Site-specific mutations of nicotinic acetylcholine receptor at the lipid-protein interface dramatically alter ion channel gating

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INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) is a postsynaptic, integral membrane protein consisting of five subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ (Pradier and McNamee, 1991). cDNA cloning reveals that each subunit has four homologous hydrophobic segments (M1, M2, M3, and M4) that are believed to span the lipid bilayer. There is strong evidence that M2 is the ion channel-lining domain, but the specific functions of the other hydrophobic segments are not well characterized. In this report, the role of the M4 segment in ion channel function was investigated by using site-directed mutagenesis of a specific cysteine residue (αCys418) in the M4 region of *Torpedo californica* α subunit. The M4 region is believed to be located at the lipid-protein interface based primarily on chemical labeling studies (Giraudat et al., 1985), and we have examined both the labeling and mutation of cysteine residues in these domains (Pradier et al., 1989; Li et al., 1990). We show here that the mutation on the α subunit alters ion channel gating in a dramatically different way than previous mutations.

MATERIALS AND METHODS

Site-specific mutation of *Torpedo* nAChR α subunit Cys418 to Trp was carried out using polymerase chain reaction (PCR) techniques (Ho et al., 1989). Wild type and mutant RNA transcripts were synthesized in vitro and injected into *Xenopus laevis* oocytes as described (Pradier et al., 1989). The functional consequences of the mutations were initially analyzed by ^{125}I-α-bungarotoxin binding to intact oocytes and voltage clamping two to three days after injection (Li et al., 1990). Single channel properties were measured by patch clamp techniques using outside-out patches.

RESULTS

Binding of iodinated α-bungarotoxin showed that the mutation from αCys418 to αTrp418 gave rise to surface

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nAChR expression levels similar to those of the wild-type receptor (Table 1). The results are consistent with our previous analyses of Cys mutations on γ subunit, using both Western blot analyses and toxin binding, and indicate that the mutations do not alter nAChR biosynthesis, assembly, and transport to the cell surface (Li et al., 1990).

However, the acetylcholine (ACh)-induced current responses of the mutant were dramatically different from the wild type receptor as measured using voltage clamping techniques (Table 1). The αCys418 to αTrp418 mutation "increased" the nAChR normalized channel activity (measured as the ACh-induced conductance per femtomole of surface toxin sites) by > 40-fold (Table 1). In contrast, mutations on the γ subunit to either Ser or Trp "decreased" the normalized activity by $\sim 50\%$ (Li et al., 1990). Control experiments using oocytes injected with mRNA mixtures omitting either the α or the γ subunit showed that the functional alterations observed for mutant nAChRs were not due to incompletely assembled nAChR lacking the *Torpedo* α or γ subunit.

The mutant receptors displayed linear current-voltage relationships with a reversal potential of 0 mV similar to the wild-type receptor, indicating that the mutations did not alter the selective permeability of the ion channel (data not shown). In addition, the αCys418 mutant did not alter the inhibition of the receptor by noncompetitive blockers such as phencyclidine and tetracaine, suggesting that the mutation did not act by physically blocking the ion permeability pathway. The mutation did not change the acetylcholine Hill coefficient nor the IC50 for the competitive antagonist *d*-tubocurare, suggesting that the mutation altered the coupling between ACh binding and channel opening (data not shown).

Single channel analysis of the receptors in excised outside-out patches from *Xenopus* oocytes revealed that the αCys418 to αTrp418 mutant dramatically altered channel open times without affecting the single channel conductance (Fig. 1). The mutation of αCys418 to αTrp418 increased channel open time ~ 20-fold. In contrast, the γCys451 to γTrp451 mutation decreased open times by $\sim 40\%$. In both cases, the single channel

nAChR	Whole-cell current[‡]	^{125}I-BGT binding[§]	Normalized activity[∥]
	nA	fmol/oocyte	μS/fmol
Wild type			
0.2 μM ACh	7 ± 1 (6)	2.2 ± 0.2 (6)	0.04 ± 0.01 (6)
1.0 μM ACh	177 ± 21 (6)	2.2 ± 0.2 (6)	1.02 ± 0.10 (6)
αTrp418			
0.2 μM ACh	277 ± 25 (17)	2.3 ± 0.2 (17)	1.66 ± 0.19 (17)

*Data are given as means ± SEM. Numbers in parentheses indicate
the number of oocytes tested. [‡]Whole-cell currents are responses to 0.2
or 1 μM ACh (Vh = −80 mV) measured 41–46 h after injection of
transcripts. [§,125]I-BGT binding was measured on the same oocytes
immediately after the electrophysiological recordings by incubating
oocytes individually in 1 nM ^{125}I-BGT in MOR2 solutions. [∥]Normalized
activity is expressed in ACh-induced conductance per fmol of surface
BGT binding sites.

data are qualitatively consistent with the whole-cell
voltage clamping data.

DISCUSSION

The combination of site-directed mutagenesis and elec-
trophysiologic measurements of wild type and mutant
acetylcholine receptors expressed in *Xenopus* oocytes
has proved to be exceedingly powerful in studying the
molecular mechanism of nAChR ion channel function
(Imoto et al., 1988). We have focused on the role of
cysteine residues in modulating ion channel function
based previous analyses of the effects of sulfhydryl
alkylating agents on receptor function (Pradier et al.,
1989; Yee et al., 1986). The discovery that the change of
a single Cys residue in the α subunit M4 domain
"increased" the ion channel response to acetylcholine
40-fold was both unexpected and intriguing. The single
channel data indicate that the mutation increases open
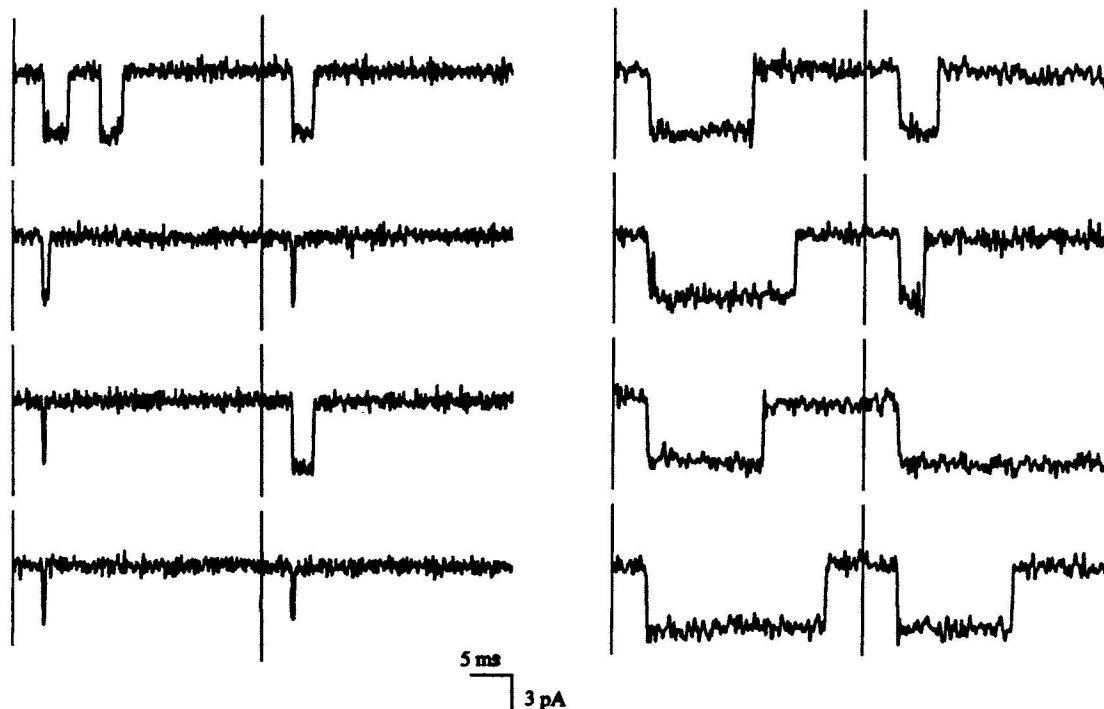
WILD TYPE

α-418trp

FIGURE 1 Patch clamp recordings of single channel currents from wild type (*left*) and αTrp418 mutant (*right*) *Torpedo californica* acetylcholine
receptors expressed in *Xenopus laevis* oocytes. Channel openings are downward. The records show sequential channel currents but are not
continuous with the vertical lines marking individually triggered epochs. Recordings were made from outside-out patches at a holding potential of
−80 mV. The pipet solution contained 80 mM KF, 20 mM KCl, 10 mM K-EDTA, 10 mM Hepes, pH 7.2, and the bath solution contained 1 μM
acetylcholine, 100 mM KCl, 1 mM MgCl₂, 10 mM Hepes, pH 7.2. The records from the wild type channels were filtered at 3 KHz and those from the
mutant channels filtered at 2 KHz.

channel lifetime suggesting that the closing rate is significantly decreased. Thus, the mutation is affecting a key aspect of ion channel gating.

The M4 domain is presumed to be at the lipid-protein interface and there is no evidence to suggest that M4 directly participates in the ion channel pathway. The large effects reported here emphasize the global linkages among subunits and subunit domains that are likely to be essential for normal channel function. The αCys418 is located in the middle of the presumed transmembrane M4 alpha helix. This M4 Cys residue is highly conserved in the α subunit of both muscle type and neuronal nAChRs across all species, suggesting that it may play an important role in gating. Efforts are underway to examine the effects of comparable mutations on other subunits and to define the molecular constraints associated with the mutations by introducing a variety of amino acid residues in place of the cysteines.

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