brief communication

Mutations affecting agonist sensitivity of the nicotinic acetylcholine receptor

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ABSTRACT The nicotinic acetylcholine receptor (AChR) is a pentameric transmembrane protein ($\alpha_2\beta\gamma\delta$) that binds the neurotransmitter acetylcholine (ACh) and transduces this binding into the opening of a cation selective channel. The agonist, competitive antagonist, and snake toxin binding functions of the AChR are associated with the α subunit (Kao et al., 1984; Tzartos and Changeux, 1984; Wilson et al., 1985; Kao and Karlin, 1986; Pederson et al., 1986). We used site-directed mutagenesis and expression of AChR in *Xenopus* oocytes to identify amino acid residues critical for ligand binding and channel activation. Several mutations in the α subunit sequence were constructed based on information from sequence homology and from previous biochemical (Barkas et al., 1987; Dennis et al., 1988; Middleton and Cohen, 1990) and spectroscopic (Pearce and Hawrot, 1990; Pearce et al., 1990) studies. We have identified one mutation, Tyr^{190} to Phe (Y190F), that had a dramatic effect on ligand binding and channel activation. These mutant channels required more than 50-fold higher concentrations of ACh for channel activation than did wild type channels. This functional change is largely accounted for by a comparable shift in the agonist binding affinity, as assessed by the ability of ACh to compete with α-bungarotoxin binding. Other mutations at nearby conserved positions of the α subunit (H186F, P194S, Y198F) produce less dramatic changes in channel properties. Our results demonstrate that ligand binding and channel gating are separable properties of the receptor protein, and that Tyr^{190} appears to play a specific role in the receptor site for acetylcholine.

INTRODUCTION

The α subunits of the AChR are known to contribute to the ligand binding site of the receptor. Affinity labeling with analogues of ACh or with other cholinergic ligands has identified several amino acid residues in the α subunit near the ACh binding site. Particular attention has been focused on the two conserved cysteines at positions 192 and 193, and the surrounding region (Fig. 1). Peptides from this region bind α-BgTx with moderate affinity (Wilson et al., 1985; Barkas et al., 1987; Oblas et al. 1986). Two cholinergic affinity labels, bromoacetylcholine and 4-(*N*-maleimido)benzyltrimethylammonium iodide (MBTA), react with Cys^{192} and Cys^{193} of DTT-treated receptor protein (Kao et al., 1984; Kao and Karlin, 1986; Silman and Karlin, 1969). Nicotine and *p*-(*N*,*N* dimethylamino)benzenediazonium fluoroborate (DDF) react with the conserved tyrosine residues at positions 190 and 198 (Dennis et al., 1988; Middleton and Cohen, 1990). Lophotoxin analogue-1, a coral-derived toxin that irreversibly blocks cholinergic ligand binding, also reacts with tyrosine 190 (Abramson et al., 1989).

There is, however, little information about the functional role of these and other amino acid residues in this region. Mishina and colleagues (Mishina et al., 1985) mutated the two conserved cysteine residues at positions 192 and 193 to serine. These mutant proteins exhibited a 10- to 30-fold reduction in binding affinity for carbamylcholine, consistent with a location near the binding site. However, the mutant proteins did not function as ligand-activated channels, leaving open the question of how extensively the mutations disrupted the protein structure. We have investigated the role of several other conserved residues of the AChR α subunit in the region surrounding positions 192 and 193. We prepared RNA coding for AChRs mutated at α subunit positions 186 (His→Phe), 190 (Tyr→Phe), 194 (Pro→Ser), and 198 (Tyr→Phe), and expressed them in *Xenopus* oocytes to assess the role of these residues in receptor function (Fig. 1). These data (Tomaselli et al., 1991) and similar data for the *Torpedo* receptor (O'Leary and White, 1991) have been presented in preliminary form.

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EXPERIMENTAL PROCEDURES

Mutagenesis and in vitro RNA transcription

The pSP64-based plasmids with cDNAs for the murine (BC3H-1) α, β, γ, and δ subunits of the nicotinic AChR

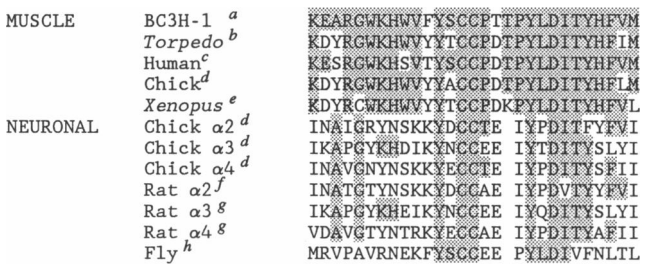

FIGURE 1 The amino acid sequences of α subunits from muscle and neuronal tissue. The sequences are compared with the murine BC3H-1 cell line nicotinic receptor sequence. Shaded areas indicate identity of the amino acid with the corresponding residue in the BC3H-1 sequence. The numbered residues were mutated as follows: H186F, Y190F, P194S, and Y198F. Abbreviations for the amino acid residues are: A, ala; C, cys; D, asp; E, glu; F, phe; G, gly; H, his; I, ile; K. lys; L, leu; M, met; N, asn; P, pro; Q, gln; R, arg; S, ser; T, thr; V, val; W, trp; and Y, tyr. References for sequences: [a]Boulter et al., 1985; [b]Noda et al., 1982; [c]Noda et al., 1983; [d]Nef et al., 1988; [e]Baldwin et al., 1988; [f]Wada et al., 1988; [g]Boulter et al., 1987; [h]Bossy et al., 1988.

were generously provided by Dr. J. Boulter of the Salk Institute. The α subunit cDNA was excised with EcoRI and subcloned into pBluescript KS$^-$ (Stratagene, La Jolla, CA) for oligonucleotide-directed mutagenesis. Missense mutations were introduced using dut$^-$ and ung$^-$ selection by the method of Kunkel (Kunkel, 1985). The entire cDNA of the α subunit was sequenced (Sanger et al., 1977) to confirm the nucleotide changes and to guard against stray mutations. The EcoRI fragments of the α subunit mutants were cloned back into pSP64 for in vitro transcription.

RNAs encoding the wild type and mutant receptor subunits were transcribed in a similar fashion. Plasmids containing the wild type and mutant subunit cDNA were linearized using Sca I. The linearized plasmid (2–4 μg) was incubated in 10 mM NaCl, 6 mM $MgCl_2$, 40 mM Tris (pH 7.5), 1 mM dithiothreitol, 2 mM spermidine, NTPs (1 mM each except GTP which was 0.2 mM), 0.6 mM diguanosine triphosphate (Pharmacia, Piscataway, NJ), 30 U SP6 RNA polymerase, and 100 U RNasin (Promega, Madison, WI) at 37° for 1 h. An additional 30 U of SP6 RNA polymerase and 100 U RNasin were added after an hour and the incubation was continued for another hour. The template DNA was removed with 4 U of RNase-free RQ1 DNase (Promega, Madison, WI) for 15 min at 37°. The RNA was extracted with phenolchloroform and chloroform, precipitated from isopropanol then resuspended in DEPC-treated water at a concentration of ~0.2 μg/μl. Subunit RNA was mixed in a ratio of 2α:1β:1γ:1δ for later injection into oocytes.

Preparation and microinjection of oocytes

Adult female, HCG-primed *Xenopus laevis* (Xenopus I, Ann Arbor, MI) were anesthetized by immersion in 0.17% aminobenzoic acid ethyl ester and several lobes of ovary were removed through a small abdominal incision. Individual stage V or VI oocytes were isolated by manual dissection followed by incubation in 0.5–2 mg/ml collagenase (type IA; Sigma Chemical Co., St. Louis, MO) in calcium-free saline (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes pH 7.6) for 1–2 h. The oocytes were allowed to recover in amphibian saline (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes pH 7.6) before injection.

Selected oocytes were injected with 25–50 nl of RNA solution using a positive displacement injector (Drummond Instruments, Broomhall, PA) through glass bores with tip diameters of 20–40 μm. The injected oocytes were incubated for at least 48 h in amphibian saline supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco BRL, Gaithersburg, MD), 0.5 mM theophylline, and 2 mM Na pyruvate.

Electrophysiology

The oocytes were screened for ACh-induced current by two microelectrode voltage clamp as previously described (Tomaselli et al., 1990). Whole oocytes were continuously perfused with ACh at a concentration that elicited near maximal current response for the channel variant being tested. Oocytes with sufficient whole cell current (>0.5 μA at −30 mV holding potential) were then patch clamped. The oocytes were devitellinized in preparation for patch clamp recording as previously described (Methfessel et al., 1986). Excised outside-out patches (Hamill et al., 1981) were obtained in symmetric pipette and bath solutions (in mM): 97 KCl, 1 $MgCl_2$, 0.2 mM EGTA, 5 K-Hepes, pH 7.5. Patches were continuously perfused and currents were elicited by the application of the bath solution containing the desired concentration of ACh.The patch was positioned in a custom-designed bath at the convergence point of streams of control and ACh-containing solution. Rapid solution changes (1–2 ms) were made by computer-controlled solenoid switches as previously described (Maconachie and Knight, 1989). ACh was applied in 400 ms pulses being delivered no more frequently than every 5 s. Currents were recorded by a List EPC7 patch clamp amplifier (List-electronic, Darmstadt, West Germany). During pulsatile application of ACh the currents were digitized at 2.5 kHz and filtered at 1 kHz (8-pole Bessel, −3 dB). The level of channel activity varied from patch

to patch; ensemble averages were compiled from 5–32 individual records. The peak of the ensemble average current at a given ACh concentration was corrected for ACh block using the measured single channel current. The ensemble average currents were then normalized to the maximal corrected current elicited from that patch and are plotted against the log of the ACh concentration. The single channel current voltage relationships were obtained by continuous application of ACh at the desired concentration with rampwise or steady-state voltage changes. Under these conditions the currents were digitized at 10 kHz and filtered at 2 kHz (8-pole Bessel, −3dB). All recordings were done at room temperature 22–25°C.

Toxin and competition binding

Whole oocyte binding was performed on 5–20 oocytes injected with wild type or mutant AChR channel RNA and a similar number of naive oocytes. The cells were incubated in (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes pH 7.6, 0.1% bovine serum albumin and saturating [^{125}I]-α-BgTx (1,000 counts/fmol) for 2–4 h, then washed with the same solution minus the labeled toxin. The oocytes were counted on an LKB model 1282 gamma counter. The number of counts per oocyte were corrected for nonspecific binding by subtracting the average number of counts bound to uninjected oocytes. The density of receptors in the oocyte surface membrane is estimated as fmols of bound toxin per oocyte.

Membrane preparation and toxin binding assays were performed as previously described (McLaughlin and Hawrot, 1989). Briefly, oocyte membranes were prepared fresh or in some cases from previously frozen cells. 100–200 oocytes were homogenized in 0.1 ml/oocyte of membrane binding buffer (in mM: 140 NaCl, 20 Na phosphate pH 7.5, 1 EDTA, 1 EGTA, 0.5 phenylmethylsulfonyl fluoride, and 200 U/ml aprotinin). Homogenates were centrifuged for 30 min at 20,000 × *g*, pellets were then resuspended in 0.04 ml/oocyte of membrane binding buffer and stored at −80°C.

Homogenized membranes (0.16 ml) were incubated for 3 h at room temperature in a final volume of 0.22 ml with 2.5 nM ^{125}I labeled-α-BgTx and the desired concentration of ACh. The samples were then diluted to 6 ml with cold Tris-buffered saline (in mM: 150 NaCl, 50 Tris-HCl pH 7.6) and filtered through Whatman GF/F filters and washed. The filters were counted on an LKB model 1282 gamma counter to determine the level of [^{125}I]-α-BgTx bound. Correction for nonspecific binding was made using a parallel protocol done in the presence of 1.25 μM unlabeled α-BgTx.

RESULTS AND DISCUSSION

Each mutant was assayed electrophysiologically to determine its functional response to ACh. Outside-out patches from injected oocytes were placed in a stream of bathing solution that could be switched rapidly (within 1–2 ms) to a solution containing a known, constant concentration of ACh. Patches from oocytes expressing the wild type or mutant channels typically contained many channels (~5–50) that opened rapidly in response to ACh application (Fig. 2). A dose-response curve for each channel variant was made by compiling ensemble averages of the responses to repeated presentations of each concentration studied, and measuring the peak of the current response (Fig. 2, *A* and *B*). All of the mutant channels respond to ACh, but the Y190F mutant is dramatically altered in its dose response and requires millimolar concentrations of ACh for channel opening (Fig. 2 *C*; Table 1).

This change in the sensitivity of the Y190F mutant could be due either to a change in the binding affinity of the receptor for ACh or to a change in the energetics of opening of the channel after binding. To distinguish between these possibilities, we assessed the ACh binding affinity of the mutants by measuring the ability of ACh to compete for [^{125}I]-α-bungarotoxin binding. The affinity of toxin itself is relatively unaffected by the mutations (Table 1), but the ability of ACh to prevent toxin binding is dramatically altered in the Y190F mutant. As for channel activation, the concentration of ACh required for competition in the Y190F mutant is several hundred-fold higher than that for wild type (Fig. 3). Thus, the entire physiological effect of the mutation can be explained by its effect on binding, without postulating an effect on transduction.

Binding of ACh to the AChR has not only the short-term consequence of opening the channel, but also the longer-term consequence of receptor desensitization. Desensitization reflects the closure of channels in spite of prolonged exposure to ACh, and it is also accompanied by an increase in ACh binding affinity (Katz and Thesleff, 1957; Sine and Taylor, 1979; Sine and Taylor, 1980). Two observations on the mutant channels indicate that the Y190F mutation affects ACh binding to the resting and the desensitized states of the channel in parallel. The first indication is that ACh competition with toxin binding, which is measured in long incubations and thus reflects binding to the desensitized state, parallels the results on the rapid physiological effects of ACh. The second indication comes from direct observation of desensitization rates during the

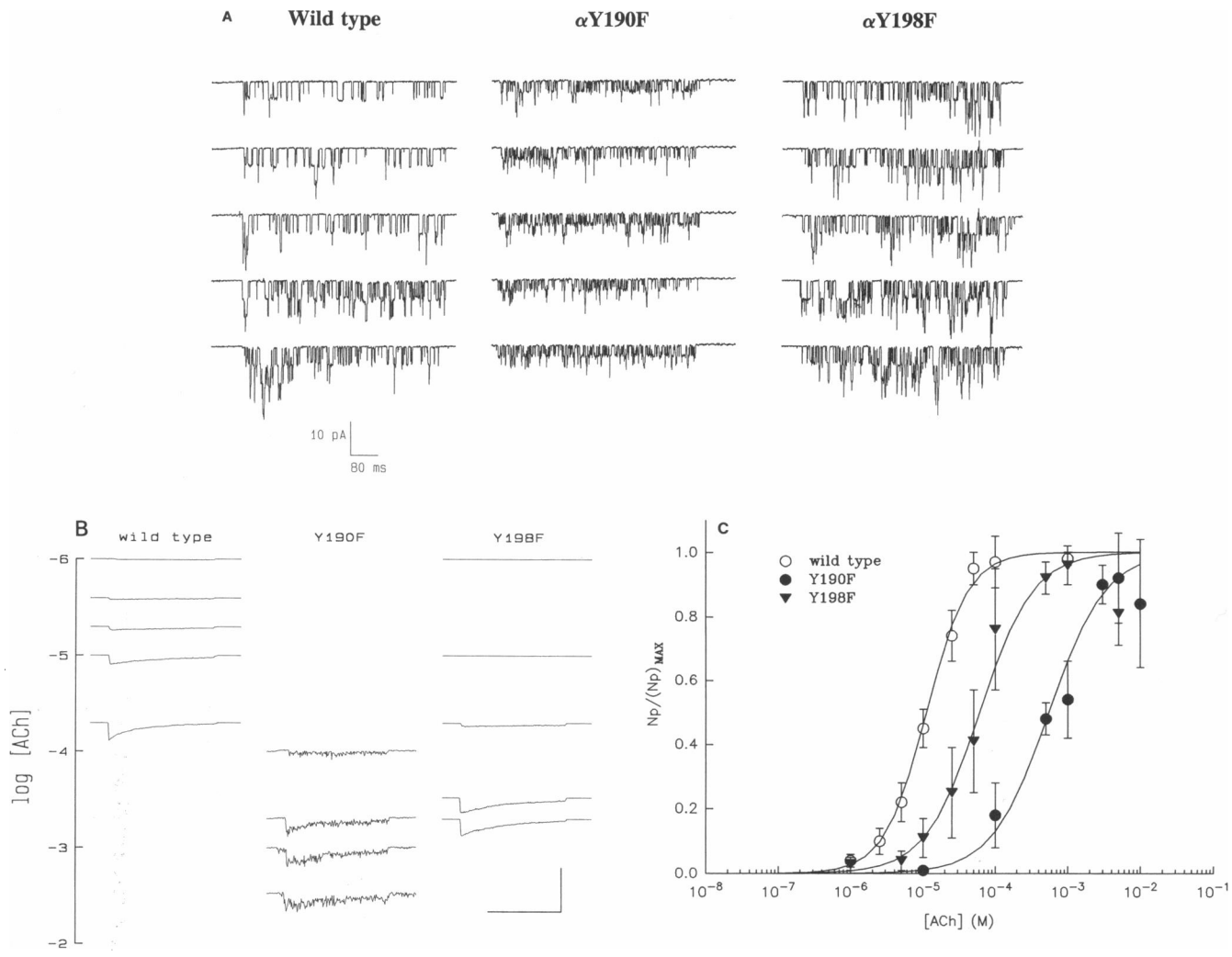

FIGURE 2 Physiological responses to a ACh. (*A*) Single channel current responses to the application of 1 μM (wild type), 10 μM (Y198F), or 1 mM (Y190F) ACh. Patches were held at a potential of −100 mV. These records were selected to show resolved single channel currents; most patches contained many more channels. (*B*) Ensemble average currents obtained at a series of ACh concentrations for wild type (1, 2.5, 5, 10, and 50 μM), Y190F (0.1, 0.5, 1, 3, and 5 mM) and Y198F (1, 10, 50, 300, and 500 μM). The vertical position of each of the averages is indicative of the ACh concentration that elicited that current. The vertical scale bar for each of the channel variants is 200 pA (wild type), 5 pA (Y190F), and 80 pA (Y198F). The horizontal scale bar represents 300 ms. (*C*) A plot of the normalized current corrected for ACh block against the log of the ACh concentration. Each point represents the mean of the peak of the ensemble average current corrected for ACh block and normalized to the maximal current elicited in that patch for wild type (○), Y190F (●), and Y198F (▼). The smooth curves are fits to the data generated as described in Table 1. The Hill coefficients for each of the channel types were optimized and had values of 1.5 ± 0.09 (wild type), 1.12 ± 0.1 (Y190F), and 1.17 ± 0.09 (Y198F).

physiological exposure to ACh. Desensitization is typically faster at higher concentrations of agonist (Fig. 2*B*). Desensitization of the Y190F mutant, however, remains slow even at concentrations that produce rapid desensitization in the wild type channel; rapid desensitization of the Y190F mutant occurs only at the millimolar concentrations required for maximal activation of the mutant. Thus, desensitization appears to be affected in parallel with activation. This observation argues against the proposed existence of separate ACh binding sites for activation and desensitization (Dunn and Raftery, 1982).

The Y190F mutant specifically affects the ligand binding properties of the AChR, and does not affect its open channel properties. The single-channel current voltage relationship is unaltered in the mutant (Fig. 4*A*). At high concentrations, ACh not only opens the AChR channels, it also acts to block current through the open channel (Sine and Steinbach, 1984). Such blockade

TABLE 1 **Affinity constants of wild type and mutant channels**

Receptor	Surface binding	α-BgTx K_d	EC_{50}	IC_{50}
	fmol/oocyte	*nM*	*μM*	*μM*
Wild type	1.5 ± 1.1 (7)	0.54 ± 0.1	11.0 ± 0.7 (6)	4.4 ± 2.0 (3)
H186F	1.9 ± 0.8 (5)	0.37 ± 0.1	21.8 ± 1.2 (7)	3.2 ± 1.4 (3)
Y190F	0.7 ± 0.3 (7)	0.49 ± 0.2	548 ± 52 (5)	338 ± 98 (3)
P194S	2.1 ± 1.5 (3)	0.66 ± 0.2	10.2 ± 0.1 (7)	3.6 ± 1.8 (3)
Y198F	0.9 ± 0.4 (4)	0.44 ± 0.1	60.3 ± 8.1 (5)	23.8 ± 4.8 (3)

The EC_{50} and IC_{50}s were estimated from a weighted least squares fit of the mean normalized current and percent of control α-BgTx binding, respectively, to the function $f = 1 - 1/(1 + ([ACh]/x)^n)$, where x is the EC_{50} or IC_{50} and n is the Hill coefficient (see Figs. 2 and 3 legend). The normalized current and percent of control binding are determined as described in Figs. 2 and 3, respectively. The EC_{50} and IC_{50}s are presented as the mean and standard deviation. In parentheses are the number of patches or membrane preparations for each channel variant.

The affinity of the receptors for toxin is similar as illustrated by the K_ds. The K_ds were determined by competition of labeled α-BgTx by unlabeled toxin values are the mean of three determinations. The data were normalized and fit as described for agonist competition of toxin binding.

appears as a voltage-dependent reduction in the open channel current at high ACh concentration. This reduction is identical in the wild type and mutant channels (Fig. 4 *B*).

The other mutations of conserved residues in this region have smaller or no effect on binding and activation by ACh. For each of the other mutants, the EC_{50}s and IC_{50}s are within an order of magnitude of the wild type values. The conserved proline residue at position 194 may conformationally constrain this region of the receptor, but the absence of an effect of the P194S mutation rules out a critical role for this residue. The basic residue His^{186} has been suggested to function in a charge relay system essential to ligand binding (Pearce and Hawrot, 1990; Pearce et al., 1990); however, mutation of this residue to Phe produces no change in binding

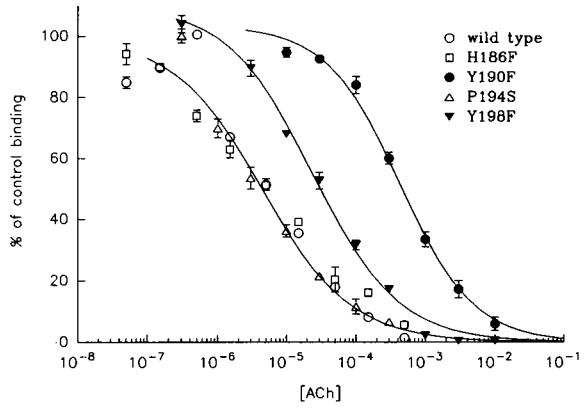

FIGURE 3 Concentration dependence of ACh inhibition of steady-state α-BgTx binding for the wild type receptor and the mutant receptor channels. Toxin binding at each ACh concentration was normalized to the [^{125}I]-α-BgTx binding in the absence of agonist. The normalized binding is the mean of at least three determinations at each ACh concentration for wild type (○), H186F (□), Y190F (●), P194S (△), and Y198F (▼). The data were fit and the IC_{50}s were determined as described in Table 1. Hill coefficients ranged from 0.7 to 1.3 for both wild type and mutant receptors.

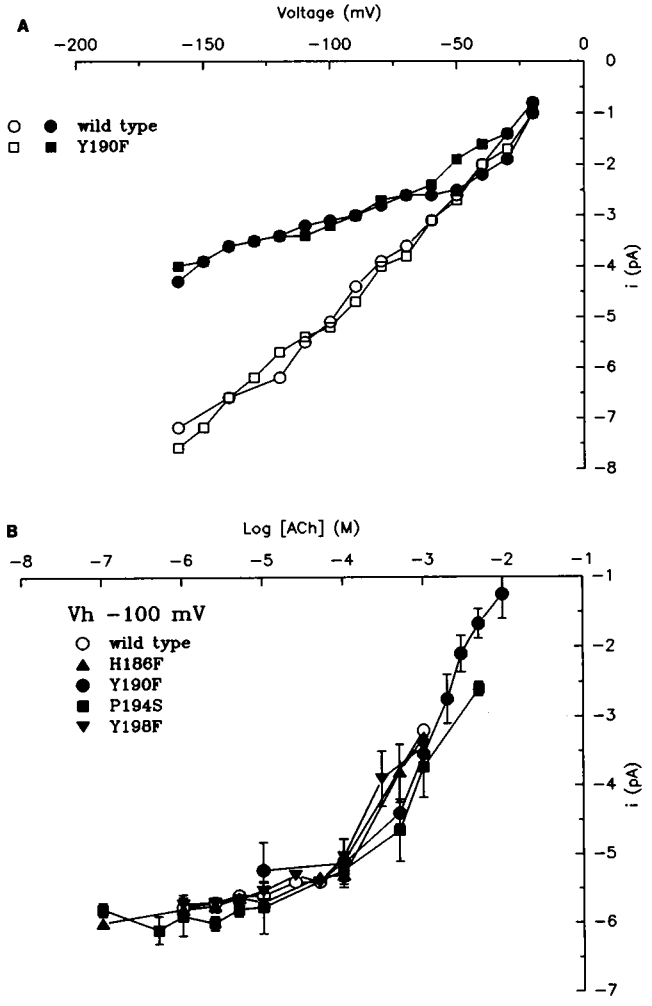

FIGURE 4 Open channel properties of the AChR are not altered by the mutations. (*A*) Representative single channel current voltage relationships for the wild type (○, ●) and Y190F (□, ■). ACh at 100 μM (*open symbols*) and 1 mM ACh (*solid symbols*). The conductances in the negative voltage range for both channel variants are identical. (*B*) Single channel current amplitude as a function of the ACh concentration for all channel variants at a holding potential of −100 mV: wild type (○), H186F (▲), Y190F (●), P194S (■), and Y198F (▼).

and only a small change in physiological response. Thus it is possible to alter conserved residues in this region without producing a nonspecific effect on binding and activation, supporting the notion that Tyr^{190} plays a particular and important role in these processes. The smaller but significant effect of the Y198F mutation also implicates this residue in binding and channel activation.

Another suggestion that either Tyr^{190} or Tyr^{198} might play an interesting role in ACh binding comes from spectroscopic studies of synthetic peptides from this region of the AChR that bind α-BgTx. The intrinsic fluorescence spectra of the peptides suggest the presence of a negatively charged tyrosinate (phenolate) ion at neutral pH (Pearce and Hawrot, 1990; Pearce et al., 1990). The authors of this work have suggested that one of these tyrosines might therefore be a good candidate for the hypothetical "anionic subsite" of the AChR binding site proposed to interact with the positively charged, quaternary ammonium moiety common to most cholinergic ligands. Is this proposal consistent with our observation that mutation of Tyr^{190} to Phe (which cannot ionize) produces a several hundred-fold shift in ACh binding? Perhaps. One might expect at first that removing a critical charge–charge interaction might have an even greater effect on binding affinity. The observed shift in the dose response corresponds to a change in binding energy of ~2–3 kcal/mol. This energy is comparable, for example, to the coulombic interaction energy of two point charges at a distance of 6–7 Å in a medium of dielectric constant 20. Thus the magnitude of the effect may be consistent with the proposed model.

We attempted to test this hypothesis that Tyr^{190} is the anionic subsite of the AChR by substituting other amino acids at this position, neutral glutamine or negatively charged glutamate. Both of these mutations produce shifts in the dose-response similar to that produced by the Y190F mutation (data not shown). It thus appears that tyrosine is unique in its ability to promote ACh binding at this position. This could mean that tyrosine, because of its size, its shape, and the hydrogen-bonding properties of its hydroxyl group, is critical for producing the proper folding of the ACh binding pocket. Alternatively, both the negative charge of the proposed tyrosinate and the aromatic character of tyrosine may be involved in the interaction between ACh and Tyr^{190}. Dougherty and Stauffer have suggested that aromatic residues are especially well suited for binding with the diffuse charge of quaternary ammonium moieties; their host-guest experiments with aromatic macrocycles support this notion, as do the known structures of several proteinaceous binding sites for quaternary amines (Dougherty and Stauffer, 1990). The recent discovery of a tyrosine residue critical for the high affinity blockade of potassium channels by tetraethylammonium may provide another such example (MacKinnon and Yellen, 1990).

We have observed a dramatic and specific effect of mutations of Tyr^{190} on the ACh binding and activation of the nicotinic AChR. Much higher concentrations of ACh are required to activate the mutant, and this change in sensitivity can be entirely accounted for by a change in the binding affinity of ACh. These results suggest that Tyr^{190} plays a critical role in producing the binding pocket for ACh. Determining the exact role of this and other residues in ACh binding will of course require further studies using both mutagenesis and structure determination.

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