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The Acetylcholine Receptor of the Neuromuscular Junction Recognizes Mecamylamine as a Noncompetitive Antagonist

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SUMMARY

The secondary amine, mecamylamine, interacts with the nicotinic receptor ionic channel complex as a noncompetitive antagonist. Mecamylamine (1–10 μM) blocked indirect muscle twitches with no discernible effect on the membrane potential, overshoot, or amplitude of the action potential. It also produced a voltage- and concentration-dependent depression of the peak amplitude of the endplate currents (EPC) and induced nonlinearity in the current-voltage relationship. The decay time constant of the EPC (τ_{EPC}) was significantly shortened. The linear relationship between the reciprocal of τ_{EPC} and the drug concentration suggested an open channel blockade. Patch-clamp studies, in agreement with the noise analysis results, revealed that mecamylamine (1–8 μM) shortened the lifetime of the open channels. Further, the single channel studies showed that at high concentrations mecamylamine reduced the double exponential nature of the distribution of open times characteristic of channels recorded from myoballs. Closed times had a complex distribution that could not be fitted to a single exponential function because of the presence of short closures or "flickers" during the open state. Although the frequency of channel openings progressively decreased with increasing drug concentration, the single channel conductance remained unchanged at all the concentrations tested. Biochemical studies showed that mecamylamine (up to 100 μM) did not block [^{3}H]acetylcholine binding to the nicotinic receptor of the *Torpedo* electroplax, but inhibited the binding of [^{3}H]perhydrohistionicotoxin to its channel site, both in the resting and the activated state. These results suggested that, at the nicotinic receptors of the neuromuscular junction, mecamylamine acted as a noncompetitive blocker, binding primarily to the receptor's open channel conformation. Most of the alterations of EPCs were consistent with the predictions of a sequential model for open channel blockade. Biochemical and patch-clamp results, however, could not be fully explained by this model and provided some evidence of the existence of additional blocked states most likely through pathways into desensitized species. In contrast to a competitive antagonism of acetylcholine receptors reported at autonomic ganglia, there was no such action of the drug at the neuromuscular junction; thus, mecamylamine is a useful tool to characterize the nicotinic receptors from different synapses.

INTRODUCTION

Mecamylamine (*N*-2,3,3-tetramethylbicyclo[2.2.1] heptan-2-amine) (*inset* of Fig. 1) is a secondary amine with a p*Ka* of 11.4 (1) which "blocks ganglionic transmission by occupying receptor sites and stabilizing the postsynaptic membranes against the actions of acetylcholine liberated from presynaptic nerve endings" (2). Its effects on ganglionic mediated responses are qualitatively similar to those exhibited by the quaternary ammonium compounds, hexamethonium and pentolinium. In addition to its competitive antagonism of ganglionic transmission (3, 4), the attenuation of indirectly elicited

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twitches of frog rectus abdominis muscles suggested a weak curare-like effect on myoneural junctions (3). Recently, the molecular actions of mecamylamine were studied in more detail on the parasympathetic neurons of rat submandibular ganglion (5). These studies disclosed that the blockade induced by mecamylamine of (ACh)[5]-activated currents was voltage-independent and decreased with increasing agonist concentration. This effect was interpreted as a competitive antagonism by the drug of the nicotinic receptor, thus preventing the binding of the agonist to its recognition site. Mecamylamine also interacts with nicotinic receptors of the central nervous system, antagonizing the nicotine-induced convulsions in mice and Renshaw cell unit potentials in cats (6, 7).

In light of the varied effects of mecamylamine on different nicotinic synapses, we decided to investigate the nature of mecamylamine's actions on the AChR of frog and mammalian skeletal muscles, as well as its effects on the binding of ligands to *Torpedo* AChR. To this end, (i) voltage-clamp studies were performed on the frog neuromuscular junction, (ii) single channel currents were recorded from cultured neonatal rat muscles, and (iii) binding assays, using receptor and ionic channel ligands, were performed on *Torpedo* electric organ membranes. The present results, consonant with the preliminary evidence (8), support the hypothesis that, at the nicotinic receptors of the neuromuscular junction, mecamylamine acts as a noncompetitive antagonist of the nicotinic AChR complex. Thus, a question is raised regarding the functional dissimilarities between the peripheral nicotinic receptors located at the skeletal neuromuscular junction and those located at the ganglionic synapses and at certain areas of the central nervous system. Mecamylamine appears to differentiate between subtypes of the nicotinic AChR (9) at various cholinergic synapses.

METHODS

Tissue preparations and solutions. Muscle twitch, EPC, and noise analysis experiments were performed on a sciatic nerve-sartorius muscle preparation of the frog *Rana pipiens* at room temperature (20–22°). Normal frog Ringer's solution used in these experiments had the following composition (millimolar): NaCl 116; KCl, 2; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; NaH_2PO_4, 0.7. The solution, saturated with O_2, had a pH of 6.9–7.0. The dissection and mounting of the preparations for muscle twitch, EPC, and noise analysis experiments are described elsewhere (10, 11).

Patch-clamp studies were performed on rat myoballs at a temperature of 10°. The cells were cultured from hind limb muscles of neonatal rats (Dublin, VA (Sprague-Dawley) Dominion Laboratories) according to procedures described elsewhere (12). Immediately upon removal of culture dishes from the incubator, the nutrient medium was replaced by a modified Hanks' salt solution with the following composition (millimolar): NaCl, 137; KCl, 5.4; $NaHCO_3$, 4.2; $CaCl_2$, 1.3; $MgSO_4$, 0.81; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; D-glucose, 5.5; HEPES, 10. The pH of this solution was 7.2, and the osmolarity was adjusted to 340 mosM with sucrose. All the solutions for patch-clamp, as well as for noise analysis experiments, contained tetrodotoxin (0.3 μM) to inhibit spontaneous cell contractions.

[5] The abbreviations used are: ACh, acetylcholine; AChR, acetylcholine receptor-ionic channel complex; α-BGT, α-bungarotoxin; H_{12}-HTX, perhydrohistrionicotoxin; EPC, endplate current; τ_{EPC}, decay time constant of EPC; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

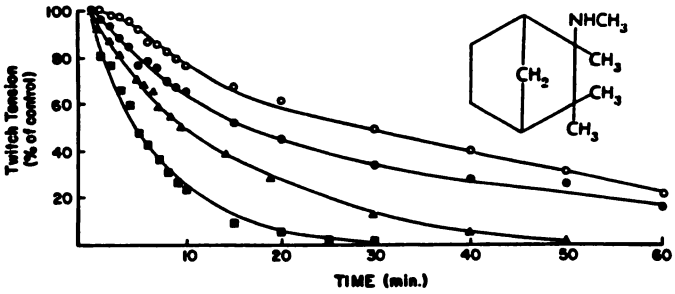

FIG. 1. *Concentration- and time-dependent effect of mecamylamine on the nerve-elicited twitches of frog sartorius muscle.*

Indirect twitch tension expressed as a percentage of control values is plotted against time (in minutes) for 1 (○), 2 (●), 4 (△), and 10 (■) μM mecamylamine. Each point represents the mean ± SE of observations made in three muscles.

For binding studies, the electric organ of *Torpedo californica* (Pacific Bio-Marine Laboratories, Venice, CA), stored at −90°, was homogenized in 2 volumes of *Torpedo* Ringer's solution (millimolar composition): NaCl, 154; Na_2HPO_4, 5; EDTA, 1; diisopropyl fluorophosphate, 0.1; phenylmethylsulfonyl fluoride, 0.1; NaN_3, 0.02% (pH 7.0), using a Polytron blender and centrifuged for 10 min at 3,000 rpm (4°) in a Sorvall 85-34 rotor. The supernatant fraction was saved at 2°, and the pellet was rehomogenized in the same volume of buffer and centrifuged for 10 min at 3,000 rpm. Supernatant fractions were then combined and centrifuged at 17,000 rpm (30,000 × *g*) for 60 min. The pellets (1 g of original tissue) were suspended in 1 ml of *Torpedo* Ringer's solution (final volume).

Muscle twitch recordings. Twitches of the sartorius muscle were indirectly elicited by stimulating the sciatic nerve (supramaximal rectangular pulses with duration of 0.05–0.2 msec) at a rate of 0.2 Hz and were recorded on a Grass ink-writing polygraph.

Endplate current recordings. For experiments on nerve-evoked EPCs, sartorius muscles were glycerol-treated (600 mM glycerol for 60 min and subsequent washing with normal physiological solution) in order to abolish excitation-contraction coupling. EPCs were elicited every 3 sec by a supramaximal voltage pulse (0.05-msec duration) and were recorded using the conventional two-electrode voltage-clamp technique described elsewhere (10, 11). Membrane voltage was changed from a holding potential, close to the resting potential, in 10-mV steps, covering the range from +50 to −150 mV. The DC current and voltage traces after amplification were sent on-line to a digital computer (PDP 11/40, Digital Equipment Corp., Marlboro, MA), digitized at 10 kHz, and stored for later analysis. Mecamylamine (2–16 μM) was dissolved in the bathing medium, and the recordings were begun 30 min after addition of the drug. τ_{EPC} was determined by linear regression on the logarithms of the EPC decay points between 80 and 20% of the peak current.

Acetylcholine-induced endplate current fluctuation analysis. Noise analysis experiments were carried out in muscles, using the same voltage-clamp circuit as that used for EPC recordings. EPCs were induced by microiontophoresis of ACh from a low resistance electrode containing 3 M ACh positioned about 25–50 μm above the junctional region. Usually, currents of 20–120 nA were induced for 30 sec using a constant current source (Howland current pump), with adequate braking current applied to prevent the agonist leakage. The currents were amplified, filtered to 1–800 Hz by a Krohn-Hite 3700 band-pass filter, and recorded on FM tape (Racal-Milgo, Miami, FL) along with an adequate portion of base-line current noise for later computer analyses. Spectral analysis, described previously (11, 13), provided single channel conductance (γ) and channel lifetime (τ_I).

Single channel current recordings. ACh-activated single channel currents were recorded using patch-clamp technique (14). Microelectrodes were pulled in two stages from microhematocrit tubes (length =

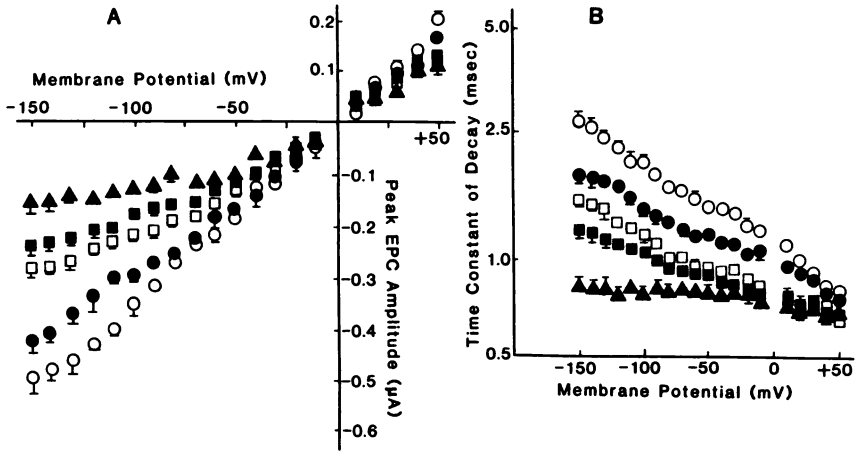

FIG. 2. *Effects of mecamylamine on the peak amplitude and decay time constant (τ_{EPC}) of the endplate currents*

A. Relationship between the EPC peak amplitude and the membrane potential under control conditions (○) and after 30–60 min of exposure to mecamylamine at 2 (●), 4 (□), 8 (■), and 16 (▲) μM. B. Relationship between the logarithm of τ_{EPC} and membrane potential under control conditions and in the presence of different concentrations of mecamylamine (symbols are the same as A). Each point represents the mean ± SE of 5–20 fibers from 5–8 muscles. Where no bar appears, the values are too small to be shown.

75 mm; inner diameter = 1.1–1.2 mm) using a vertical electrode puller (David Kopf Instruments, Tujunga, CA). The tips of the microelectrodes were heat-polished, and their shanks were coated with Sylgard. The resistance of these micropipettes ranged from 2 to 6 megaohms when they were filled with normal Hanks' solution. Patch microelectrodes were filled with solutions of ACh (0.2 μM), either alone or combined with mecamylamine (1–8 μM). Single channel currents were recorded from both cell-attached and inside-out patches. An LM-EPC-5 patch clamp (List Electronics, West Germany) was used to measure the currents, which were low-pass filtered to 3 kHz (second-order Bessel), and the data were stored on FM magnetic tape for computer analysis. The data were sent to the computer through a fourth-order Butterworth low-pass filter (2–3 kHz) to improve the signal-to-noise ratio and were digitized at 10 kHz by an LPS-11 12-bit analog to digital converter. Single channel currents were analyzed, as described previously (12), on a PDP 11/40 minicomputer. Histograms of closed and open times were fitted to a single exponential function, or, if the fit was considered inadequate (correlation coefficient < 0.95), to a double exponential function using a nonlinear regression program.

Binding assays. [^{3}H]ACh (specific activity 90 mCi/mmol, New England Nuclear) binding was measured by equilibrium dialysis using tissue sample volume of 250 μl, bath volume of 10 ml, and sample aliquots of 50 μl. [^{3}H]ACh (0.01 or 0.1 μM) and drugs to be tested were placed in the buffered Krebs-Ringer bath containing 0.1 mM diisopropyl fluorophosphate and were equilibrated with the tissue sample, placed inside a dialysis sac, and shaken in the bath for 4 hr at 23°. Controls for nonspecific binding were obtained from *Torpedo* membranes preincubated with 10 μM *Naja* α-neurotoxin.

[^{3}H]H_{12}-HTX (specific activity 21 Ci/mmol) binding to *Torpedo* membranes was measured by a filter assay adapted from that previously reported (15). In this method, [^{3}H]H_{12}-HTX (2 nM) is incubated with *Torpedo* membrane in a final volume of 1 ml at 21° for either 30 sec or 120 min in the presence or absence of agonist, respectively.

Binding of ^{125}I-α-BGT (specific activity 16.9 μCi/μg, New England Nuclear) to *Torpedo* membranes was measured by the ion exchange minicolumn method. In this method, *Torpedo* ACh receptors (1 nM) present in 200 μl of Na_2HPO_4 buffer (pH 7.4) were incubated with ^{125}I-α-BGT (10 nM) for 40 sec, and 100 μl of the incubation mixture was transferred to a Whatmann CM52 cation exchanger minicolumn. The reaction was terminated immediately upon addition of the sample to the column, which was washed with 0.5 ml of buffer to separate bound from free toxin. Radioactive contents of the column were counted in a TriCarb auto γ-counter. The binding rate of ^{125}I-α-BGT to *Torpedo* membranes in 40 sec was used as a measure of the initial rate of toxin binding (16). Specific binding of ^{125}I-α-BGT was determined by measuring the amount bound to *Torpedo* membranes which had been preincubated with 10 μM *Naja* α-neurotoxin and by subtracting this value from the ^{125}I-α-BGT bound to untreated *Torpedo* membrane.

Statistical analysis. Where applicable, Student's unpaired *t* test or analysis of variance was used to compare data from control and experimental conditions. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of mecamylamine on nerve-elicited twitch tension of frog sciatic nerve-sartorius muscle preparation. The time course of mecamylamine-induced depression of the evoked muscle twitches is shown in Fig. 1. Exposure of the preparation for 30 min to 10 μM mecamylamine caused complete blockade of the neuromuscular transmission, while at concentrations of 1, 2, and 4 μM, twitch tension was reduced to 50, 35, and 15% of control values, respectively. Continuous drug exposure resulted in a progressive decrement of muscle responses, ultimately leading to a complete block. Neither the resting membrane potential nor the directly evoked action potential was affected by any concentration of mecamylamine tested, indicating that the blockade did not involve membrane excitability. The blockade of twitch was reversible upon washing for up to 1 hr.

Concentration-dependent effect of mecamylamine on nerve-evoked endplate currents. Exposure to mecamylamine (2–16 μM) for 30 min induced a voltage-dependent depression of the EPC peak amplitude (Fig. 2). The magnitude of this depression was more pronounced at hyperpolarized potentials, such that in contrast to control conditions, a nonlinear current-voltage relationship was observed in the presence of mecamylamine (Fig. 2A). In addition, mecamylamine significantly shortened τ_{EPC} (Fig. 2B), and increasing concentrations of the drug produced an apparent loss of the voltage sensitivity seen under control conditions. The slope of the semilogarithmic plot of the τ_{EPC} versus voltage was changed from

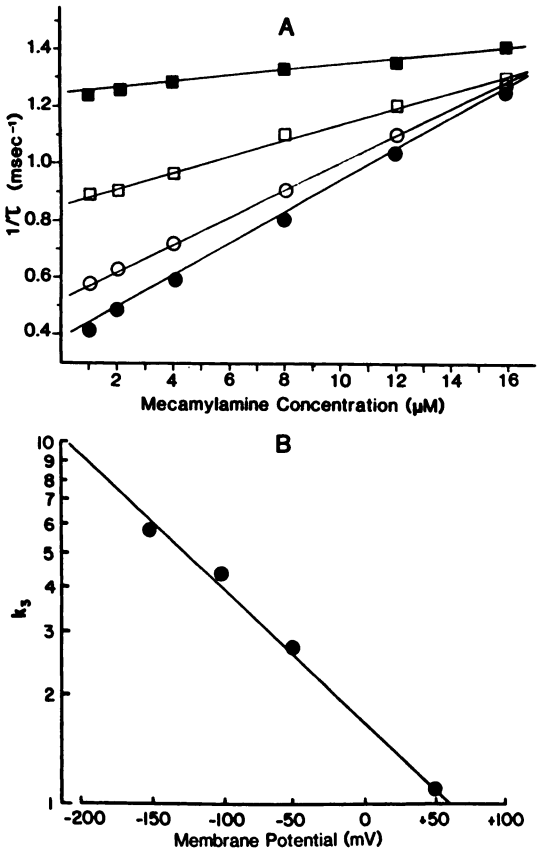

FIG. 3. *Relationship between the reciprocal of τ_{EPC} and mecamylamine concentration (A) and voltage-sensitivity of the forward rate constant for mecamylamine blockade (B).*

A. Membrane potentials are +50 (■), −50 (□), −100 (○), and −150 (●) mV. B. Forward rate constants ($k_3 \times 10^7$ sec^{-1}M^{-1}) were determined from the slopes of the plots of the reciprocal of the time constant of decay versus mecamylamine concentration (A).

8×10^{-3} sec V^{-1} under control conditions to 9×10^{-4} sec V^{-1} in the presence 16 μM mecamylamine (Fig. 2B). This finding is consistent with an opposite voltage dependence of the rate constant of decay (k_{-2}) in the absence of drug and with that of the blocking reaction (k_3) (see "Discussion"). Under all experimental conditions, the decay of the EPCs remained a single exponential function of time. There was a linear relationship between the reciprocal of τ_{EPC} and the mecamylamine concentration (2–16 μM) (Fig. 3A). The exponential dependence on voltage of the rate constant k_3 is seen in Fig. 3B.

Effect of mecamylamine on acetylcholine-induced endplate current fluctuations. The effect of mecamylamine (2–12 μM) on the mean open time and conductance of ACh-activated channels was determined from fluctuation analysis performed on the endplate region of the frog sartorius muscle. The power spectra disclosed only one component and showed a shortening of the channel lifetime (Table 1). In addition, increasing concentrations of mecamylamine produced a decrease in the voltage dependence of the channel open time, in a manner similar to that observed with τ_{EPC}. Single channel conductance was not significantly altered at these concentrations of mecamylamine.

TABLE 1

Effects of mecamylamine on single channel conductance (γ) and lifetime (τ_1) determined from fluctuation analysis performed in frog muscle postjunctional region at various membrane potentials (E_m)

	n^a	E_m	γ	τ_1
		mV	*pS*	*msec*
Control				
	9	−70	23.7 ± 0.77	0.98 ± 0.08
	14	−80	21.3 ± 1.16	1.17 ± 0.07
	10	−90	20.7 ± 1.17	1.32 ± 0.07
	7	−100	21.9 ± 3.13	1.59 ± 0.09
Mecamylamine				
2.0 μM	4	−70	19.5 ± 1.85	0.92 ± 0.03
	6	−80	23.7 ± 2.62	1.01 ± 0.08
	10	−90	20.7 ± 1.23	1.14 ± 0.05
	13	−100	20.3 ± 2.19	1.37 ± 0.03
4.0 μM	10	−75	22.6 ± 2.21	0.90 ± 0.02
	5	−80	20.2 ± 2.33	0.93 ± 0.03
8.0 μM	9	−80	17.1 ± 2.33	0.81 ± 0.04[b]
	3	−90	26.2 ± 2.71	0.90 ± 0.09[b]
	7	−110	23.0 ± 3.01	1.22 ± 0.07[b]

[a] *n*, number of spectra.
[b] $p < 0.05$.

Effect of mecamylamine on ACh-activated single channel currents. Single channel currents were recorded from rat myoballs with microelectrodes containing either ACh (0.2 μM) alone or combined with mecamylamine (1–8 μM) (Fig. 4). ACh-activated channel currents were similar to those previously reported by Akaike *et al.* (12) and Aracava *et al.* (17). In rat myoballs, channel openings with three different conductance states, ~10, 20, and 33 pS, were observed. The intermediate current level was the most prevalent and corresponded to about 90% of the total opening events recorded (Fig. 4A). The plot of these channel currents versus holding potential obtained from inside-out patches showed a linear relationship between −40 and −160 mV, and the estimated reversal potential was close to 0 mV. In addition, the open time histograms showed an excessive number of very fast events (<2 msec) that accounted for a departure from a single exponential distribution (Fig. 5A). The double exponential distribution of these open times suggests the existence of at least two open states. The closed time histograms also showed two distinct components (Fig. 6), the fast one representing the very short closures (<8 msec) during the open state (intra-burst gaps) and the slow component corresponding to the long intervals (second range) between two successive events. The frequency of opening events appeared to be voltage-sensitive, with the probability of channel opening higher at more negative potentials; however, we observed large variations in the channel-opening frequency from one myoball culture to another, presumably related to the heterogeneity in the density of AChRs or their distribution in clusters.

Mecamylamine, when included in the patch pipette with ACh (0.2 μM), produced a concentration-dependent shortening of the mean channel open time (Figs. 4 and 5). The relative proportion of the fast component of the open time histograms was decreased with increasing concentrations of mecamylamine such that, at 8 μM, the distribution of the open times was fit to a single expo-

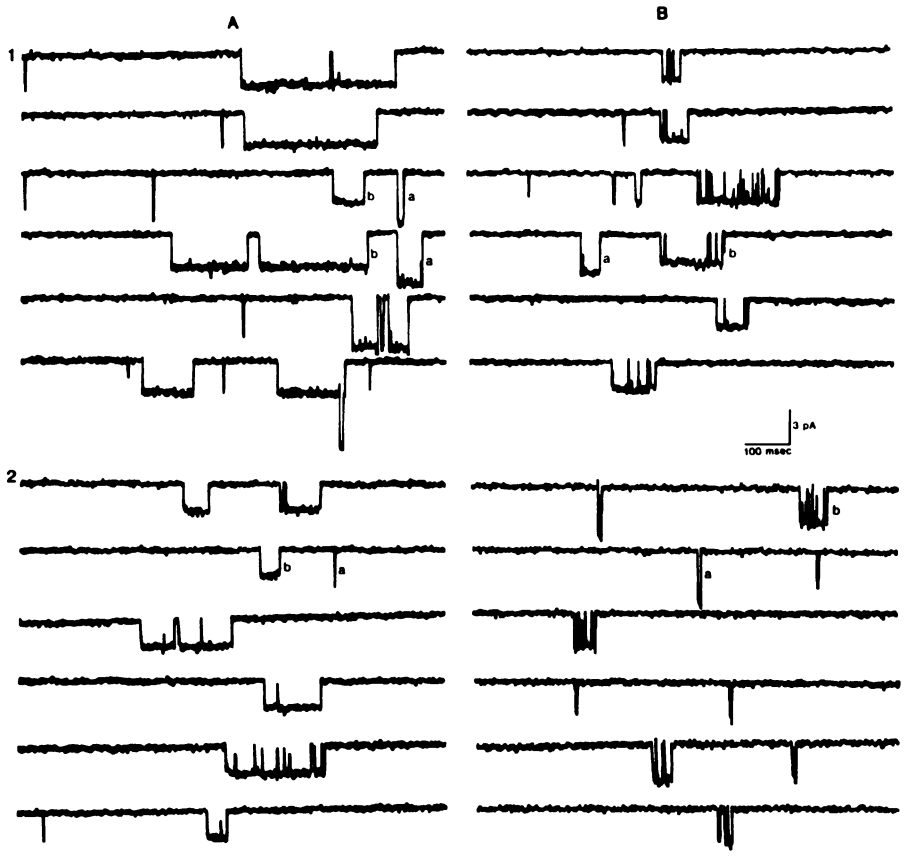

FIG. 4. *Samples of ACh-activated single channel currents recorded in the absence and presence of mecamylamine*
The microelectrode contained either ACh at 0.2 μM alone (A1) or combined with mecamylamine at 1 (A2), 4 (B1), or 8 (B2) μM. *a* and *b* represent channel openings with conductance of 33 and 20 pS, respectively.

nential function (correlation coefficient of 0.96). It should be noted that the loss of the fast component could be due in part to an inability of instrumentation to resolve very rapid events. The effects of mecamylamine on the shortening of the channel open state were more prominent at more negative potentials. At −80 mV, mecamylamine concentrations of 1, 2, and 8 μM reduced the mean open times by 33, 57, and 78% of the control values, respectively, while at −120 mV, they decreased the mean open times by 41, 66, and 86% of the control values, respectively (Fig. 7A). The plots of the reciprocal of mean channel open time versus mecamylamine concentration showed a linear relationship, suggesting a block of the open conformation of ACh-activated channels (Fig. 7B). Also, mecamylamine induced the appearance of events with "flickering" activity during the open state. Some of these events are shown in Fig. 4. The histograms of the short closures within a burst showed, under control conditions, a mean of 0.3–0.5 msec and a distribution that was fit to a single exponential function. In the presence of mecamylamine (1–8 μM), although the number of events was limited, the histograms of the fast closed times appeared to be fit by a double exponential function, the faster of the two resembling that seen under control conditions, and an additional slower component with a time constant of about 1.5–2.2 msec (*insets* of Fig. 6). Mecamylamine at all concentrations tested did not change the single channel conductance or reversal potential (Fig. 8).

Effects of mecamylamine on the binding of receptor and ionic channel ligands to Torpedo electric organ membranes. Mecamylamine, at concentrations of 1.0–100 μM, did not block the binding of 0.1 μM [^{3}H]ACh to *Torpedo* ACh receptors, but rather slightly increased it. Under the same conditions, *d*-tubocurarine inhibited [^{3}H]ACh binding in a concentration-dependent manner (Fig. 9). At lower concentration of [^{3}H]ACh (specific activity 2.6 Ci/μmol Amersham Corp., Arlington Heights, IL) (10 nM), mecamylamine (0.01–100 μM) did not significantly affect ACh binding. Saturation isotherms of [^{3}H]ACh binding, in the absence and presence of 1 μM mecamylamine, showed similar K_d and B_{max} values (data not shown). We tested the effect of this agent on the specific binding of [^{3}H]H_{12}-HTX under experimental conditions where the AChRs would be in either resting or activated states. In the absence of agonist, AChRs, mostly in the resting state, bound [^{3}H]H_{12}-HTX slowly, reaching equilibrium after 120 min. In the presence of saturating concentrations of an agonist (e.g., carbamylcholine, 100 μM), when AChRs would be mostly in the activated and/or desensitized states, maximal binding of [^{3}H]H_{12}-HTX occurred within seconds. Mecamylamine inhibited the binding of [^{3}H]H_{12}-HTX in the presence and absence of agonist (Fig. 10).

The binding of certain channel blockers to AChRs

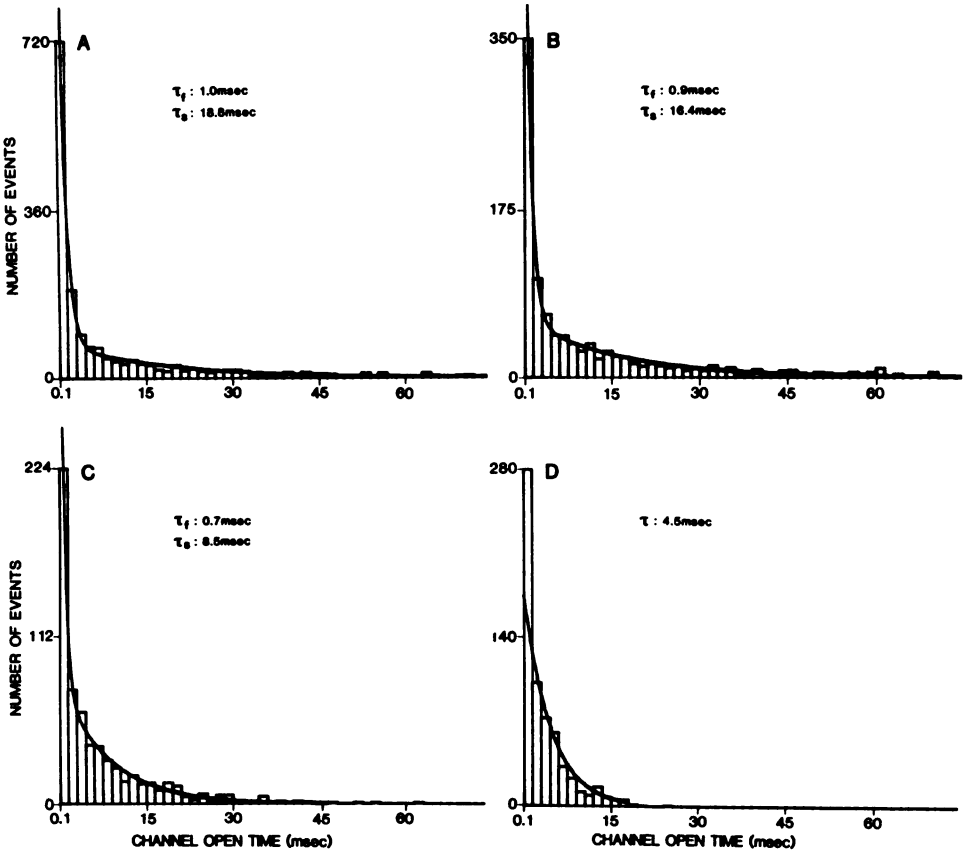

FIG. 5. *Open time histograms of ACh-activated single channels in the absence and presence of mecamylamine*

Channel currents were recorded with a patch pipette containing ACh at 0.2 μM alone (A) or combined with mecamylamine at 1 (B), 4 (C), and 8 (D) μM. The histograms represent 1790 (A), 1050 (B), 639 (C), and 634 (D) events analyzed. In A–C, the time constants of the fast (τ_f) and slow (τ_s) components (amplitude ratio of 20, 12, and 6.5 for A–C, respectively) were estimated from the best fit to a double exponential function. In D, a single exponential was fitted with a correlation coefficient of 0.96.

induces or enhances receptor desensitization, which may be detected using an α-BGT binding rate assay. In this assay, preincubation of *Torpedo* membranes with carbamylcholine for 30 min increased the agonist-induced inhibition of ^{125}I-α-BGT binding in comparison to the measurements made after 40 sec of simultaneous exposure to both ligands, reflecting an increased receptor affinity for the agonist (Fig. 11). Incubation of *Torpedo* membranes with mecamylamine (5 and 15 μM) for 30 min, prior to a 40-sec exposure to carbamylcholine and α-BGT, had no effect on the concentration-response curve of the agonist. However, at a high concentration (100 μM), mecamylamine, which inhibited 18% of ^{125}I-α-BGT binding in the absence of carbamylcholine, potentiated the inhibition of α-BGT binding produced by a 40-sec exposure to carbamylcholine (Fig. 11). This shift in the concentration-response curve was equivalent to preincubation with carbamylcholine for 30 min in the absence of mecamylamine. In other words, it appeared that mecamylamine at high concentrations (100 μM) increased the receptor's affinity for carbamylcholine.

DISCUSSION

In the frog sartorius preparation, mecamylamine (2–16 μM) blocked neuromuscular transmission, producing a voltage- and concentration-dependent depression of the EPC peak amplitude and a shortening of τ_{EPC}. Most of the effects of mecamylamine on EPCs can be predicted by the sequential model (18, 19) represented by the following:

$$nA + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_nR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_nR^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} A_nR^*D \quad (D)$$

where nA represents n agonist molecules, R is the resting state of the nicotinic receptor-ionic channel complex, A_nR is the agonist-bound closed state, A_nR^* is the state with the channel in open conformation, and A_nR^*D is the blocked nonconducting state in the presence of the drug, D. The rate constants are as indicated. According to this model, one assumes that, in the presence of an open channel blocker, EPC decay is governed by both k_{-2} and k_3. The opposite voltage dependence of these two rate constants resulted in an apparent loss of the voltage sensitivity of τ_{EPC} with increasing concentrations of mecamylamine, shown in the Fig. 2B. In addition, if the unbinding rate constant (k_{-3}) is considered negligible, the EPC decays should be adequately described by a single exponential function of time and τ_{EPC} should be shortened according to the following expression:

$$1/\tau_{EPC} = (k_{-2} + [D]\, k_3)$$

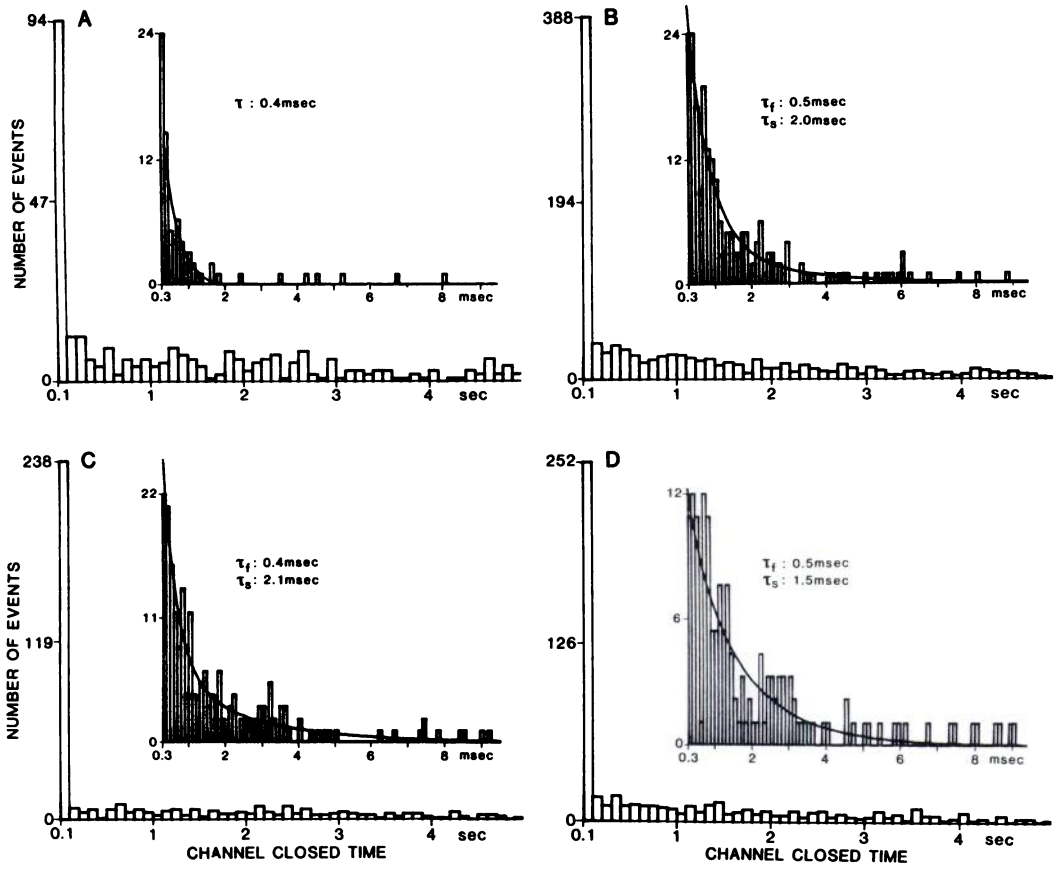

FIG. 6. *Histograms of the closed times of ACh-induced channel currents in the absence and presence of mecamylamine*

Single channel currents were recorded with the pipette containing ACh at 0.2 μM alone (A) or combined with mecamylamine at 1 (B), 4 (C), or 8 (D) μM. The histograms of the total closed times (seconds scale) are composed of at least two components. *Insets*: The histograms represent an expansion of the first bin (millisecond scale) of the plots of the total closed times and contain 75 (A), 218 (B), 196 (C), and 151 (D) events. In A the *solid line* represents the fit of the distribution to a single exponential (correlation coefficient = 0.95) and in B–D to a double exponential function with an amplitude ratio of 7, 6, and 0.5, respectively.

where k_{-2} is the rate constant of decay under control conditions (i.e., in the absence of the drug), and k_3 is the second-order, forward rate constant for drug binding. Consistent with the predictions of the sequential model, a linear relationship between $1/\tau_{EPC}$ and mecamylamine concentration (up to 16 μM), and an exponential dependency on voltage of the estimated forward rate constant of drug binding (k_3) were observed (Fig. 3). From the voltage sensitivity of k_3 described by a Boltzmann distribution, one can estimate the location of the energy barrier for mecamylamine interactions within the membrane electric field as follows: $k_3(V) = k_3(0)\ \exp(-neV\delta/kT)$, where $k_3(V)$ is the rate constant at a given potential, V, $k_3(0)$ is the rate constant at 0 mV, ne is the drug charge, k is the Boltzmann's constant, T is the absolute temperature, and δ is the fraction of the membrane potential that influences k_3. For mecamylamine δ was determined to be 0.18.

Noise analysis and single recordings revealed a concentration-dependent shortening of the open state of the ionic channels without change in single channel conductance, in agreement with the sequential model described earlier. Also, biochemical results showed that mecamylamine is a noncompetitive antagonist of the nicotinic receptor, binding to the [^{3}H]H_{12}-HTX site at the ionic channel of activated AChR. Mecamylamine binds as well to the resting or unliganded AChR (Fig. 10), and at very high concentrations (>100 μM) there was evidence of enhancement of agonist-induced receptor desensitization (Fig. 11). Mecamylamine did not induce the marked time-dependent depression of the peak EPC amplitude characteristic of blockade of closed conformation or desensitization of the nicotinic receptor and clearly observed with drugs such as H_{12}-HTX (20) and meproadifen (21). However, patch-clamp recordings revealed details that, taken together with the biochemical findings, suggest the existence of pathways for mecamylamine actions other than that described by the sequential model for open channel blockade. In the absence of drug, the histograms of the short closures within a burst could be fitted to a single exponential function, with a mean of 0.3–0.5 msec (inset of Fig. 6A). These short gaps have been reported in different preparations, such as frog skeletal muscles (22), cultured skeletal muscle of rat (17), and human (23), as well as reconstituted AChR in lipid bilayers (24). These have been interpreted to reflect the fast transitions between the agonist-bound closed conformation and the open state of the AChR

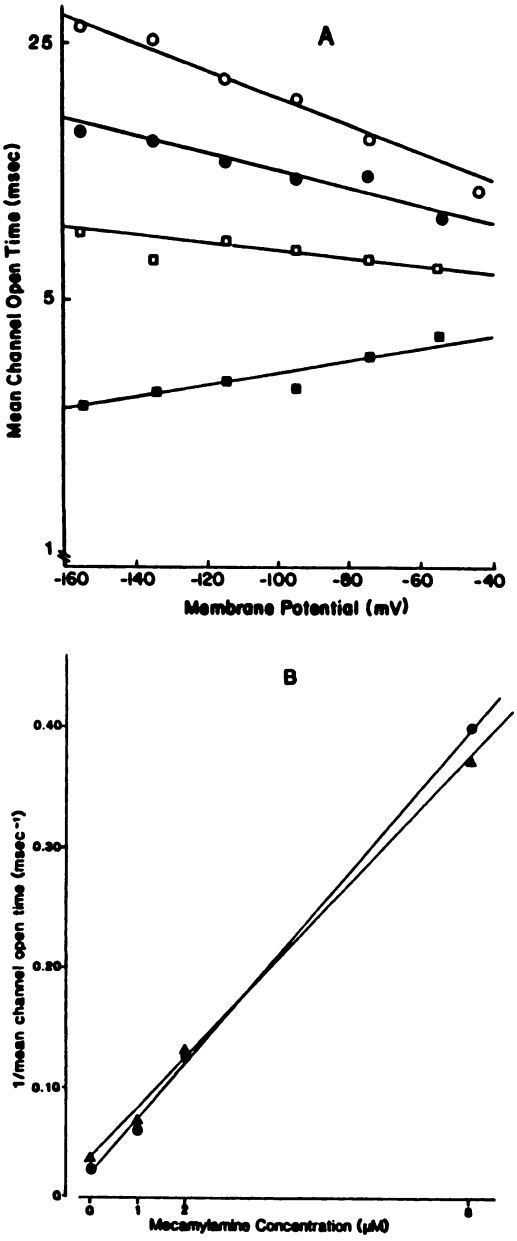

FIG. 7. *Concentration-dependent shortening of mean channel open time produced by mecamylamine and reciprocal of the mean channel open time versus mecamylamine concentration*

A. Single channel currents were recorded either from cell-attached or inside-out patches with micropipettes containing ACh at 0.2 μM alone (○) or combined with mecamylamine at 1 (●), 2 (□), and 8 (■) μM. Temperature was 10° B. Relationship of reciprocal of the mean channel open time versus mecamylamine concentration. Holding potentials: −140 mV (▲) and −160 mV (●).

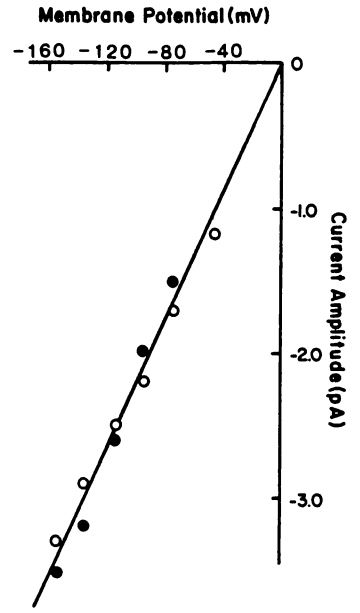

FIG. 8. *Current-voltage relationship of ACh-activated channel currents in the absence and presence of mecamylamine*

Single channel currents were recorded with pipettes containing ACh at 0.2 μM alone (○) or combined with mecamylamine at 2 μM (●).

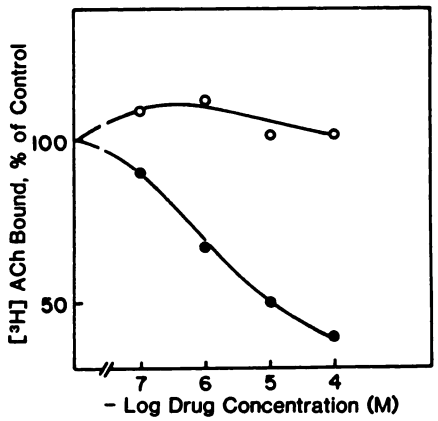

FIG. 9. *Comparative effects of mecamylamine and d-tubocurarine on the binding of [³H]ACh to T. californica membranes*

Inhibition of [³H]ACh (0.1 μM) binding in the presence of varied concentrations of mecamylamine (○) or *d*-tubocurarine (●) is plotted as percentage of control values.

complex (22). In the presence of mecamylamine, events with an increased number of short gaps relative to control were recorded, and an additional slow component in the histograms of the fast closed times was observed (Figs. 6, B–D). It has been reported that the local anesthetic QX 222 (25) induced multiexponential distributions of the closed times and increased the number of gaps to a magnitude 1 to 2 orders higher than that observed in the absence of the drug, which may form the basis for the appearance of multiple decays of the EPCs (18). In the case of mecamylamine, the number of gaps per burst was slightly increased at concentrations up to 4 μM; however, at 8 μM, this effect was less clear, an observation which would not be expected based on the sequential model. As reported for QX 222 (25), probably mecamylamine induces multiple classes of blocked states (closed channel blockade and/or desensitized states) that are more clearly discernible under patch-clamp conditions and are detected by the binding assays. In EPC experiment, the conditions of agonist release and drug application probably diminished the contribution of the pathways into desensitized or other blocked states, such that most of the alterations observed resulted mainly from the open channel blockade. Similar findings have been reported for the anticholinesterase agent physostigmine (26),

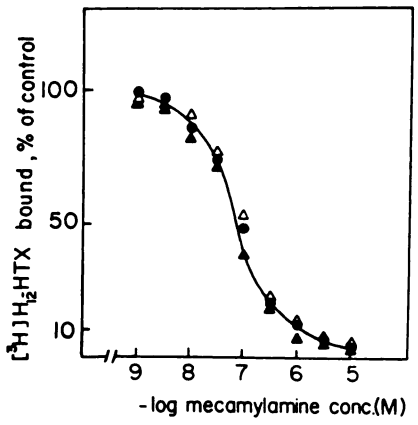

FIG. 10. *Inhibition of [^{3}H]H$_{12}$-HTX binding to Torpedo membranes produced by mecamylamine*

Torpedo membranes (50 μl of a preparation containing 1 μM receptor sites), pretreated with 10 μM *Naja* α-neurotoxin for 60 min (△) or untreated (▲), were added to 950 μl of buffer containing [^{3}H]H$_{12}$-HTX and mecamylamine and then, after a 120-min incubation, the amount bound was measured. A similar untreated membrane preparation (50 μl) was added to 950 μl of buffer containing [^{3}H]H$_{12}$-HTX, mecamylamine, and carbamylcholine and the amount bound was measured after 30 sec (●). The concentration of [^{3}H]H$_{12}$-HTX was 2 nM, carbamylcholine was 100 μM, and mecamylamine was as indicated.

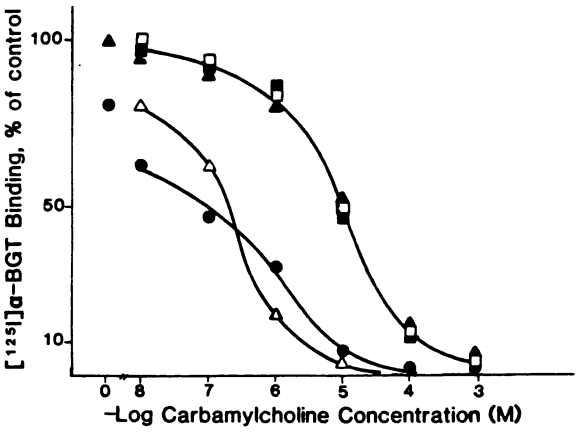

FIG. 11. *Effect of mecamylamine on the carbamylcholine-induced inhibition of ^{125}I-α-BGT binding to ACh receptor sites of Torpedo membranes*

The inhibition of ^{125}I-α-BGT (10 nM) was determined by incubating the *Torpedo* ACh receptors (1 nM) with the indicated concentrations of carbamylcholine for 40 sec (□) or 30 min (△). The effect of 5 (▲), 15 (■), and 100 (●) μM mecamylamine was tested by incubating the membranes with the drug for 30 min prior to addition of ^{125}I-α-BGT and the various concentrations of carbamylcholine. The amount of ^{125}I-α-BGT bound in 40 sec was determined. 100% binding represents ^{125}I-α-BGT bound in absence of carbamylcholine.

whose multiple effects (open channel blockade, agonist, and desensitizing properties) could be more clearly discerned in single channel current rather than in EPC recordings.

The present studies disclosed differences between the nicotinic receptors of the *Torpedo* electric organ membrane, and of the neuromuscular junctions of the frog and mammalian, and those located at the ganglia and some regions of the central nervous system. In the nicotinic receptors of the autonomic ganglia (3–5), mecamylamine appears to act as a competitive antagonist of the ACh receptor. The biochemical data showed that mecamylamine did not bind to the agonist recognition site (Fig. 9) but, in agreement with the electrophysiological findings, revealed a significant interaction with the site of the ionic channel (Fig. 10). The studies of the AChR from different areas (central nervous system, autonomic ganglia, Renshaw cell, etc.) have shown, in terms of its ligand affinities, that the AChRs from the optic tectum are more similar to that from the neuromuscular junction than to those from the autonomic ganglia (27). For example, at the AChR located in the central nervous system, such classical neuromuscular antagonists as decamethonium and *d*-tubocurarine are strong ligands, and hexamethonium, a ganglionic competitive blocker, is weak, whereas at the ganglia the opposite is found. Recently, patch-clamp studies have revealed some distinct kinetics properties of ACh-activated channels in chick ciliary ganglion neurons in comparison to those from the neuromuscular junction (28). Studies based on the specific binding of α-BGT and its ability to block physiological responses have demonstrated that the binding of the toxin does not block activation of these receptors at the ganglionic nicotinic synapses (29, 30). Similar findings have been reported for central nicotinic receptors where α-BGT seems to bind to a site distinct from the ACh-binding site (27). Certain characteristics, such as molecular weight, polypeptide composition, and amino acid sequence of the subunits, could account for the similarities and differences observed between the peripheral, ganglionic, and central nicotinic receptors (27, 31–33).

In summary, the studies with mecamylamine have shown a noncompetitive blockade of the AChR activation in the nicotinic synapse of the frog mammalian skeletal muscles and in the *Torpedo* electroplax membranes, primarily through an interaction with the open conformation of the ionic channel of the nicotinic receptor. In these synapses, mecamylamine did not bind to the agonist recognition site, in contrast to its competitive-mechanism on the ganglionic nicotinic AChR. Thus, taken together the present electrophysiological and biochemical evidence for a noncompetitive antagonism of mecamylamine at the neuromuscular junction reveals dissimilarities between the nicotinic receptors of the neuromuscular and ganglionic cholinergic synapses.

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