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Ligand–receptor interactions in the nicotinic acetylcholine receptor probed using multiple substitutions at conserved tyrosines on the α subunit

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Abstract

Affinity labeling studies have identified several conserved tyrosine residues in the α subunit of the nicotinic acetylcholine receptor (αY93, αY190, and αY198) as being in or near the ligand binding site. Mutagenesis studies from several laboratories have shown that substitution of phenylalanine for tyrosine at these positions reduces the apparent affinity for ACh. We have examined this apparent reduction in affinity further through the use of multiple substitutions at each position. Substitution of either phenylalanine, tryptophan, or serine resulted in an apparent decrease in agonist affinity, but the degree of reduction depended on both the position and the nature of the substitution. Analysis of the effects of each substitution suggests that each residue interacts with the quaternary N of ACh, and that each residue may make a different type of interaction with the agonist.

Key words: Nicotinic acetylcholine receptor; Acetylcholine binding site; Site-directed mutagenesis

1. Introduction

The nicotinic acetylcholine receptor (AChR) is a multisubunit transmembrane glycoprotein involved in synaptic transmission at both the neuromuscular junction and the central nervous system. The muscle-type receptor is a pentameric complex consisting of four homologous subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ [1]. Although the neuronal subtypes are not as well characterized, they, too, appear to be pentameric complexes containing two α and three non-α or β subunits [2]. All α subunits cloned to date from muscle and neuronal tissue exhibit a high degree of homology, suggesting that they share common structural features.

A number of elegant affinity labeling studies have identified three conserved tyrosines on the α subunit as forming at least part of the ligand-binding domain of the receptor; they are located at position 93 (αY93), 190 (αY190), and 198 (αY198) (the notation used is subunit/amino acid/position in the *Torpedo* subunit) (reviewed in [3,4]). Subsequent mutagenesis studies using α subunits from neuronal [5], mouse muscle [6], and *Torpedo* electroplax [7,8] AChRs have demonstrated that replacement of these tyrosines by phenylalanine leads to an apparent reduction in agonist affinity, as measured by shifts in dose–response curves for currents elicited by bath application of ACh to voltage-clamped *Xenopus* oocytes expressing either wild-type or mutant AChRs.

In this report, we have examined the interaction of ACh with these three conserved tyrosines in the mouse muscle AChR in greater detail by making multiple substitutions at each position and examining the functional consequences of each change. In all cases, replacement of the tyrosine by phenylalanine, tryptophan, or serine leads to a reduction in the apparent affinity of ACh for the receptor; however, the exact effect at each position depends upon the chemical nature of the substitution.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

Mouse muscle AChR subunit cDNAs were obtained from Dr. J.P. Merlie (Washington University; α subunit), N. Davidson (California Institute of Technology; β and δ subunits), and S. Heinemann (Salk Institute; γ subunit). The α subunit was subcloned into the pALTER-1 vector (Promega, Madison, WI).

Mutagenesis of the α subunit was carried out using the commercially-available ALTERED SITES system (Promega, Madison, WI). The mutagenic primers were 17–21 nucleotides long and were synthesized using an Applied Biosystems Model 391 oligonucleotide synthesizer. Mutations were confirmed by sequence analysis using the Sanger dideoxy termination method (Sequenase, US Biochemicals, Cleveland, OH) through the entire coding region in order to verify that only the desired nucleotide changes were present.

2.2. In vitro transcription and expression in Xenopus oocytes

Plasmid DNAs were linearized with the appropriate restriction enzymes and transcribed in vitro using SP6 RNA polymerase as described previously [9]. Oocytes were harvested from mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) and the adhering follicle cell layer was removed using collagenase (Type IA, Sigma). Isolated, follicle-free oocytes were maintained in SOS (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin. AChR subunit RNAs were mixed in a molar stoichiometry of 2:1:1:1 ($\alpha:\beta:\gamma:\delta$) and 8–15 ng were injected into the oocyte cytoplasm. Oocytes were maintained in SOS supplemented with pyruvate and gentamicin at 19°C for 18–72 h prior to recording.

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Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PTMA, phenyltrimethylammonium

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2.3. Electrophysiology

Currents elicited by bath application of various concentrations of ACh were measured using a standard two-microelectrode voltage clamp (either a GeneClamp 500 (Axon Instruments, Foster City, CA) or an OC-725 (Warner Instruments, Hamden, CT)) at a holding potential of −70 mV. Electrodes were filled with 3 M KCl and had resistances of 0.5–3 MΩ. The recording chamber was continuously perfused with a low-Ca^{2+} saline (100 mM NaCl, 2 mM KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.6) containing 300 nM atropine to block any residual muscarinic ACh receptors that remained after removal of the follicle cells. The low Ca^{2+} saline was used in order to reduce receptor desensitization, which is enhanced by external Ca^{2+} [10,11].

ACh blocks AChR channels in a voltage-dependent fashion, with a dissociation constant on the order of 5 mM at −70 mV [12]. Such a block would reduce the amplitude of the macroscopic currents at high ACh concentrations and negative membrane potentials. At −70 mV this block does not become significant until the ACh concentration exceeds 2 mM; therefore, in this study, ACh concentrations were limited to ≤ 1 mM.

2.4. Data analysis

Macroscopic dose–response curves from individual oocytes were fit to Eqn. **(1a)** using a Levenberg-Marquart algorithm from a commercially-available software package (Igor, Wave Metrics, Oswego, OR):

$$I = \frac{I_{max}}{1 + (EC_{50}/[A])^n} \quad \textbf{(1a)}$$

I and I_{max} are the currents at a given ACh concentration, and the maximal value, respectively, EC_{50} is the concentration of ACh required to obtain half-maximal current, and n is the apparent Hill coefficient. Data from individual oocytes were then normalized to the value of I_{max} determined for each oocyte, and then the normalized data from 3–7 oocytes were fit to Eqn. **(1b)** to obtain final estimates of EC_{50} and n.:

$$\theta = [1 + (EC_{50}/[A])^n]^{-1} \quad \textbf{(1b)}$$

where θ is the normalized current (I/I_{max}).

3. Results and discussion

Replacement of one of the conserved tyrosines (αY93, αY190, and αY198) on the α subunit by phenylalanine, tryptophan, or serine leads to an apparent reduction in ACh affinity, as measured by shifts in the dose–response curves for ACh-elicited currents. Fig. 1 shows the effects of phenylalanine substitution at positions αY93 (αY93F), αY190 (αY190F), and αY198 (αY198F). When compared on a μA/fmol receptor basis, all mutations described in this report give essentially the same maximum currents; however all three mutations lead to a shift in the dose response curve towards higher concentrations. Wild-type receptors have an EC_{50} value for ACh on the order of 20 μM, while those for αY93F, αY190F, and αY198F are 350, 810, and 88 μM, respectively. It can be seen that the same substitution at different positions decreases the apparent affinity for ACh from 4- to 40-fold, depending upon the position of the substitution.

Figs. 2 and 3 compare the effects of multiple substitutions at positions αY93 (Fig. 2) and αY198 (Fig. 3). While replacement of tyrosine by phenylalanine, tryptophan, and serine all lead to a reduction in the apparent affinity of ACh, the actual magnitude of the reduction depends on both the position and the actual substitution. For example, replacement of αY93 by phenylalanine (αY93F), tryptophan (αY93W), or serine (αY93S) all

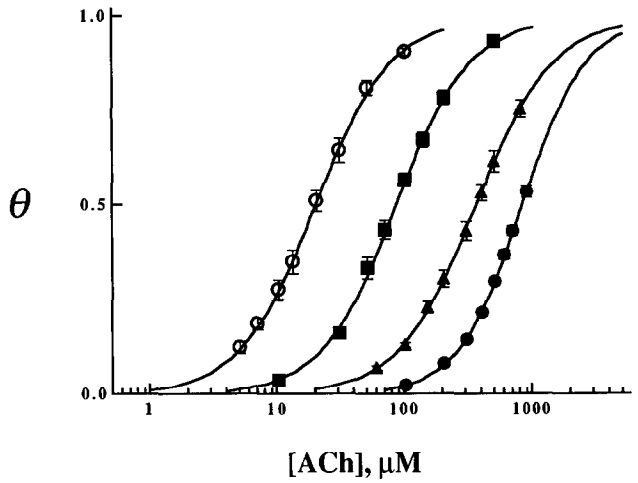

Fig. 1. Dose–response curves for phenylalanine for tyrosine substitutions. Oocytes expressing wild-type or mutant AChRs were voltage-clamped at −60 mV and the currents elicited by bath application of various concentrations of ACh were measured. Currents from individual oocytes were normalized as described in the text. Each point represents the mean ± S.E.M. of 5–7 determinations. The smooth curves are described by Eqn. **(1b)** using parameters listed in Table 1. The symbols used are: WT (○), αY93F (▲), αY190F (●), and αY198F (■).

reduce the apparent affinity by 20–30-fold. On the other hand, replacement of αY198 by another aromatic amino acid (αY198F and αY198W) has a small effect (an approximate 5-fold decrease in affinity), while replacement by a non-aromatic amino acid (αY198S) leads to a much larger change (30-fold decrease in affinity).

Table 1 summarizes the results of all of the substitutions at positions αY93, αY190, and αY198. We were unable to detect currents elicited by ACh at concentrations up to 1 mM from oocytes expressing αY190S receptors. However, using the binding of ^{125}I-α-bungarotoxin (a competitive antagonist) or ^{125}I-mAb 210 (which recognizes residues 67–76 on the α subunit) to detect the presence of receptor complexes on the oocyte surface, we found that αY190S receptors were present on the oocyte surface at levels similar to those of the other receptors in this study (data not shown). Presumably, they are either unable to couple agonist binding to channel opening and are thus non-functional, or their apparent affinity has been reduced to such an extent that 1 mM ACh (the upper limit used in this study) was too low elicit detectable currents.

ACh can be considered to be made up of two domains – the choline moiety, which contains the quaternary N, and the acetyl moiety, which contains a carbonyl group. If ACh interacts with αY93, αY190, and αY198, then one or both parts of the ACh molecule could interact with these residues. In order to determine which part of the ACh molecule the tyrosines may interact with, we used the partial agonist phenyltrimethylammonium (PTMA) in which the acetyl moiety is replaced by a phenyl ring. In contrast to *Torpedo* AChRs, from which

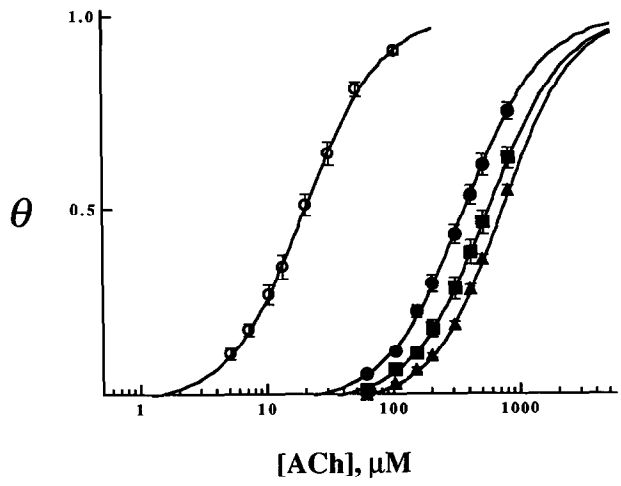

Fig. 2. Dose–response curves for substitutions at αY93. Dose–response data were collected and analyzed as described in the legend to Fig. 1. Each point represents the mean ± S.E.M. of 5–7 determinations. The symbols used are: WT (○), αY93F (▲), αY93W (●), and αY93S (■). Note that all three substitutions have essentially the same effect.

we were unable to detect PTMA-evoked currents from mutant receptors [7], we were able to record small PTMA-evoked currents from mouse AChRs containing mutations at positions αY93, αY190, and αY198. This is most likely due to the fact that macroscopic currents recorded from mouse AChRs expressed in *Xenopus* oocytes are much larger and desensitize more slowly compared to their *Torpedo* counterparts at a given ACh concentration, allowing the detection of much smaller fractional currents. Fig. 4 shows the EC_{50}'s for PTMA-elicited currents from WT and the substitutions at positions αY93 and αY198 plotted against the EC_{50} value for ACh. Although we could detect currents elicited by PTMA from αY190F and αY190W receptors, they were too small to accurately determine EC_{50} values. The plot shows that each substitution has a similar effect on the EC_{50} values for both agonists. Since the only portion that the two agonists have in common is the quaternary N, this strongly suggests that if αY93 and αY198 directly interact with agonists, they most likely do so at the quaternary N. It must be emphasized, however, that these data do not prove that ACh interacts directly with the tyrosines under study here, but they could instead play an indirect role in coupling ligand binding to channel opening. However, the fact that in the *Torpedo* AChR changes in the affinity of the competitive antagonist curare are similar to those of the ACh EC_{50} for αY93F, αY190F, and αY198F receptors suggests that these residues are involved in ligand–receptor interactions [8], rather than coupling ligand binding to channel opening as we previously suggested [7].

Tyrosine can, in principle, make two types of interactions with the quaternary N of ACh. First, if the local environment of a tyrosine is such that the pK_a of the tyrosine hydroxyl is reduced from its normal value of 9.7 to a value closer to 7.6 (the pH used in the experiments), a significant fraction of the tyrosine could be in the anionic tyrosinate form and make a salt bridge-type interaction with the quaternary N of ACh. While this may seem unlikely, the fact that αY190 in the *Torpedo* AChR forms a covalent adduct with lophotoxin analog-1 led Abramson et al. [13] to conclude that αY190 is an excellent nucleophile, which is consistent with this notion. Second, tyrosine (and other aromatic groups) can form a cation–

Table 1
Macroscopic dose–response relationships for wild type and mutant AChRs

Receptor	EC_{50}	n	EC_{50} ratio (mutant/WT)	$\Delta(\Delta G)$, kcal/mol
WT	19.3 ± 0.6	1.5 ± 0.1	1	–
αY93F	353.2 ± 16.4	1.4 ± 0.1	18	1.7
αY93W	705.8 ± 43.2	1.6 ± 0.1	36	2.1
αY93S	545.4 ± 38.7	1.4 ± 0.1	28	2.0
αY190F	812.3 ± 36.0	1.6 ± 0.1	42	2.2
αY190W	208.3 ± 13.8	1.6 ± 0.1	11	1.4
αY190S	ND	ND	ND	–
αY198F	88.1 ± 2.9	1.5 ± 0.1	4	0.8
α198W	124.1 ± 1.7	1.6 ± 0.1	6	1.0
α198S	635.0 ± 57.1	1.9 ± 0.2	34	2.1

Concentrations of ACh required for half-maximal current (EC_{50}) and the apparent Hill coefficients (n) were estimated from fits of dose–response data to Eq. (**1b**) as described in the text. Each value is the mean ± S.E.M. of 5–7 determinations. ND, no current detected. The change in free energy due to the mutation, $\Delta(\Delta G)$, is defined as:

$$\Delta(\Delta G) = RT \ln \frac{EC_{50}\,(\text{mut})}{EC_{50}\,(\text{WT})}$$

where EC_{50}(mut) and EC_{50}(WT) are the EC_{50} values for the mutant and wild-type receptors, respectively.

π interaction between the unpaired π-electrons of the aromatic ring and the quaternary N of ACh [14]. If a charge–charge interaction takes place, then replacement of tyrosine by phenylalanine, tryptophan, or serine should remove this interaction, and each substitution should give a similar reduction in affinity. On the other hand, if tyrosine is involved in a cation–π interaction, then the serine substitution should eliminate this interaction. Phenylalanine and tryptophan are capable of making cation–π interactions, and thus substitution of these residues should either have no effect or a much smaller (due to less-than-optimal geometry compared to tyrosine) effect than replacement by serine.

With this logic in mind, we can make some speculations as to the types of interactions that may take place in the ligand binding site. In the case of the mutations at αY93, the fact that all three substitutions have essentially the same effect ($\Delta(\Delta G)$ approximately 2 kcal/mol) suggests that this residue makes an interaction that is rather specific to tyrosine, such as a salt bridge. The substitutions at αY198 indicate that a different type of interaction may take place at this position. Substitution by aromatic amino acids has a much smaller effect than replacement by serine ($\Delta(\Delta G)$ approximately 1 kcal/mol for the aromatic substitutions vs. 2 kcal/mol for serine). This is consistent with the notion that αY198 makes an interaction that depends upon the aromatic nature of the side chain such as cation–π interactions. The reduction in apparent affinity after substitution by phenylalanine or tryptophan at αY198 may be due to a less-than-optimal geometry which could result in a weaker cation–π interaction. In the case of αY190, the lack of information about the underlying cause of the lack of a response of αY190S mutations (i.e. non-functional or a very low apparent affinity) limits our ability to speculate about the types of interactions (if any) that this residue may make with the agonist.

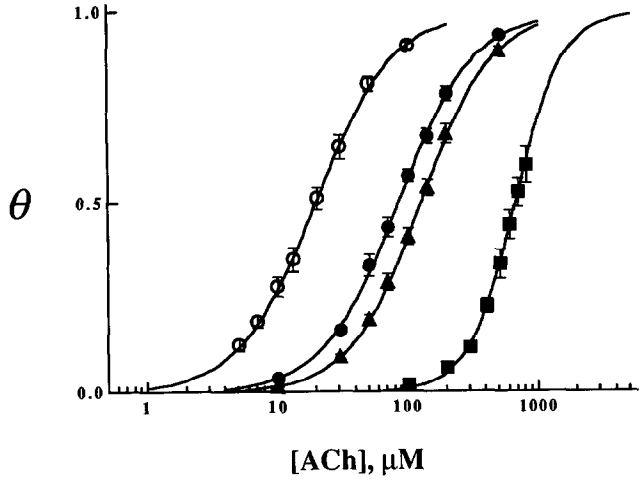

Fig. 3. Dose–response data for substitutions at αY198. Dose–response data were collected and analyzed as described in the legend to Fig. 1. Each point represents the mean ± S.E.M. of 5–7 determinations. The symbols used are: WT (○), αY198F (▲), αY198W (●), and αY198S (■). Note that the aromatic substitutions (αY198F and αY198W) have a smaller effect than the serine substitution.

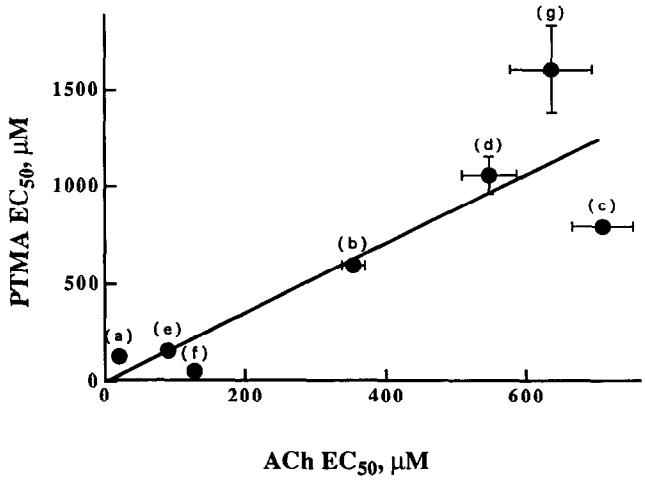

Fig. 4. Relationship between EC_{50}s for ACh and PTMA activation. Dose–response data were collected using ACh and PTMA as agonists and the EC_{50} values were determined as described in the text. Each point represents the mean ± S.E.M. (in both x and y directions) of 5–7 determinations. The receptors are identified as follows: (a), WT; (b), αY93F; (c), αY93W; (d), αY93S; (e), αY198F; (f), αY198W; (g), αY198S. The straight line is for illustrative purposes only, and has no theoretical significance.

The overall picture of the binding site that has emerged from this and other studies is one in which a number of aromatic amino acids play an important role in determining ligand–receptor interactions. This is similar to the situation in acetylcholinesterase, in which analysis of the structure at atomic resolution has shown that the substrate binding site is in what has been termed the 'aromatic gorge' [15]. Although our current pictures of the structure of the AChR are not of sufficient resolution to determine the arrangement of amino acid side chains in the ligand binding site [16], the combination of site-directed mutagenesis and analysis of the functional consequences of the changes does provide some insight into the interactions that take may place during the process of the transduction of ligand binding to channel opening. Further analyses of this type, employing a wider variety of ligands of defined structure, should enable us to provide a better picture of the structure of the ligand-binding site.

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References

[1] Karlin, A. (1991) Harvey Lect. 85, 71–107.
[2] Role, L.W. (1992) Curr. Opin. Neurobiol. 2, 254–262.
[3] Karlin, A. (1993) Curr. Opin. Neurobiol. 3, 299–309.

[4] Changeux, J.-P., Galzi, J.-L., Devillers-Thiery, A. and Bertrand, D. (1992) Quar. Rev. Biophys. 25, 395–432.
[5] Galzi, J.-L., Bertrand, D., Devillers-Thiery, A., Revah, F., Bertrand, S. and Changeux, J.-P. (1991) FEBS Lett. 294, 198–202.
[6] Tomaselli, G.F., McLaughlin, J.T., Jurman, M.E., Hawrot, E. and Yellen, G. (1991) Biophys. J. 60, 721–727.
[7] O'Leary, M.E. and White, M.M. (1992) J. Biol. Chem. 267, 8360–8365.
[8] O'Leary, M.E., Filatov, G.N. and White, M.M. (1994) Am. J. Physiol. 266, C648–C653.
[9] Buller, A.L. and White, M.M. (1988) Proc. Natl. Acad. Sci. USA 85, 8717–8721.
[10] Manthey, A.A. (1966) J. Gen. Physiol. 49, 963–976.
[11] Miledi, R. (1980) Proc. R. Soc. Lond. B. 209, 447–452.
[12] Sine, S.M. and Steinbach, J.H. (1984) Biophys. J. 46, 277–284.
[13] Abramson, S.N., Pi, Y., Culver, P. and Taylor, P. (1989) J. Biol. Chem. 264, 12666–12672.
[14] Dougherty, D.A. and Stauffer, D.A. (1990) Science 250, 1558–1560.
[15] Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I. (1991) Science 253, 872–879.
[16] Unwin, N. (1993) J. Mol. Biol. 229, 1101–1124.