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The Reversible Cholinesterase Inhibitor Physostigmine Has Channel-Blocking and Agonist Effects on the Acetylcholine Receptor-Ion Channel Complex

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SUMMARY

The actions of the carbamate cholinesterase inhibitors, physostigmine (Phy) and physostigmine methiodide (MetPhy), were studied on the acetylcholine receptor-ion channel complex (AChR) of skeletal muscles. Low concentrations of these agents produced cholinesterase inhibition which resulted in potentiation of nerve-elicited muscle twitches and an increased peak amplitude and prolongation of the decay time constant (τ_{EPC}) of endplate currents (EPCs) elicited in frog (*Rana pipiens*) sartorius muscles. However, increasing concentrations of Phy depressed the peak amplitude and shortened the decay phase of the EPC with an apparent loss in the voltage dependence of τ_{EPC}. At higher concentrations and depolarized potentials, EPC decays were double exponential. The effects of both Phy and MetPhy on the postsynaptic AChR complex were also evident in preparations pretreated with diisopropylfluorophosphate. Under these conditions, a linear relationship between the reciprocal of τ_{EPC} and the concentration of these agents was observed. Single channel studies revealed that Phy (20–600 μM) shortened channel lifetime and decreased channel conductance at very high concentrations. In addition, Phy (0.5 μM) induced the appearance of channel openings with conductance similar to that of acetylcholine. High concentrations (>50 μM) of this agent activated channel openings with decreased conductance. Similar results were obtained with MetPhy. Thus, the reversible cholinesterase inhibitors Phy and MetPhy altered the properties of the AChR by interacting as agonists capable of inducing desensitization and blockade.

INTRODUCTION

Phy,[3] a reversible inhibitor of ChE, was isolated as a pure alkaloid by Jöbst and Hesse as early as 1864 (1). Phy readily penetrates the blood-brain barrier and so exerts both peripheral and central cholinomimetic actions. The drug has been widely used as a diagnostic aid, as an antagonist of acute toxic cholinolytic psychosis, and in the treatment of glaucoma. Additionally, it has been recommended as a prophylactic agent against exposure to irreversible inhibitors of ChE (2–4).

In spite of its remarkably long use in research and its clinical applications, the pharmacological actions of Phy on the neuromuscular nicotinic receptor and its ionic channel are practically unknown. Although the actions of Phy have been attributed primarily to a reversible inhibition of ChE, several lines of evidence suggest that this agent (as well as other carbamates such as neostigmine) has quite diverse actions on the pre- and postsynaptic regions of the neuromuscular junction (5–7). Recently, we have shown that pyridostigmine, another carbamate inhibitor of ChE, interacts with the AChR macromolecule as a weak agonist, enhances desensitization, and increases the affinity of other agonists for the nicotinic receptor (8–10). Some of these effects were also observed in our initial studies with Phy (11–13) and apparently did not result from an increased ACh persistence at the junctional region.

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[3] The abbreviations used are: Phy, physostigmine; MetPhy, physostigmine methiodide; ACh, acetylcholine; ChE, cholinesterase; AChR, acetylcholine receptor-ionic channel complex; DFP, diisopropylfluorophosphate; EPC, endplate current; τ_{EPC}, decay time constant of EPC.

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The objective of the present study is to reveal, through voltage clamp and patch clamp techniques, the specific mechanism(s) involved in the interaction of Phy and its quaternary analog MetPhy on the junctional nicotinic AChR complex. MetPhy was used to determine whether the site of action of these drugs was on the extracellular or intracellular segments of the AChR complex and whether the charged or uncharged form is responsible for the effects observed on neuromuscular transmission. Additionally, studies were performed after treatment of the neuromuscular preparations with an irreversible organophosphate ChE inhibitor in order to reveal direct interactions of these agents with the AChR macromolecule.

MATERIALS AND METHODS

Tissue preparation and solutions. Indirectly elicited muscle twitch, EPC, and noise analysis experiments were performed at room temperature (20–23°) on the sartorius muscles of the frog, *Rana pipiens.* In these experiments, normal Ringer's solution for frog muscles had the following composition (mM): NaCl, 115; KCl, 2; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; and NaH_2PO_4, 0.7. This solution was bubbled with 100% O_2, and the pH was maintained at 6.9–7.1. The details for the dissection and mounting of the nerve-muscle preparation were as previously described (9, 14).

Patch clamp studies were performed at 10–11° on single fibers isolated from interosseal and lumbricalis muscles of the hind leg toe of the frog *R. pipiens.* The procedure of obtaining single fibers was previously described (15). Briefly stated, the dissected muscles were treated with collagenase (1 mg/ml) (16) for 90–120 min and then with protease (0.2 mg/ml) for 20–30 min, at room temperature (20–22°). The isolated single fibers were kept at 2–5° prior to the experiments. An adhesive mixture composed of parafilm (30%) and paraffin oil (70%) was used to immobilize the single muscle fiber on the bottom of the recording mini-chamber. Composition of the physiological solution for the single muscle fibers was (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3.0. This solution was saturated with O_2 (100%) and the pH adjusted to 7.2.

Solutions of physostigmine (1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethyl-pyrrolo[2,3-b]indol-5-ol methylcarbamate) sulfate (Sigma Chemical Co., St. Louis, MO), physotigmine 1-methiodide, and DFP (Sigma) were prepared before each experiment. MetPhy was prepared by a standard method as described elsewhere (17) and recrystallized from acetone. There was no Phy present (<0.01%) based on thin layer chromatographic analysis. The methiodide is hygroscopic and was stored under anhydrous conditions. α-Bungarotoxin and Naja α-toxin were kindly provided by Dr. M. Eldefrawi (University of Maryland, Baltimore, MD). Tetrodotoxin (0.3 μM, Sankyo) was added to solutions used in noise analysis and patch clamp experiments.

Electrophysiological techniques. Muscle twitch experiments were performed on sciatic nerve-sartorius muscle preparations. Muscle twitches were evoked by passing supramaximal square-wave pulses of 3 msec duration through a platinum bipolar electrode attached to the sciatic nerve. Direct muscle stimulation was also performed, and both direct and indirect muscle twitches were alternatively recorded at intervals of 5 sec. The muscles were allowed to equilibrate in the stimulated condition for 30–90 min. In all experiments, the duration of drug exposure ranged from 30–40 min. Muscle action potentials were elicited by passing 30-msec depolarizing pulses through a microelectrode inserted into a surface fiber about 50 μM from the recording electrode.

For EPC experiments, frog sciatic nerve-sartorius muscle preparations were treated with 400–600 mM glycerol for 60 min to disrupt excitation-contraction coupling (18). The voltage clamp circuit was similar to that described by Takeuchi and Takeuchi (19) and modified by Kuba *et al.* (20). Intracellular recordings were taken from surface fibers, using electrodes filled with 3 M KCl with resistance in the range of 3–4 megaohms. EPCs were elicited at various holding potentials using 10-mV conditioning step changes in both depolarizing and hyperpolarizing directions throughout the range of +60 to −150 mV. At the end of each 3-sec duration conditioning step, the nerve was stimulated at 0.3 Hz to generate the EPCs. The EPC waveforms were displayed on an oscilloscope, sent on-line to a computer (PDP 11/40 from Digital Equipment Corp., Maynard, MA) at a digitizing rate of 10 kHz, and stored for later analyses. Decay time constants (τ_{EPC}) and peak amplitudes were obtained directly from digitized EPC data. The data of decay phase from at least eight EPCs were fit by either a single or a double exponential function; in the first case, τ_{EPC} was calculated by linear regression on the logarithms of the data points (20–80%) against the time; in the second case, the Marquardt-Levenberg algorithm was used to determine the best fit and to resolve the two components of EPC decays according to the following equation:

$$I(t) = A \exp^{-t/\tau_f} + (100 - A)\exp^{-t/\tau_s}$$

where $I(t)$ is the current taken as percentage of peak amplitude at time t; τ_f and τ_s are the time constants of the fast and slow components, respectively; and A is the contribution of the fast decay to the total current (9). When the per cent of one component exceeded 85 ± 5% of the total current, the decay was considered to be single exponential.

The experiments on EPC fluctuation analysis were performed on the junctional region of surface fibers of frog sartorius muscles using the techniques described elsewhere (9, 21). Current fluctuations were produced by microiontophoresis of ACh from a low resistance electrode containing 3 M ACh and positioned about 100 μm above the surface of the muscle. Currents of 20–120 namp were induced for a period of 30 sec using a constant current source (Howland current pump) with an adequate braking and adjustment of the iontophoretic current to achieve a steady, sustained current. The power density spectra (9, 22), fitted to a single Lorentzian function, provided single channel conductance (γ) and lifetime (τ_l).

Single channel recordings were obtained using the patch clamp technique (23). The microelectrodes for these studies were prepared on a vertical electrode puller (David Kopf Instruments, Tujunga, CA) using microhematocrit capillary tubes as described previously (10). The resistance of these microelectrodes ranged from 2 to 5 megaohms. The patch electrodes were filled with solution containing ACh, Phy, or MetPhy either alone or in combination. Single channel currents were recorded from the perijunctional region of single fibers using an LM-EPC-5 patch clamp system (List-Electronic, Darmstadt, West Germany), filtered at a bandwidth of 3 kHz, and stored on an FM magnetic tape. Data were sent to the computer (PDP 11/40 or PDP 11/24) and digitized at 10 kHz. Automated computer analyses of single channel currents, as previously described (10), provided channel conductance, and open and closed time histograms. Open time histograms represent the distribution of the time intervals between a downward step (channel opening) and the following upward step (channel closing or blockade). An opening was considered a burst when separated from the next opening event by an interval longer than 8 msec. Fast closed time histograms include only the short closures (intraburst gaps) with durations briefer than 8 msec.

Statistical analysis. The data are expressed as mean ± standard error. The two-tailed Student's t test was used for statistical comparisons. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Potentiation and depression of muscle twitch tension induced by Phy. Phy (0.02–20 μM) potentiated indirect muscle twitches such that, at 20 μM, twitch tension was 117% of the control values. At higher concentrations, however, a concentration-dependent depression of the muscle twitch tension was observed. The IC_{50} for depression of the indirect muscle twitch was about 200 μM, and complete blockade occurred at 2 mM. Following complete

blockade, a partial recovery (74% of the control values) was achieved after repetitive washing of the preparation for 60 min (8).

Neither Phy (200 μM) nor its quaternary analog MetPhy (200 μM) had any effect on the membrane potential for the surface muscle fibers recorded at the extrajunctional region. Similarly, no significant effect was observed on the threshold, amplitude, rate of rise, or half-decay time of directly elicited action potentials.

Potentiation and blockade of the EPC induced by Phy and MetPhy. The voltage dependence of the EPC peak amplitude and τ_{EPC} under control conditions and during exposure to Phy are shown in Fig. 1. In physiological solution, the relationship between the transmembrane potential and the EPC peak amplitude was linear, although in some instances the peak amplitude underwent a slight nonlinearity at very hyperpolarized potentials (−120 to −180 mV) (20, 24). At low concentrations of Phy (0.2–2 μM), the peak amplitude of the EPC was increased (Fig. 1A) and τ_{EPC} was prolonged while maintaining the same voltage dependence observed under control conditions (Fig. 1B). At high concentrations, Phy produced a concentration-dependent depression of the EPC peak amplitude with no change in its voltage dependence. When EPCs were elicited at shorter time intervals (every 0.5 sec instead 3 sec) in the presence of 200 μM Phy, the depression of the EPC peak amplitude was potentiated. Phy (20–200 μM) accelerated EPC decays, and the voltage sensitivity of τ_{EPC} was progressively decreased with increasing concentrations of this drug. At 200 μM Phy, an inversion in the sign of the slope of this relationship was observed. At negative holding potentials (−20 to −150 mV), and with all concentrations of Phy tested, the EPCs decayed as a single exponential function of time (Figs. 1B and 2A). However, in the presence of 200 μM Phy, at holding potentials between +20 and +60 mV, double exponential decays became discernible (Fig. 2B). For example, at a membrane potential of +60 mV, the EPC decays consisted of a fast component with a time constant, τ, of 0.79 ± 0.12 msec (74.0 + 4.0% of peak current, n = 12) and a slow component with a τ of 5.45 ± 0.7 msec (26.0 ± 4.0% of peak current). All of these effects were reversible and, after washing (1–2 hr), both EPC peak amplitude and decay recovered nearly to values for control conditions.

MetPhy (1–10 μM), similar to Phy, caused potentiation of the EPC peak amplitude and prolongation of τ_{EPC}. Increasing concentrations of MetPhy (up to 100 μM), produced a dose-dependent depression of EPC peak amplitude and shortening of τ_{EPC}. The linear current-voltage relationship was maintained throughout the voltage range tested (Fig. 3). At 100 μM MetPhy, double exponential decays of the EPCs were identified at membrane potentials between −60 and +60 mV. Thus, at −60 mV membrane potential, the fast component of the EPC decays had a τ of 1.44 ± 0.13 msec (72.0 ± 5.0% of peak current) and the slow component had a τ of 29.76 ± 2.55 msec (28.0 ± 5.0% of peak current). At a membrane potential of +60 mV, both τ_f and τ_s were shortened to 0.81 ± 0.06 msec (42.0 ± 3.0% of peak current) and 9.31 ± 0.23 msec (58.0 ± 3.0% of peak current), respectively. At 200 μM MetPhy, double exponential decays with smaller τ_f and τ_s were only discernible at positive potentials.

To describe more clearly the interactions of Phy and MetPhy with sites associated with the AChR macromolecule, the EPC experiments were performed on preparations in which the ChEs were previously inhibited. The muscles were pretreated with DFP (1 mM) for 60 min and subsequently washed for 60 min to remove excess

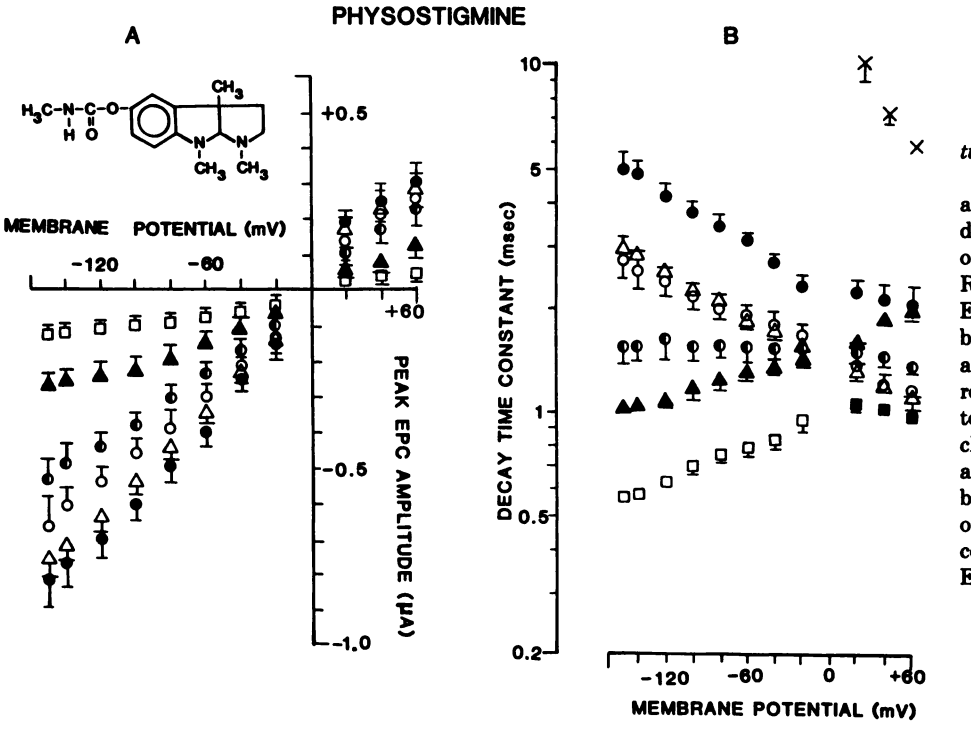

FIG. 1. *Effects of Phy on the peak amplitude and decay time constant of the EPCs*

A: Relationship between the EPC peak amplitude and the membrane potential under control conditions and in the presence of Phy. *Inset*: Chemical structure of Phy. B: Relationship between the logarithm of the EPC decay time constant (τ_{EPC}) and membrane potential under control conditions and in the presence of Phy. Each *point* represents the mean (± standard error) of 8 to 24 surface fibers from at least four muscles. ○, control; ●, 0.2; △, 2; ◐, 20; ▲, 60; and □, 200 μM Phy. At membrane potentials between +20 and +60 mV, in the presence of 200 μM Phy, ■ and × represent the time constants of the fast and slow phases of EPC decays, respectively.

DFP (20). EPCs elicited in DFP-treated muscles showed an increased peak amplitude and a prolongation of the decay phase (Fig. 4). Under these conditions, exposure of the preparation to low concentrations (0.2–2.0 μM) of either Phy or MetPhy did not significantly affect the properties of the EPC amplitude or the decay of the EPCs. Increasing concentrations of these drugs resulted in a depression of the EPC peak amplitude while a linear current-voltage relationship was maintained. In addition, both Phy and MetPhy induced a concentration-dependent decrease of τ_{EPC} and, similar to the effect of these drugs on untreated muscles, at high concentrations of these agents, double exponential decays of the EPC became noticeable at positive membrane potentials (Fig. 4). Phy induced double exponential decays only at positive membrane potentials (between +20 and +60 mV), whereas with MetPhy this effect was discernible at potentials between −60 and +60 mV. For example, at a −60-mV membrane potential, in the presence of MetPhy (100 μM), the EPCs decayed with a τ_f of 0.94 ± 0.02 msec (91.0 ± 1.0% of peak current) and a τ_s of 32.28 ± 9.45 msec (9.0 ± 1.0% of peak current). At +60 mV, τ_f was reduced to 0.69 ± 0.08 msec (44.0 ± 4.0% of peak current) while τ_s was reduced to 2.78 ± 0.19 msec (56.0 ± 4.0 of peak current). Fig. 5 shows the decreasing contribution of the fast phase of the EPC decay as the membrane was depolarized. According to a sequential model for open channel blockade (see "Discussion"), the single exponential decays result from the slow dissociation of the blocker from its binding site. Thus, at membrane potentials between −20 and −150 mV for Phy and −150 and −70 mV membrane potentials for MetPhy, plots of the reciprocal of τ_{EPC} versus drug concentration were linear as expected from this model (Fig. 4C). The predicted exponential dependency of the rate constant of the blocking (k_3) reaction is shown in the *inset* of the Fig. 4C.

Effects of Phy and MetPhy on ACh-induced EPC Fluctuations. The effects of Phy and MetPhy on single channel lifetime and conductance were determined from fluctuation analysis on voltage-clamped surface fibers of sartorius muscles. Phy, at concentrations higher than 20 μM, markedly shortened channel lifetime, whereas with MetPhy a significant effect was seen at 100 μM (Table 1). In addition, both agents at concentrations higher than 100 μM significantly decreased single channel conductance.

Effects of Phy and MetPhy on the ACh-induced single channel currents. Patch clamp studies of the perijunctional region of the frog skeletal muscle fiber revealed a number of important features regarding the actions of Phy and MetPhy on the nicotinic AChR complex. Square-shaped currents were activated by ACh (0.3 μM) placed inside the patch pipette (Fig. 6), and the single channel conductance was estimated to be 31.2 pS at 10°. A small percentage (<5%) of low conductance channel openings (20 pS) were recorded in 2 of 13 cells examined. The computer analysis was based on events with larger amplitude since they were consistently observed in all of the recordings. The histograms of the channel open times were fitted to a single exponential function and disclosed a mean of 10.5 msec at a membrane potential of −100 mV (Table 2). The open state was prolonged at hyperpolarized potentials. Some of the characteristics of the ACh channels of these isolated muscle fibers have been reported previously (15).

Single channel currents were recorded with a pipette filled with various concentrations (0.1–600 μM) of Phy together with ACh (0.3 μM). Whereas the current noise

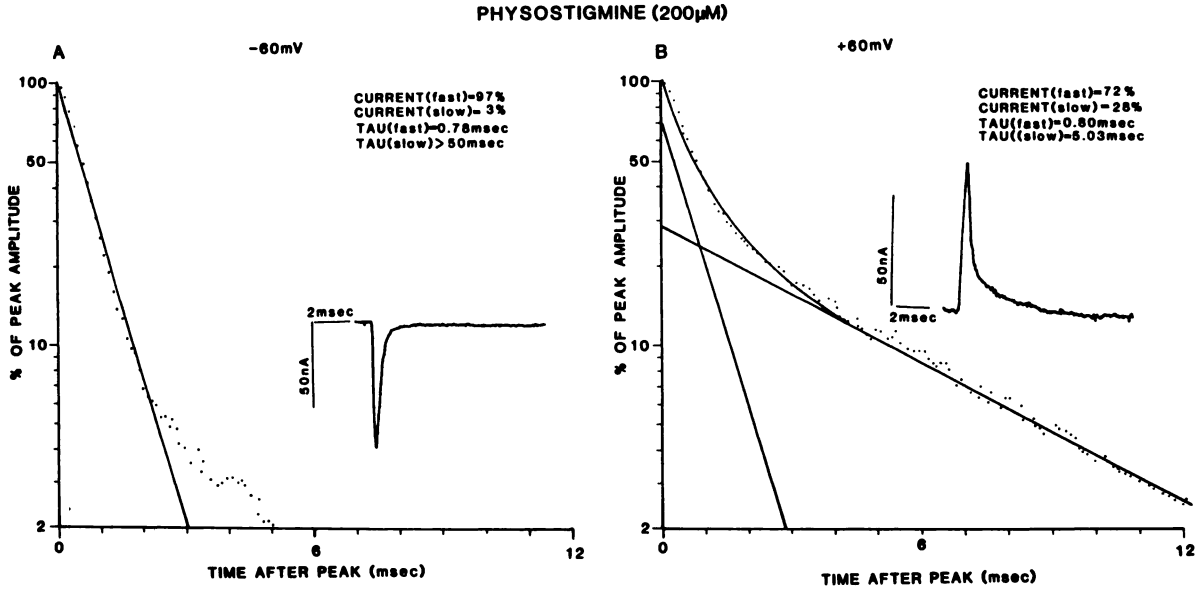

FIG. 2. *Voltage-dependent effect of Phy (200 μM) on the decay phase of the EPCs*

The decay phase of the EPCs recorded from a single cell was plotted as a percentage of the peak amplitude versus time on a semilogarithmic scale. The *solid lines* represent the computer-generated best fit and the resolution of fast and slow components of the EPC decays. When one component exceeded 85 ± 5% of the total current amplitude, the decay was considered to be single exponential and linear regression was used on the logarithms of the data points from 80% to 20% (see "Materials and Methods"). EPCs were recorded at membrane potentials of −60 mV (A) and +60 mV (B), as were the samples of EPCs in the *insets*.

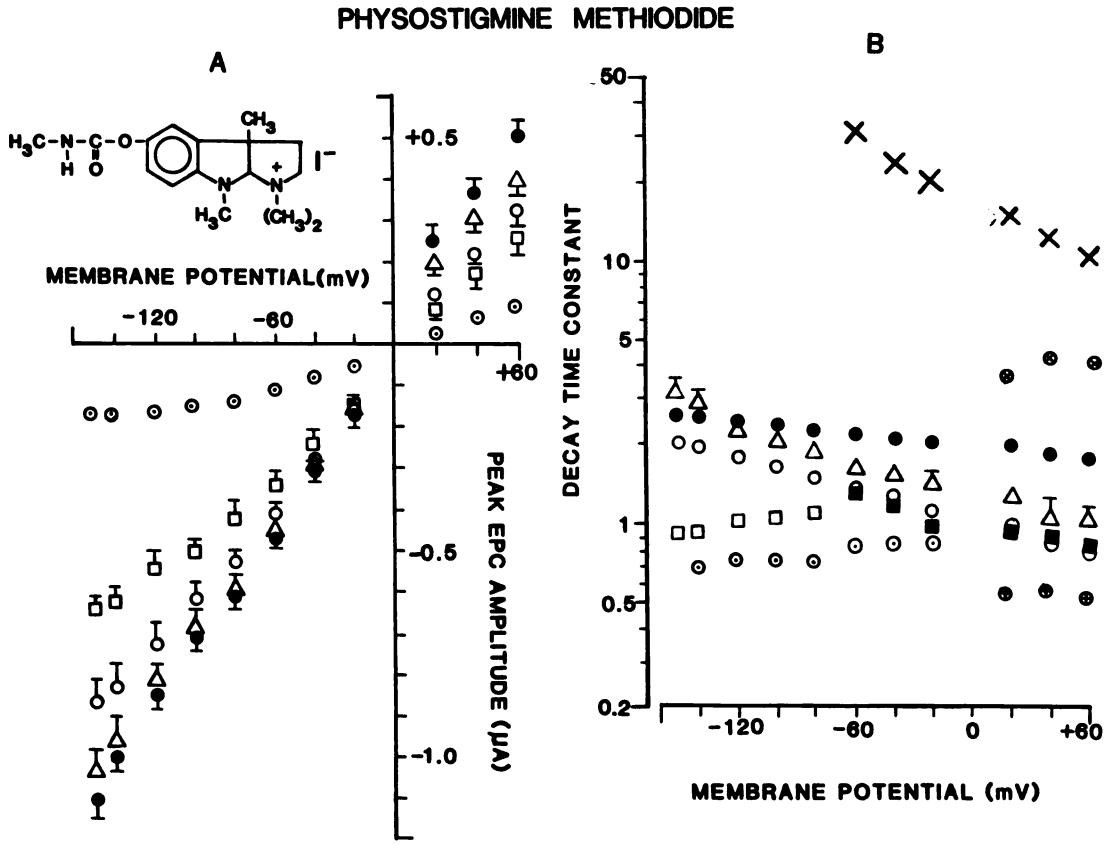

FIG. 3. *Effects of MetPhy on peak amplitude and decay time constant of EPCs*

A: Relationship between the EPC peak amplitude and the membrane potential under control conditions and in the presence of various concentrations of MetPhy. *Inset*: Chemical structure of MetPhy. B: Plot of the logarithm of the EPC decay time constant (τ_{EPC}) versus membrane potential under control conditions and in the presence of MetPhy. Each *point* represents the mean (± standard error) of 8 to 24 surface fibers from at least four muscles. ○, control; ●, 1; △, 10; □, 100; and ⊙, 200 μM MetPhy. ■ and × represent time constants of the fast and slow phases of the EPC decays induced by 100 μM, and ⊕ and ⊗ by 200 μM MetPhy, respectively.

level of the open and closed states were similar for ACh-activated channels, in the presence of Phy the current level during the channel open state was irregular and interrupted by many short channel closures or gaps (Fig. 7). In contrast to burst times, which remained essentially unchanged under control conditions and in the presence of Phy, the analysis of the open times revealed a significant shortening at concentrations of 20 to 200 μM (Fig. 8, Table 2); however, at higher concentrations of Phy, no further decrease was observed. Thus, in contrast to the predictions of the sequential model described later, the plots of the reciprocal of mean channel open time versus drug concentration showed a clear departure from linearity. The histograms of the channel open times, similar to control conditions, showed a single exponential distribution at all concentrations of Phy tested (Fig. 8). In addition, at this concentration range a decrease in current amplitude was observed. Single channel conductance determined from the slope of the current-voltage relationship was estimated to be 18.6 pS with 200 μM Phy. Increasing the concentrations (up to 600 μM) did not produce additional changes in channel conductance (Table 2). Similar effects were seen with MetPhy (Fig. 9) which, at concentration of 200 μM, produced a decrease in the single channel conductance to 17.5 pS and a shortening of the mean open time to 4.3 ± 0.3 msec ($n = 6$) at −100 mV membrane potential.

The effect of Phy on channel closed times was also analyzed. The distributions of closed times of channels activated by ACh alone and when combined with different concentrations of Phy disclosed the presence of two components, a fast one corresponding to the "flickers" inside the burst (intraburst gaps) and a slow component related to the long-lasting closures between the bursts (interburst gaps). Although the number of openings per burst was progressively increased with Phy concentration, i.e., from 1.5 under control conditions to 4.5 in the presence of 600 μM, the time constant of the fast component was not significantly changed (Table 2).

To determine whether there are sites at the intracellular portion of the nicotinic AChR for Phy interactions, we superfused MetPhy into the bathing medium under inside-out patch condition. Phy, having a pK_a of 6.1, is mostly in uncharged form (90%) at the experimental pH of 7.0–7.1, therefore having a great possibility of diffusing through the cell membrane. MetPhy, carrying a permanent positive charge, would be more suitable to discriminate the location of Phy site(s) at the AChR. Since cell-free patches are difficult to obtain in mature muscle fibers, we opted to perform this series of experiments in cultured rat myoballs (see Refs. 10 and 25 for the details of the culturing procedure and single channel recordings). Gigaohm seals were achieved with a micropipette

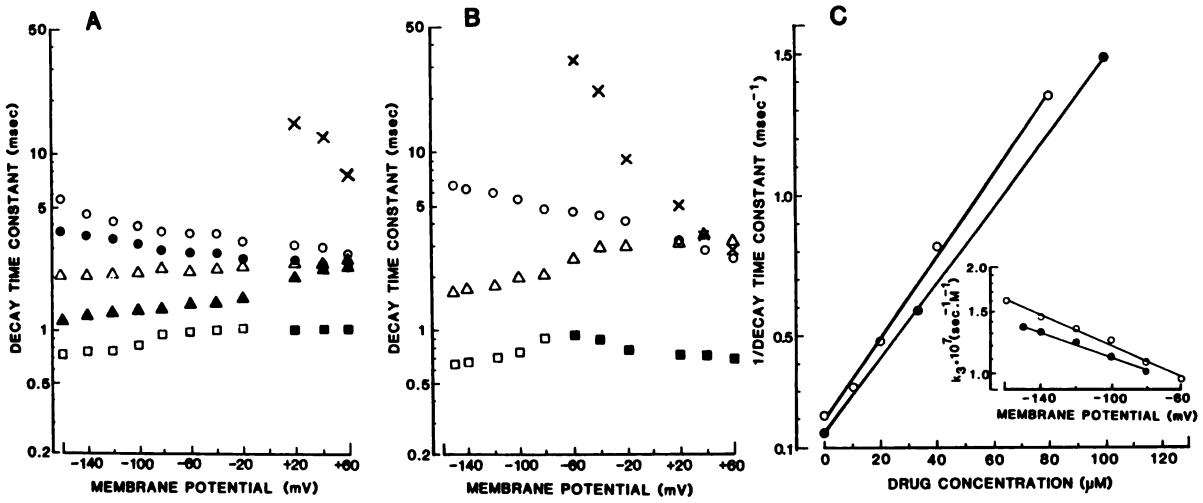

FIG. 4. *Effect of Phy and MetPhy on the decay time constant of the EPCs following pretreatment with DFP*

Relationship between the logarithm of the decay time constant of the EPCs and membrane potentials under control conditions and in the presence of Phy (A) and MetPhy (B). The preparation was treated with DFP (1 mM, see "Results") before exposure to either Phy or MetPhy. Each *point* represents the mean (± standard error) of 8 to 24 surface fibers from two to four muscles. A: DFP-treated muscles before (○) and after exposure to 10 (●), 20 (△), 40 (▲), and 80 μM (□) Phy. At membrane potentials between +20 and +60 mV, ■ and × represent the time constants of the fast and the slow phases of the EPC decays, respectively, induced by 80 μM Phy. B: DFP-treated muscles before (●) and after exposure to 33 (△) and 100 (□) μM MetPhy. At a membrane potential range of −60 to +60 mV and in the presence of 100 μM MetPhy, ■ and × represent the time constants of the fast and slow phases of the EPC decays, respectively. C: Reciprocal of decay time constant of the endplate currents versus concentration of Phy (○) and MetPhy (●) obtained from A and B. *Inset*: Voltage sensitivity of the forward rate constant of the blocking reaction (k_3) in the presence of Phy (○) and MetPhy (●) plotted on a semilogarithmic scale.

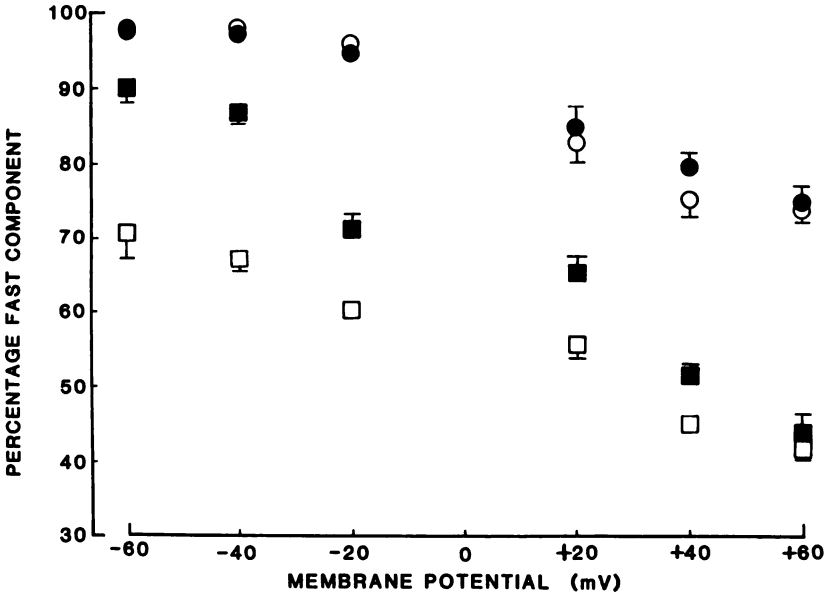

FIG. 5. *Voltage dependence of the relative magnitude of the EPC fast decay phase in the presence of Phy and MetPhy after treatment with DFP*

The contribution of the fast component of the τ_{EPC} to the total current (percentage), in the presence of 200 μM Phy (○) and 100 μM MetPhy (□) in normal muscles, and in the presence of 80 μM Phy (●) and 100 μM MetPhy (■) in DFP-pretreated muscles, is plotted at different membrane potentials.

containing ACh (0.1–0.2 μM) and inside-out patches obtained according to previous description (23). The channel openings recorded had a conductance of 20–22 pS and an average open time of 18 msec (−100 mV) at 10°. MetPhy (100 μM) was slowly superfused into the bathing medium through a tube ending in a capsule (volume of ~20 μl) which had a small opening of 25–50 μm diameter. The micropipette containing the inside-out patch was then carefully moved into this capsule and single channel currents were recorded for 60 min at different membrane potentials. After this period of exposure, MetPhy did not change either the amplitude or the duration of the single channel currents. Assuming a close similarity between the tertiary and the quaternary compounds, this finding suggested that there is no site for the interactions of Phy at the cytoplasmic segments of the nicotinic AChR.

Agonist effect of Phy. Preliminary studies with Phy (0.5 μM) present alone inside the patch micropipette showed that this agent had an agonist effect, activating ionic currents in frog single muscle fibers (Fig. 10, *right*). These currents were blocked by previous exposure of the fibers to Naja α-toxin (2 μM) or α-bungarotoxin (5 μg/ml) or by mixing the solutions of one of these toxins and Phy inside the pipette. The amplitude of Phy-evoked channel openings was linearly related to the voltage, and channel conductance was estimated from the current-

TABLE 1

Effects of Phy and MetPhy on ACh-induced EPC fluctuations[a]

Conditions	Channel conductance (γ)	Mean channel lifetime (τ_I)
	pS	*msec*
Control	26.3 ± 0.2	1.01 ± 0.07
Phy (200 μM)	18.9 ± 1.6[b]	0.32 ± 0.04[b]
MetPhy (100 μM)	17.6 ± 0.9[b]	0.59 ± 0.07[b]

[a] Each number refers to 5–10 spectra from at least three sartorius muscles. All of the holding membrane potentials were between −70 and −80 mV.

[b] Statistically significant at $p < 0.05$.

voltage relationship to be 29.0 pS. The channel open times showed a single exponential distribution and a mean of 9.6 msec at −80 mV membrane potential (Fig. 10, *left*). Thus, at low concentrations, Phy generated channel openings similar to those of ACh-activated channels. Increasing concentrations of Phy decreased current amplitude such that, at a concentration of 50 μM, the channel conductance was estimated to be 18.0 pS (12). At these concentrations of Phy, channel open times were slightly shortened, and the recordings exhibited altered events with a baseline during the open state resembling that of single channel currents activated by the combination of ACh and Phy (see Fig. 7 and Ref. 12). MetPhy (50–100 μM) showed similar agonist effects activating events with irregular and noisier currents during the open state (Fig. 11, *right*). Channel open times had a single exponential distribution and a mean of 9.9 msec at −120 mV membrane potential (Fig. 11, *left*).

DISCUSSION

Phy and MetPhy (>20 μM) caused a concentration-dependent shortening of τ_{EPC} with an apparent loss of its voltage dependence relative to the control conditions (Figs. 1 and 3). Moreover, additional alterations such as double exponential decays became evident as the concentration of these agents was increased. The double exponential decays were gradually discernible as the potentials were shifted to a more positive range. Patch clamp recordings in the presence of either Phy or MetPhy in combination with ACh showed events with increased "flickering" and an irregular current noise during the open state. Both drugs caused shortening of the mean channel open time and a decrease in conductance. In addition, Phy and MetPhy acted as agonists, i.e., single channel events were recorded when they were present alone in the patch micropipette. These openings were attributed to agonist effect because no spontaneous openings similar to those observed in cultured myotubes (26) were seen in our patch clamp recordings of muscle fibers isolated from adult frogs.

The shortening of τ_{EPC} suggested an interaction of these anti-ChE agents with the open channel conformation. Most of the data derived from EPC experiments could be fit to a sequential model (22, 27). A modified sequential model (28), used to explain the two components of the EPC decay phase and the mechanisms involved in the blockade of the ionic channels by Phy and MetPhy, is represented as follows:

$$\begin{array}{c}\text{diffusion}\\ \uparrow\\ n\text{ACh}\\ \downarrow\\ \text{hydrolysis}\end{array}$$

$$+\ R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ACh}_nR \underset{k_{-2}(V)}{\overset{k_2(V)}{\rightleftharpoons}} \text{ACh}_nR^* \underset{D\,k_{-3}(V)}{\overset{D\,k_3(V)}{\rightleftharpoons}} \text{ACh}_nR^*D$$

In this model, R is the receptor, which interacts with n molecules of the transmitter, ACh, to form an agonist-bound but nonconducting species, ACh_nR. This species undergoes a conformational change to form a conductive state, ACh_nR^*. ACh_nR^*D is the species blocked by drug, D, which is assumed to have no conductance. k_3 and k_{-3} are the forward and backward rate constants for the blocking reaction, respectively, and V indicates the steps which are voltage sensitive. This model has been used to describe the actions of some local anesthetics such as QX-222 (22, 27) and bupivacaine (25, 29), as well as actions of anticholinergic agents such as atropine and scopolamine (28).

Under control conditions, τ_{EPC} reflects the lifetime of

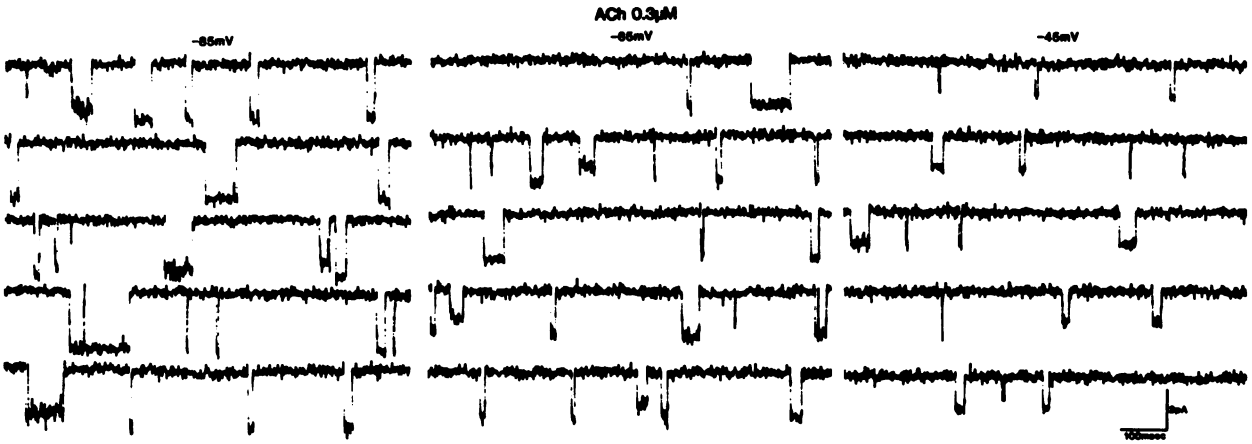

FIG. 6. *Samples of ACh-activated channel currents*
Single channel currents were activated from an isolated fiber of the interosseal muscle by ACh (0.3 μM).

TABLE 2

Effects of Phy on ACh-activated single channel currents

	Conductance[a]	Mean open time[b]	Mean fast closed time[b]
	pS	*msec*	
Control (ACh 0.3 μM)	31.2	10.6 ± 0.9 (n = 6)	0.23 ± 0.04 (n = 4)
ACh (0.3 μM) + Phy at:			
20 μM		6.4 ± 0.4 (n = 3)[c]	0.25 ± 0.02 (n = 3)
50 μM	29.2	5.4 ± 0.9 (n = 3)[c]	0.27 ± 0.01 (n = 6)
100 μM	22.7	4.5 ± 0.2 (n = 3)[c]	0.25 ± 0.04 (n = 4)
200 μM	18.6	4.3 ± 0.2 (n = 4)[c]	0.31 ± 0.02 (n = 3)
600 μM		4.8 (n = 2)	0.20

[a] Channel conductance was estimated from the slope of the current-voltage relationship between −60 and −150 mV.
[b] The channel open times and fast closed times were measured at membrane potentials of −90 to −100 mV.
[c] Statistically significant at $p < 0.05$.

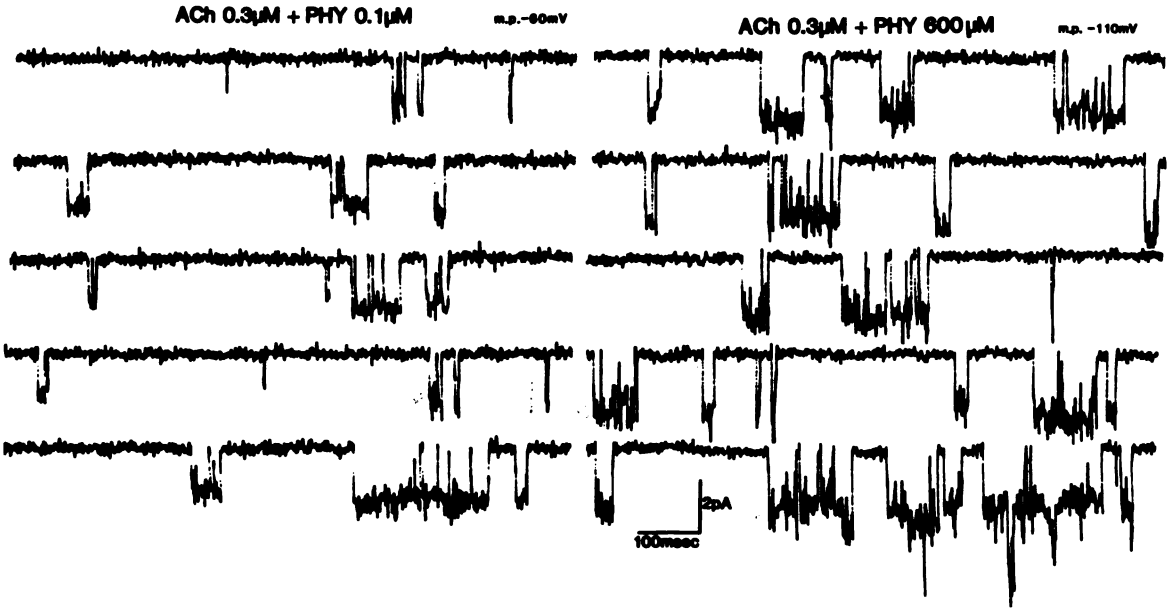

FIG. 7. *Samples of ACh-activated channel currents in the presence of Phy*
Single channel currents were recorded with 0.1 (A) and 600 (B) μM Phy included in patch pipette solution together with ACh (0.3 μM).

open ion channels (21) and is dependent on the rate constant for spontaneous channel closure k_{-2} described by the equation $k_{-2} = B \exp^{AV}$. Under conditions where the ChE is irreversibly inhibited, k_{-2} is roughly increased by a factor of 2 due to a doubling in the constant, B, with no change of its voltage dependence (20, 24). Therefore, assuming that ChE inhibition and the characteristics and number of receptors available remained unchanged, the blocking phase of the action of Phy was considered relative to this "new control condition" (i.e., after DFP treatment) with the new rate constant k'_{-2} being a multiple of the "original" k_{-2}. The sequential model was used to interpret the results obtained under these experimental conditions. The progressive shortening of τ_{EPC} with increasing concentrations of either Phy or MetPhy is reasonably well predicted by this model. In the presence of a blocker, the channel egresses from the open state by two routes: by spontaneous closure and by drug blockade, these processes being governed by the rate constants k'_{-2} and k_3, respectively. The opposite voltage dependence of these rate constants accounts for the apparent loss in the voltage sensitivity of τ_{EPC} seen with increasing concentrations of the blocking agent. In this model, the appearance of single or double exponential decays is solely determined by the magnitude of the rate constant for the unblocking reaction (k_{-3}) (28). When k_{-3} is negligible, AChR*D is sufficiently stable so that the EPCs will decay as a single exponential function of time; however, as k_{-3} becomes appreciable, more rapid dissociation of drug will restore and accumulate AChR*, producing slower diminution of its concentration and consequently leading to appearance of a slow decay component. In the case when $k_{-3} \simeq 0$, the decay is accelerated according to the expression $1/\tau_{EPC} = k_{-2} + [D]\, k_3$. For Phy at membrane potentials ranging from −150 to −10 mV and for MetPhy from −150 to −70 mV, single exponential decays were observed suggesting that, at this voltage range, the magnitude of the rate constant k_{-3} is not appreciable. Accordingly, the plots of $1/\tau_{EPC}$ versus concentration of the drug are expected to display a linear relationship where the slopes represent the blocking rate constant k_3. Such an effect was seen in DFP-pretreated muscles with both Phy and MetPhy up to 100 μM (see Fig. 4C). The exponential voltage dependence of k_3 for both drugs is

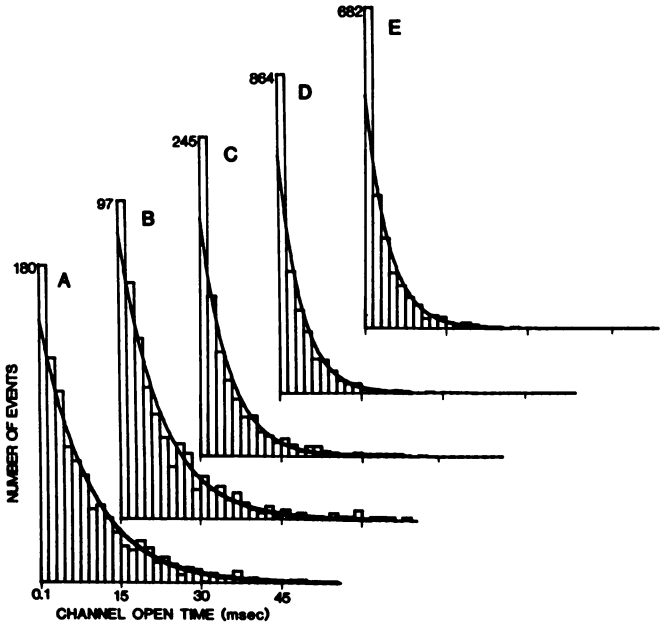

FIG. 8. *Open time histograms of the channels activated by ACh in the absence and presence of Phy*

Histograms correspond to channels activated by ACh (0.3 μM) in the absence (A, 959 events) or presence of Phy at 0.1 (B, 474 events), 20 (C, 728 events), 200 (D, 1980 events), or 600 (E, 1628 events) μM concentration. The mean channel open times determined from the fit of the distributions to a single exponential function (correlations >0.97) are: 9.1 (A), 7.6 (B), 5.2 (C), 4.0 (D), and 4.2 msec (E).

shown in the *inset* of Fig. 4C. The k_3 values, which increased as membrane potential became more negative, ranged from 1.5×10^7 at −140 mV to 1.1×10^7 M^{-1} sec^{-1} at −80 mV for Phy and from 1.3×10^7 at −140 mV to 1.0×10^7 M^{-1} sec^{-1} at −80 mV for MetPhy. These values were similar to those found for the variety of other compounds that seem to block the ACh-activated ion channel (30, 31). The slight voltage dependence of k_3 (*inset* of Fig. 4C) indicated that the effects of both MetPhy and Phy resulted from their interactions with a site located close to the outer surface of the AChR. A similar external binding site has been proposed for drugs such as the local anesthetic bupivacaine and its quaternary analog (25, 29), phencyclidine methiodide, and piperocaine methiodide (17).

In addition, both Phy and MetPhy at high concentrations induced double exponential decays of the EPCs which were more clearly discernible at positive membrane potentials. The double exponential decays of the EPCs induced by MetPhy were apparent over a larger range of potentials (−60 to +60 mV), whereas Phy induced this effect only at positive potentials. As predicted by the sequential model, in the presence of either Phy or MetPhy, the hyperpolarization caused the terminal component to decay more slowly and its relative magnitude to become gradually suppressed (Fig. 5). However, according to the predictions of this model, the blocked state has no conductance, so that channels with only one conductance state similar to that of control conditions are detected; furthermore, the magnitude of the slow component of the EPC decays is predicted to decrease with increasing concentrations of the blocker which was not clearly observed with either Phy or MetPhy. Indeed, the double exponential decays only became discernible at high concentrations of these agents, and a decreased single channel conductance was observed in the presence of high concentrations of either Phy or MetPhy. Multiple effects of these agents on the nicotinic neuromuscular AChR, such as an agonistic and desensitizing effect altering and/or reducing the repeated activation of the receptors (see below), could account for the difficulties to describe all the data in terms of a simple sequential model.

Single channel recordings revealed additional details of the effects of both Phy and MetPhy on the AChR complex. First, although both Phy and MetPhy completely blocked the EPCs at concentrations higher than 300 μM (Figs. 1 and 3), we have been able to record single channel currents when Phy, together with ACh, was present inside the patch microelectrode at concentrations as high as 600 μM (Fig. 7). Second, the square shape typical of ACh-activated single channel currents was no longer evident in the presence of either Phy or MetPhy. The current during the open state showed an irregular and broader baseline interrupted by more short closures than in control conditions (number of openings per burst increased from 1.3 in control to 4.7 in the presence of 600 μM Phy). These altered currents were recorded in the presence of Phy at concentrations as low as 0.1 μM (Fig. 7). Thus, interesting questions are raised insofar as the nature of these single channel currents is concerned. An effect of Phy on the lipid phase or on the interface lipid-protein, unspecifically altering the ionic flux through the channels, cannot be eliminated. The persistence of the altered channel events at all concentrations of Phy or MetPhy, alone or in combination with ACh, gives some credence to this hypothesis. Alternatively, these altered events could result from a blockade of the open channels concomitant with a decrease in conductance, as reported for ACh at high concentrations (32). However, the sequential model presented for open channel blockade could not explain the full spectrum of alterations disclosed by the single channel recordings. The mean channel open time was shortened from a control value of 9.2 msec to 4.1 msec at 200 μM Phy but the plot of the reciprocal of this parameter versus drug concentration was not linear. Indeed, at higher concentrations, the open times decreased no further, or even showed a slight tendency to increase at 600 μM. The analysis of the current amplitude disclosed similar behavior. Channels activated in the presence of a low concentration of Phy (0.1 μM) together with ACh had a conductance similar to those activated by ACh alone. However, higher concentrations of Phy, up to 200 μM, decreased channel conductance with no additional changes at much higher doses. Also, the analysis of the duration of the short closures within a burst did not show the appearance of a new slow component corresponding to the blocked state, clearly observed with mecamylamine (33) and QX-222 (34). Furthermore, the frequency of single channel events remained quite high at 600 μM Phy. It is conceivable that what we could be seeing in patch-clamp recordings is a mixture of ACh- and Phy-

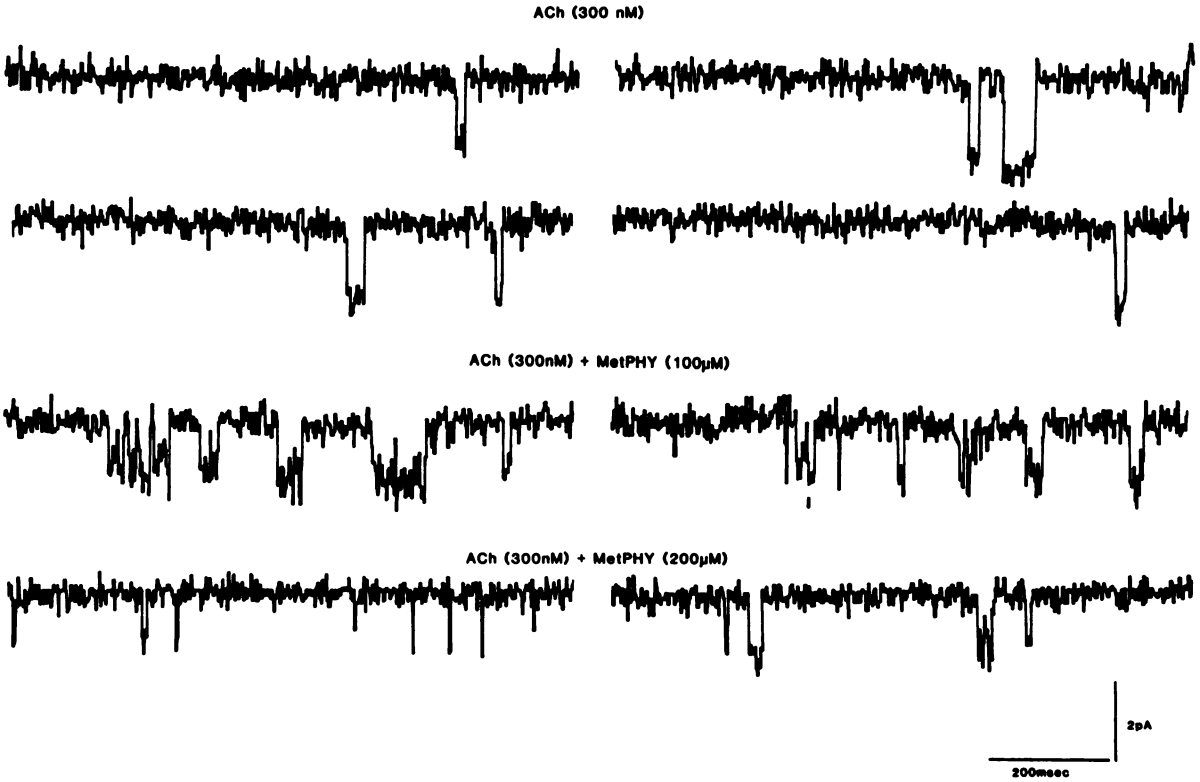

FIG. 9. *Samples of ACh-activated channel currents in the presence of MetPhy*
Single channel currents were recorded from the interosseal muscle fiber at −80 mV membrane potential with a pipette containing either ACh (0.3 μM) alone or combined with MetPhy (100–200 μM).

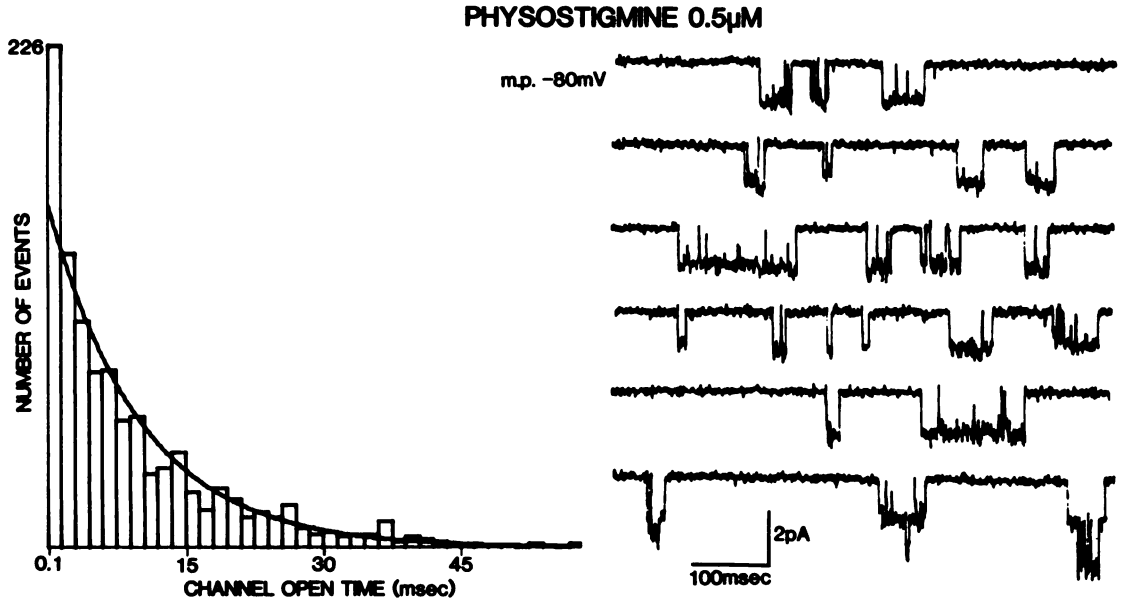

FIG. 10. *Single channel currents activated by Phy*
Samples of the recordings obtained from frog muscle fiber with a patch electrode filled with solution containing only 0.5 μM Phy (*right*) and the corresponding histogram of the channel open times (*left*). The histogram contains 1088 events, and the mean channel open time, determined from the fit to a single exponential function (correlation coefficient = 0.96), is 9.6 msec.

activated channels, the latter becoming predominant at high Phy concentrations. The channels opened by Phy itself might exhibit distinct gating kinetics and desensitize differently. This could explain the unexpected persistence of opening events at high concentrations of Phy as well as a lack of shortening of the mean channel open time at very high concentrations. Preliminary results (12) have shown that the lifetime of Phy-activated channels is approximately equal to that of ACh-activated channels at low concentrations (0.5 μM) but at concentrations of Phy of 50 μM and above, the lifetime is shortened to about 7 msec. Since the conductance of

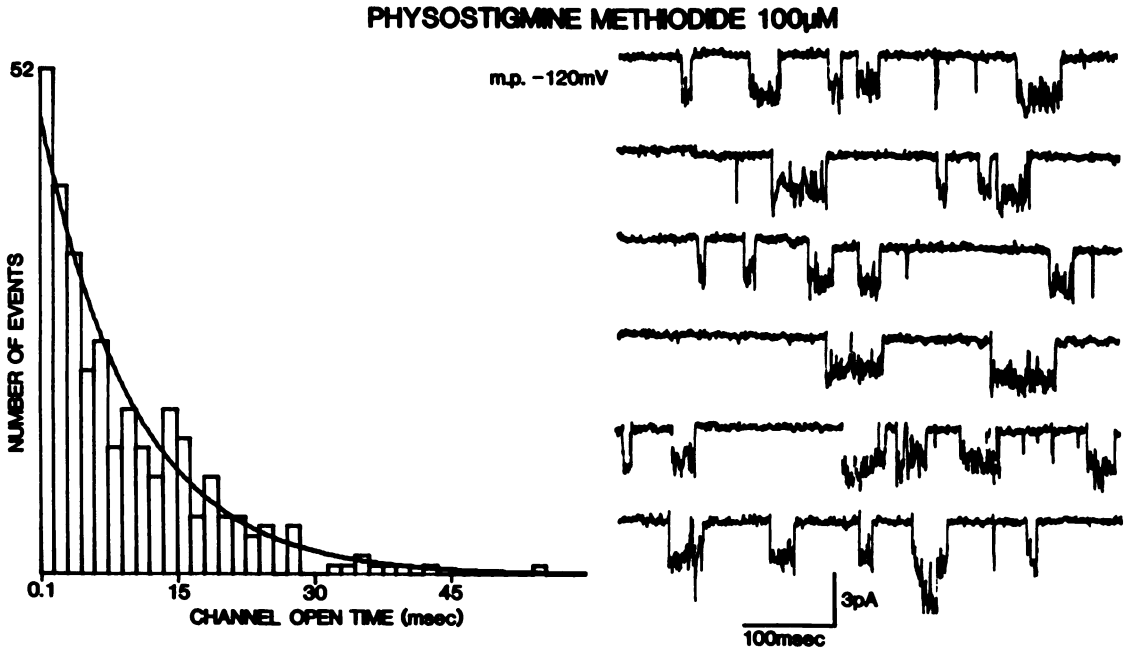

FIG. 11. *Channel currents activated by MetPhy*

Samples of channel currents activated by MetPhy (100 μM) placed inside the patch pipette (*right*) and the corresponding channel open time histogram containing 307 events (*left*). The mean channel open time, (correlation coefficient = 0.96), is 9.9 msec.

channels activated by Phy and by ACh plus Phy is approximately equal over the concentration ranges studied here, it was not possible to separate the two different channel types.

The present and earlier studies have revealed that many carbamate ChE inhibitors have significant direct effects on the nicotinic AChR complex. In spite of their common anticholinesterasic properties, there are differences in the actions of these ChE inhibitors. In agreement with the electrophysiological results (this paper and Refs. 9, 10, and 12), binding studies performed on *Torpedo* membranes have shown that Phy and MetPhy as well as neostigmine and pyridostigmine interact noncompetitively with the activated state of AChR as judged from the inhibition of [^{3}H]-perhydrohistrionicotoxin binding to the ionic channel sites (9, 10, 13); Phy was one of the most powerful agents in this sense. The negligible effect of these carbamates on binding of [^{3}H]-perhydrohistrionicotoxin in the absence of agonist was suggestive of minimal interaction with the closed or resting conformation of the AChR. In addition, these carbamates displaced the binding of [^{3}H]ACh to the receptor site, thus indicating an agonist effect at the AChR (13) in accordance with patch-clamp data (this paper; Refs. 10 and 12). Although data obtained on *Torpedo* membranes are qualitatively similar to those observed on the junctional region of skeletal muscles, it appeared that lower concentrations of these drugs were required to disclose the agonistic and channel-blocking properties on the latter. Conversely, binding assays suggest that these carbamates enhance receptor desensitization (13) which was not clearly revealed electrophysiologically, although a greater depression of the EPC peak amplitude was observed when the EPCs were elicited at higher frequency of nerve stimulation.

Thus, in addition to an anti-ChE effect on the AChR of the neuromuscular junction, both Phy and MetPhy appeared to have at least two distinct actions: i) as agonists, both agents induced channel openings similar to those activated by ACh, most likely by an interaction with ACh receptor site since this activation was blocked by either α-bungarotoxin or Naja α-toxin; and ii) as noncompetitive blockers both agents appeared to interact primarily with a site at the ACh-activated channel in its open conformation although the possibility of a desensitizing component cannot be discarded. Finally, patch-clamp studies with the quaternary analog MetPhy provided no evidence for the existence of any binding site for Phy interactions at the internal portion of AChR as judged from the absence of any effect when MetPhy was present at the cytoplasmic side of the membrane patch under inside-out configuration. Also, since MetPhy acted similarly, most likely the charged form is responsible for all of the interactions with the different sites in the AChR complex.

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