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Functional significance of aromatic amino acids from three peptide loops of the α7 neuronal nicotinic receptor site investigated by site-directed mutagenesis

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Three aromatic amino acids, Tyr^{92}, Trp^{148} and Tyr^{187} belonging to three separate domains of the α7-subunit of neuronal nicotinic acetylcholine receptor were mutated to phenylalanine, and the electrophysiological response of the resulting mutant receptors analyzed in the *Xenopus* oocyte expression system. All mutations significantly decreased the apparent affinities for acetylcholine and nicotine, and to a lesser extent, those for the competitive antagonists dihydro-β-erythroidine and α-bungarotoxin. Other properties investigated, such as the voltage dependency of the ion response as well as its sensitivity to the open channel blocker QX222, were not significantly changed, indicating that the mutations affected selectively the recognition of cholinergic ligands by the receptor protein. The maximal rates for the rapid desensitization process were slightly modified, suggesting that the contribution of Tyr^{92}, Trp^{148} and Tyr^{187} to the binding area might differ in the various conformations of the nicotinic receptor. Other mutations at nearby positions (S94N, W153F, G151D and G82E) did not affect the properties of the electrophysiological response. These data point to the functional significance of Tyr^{92}, Trp^{148} and Tyr^{187} in the binding of cholinergic ligands and ion channel activation of the nicotinic receptor, thus supporting a multiple loop model [(1990) J. Biol. Chem. 265, 10430–10437] for the ligand binding area.

Neuronal nicotinic acetylcholine receptor; Acetylcholine binding site; site-directed mutagenesis

1. INTRODUCTION

Cholinergic agonists cause the opening of the nicotinic receptor ion channel when interacting with two ACh binding sites (reviewed in [2–4]). These sites are mainly carried by the α-subunits and include a doublet of cysteine (*C*) residues (C192, C193 in *Torpedo* receptor) which react with sulfhydryl reagents after reduction of the receptor [5]. Further studies based on α-bungarotoxin binding to α-subunit peptide fragments (reviewed in [4]), and the consequences on receptor channel response of disulfide bridges reduction (reviewed in [6]) and point mutations [7,8], established that the region α180–200, containing Y190, C192 and C193, contribute to the recognition of cholinergic ligands.

Two additional peptide domains from the α-subunit, potentially involved in the binding of cholinergic ligands, were identified using the photoactivatable cholinergic ligand [^{3}H]*p*-(*N*,*N*) dimethylamino benzene diazonium fluoroborate (DDF) [9]. This compound covalently incorporated into several amino acids from the amino-terminal hydrophilic domain of the native *Torpedo marmorata* α-subunit at the level of Y190, C192, C193 and Y198 from the aforementioned α-subunit domain, as well as W149 and Y93 [1,10], suggesting a multiple loop model for the cholinergic binding area.

The relevance of these amino acids in the physiological binding of cholinergic ligands was supported by the fact that their labeling (i) was inhibited by the agonist carbamylcholine and the competitive antagonists α-bungarotoxin and *d*-tubocurarine and (ii) was not inhibited by excess of aqueous scavengers [1,10], indicating that the probe did not significantly diffuse before reacting with amino acid residues. In addition, the DDF-labeled amino acids, Y190 and Y198, are the sites of covalent incorporation for the natural cholinergic ligands lophotoxin [11] and nicotine [12], respectively. Also, these specifically labeled amino acids are strictly conserved in the sequences of all functional α-subunits from electric organ, skeletal muscle and brain, but are absent from the corresponding portions of the peripheral receptor non-α-subunits which do not carry primary binding sites for cholinergic ligands (reviewed in [4]).

Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; DHβE, dihydro-β-erythroidine; DDF, *p*-(*N*,*N*) dimethylamino benzene diazonium fluoroborate.

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	93	149	190	198
α1 Torpedo	DLVL**Y**NNAD...GI**W**TYDGTK..VY**Y**TCCPDTP**Y**LD			
	92	148	187	
α7 chick	DILL**Y**NSAD...GS**W**TYGGWS..SF**Y**ECCKE-PYPD			

Fig. 1. Amino acid sequence of α1-subunit from *Torpedo* nicotinic receptor and α7-subunit of neuronal nicotinic receptor from chick. The amino acids photo-affinity-labeled by [^{3}H]DDF in the α1-subunit and the homologous residues mutated in the α7-subunit are shown in bold.

Here we further investigate the functional contribution of these aromatic amino acids to the recognition of cholinergic ligands and gating of the receptor channel by site-directed mutagenesis and electrophysiological recordings. The amino acids corresponding to *Torpedo* Y93, W149 and Y190 (Fig. 1) were mutated on the α7-subunit of chick neuronal nicotinic AChR [13] which assembles into functional homo-oligomers, is activated by ACh concentrations comparable to those which activate muscle or electric organ receptors, and is blocked by α-bungarotoxin. The responses to ACh and nicotine, and their inhibition by the competitive antagonist dihydro-β-erythroidin (DHβE) were analyzed using the *Xenopus* oocyte expression system.

2. MATERIALS AND METHODS

2.1. *Mutagenesis*

The 1057 bp *Pst*I (NT −153)–*Bgl*II (NT 904) and the 2122 bp *Bgl* II (NT 904)–*Eco*RI (NT 3026) fragments from plasmid flip α7 delta [13], were ligated and subcloned between *Pst*I and *Eco*RI sites of Blueacript KS$^+$ vector (Stratagene) to permit single-stranded DNA synthesis. The promoter from SV40 virus was introduced into the same vector between *Not*I and *Spe*I sites. Mutants were obtained using the 'oligonucleotide-directed mutagenesis kit' (Amersham) according to the instructions of the manufacturer, and their sequence was systematically checked.

2.2. *Electrophysiology*

Xenopus oocytes were prepared, injected and recorded as previously described [14]. Recordings were usually made 2–3 days after injection. Cells were clamped at −100 mV. Inhibition of ACh responses by α-bungarotoxin were determined by measuring currents on the same oocyte before and after 30 min incubation with 50 nM α- bungarotoxin. For technical reasons, nicotine concentrations higher than 4 mM could not be tested. The nicotine responses on the W148F mutant were therefore compared to ACh responses in the same cell for estimation of the maximal value used in normalization. QX222 was a gift from Dr. Igolen, Institut Pasteur, Paris. DHβE was a gift from Merk-Sharp and Dohme All other ligands were from commercial sources.

3. RESULTS AND DISCUSSION

The aromatic amino acids Y92 and Y187 from the α7-subunit (Fig. 1) were mutated into phenylalanine or serine, and W148 was either replaced by phenylalanine or by a histidine residue. All the Y/W-to-F mutants yielded functional receptors when tested for whole-cell responses in the *Xenopus* oocyte (Fig. 2).

Dose–response curves for agonist (Fig. 3) indicated that mutation of Y187 into F resulted in a 10-fold decrease in ACh apparent affinity (Table I), a result which is in good agreement with the recently described mutation of the homologous residue from mouse muscle nicotinic receptor α-subunit [8].

Additional mutations of Y92 and W148, which be-

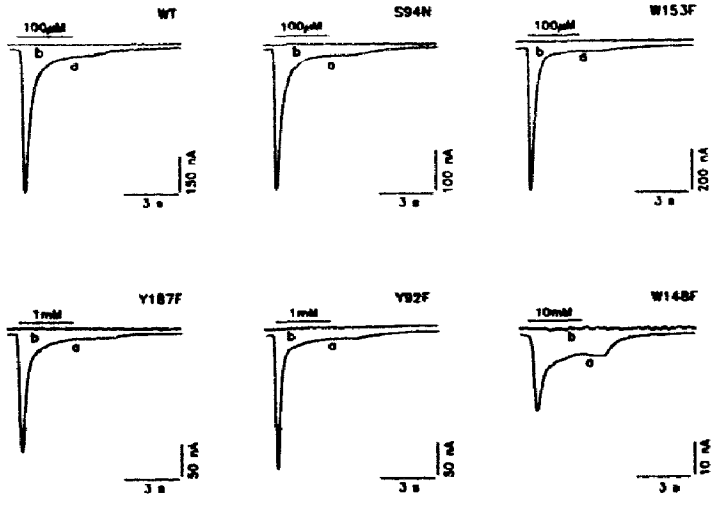
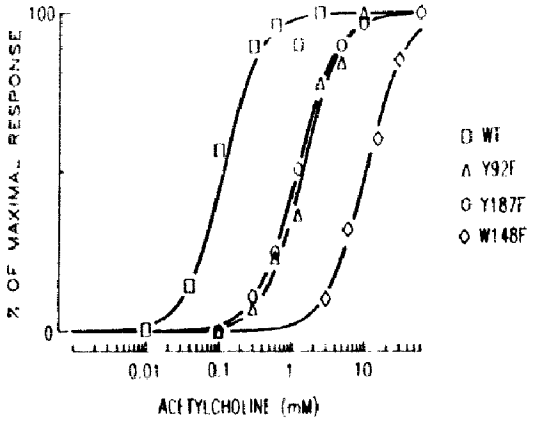


Fig. 2. Currents evoked by ACh in *Xenopus* oocytes expressing wild- type (WT) and Y92F, W148F, Y187F, S94N and W153F mutants of the α7 receptor. Cells were held at −100 mV and currents (a traces) were evoked by 3 s ACh application (horizontal bar). The concentrations indicated for Y92F, W148F, Y187F, S94N and W153F correspond to the half-maximally effective dose of ACh. Blockade of the responses by α-bungarotoxin is shown (b traces).



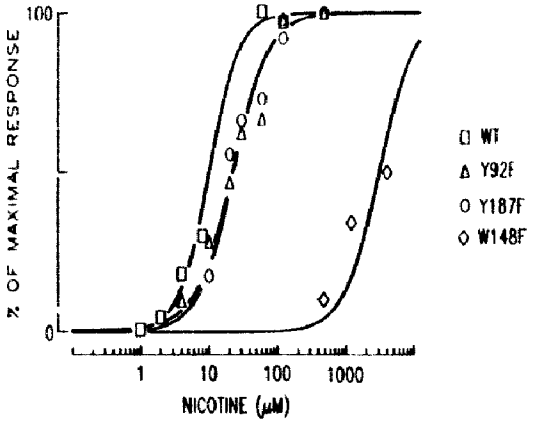


Fig. 3. ACh and nicotine dose response relationships of α7 wild-type (WT) and Y92F, W148F and Y187F receptors. Responses evoked by 3 s of increasing ACh or nicotine concentrations were measured in 2–8 cells from several batches held at −100 mV. Peak currents were then normalized to the maximum values, averaged and plotted as a function of the logarithm of agonist concentration. Lines are the best fits obtained with the empirical Hill equation adjusted by weighted least square algorithm. EC_{50}s and Hill coefficients are given in Table I.

long to separate domains of the α-subunit, affected the sensitivity of the receptor to ACh. Indeed, apparent affinity for ACh was reduced by one order of magnitude

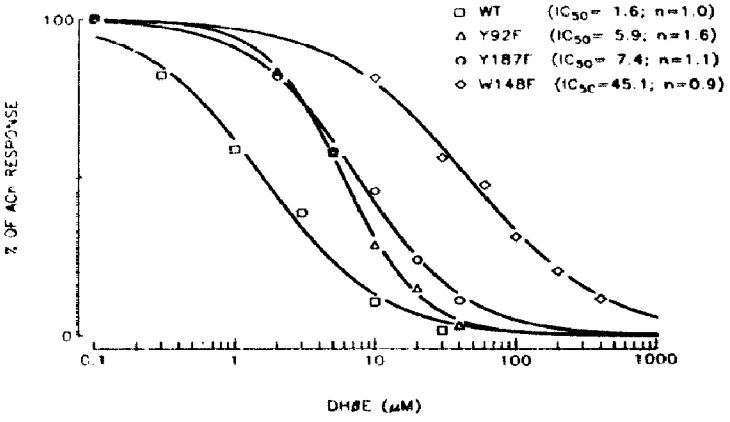


Fig. 4. Inhibition of the ACh evoked responses on the α7 wild-type (WT), Y92F, W148F and Y187F receptors by DHβE. The responses of several (3–7) oocytes expressing each receptor were perfused with half-maximally effective dose of ACh and increasing concentrations of DHβE. Peak currents were then normalized to the maximum values, averaged and plotted as a function of the logarithm of DHβE concentration. Lines are the best fits obtained with the empirical Hill equation adjusted by weighted least square algorithm. Apparent IC_{50} and Hill coefficients are given in Table I.

upon Y92-to-F mutation and by two orders of magnitude upon W148-to-F mutation (Fig. 3) without significant changes in Hill coefficients (Table I), strongly supporting the contribution of amino acids from several domains of the α-subunit to the recognition of cholinergic ligands and activation of ion permeation.

Activation of the mutant receptors by nicotine was also modified. The Y92F and Y187F mutations affected to a smaller extent the responses to this agonist than to ACh, as manifested by the 2-fold decrease in apparent affinity; on the other hand, W148F mutation led to a strong 300-fold reduction of nicotine EC_{50} (Fig. 3, Table I). These data indicate that the 2 agonists do not establish the same interactions with the set of 3 residues belonging to the cholinergic ligand binding area.

The competitive antagonists DHβE (Fig. 4) and α-bungarotoxin blocked all ACh responses in a concentration–dependent manner. The IC_{50} determined for DHβE, at the concentration of ACh which yielded half of the maximal response, decreased by ≈4-fold (as com-

Table I

Summary of the data obtained for the α7 wild-type (WT), Y92F, W148F and Y187F receptors.

Receptors	Acetylcholine		Nicotine		DHβE		Acetylcholine
	EC_{50} (μM)	nH	EC_{50} (μM)	nH	IC_{50} (μM)	nH	τ s
WT	115	1.7	10	2.0	1.6	1.0	0.20
Y92F	1408	1.7	23	1.7	5.9	1.6	0.11
W148F	10523	1.6	3057	1.7	45.1	0.9	0.32
Y187F	1186	1.6	23	1.7	7.4	1.1	0.34

Tau (τ) values determined at saturating concentrations of ACh are given. nH, Hill coefficients.

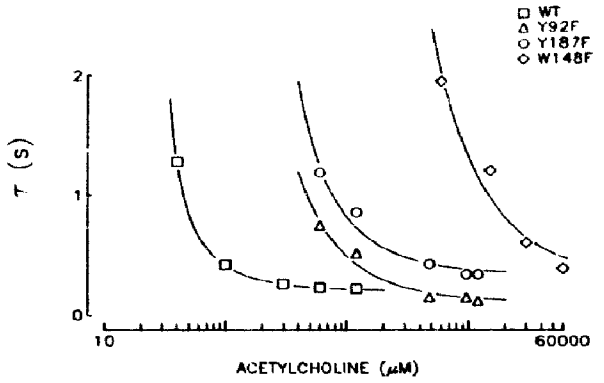


Fig. 5. Desensitization of wild-type and mutated receptors as a function of the agonist concentration. ACh evoked currents were recorded in oocytes expressing wild-type (WT) or Y92F, W148F or Y187F mutants for several agonist concentrations. Cells were clamped at −100 mV and ACh was applied for 3 s, once every min. The time course of the responses could be approximated by a single exponential with the equation $y(t)=\alpha\, e^{(-t/\tau)}$ adjusted using weighted least square algorithm. The time constant of the exponential is plotted as a function of the logarithm of ACh concentration. Each point represents the mean value from 4 cells. Standard deviations, within the symbol size, were ommitted for clarity. At saturating concentrations of ACh, the maximal rate of desensitization was characterized by the limit values of the time constant τ given in Table I.

pared to wild-type) with Y92F and Y187F mutants and by ≈30-fold with the W148F mutant receptor (Table I).

Similarly, the potency of α-bungarotoxin to inhibit ACh responses was reduced by the Y92F, W148F and Y187F mutations. A concentration of 10 nM α-bungarotoxin did not fully inhibit the responses of the mutant receptors while quantitatively blocking those of the wild-type receptor [13]. The complete block of all mutant responses (Fig. 2, **b** traces) was however obtained with 50 nM α-bungarotoxin. Such effects are consistent with experiments on *Torpedo* α180–200 peptide fragments reporting that the mutation of Y190 (homologous to α7 Y187) to F, A or G, decreases α-toxin affinity [15–17].

As observed for mutations near amino acid Y187 in mouse muscle receptor [8], several mutations near Y92 and W148 of the α7-subunit, like S94N and W153F (Fig. 2), G82E and G151D (not shown), did not cause significant changes on the physiological properties of the receptor. The EC_{50}s of ACh for the S94N (75 μM), W153F (120 μM) and wild-type (115 μM) receptors did not significantly differ.

To further determine if the alterations in the dose–response relationships resulted from selective alterations of the ligand-binding properties of the Y92F, W148F and Y187F receptors, several features of the ion channel permeability response were analyzed. The I–V relation of the mutant and wild-type receptor responses were similar (not shown), indicating that the voltage-sensitivity of the ion channel was not significantly altered. Also, the open channel blocker QX222, formerly used to probe the structure of the ion channel [18], inhibited in a voltage-dependent manner ion flow through mutant and wild-type receptor channels to the same extent (not shown).

To test for a possible influence of the mutations on the desensitization properties of the receptor, the time constants for response decay were measured during steady application of ACh. The ACh concentrations required to reach desensitization kinetics similar to those of the wild-type receptor were shifted by approximately one order of magnitude for Y92F and Y187F and by two orders of magnitude for W148F receptors (Fig. 5). In addition, slight but significant changes in the time constants were observed at saturating concentrations of ACh, which reflected a reduction of the maximal speed of the rapid desensitization process in Y187F and W148F receptors and an acceleration of this process in the Y92F receptor. These changes in the maximal speed of desensitization suggest that the amino acids Y92, W148 and Y187 are involved in the structural reorganizations occurring upon transition from the active to the desensitized conformation of the receptor. Such observations have already been reported with *Torpedo* nicotinic receptor under conditions of photo-affinity labeling by [^{3}H]DDF in the resting and desensitized conformations [19].

Several lines of evidence are accumulating in favor of the contribution of aromatic rings to the binding of large, diffusely charged, quaternary ammonium ions, in particular in the case of proteins interacting with ACh or its derivatives. [^{3}H]DDF photo-affinity labeling experiments have led to the identification of tyrosine and trytophan residues as major sites of specific incorporation [1,10]. Aromatic amino acids are also found around the quaternary ammonium moiety in the binding sites of anti-phosphorylcholine antibody [20], acetylcholinesterase [21] as well as in three-dimensional models of muscarinic AChR [22].

Here, none of the mutants, in which Y92 and Y187 were replaced by serines or W148 by a histidine, responded to ACh at concentrations as high as 100 mM. Although disruption of subunit assembly by these mutations cannot be ruled out, the complete abolition of receptor responses observed upon removal of the aromatic rings (Y-to-S mutations) support the view that such aromatic amino acids are indeed functionally relevant to the activation of the nicotinic AChR and that their aromatic properties are at least as (if not more) important as their polar character. Such conclusions are supported by the fact that strictly aromatic macrocycles [23,24] can bind quaternary ammonium-containing molecules with affinities in the micromolar range. The presence of aromatic side-chains may thus be a general feature of binding sites for quaternary ammonium ligands.

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