-affinity site for noncompetitive blockers of the acetylcholine receptor: [^{3}H]chlorpromazine labels homologous residues in the β and δ chains. *Biochemistry* **26:**2410–2418

Giraudat, J., Gali, J., Revah, F., Changeus, J.-P., Haumont, P., Lederer, F. 1989. The noncompetitive blocker [^{3}H]chlorpromazine labels segment M2 but not segment M1 of the nicotinic acetylcholine receptor alpha-subunit. *FEBS Lett* **253:**190–198

Hucho, F., Oberthur, W., Lottspeich, F. 1986. The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices MII of the receptor subunits. *FEBS Lett* **205:**137–142

Imoto, K., Methfessel, C., Sakmann, B., Mishina, M., Mori, Y., Konno, T., Fukuda, M., Kurosaki, M., Bujo, H., Fujita, Y., Numa, S. 1986. Location of a δ subunit region determining ion transport through the acetylcholine receptor. *Nature* **324:**670–674

Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J.M., Bujo, H., Mori, Y., Fukuda, M., Numa, S. 1988 Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. *Nature* **335:**645–648

Imoto K., Konno, T., Nakai, J., Wang, F., Mishina, M., Numa, S. 1991. A ring of uncharged polar amino acids as a component of channel constriction in the nicotinic acetylcholine receptor. 1991. *FEBS Lett.* **289:**193–200

Karlin, A., Akabas, M.H. 1995. Toward a structural basis for the function of nicotinic acetylcholine receptors. *Neuron* **15:**1231–1244

Lasalde, A.J., Tamamizu, S., Butler, D.H., Vibat, C.R.T., Hung, B., McNamee M.G. 1996. Tryptophan substitutions at the lipid-exposed transmembrane segment M4 of Torpedo californica acetylcholine receptor govern channel gating. *Biochemistry* **35:**14139–14148

Lee, Y.-H., Li, L., Lasalde, J., Rojas, L., McNamee, M., Ortiz-Miranda, S.I., Pappone, P. 1994. Mutations in the M4 domain of *Torpedo californica* acetylcholine receptor dramatically alter ion channel function. *Biophys. J.* **66:**646–653

Li, L., Lee, Y.-H., Pappone, P., Palma, A., McNamee, M.G. 1992. Site-specific mutations of nicotinic acetylcholine receptor at the lipid-protein interface dramatically alter ion channel gating. *Biophys J* **62(1):**61–63

Li, L., Schuchard, M., Palma, A., Pradier, L., McNamee, M.G. 1990. Functional role of the cysteine 451 thiol group in the M4 helix of the gamma subunit of the *Torpedo californica* acetylcholine receptor. *Biochemistry* **29:**5428–5436

Mishina, M., Kurosaki T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M. and Numa, S. 1984. Expression of functional acetylcholine receptor from cloned cDNAs. *Nature* **307:**604–608

Méthot, N., Baezinger, J.E. 1998. Secondary structure of the exchange-resistance core from the nicotinic acetylcholine receptor probed directly by infrared spectroscopy and hydrogen/deuterium exchange. *Biochemistry* **37 (42):**14815–14822

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., Numa, S. 1982. Primary structure of alpha-subunit precursor of *Torpedo* californica acetylcholine receptor deduced from cDNA sequence. *Nature* **299:**793–797

Ortiz-Miranda, S.I., Lasalde, J.A., Pappone, P.A., McNamee, M.G. 1997. Mutations in the M4 domain of the *Torpedo californica*

nicotinic acetylcholine receptor alter channel opening and closing. *J. Membrane Biol.* **158:**17–30

Pradier, L., McNamee, M.G. 1992. The Nicotinic Acetylcholine Receptor. In: The structure of Biological Membranes. pp. 1047–1106. Telford, Caldwell, NJ

Tamamizu, S., Lasalde, J.A., McNamee, M.G. 1996. Tryptophan substitutions at the M4 transmembrane segment of the *Torpedo,* mouse and neuronal acetylcholine receptor increment open time constant. *Society for Neuroscience Abs.* **501.1:**125*a* (*Abstr.*)

Unwin, N. 1993. The nicotinic acetylcholine receptor at 9Å resolution. *J. Membrane Biol.* **229:**1101–1124

Unwin, N. 1995. Acetylcholine receptor channel imaged in the open state. *Nature* **373:**37–43

Villarroel, A., Sakmann, B. 1992. Threonine in the selectivity filter of the acetylcholine receptor channel. *Biophy. J.* **62:**196–205

Villarroel, A., Herlitze, S., Sakmann, B. 1991. Amino acids responsible for differences in conduntance between adult and fetal forms of the acetylcholine receptor. *Biophys. J.* **59:**34a (*Abstr.*)

White, B.H., Cohen, J.B. 1992. Agonist-induced changes in the structure of the of the acetylcholine receptor M2 regions revealed by photoincorporation of an uncharged nicotinic noncompetitive agonist. *J. Biol. Chem.* **267:**15770–15783