Reaction of Tetraethylammonium with the Open and Closed Conformations of the Acetylcholine Receptor Ionic Channel Complex

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ABSTRACT   The effect of tetraethylammonium (TEA) bromide on the neurally and iontophoretically evoked endplate current (EPC) of frog sartorius muscle was investigated using voltage-clamp and noise analysis techniques, and its binding to the acetylcholine (ACh) receptor ionic channel complex was determined on the electric organ of *Torpedo ocellata*. TEA (250–500 μM) produced an initial enhancement followed by a slow decline in the amplitude of the endplate potential and EPC, but caused only depression in the amplitude of the miniature endplate potential and current. In normal ringer's solution, the EPC current-voltage relationship was approximately linear, and the decay phase varied exponentially with membrane potential. Upon addition of 50–100 μM TEA, the current-voltage relationship became markedly nonlinear at hyperpolarized command potentials, and with 250–2000 μM TEA, there was an initial linear segment, an intermediate nonlinear segment, and a region of negative conductance. The onset of nonlinearity was dose-dependent, undergoing a 50 mV shift for a 10-fold increase in TEA concentration. The EPC decay phase was shortened by TEA at hyperpolarized but not depolarized potentials, and remained a single exponential function of time at all concentrations and membrane potentials examined. These actions of TEA were found to be independent of the sequence of polarizations, the length of the conditioning pulse, and the level of the initial holding potential. TEA shifted the power spectrum of ACh noise to higher frequencies and produced a significant depression of single channel conductance. The shortening in the mean channel lifetime agreed closely with the decrease in the EPC decay time constant. At the concentrations tested, TEA did not alter the EPC reversal potential, nor the resting membrane potential, and had little effect on the action potential duration. TEA inhibited the binding of both [^{3}H] ACh (K_i = 200 μM) and [^{3}H]perhydrohistrionicotoxin (K_i = 280 μM) to receptor-rich membranes from the electric organ of *Torpedo ocellata*, and inhibited the carbamylcholine-activated ^{22}Na$^+$ efflux from these microsacs. It is suggested that TEA reacts with the nicotinic ACh-receptor as well as its ion channel; the voltage-dependent actions are associated with blockade of the ion channel. The results are compatible with a kinetic model in which TEA

first binds to the closed conformation of the receptor-ionic channel complex to produce a voltage-dependent depression of endplate conductance and subsequently to its open conformation, giving rise to the shortening in the EPC decay and mean channel lifetime.

INTRODUCTION

The increase in endplate conductance which accompanies the interaction of acetylcholine (ACh) with its recognition site on the ACh receptor is believed to be mediated by a macromolecular entity termed the ion conductance modulator (ICM) (Albuquerque et al., 1973 a,b), ionic channel (Albuquerque et al., 1978; Eldefrawi et al., 1978), or ionophore (Sobel et al., 1978). To elucidate the sequence of events between receptor activation and the increase in endplate conductance, it is first necessary to identify and characterize the ACh receptor and its ionic channel at the molecular level. Much progress has been made in this regard by use of specific probes to study these individual components of the postjunctional membrane. Thus, α-bungarotoxin has been employed as a specific agent for the detection of the nicotinic ACh receptor (Miledi et al., 1971; Lee, 1972), and the histrionicotoxins have been used as specific probes for the cholinergic channel (Albuquerque et al., 1973 b; Lapa et al., 1975; Dolly et al., 1977; Eldefrawi et al., 1978).

Based on their alteration of the endplate current (EPC), inhibitors of ACh action can be grouped into two classes. (a) "Competitive" antagonists, such as d-tubocurarine (< 5 μM), reduce the amplitude of the EPC but do not alter its time-course, nor modify the influence of membrane potential on peak amplitude or decay rate (Albuquerque et al., 1978). (b) "Noncompetitive" or allosteric antagonists, such as belladonna alkaloids (Katz and Miledi, 1973; Adler and Albuquerque, 1976; Feltz et al., 1977), local anesthetics (Cohen et al., 1974; Adams, 1975, 1977; Katz and Miledi, 1975; Beam, 1976; Kato and Changeux, 1976; Ruff, 1977; Neher and Steinbach, 1978), amantadine (Albuquerque et al., 1978; Tsai et al., 1978), and the histrionicotoxins (Albuquerque et al., 1973 b, 1974; Dolly et al., 1977), alter both the amplitude and time-course of the EPC, and in addition, modify the voltage dependence of the decay rate, and produce a marked curvature in the synaptic current-voltage relationship. Thus, the competitive antagonists are thought to react with the ACh receptor whereas the latter agents have been suggested to act on the ionic channel, although some overlap may be expected at high concentrations as shown for d-tubocurarine (Manalis, 1977; Katz and Miledi, 1978).

Tetraethylammonium (TEA) is known to have a dual action at the amphibian neuromuscular junction. Koketsu (1958) reported that immediately following application of TEA (1,000–3,000 μM), the endplate potential (EPP) amplitudes were augmented, but eventually became depressed with continued exposure to the drug. The enhancement of the EPP amplitude was attributed to an increased transmitter release. The depression consisted of a steady decline which was independent of stimulus frequency, and a frequency-dependent component which was attributed to transient depletion of ACh. Since TEA also reduced the depolarization resulting from bath applications of ACh, the steady decline of

the EPP amplitude was considered to be curariform in nature. However, since a variety of small organic cations are known to penetrate the activated junctional membrane, whereas larger ones produce blockade of transmission (Furukawa and Furukawa, 1959; Maeno et al., 1977), the frequency-independent component of the depression may result from action of the drug on the endplate channel in addition to, or instead of, the ACh receptor. An effect on the endplate channel appears likely in view of the fact that TEA is known to inhibit ion fluxes through the delayed rectification channel of excitable membranes (Armstrong, 1966, 1971).

The present study was undertaken to obtain more information on the effect of TEA on neuromuscular transmission, and to determine its postsynaptic molecular targets, utilizing both physiological and biochemical techniques. The physiological approach consisted of examining the effect of TEA on the EPC, miniature EPC (MEPC), and EPC fluctuations in voltage-clamped frog sartorius muscle. The biochemical experiments were designed to determine the effect of TEA on the binding of [^{3}H] ACh and [^{3}H] perhydrohistrionicotoxin ([^{3}H]H$_{12}$-HTX) to the ACh receptor and its ion channel, respectively, and on the carbamylcholine-induced ^{22}Na$^+$ efflux from excitable microsacs. The binding and efflux studies were carried out on the electric organ of the electric ray, *Torpedo ocellata*, which is a rich source of the nicotinic synaptic proteins. A preliminary account of some of these findings has been reported (Adler et al., 1979).

METHODS

Drugs and Toxins

TEA bromide was purchased from J. T. Baker Chemical Co., (Phillipsburg, N. J.), and [*acetyl*-^{3}H]ACh (sp act 49.5 Ci/mol) and ^{22}Na (carrier free) from New England Nuclear (Boston, Mass.). [^{3}H]H$_{12}$-HTX (sp act 4.8 Ci/mol) was obtained by tritiation of isodihydro — HTX and its activity was tested on frog sartorius muscle (Eldefrawi et al., 1977).

Electrophysiological Techniques

All experiments were carried out in vitro at 21-23°C on isolated sciatic nerve-sartorius muscle preparations of the frog *Rana pipiens*. To eliminate muscle contraction, the preparations were treated with Ringer's solution containing 600 mM glycerol and then washed with normal Ringer's as described by Fujino et al. (1961) and Gage and Eisenberg (1967). Muscles were mounted under slight tension on a paraffin block having a plano-convex lens in the center, and perfused with normal Ringer's solution of the following composition (millimolar): NaCl, 115.5; KCl, 2.0; CaCl$_2$, 1.8; Na$_2$HPO$_4$, 1.3; and NaH$_2$PO$_4$, 0.7. TEA Ringer's was made from refrigerated stock solutions of 100 mM, and diluted to the desired concentration before use. The preparation was equilibrated with drug solution for at least 1.5-2 h prior to recording to minimize transient (presynaptic) effects on the EPC amplitude (Koketsu, 1958).

The voltage-clamp circuitry for recording EPCs was similar to that described previously (Kuba et al., 1974; Adler and Albuquerque, 1976). Two microelectrodes were inserted in the endplate region at an interelectrode distance of 50 μm, to record membrane potential and pass current, respectively. Recording electrodes were filled with

3 M KCl, and had resistances between 3 and 8 MΩ. Microelectrodes used for delivering feedback current were filled with either 3 M KCl, or a mixture of 1.7 M K citrate (pH 6.5) and 0.7 M KCl (Valdiosera et al., 1974), and had resistances between 2 and 5 MΩ. Electrodes filled with the latter mixture had better current-passing properties but shorter storage lives than those filled with KCl alone.

Three voltage sequences were employed to assess the actions of TEA on neuromuscular transmission:

LONG CONDITIONING DURATION The membrane was initially clamped to the holding potential of −50, −70, or −90 mV, and driven in 10-mV steps to the extremes of +50 and −150 mV before being returned to the holding potential. To ensure that cell deterioration at extreme levels did not contribute to the observed nonlinearity in the current-voltage relationship, data were accepted only if the EPC amplitude at the end of a run differed by < 10% from its initial value. Complications arising from a presynaptic frequency-dependent attenuation of EPC amplitudes (Koketsu, 1958) were avoided by alternating the sequence of polarizations in successive trials and by use of long intervals (3–5 s) between command steps. The EPC was elicited ~0.5 s before the end of each step. Unless specifically indicated, the EPC data were obtained with long conditioning durations.

SHORT CONDITIONING DURATION The membrane was clamped to the holding potential with a constant DC source, and shifted in ~10-mV steps for periods of 20–50 ms using rectangular pulses from an isolated stimulator. The EPC was elicited within the first 5–25 ms of the conditioning pulse every 3–5 s, and returned to the holding potential between pulses.

INSTANTANEOUS CLAMP The membrane was clamped to −70 mV, and hyperpolarized in ~10-mV steps by a rectangular pulse timed to coincide with the center of the EPC peak. The same pulse was repeated without the EPC, and this background current was subtracted to yield the EPC at the desired membrane potential. To facilitate the subtraction, the temperature was lowered to 12–15°C, thereby broadening the EPC peak.

ACh-Noise Analysis

Conventional procedures were used for the recording and analysis of endplate fluctuation data (Katz and Miledi, 1972; Anderson and Stevens, 1973). The iontophoretic pipette (2 M ACh, 50–100 MΩ) was positioned between the two voltage clamp electrodes 25–50 μm above the endplate surface. ACh was ejected with positive pulses of 5–75 nA from a constant current generator for 20–30 s; backing currents of 20 nA or less usually sufficed to prevent background ACh leakage. Clamp and iontophoretic currents were stored on an FM tape recorder (Tandberg, series 115, Oslo, Norway) and subsequently transmitted for analysis to a PDP 11/40 computer (Digital Equipment Corp., Marlboro, Mass.). The noise data were recorded simultaneously on a low-gain DC and high-gain AC channel. The high-gain signal was filtered with a Krohn-Hite 3700 band pass filter (1–800 Hz, Krohn-Hite Corp., Avon, Mass.) to exclude high frequency noise and avoid aliasing errors. The sampling rate was 2 kHz. 30 segments of 0.256 s (512 points) were obtained from each cell under control conditions and in the presence of TEA. Digitized records were monitored on a Tektronix storage oscilloscope (Tektronix, Inc., Beaverton, Ore.) and those free of obvious electrical artifacts and MEPCs were processed by a fast Fourier transform to obtain the power spectrum. The power spectrum of ACh noise was obtained from an average of 30 acceptable segments after subtraction of a comparable number of segments of base-line noise before ACh iontophoresis. The resulting spectrum was fitted by a computer-generated Lorentzian curve from which the half-power or cutoff (f_c) frequency was determined. f_c was used to calculate the mean channel lifetime

(τ_l) by the relationship $\tau_l = 1/(2\pi f_c)$. Single channel conductance (γ) was routinely estimated by two methods: (a) from the power spectrum (γ_S) using the relationship

$$\gamma_S = \frac{S(0)}{4\mu_I(V - V_{eq})\tau_I},$$ (1)

where $S(0)$ is the zero frequency asymptote of the power spectrum, μ_I is the mean ACh-induced current, V is the holding potential, and V_o is the synaptic null potential (Anderson and Stevens, 1973; Ruff, 1977); and (b) from the variance of current noise (γ_V), using the relationship

$$\gamma_V = \frac{\sigma_I^6}{\mu_I(V - V_{eq})},$$ (2)

where σ_I^2 is the variance of current noise and the other symbols are as in Eq. 1.

In view of the good agreement in the values for single channel conductance yielded by the two methods, correction of χ_V for filtering (Colquhoun et., 1977) did not seem necessary.

Treatment of Data

EPCs and command potentials were measured and analyzed after suitable amplification with the aid of a PDP 11/40 minicomputer. EPCs were sampled at a rate of 100 μs/point with an analog-to-digital converter. Time constant of the decay phase (τ_{EPC}) was determined as the decay from 20 to 80% using a regression analysis program. Acceptable EPCs had correlation coefficients of 0.98 or better. Statistical analysis was performed by means of a Student's t test. P values ≤ 0.05 were considered statistically significant. Unless stated otherwise all values are expressed as the mean $\pm$ SEM.

Biochemical Techniques

Membranes were prepared from the electric organ of *T. ocellata* (stored at $-90°C$) by homogenization (20%, wt/vol) in ice-cold solution of 90 mM KCl, 10 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4), and by centrifugation of the supernate of a 5,000 g 10-min spin for 60 min at 30,000 g. This pellet was resuspended in Krebs original Ringer phosphate solution (millimolar): NaCl, 107; KCl, 4.8; $CaCl_2$, 0.65; $MgSO_4$, 1.2; and Na_2HPO_4, 15.7; pH 7.4. 1 ml represented 1 g of the electric organ, and the membranes formed microsacs. The final protein concentration, as determined by the method of Lowry et al. (1951), ranged from 1 to 2 mg/ml, and the maximum number of binding sites for [3H]ACh and [3H]H_{12}-HTX were 0.7 and 0.5 nmol/mg protein, respectively.

Equilibrium dialysis was used to study the binding of [3H]ACh to the electric organ membranes as described previously (Eldefrawi et al., 1977). 0.5 ml of membrane preparation in a dialysis bag was shaken for 4 h at 21°C in Krebs original Ringer phosphate (25 ml) containing [3H]ACh in the absence or presence of TEA. Triplicate samples of 50 μl were then taken from each dialysis bag and bath; the excess radioactivity in the former represented the amount of ligand bound. In order to inhibit all cholinesterases without affecting the binding of ACh to its receptor, diisopropylfluoro-phosphate (DFP) was added to the membranes, at a final concentration of 1 mM, 1 h before the start of dialysis, and 100 μM DFP was present in the dialysis bath.

Centrifugal assay was used to study the binding of [3H]H_{12}-HTX to the electric organ membranes, so as to save on the amount of radiolabeled toxin. Samples (10 μl) of different concentrations of [3H]H_{12}-HTX in ethanol were added to 1-ml samples of the *Torpedo* membrane preparation, mixed, and incubated for 60 min at 21°C in siliconized

1.5-ml polyethylene microcentrifuge tubes. TEA (10 μl) was added to the membranes 30 min (at 21°C) before addition of toxin. The incubation mixture was then centrifuged at 30,000 g for 60 min, and three samples (50 μl each) were taken from the mixture before, as well as from the supernate after centrifugation, and the radioactivity was counted. Excess radioactivity in the former represented the bound toxin. Specific binding to the ionic channel was obtained after subtraction of nonspecific binding to membranes immersed in boiling water for 30 min and amounted to 10 ± 3% of control at 10 nM [^{3}H]H$_{12}$-HTX.

^{22}Na$^+$ efflux measurements were made on *Torpedo* membrane microsacs as previously described (Eldefrawi et al., 1978) using a method modified from Hess et al. (1975). To each 1 ml of microsac preparation, 25 μl of ^{22}NaCl was added and incubated at 1-2°C for 36 h. A sample of 100 μl was then diluted with 20 ml of ice-cold solution consisting of 250 mM KCl, 5 mM NaCl, 4 mM CaCl$_2$, 2 mM MgCl$_2$, and 5 mM Na$_2$HPO$_4$, pH 7. Two 1-ml samples were taken after different times, filtered on a HAWP 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.), and rinsed twice with 10 ml of ice-cold buffer. The radioactivity retained on the filters was counted in an auto-gamma scintillation spectrometer (model 5230, Packard Instrument Co., Inc., Downers Grove, Ill.).

After ~30 min, most of the nonspecific ^{22}Na$^+$ efflux subsided and a steady-state efflux was reached. At 39 min, the membrane sample was divided into two. Carbamylcholine (100 μM) was added to one resulting in an instantaneous increase in ^{22}Na$^+$ efflux, and the second acted as control. When the effect of TEA on this ^{22}Na$^+$ efflux was tested, TEA was added at 36 min after dilution and 3 min before carbamylcholine addition. Because efflux of [^{3}H]glucose, ^{36}Cl$^-$, and ^{35}SO$_4$$^{2-}$ were unaffected by the addition of carbamylcholine, the receptor-activated ^{22}Na$^+$ efflux was considered specific and not due to osmotic lysis of the microsacs (Eldefrawi et al., 1978).

RESULTS

Onset of TEA's Action on Neuromuscular Transmission

In agreement with the findings of Koketsu (1958), exposure of the sartorius muscle to TEA resulted in a marked potentiation in the amplitudes of the neurally evoked EPP and EPC. Potentiation of the EPP amplitude was observed within 5 min from the start of perfusion with TEA (500 μM), and reached a peak which was three to four times the control value within 15-20 min (Fig. 1). This potentiation occurred whether the muscles were previously treated with d-tubocurarine or detubulated as a result of glycerol treatment. The EPP amplitudes returned to control values within 60 min and decreased to ~20% of control over the next 40 min, becoming stable thereafter.

In contrast to its dual action on the EPP, TEA caused only depression of the miniature endplate potential (MEPP) or miniature endplate currents amplitudes. The depression occurred rapidly and was complete within 20 min from the onset of perfusion. No prolongation in the decay of either the spontaneous or nerve-evoked response was detected at any stage of drug exposure. Some prolongation has been reported by Koketsu (1958).

Effect of TEA on the Current-Voltage Relationship

The actions of TEA on neuromuscular transmission were most apparent when EPCs were examined over a wide range of membrane potentials. Fig. 2 shows current-voltage plots in control and in the presence of TEA at concentrations of

50–1,000 μM. The current-voltage relationship in control preparations was linear through most of its range but displayed a gentle upward curvature between −100 and −150 mV. The nonlinearity in the current-voltage relationship, although small, is well documented (Kordaš, 1969; Magleby and Stevens, 1972 a,b; Kuba et al., 1974) and has been attributed to a voltage-dependent retardation in channel opening rates (Dionne and Stevens, 1975; Mallart et al., 1976).

Addition of TEA to the bathing medium produced striking alterations in the EPC current-voltage relationship. In the presence of 50 and 100 μM TEA, the current-voltage relationship resembled control except that the curvature was more pronounced and began at more depolarized potentials. With 250, 500, and 1,000 μM TEA, the current voltage relationship consisted of a brief linear
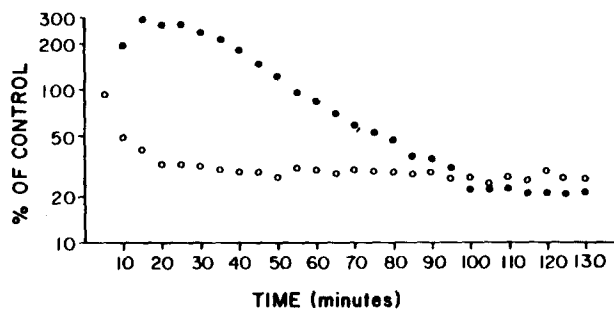
FIGURE 1. Time-course of potentiation, depression, and stabilization of the neurally evoked endplate potential (●) during the action of TEA (500 μM), in a preparation equilibrated with 2.8 μM d-tubocurarine. (O) The results of an experiment where only spontaneous MEPPs were recorded. TEA caused a rapid decrease in the MEPP amplitude. This depression reached an equilibrium at about 20 min for the MEPP and 90 min for the EPP. The abscissa shows time of incubation in presence of TEA while the ordinate (logarithmic scale) depicts percent of control amplitude. Perfusion rate was 18–20 ml per min.

segment at depolarized potentials, followed by a nonlinear segment and a region of negative conductance at hyperpolarized potentials. At the concentrations examined, TEA did not alter the EPC reversal potential (Fig. 2) nor the resting membrane potential. The falling phase of the directly elicited action potential was prolonged by < 10% even in the presence of 3,000 μM TEA, a concentration which produced almost total blockade of neuromuscular transmission.

The onset of nonlinearity in the current-voltage relationship was shifted to more depolarized membrane potentials with increasing TEA concentration (Fig. 3). In the presence of 100 μM TEA, the current-voltage relationship became nonlinear beyond −67 mV, but with 1,000 and 2,000 μM TEA, the nonlinear region encompassed all but the inside positive membrane potentials.

The marked voltage-dependent action of TEA is clearly demonstrated by the dose-response curves of Fig 4, where EPC amplitudes at −150 and +50 mV are plotted against TEA concentrations ranging from 10 to 2,000 μM. The two
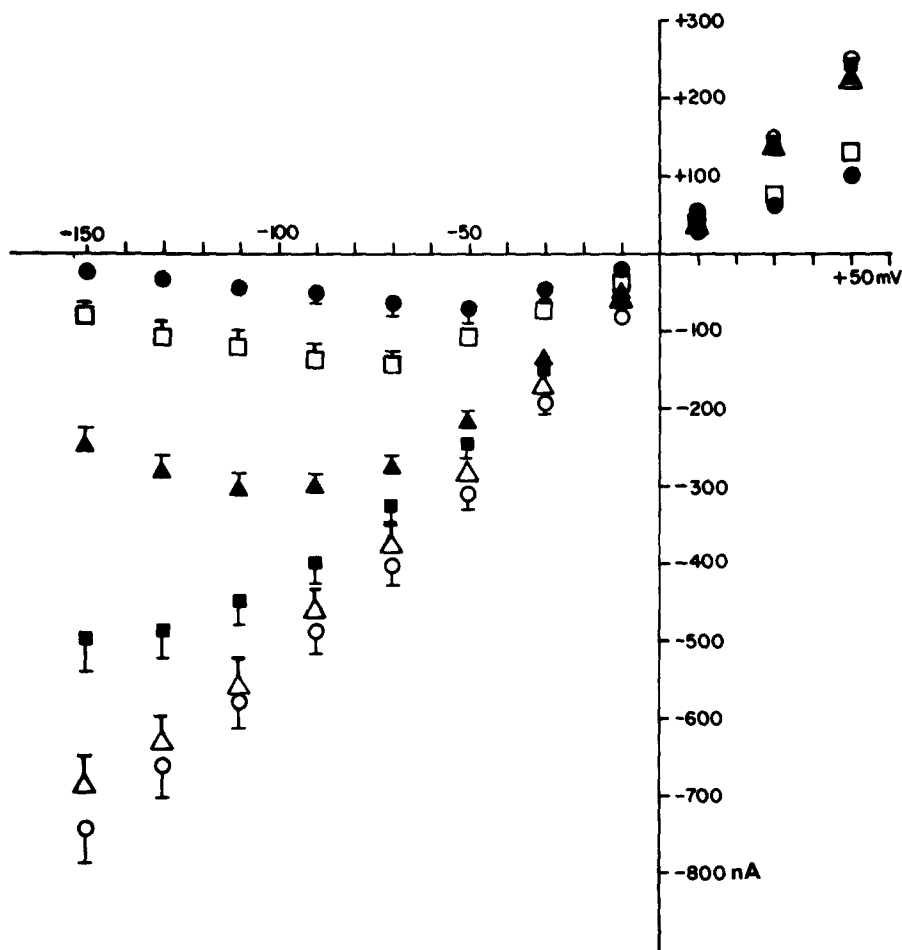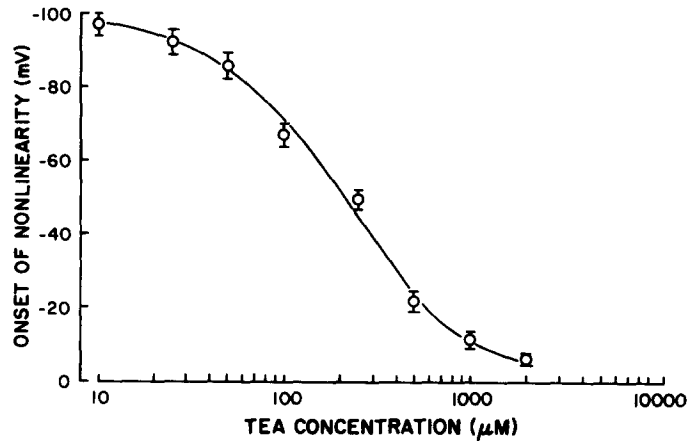
FIGURE 3.   Onset of nonlinearity in the current-voltage relationship varies with TEA concentrations. The onset voltage was obtained by plotting current-voltage relationships for each cell and noting the membrane potential where the EPC amplitude began to deviate systematically from a straight line fit to the positive potentials. Increases in drug concentration shifted the onset of nonlinearity to more depolarized membrane potentials. Each point is the mean ± SEM of 9–23 fibers in three to five muscles.
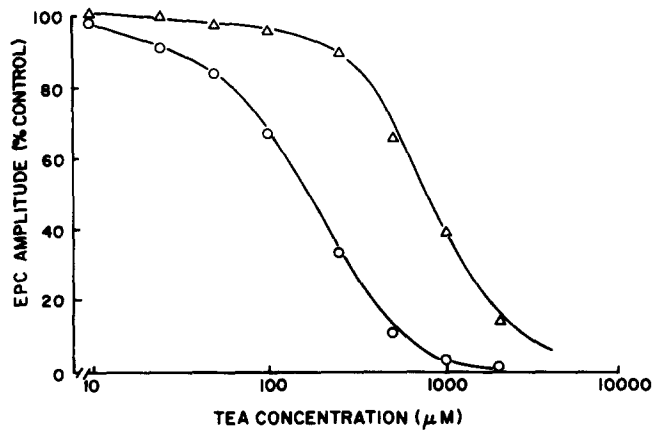

FIGURE 4.   Dose-response curves showing the voltage-dependent action of TEA where EPC amplitudes were recorded at −150 mV (O) and +50 mV (△). Each point is the mean of 9–23 fibers sampled from three to five muscles. Clearly TEA has a higher affinity for its site(s) at −150 mV than at +50 mV.

Wilcoxon, 1949). As illustrated in Fig. 5, the ED_{50} depended exponentially on membrane potential varying from 170 μM (95% confidence interval 117–247 μM) at −150 mV to 790 μM (95% confidence interval 608–1,027 μM) at +50 mV. The regression line fitted to the points gave a slope of 0.00815 mV^{-1} and a 0-mV intercept of 567 μM.

Time-Dependence of TEA Action

A possible mechanism underlying the nonlinearity in the current-voltage relationship is that TEA reacts with the endplate channel in a voltage-dependent manner, and that channels complexed with TEA fail to open when ACh receptors are activated by ACh. If the reaction between TEA and channel were to occur slowly relative to the time required to shift the membrane potential, it should be possible to obtain linear current-voltage relationships in the presence of TEA by reducing the conditioning duration. Such an effect has recently been demonstrated for histrionicotoxin (Masukawa and Albuquerque, 1978). To test
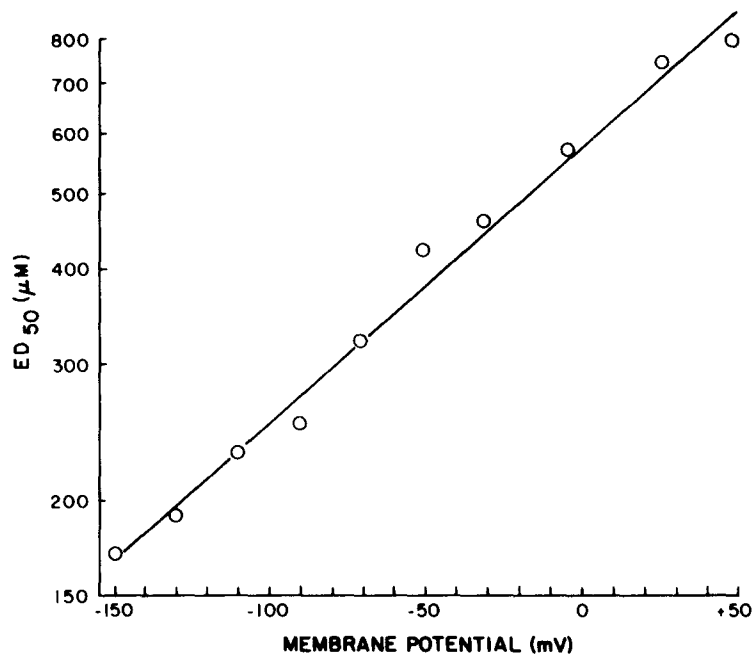

FIGURE 5. ED_{50} values obtained by probit analysis from dose-response data similar to Fig. 4 at various holding potentials. Abscissa shows the membrane potential in millivolts and the ordinate the ED_{50} in micromolar plotted on a log scale. The symbols represent the mean ED_{50} estimated from 6–23 muscle fibers.

this hypothesis, families of EPCs were recorded in TEA solutions (250 μM) by first shifting the membrane potential in 20-mV steps from the holding potential of −50 mV using 3-s intervals. The EPC was elicited 0.5 s before the end of each step making the effective conditioning duration 2.5 s. This yielded the expected nonlinear current-voltage plot with a linear region between +30 and −50 mV and marked curvature thereafter (Fig. 6). A second family was then recorded, but this time the membrane potential was displaced for only 25 ms from the holding potential; EPCs were elicited after 5 ms at 3-s intervals. As shown in Fig. 6, reduction of the conditioning duration had little effect on the current-voltage relationship. EPC amplitudes displayed essentially the same nonlinear dependence on membrane potential regardless of whether the membrane was condi-

tioned to a specified test potential for 5 ms or 2.5 s. Similar data were obtained in 29 other endplates at a variety of TEA concentrations (50–500 μM), temperatures (8–24°C), holding potentials (−30, −50, −70, and −90 mV), and conditioning durations (5 ms–10s). These results suggest that, if voltage-dependent TEA binding were responsible for the nonlinear current-voltage relationship, the reaction would have to be complete within 5 ms of a potential change.

Since brief conditioning durations did not alter the shape of the current-voltage relationship, we attempted the more complicated procedure of shifting the membrane potential instantaneously during the peak of the EPC. An example of one such experiment is illustrated in Fig. 7. EPCs were first recorded
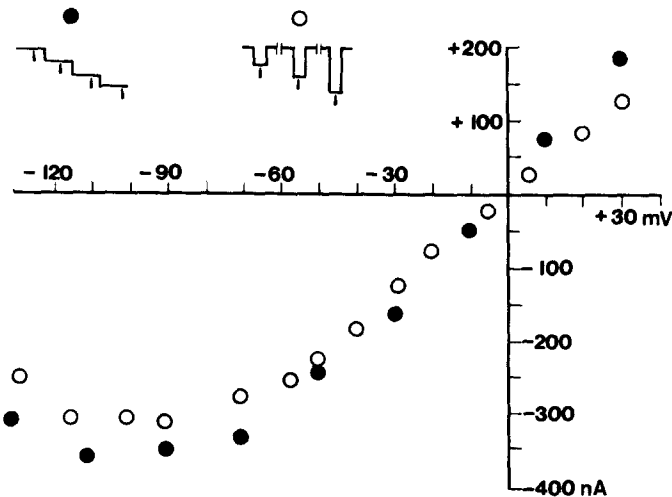

FIGURE 6. The effect of conditioning pulses of brief durations on the action of TEA (250 μM). (●) The current-voltage relationship obtained with 2.5-s conditioning durations. (O) Data recorded after 5 ms of a 25-ms conditioning pulse. Both records were from the same endplate. The insets show the respective stimulus patterns.

in the presence of 250 μM TEA by making step changes from the holding potential of −70 mV at 3-s intervals (●). At the completion of this family, EPCs were elicited at −70 mV, and during the peak, were displaced within 50 μs to new negative values. The EPC amplitude at −70 mV departed only slightly from the linear relationship seen at the more positive potentials, suggesting that approximately the same number of channels opened in response to ACh between +70 mV and −70 mV. Hence, instantaneous shifts in the membrane potential from −70 mV should give linear current-voltage relationships if the reaction of TEA with its site is appreciably longer than 50 μs, and nonlinear relationships if the reaction is already complete by 50 μs. The data in Fig. 7 clearly demonstrate that the instantaneous current-voltage relationship is nonlinear (O), and provide an upper limit of 50 μs for the completion of the voltage-dependent process.

Effect of TEA on the EPC Rise Time and Time Constant of Decay

The rise times of control EPCs and MEPCs generally show little voltage-dependence (Feltz et al., 1977). In the present study the EPC rise times ranged from 0.76 ± 0.02 ms at +50 mV to 0.83 ± 0.03 ms at −150 mV, which was not statistically significant. After addition of TEA (25–1,000 μM), EPC rise times underwent a slight, but not significant, shortening and remained relatively voltage-insensitive.

In contrast to the small effect on the EPC rising phase, TEA caused marked alterations in the EPC decay, and its dependence on membrane potential (Fig. 8). At −50 mV, TEA (50 μM) did not alter the EPC decay rate as evidence by
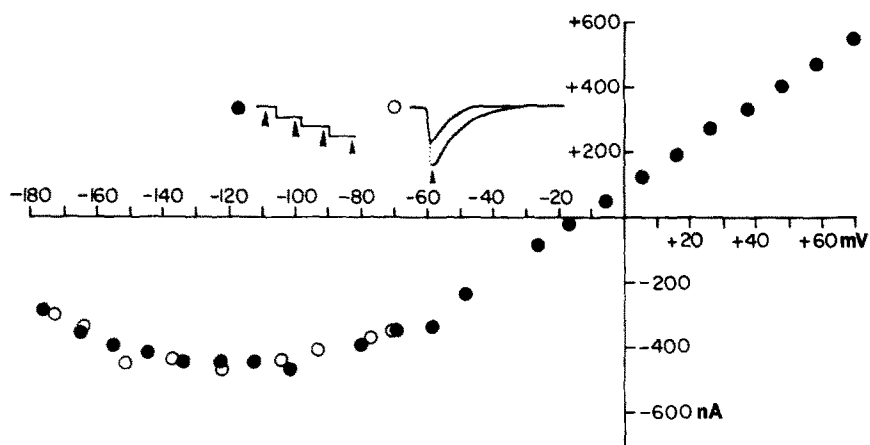

FIGURE 7.   Effect of TEA (250 μM) on the instantaneous current-voltage relationship. The closed circles were obtained with a conditioning duration of 2.5 s. The open circles were obtained from the same cell with instantaneous shifts in membrane potential. The inset illustrates the stimulus pattern schematically. Holding potential was −70 mV.

the fact that values from both control and drug-treated cells could be described by a single curve (Fig. 8 A). However, when the membrane potential was shifted to −150 mV, the decay phase of the control EPC underwent a two-fold lengthening, but the decay from the TEA-treated cell remained at the level appropriate to −50 mV (Fig. 8 B). In the example illustrated, and at all TEA concentrations and membrane potentials used, at least 95% of the total EPC decay could be adequately described by a single exponential function.

The TEA-induced alterations in the voltage-dependence of the EPC decay time constant (τ_{EPC}) are shown in Fig. 9 for a wide range of TEA concentration and membrane potentials. Prior to adding TEA, τ_{EPC} varied exponentially with membrane potential between +50 and −150 mV. In the presence of TEA, the EPC decay still varied with membrane potential at depolarized potentials but showed almost no voltage dependence in the hyperpolarized regions.

The actions of TEA on τ_{EPC} differ in several important respects from those on
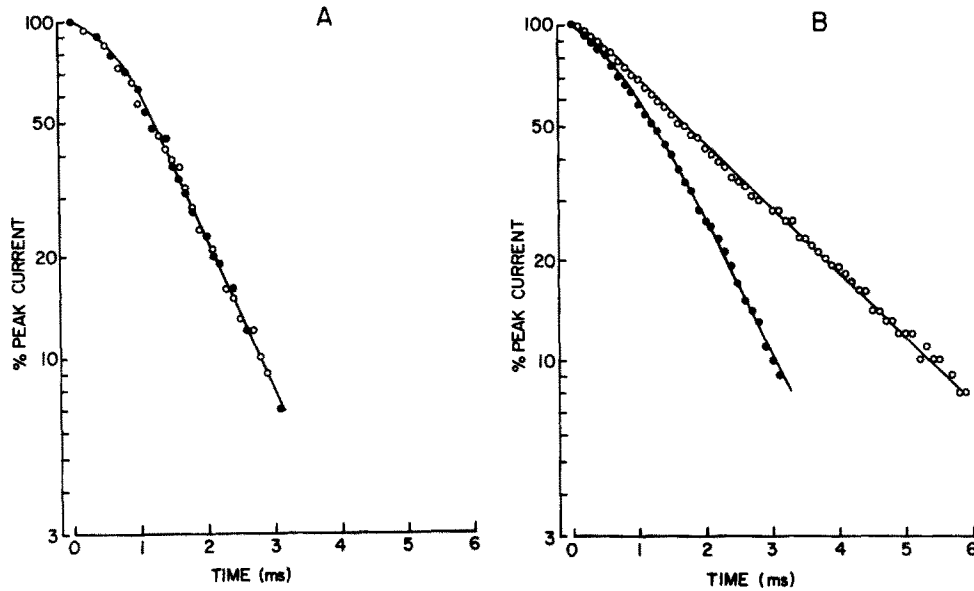
FIGURE 8.   Effects of TEA (50 μM) on the EPC decay phase. (A) Control cell (O) and one exposed to TEA for 90 min (●) at a holding potential of −50 mV. (B) The same cells after the membrane potential was shifted to −150 mV.

FIGURE 9.   The relationship between the EPC decay constant and membrane potential in control (●) and after addition of various concentrations of TEA: 50 μM (■); 100 μM (×); 250 μM (O); 500 μM ($\triangle$); 1000 μM (□). Each point represents mean values from 12 to 21 endplates from five muscles.

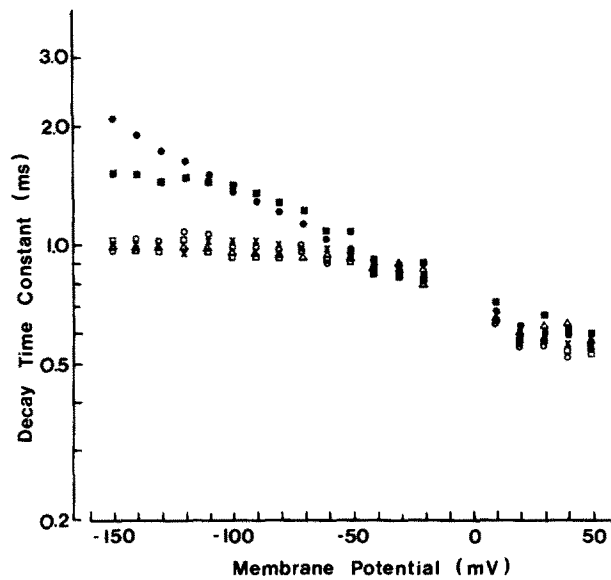the current-voltage relationship. First, the alterations on τ_{EPC} were already well developed in 50 μM TEA (Figs. 8 and 9), and could be observed with concentrations as low as 10μM, but these concentrations had little effect on the nonlinearity in the current voltage relationship (Fig. 2). Second, at hyperpolarized potentials τ_{EPC} approached 1 ms with 100 μM TEA, and did not change appreciably with a 10-fold increase in TEA concentration. In contrast, the effect of TEA on the EPC amplitude showed no tendency to saturate; EPC amplitudes underwent marked depression between 100 and 1,000 μM TEA (Figs. 2 and 4). Third, τ_{EPC} values remained in the control range between +50 and −50 mV even with 1,000 μM TEA, which produced from 61% (+50 mV) to 77% (−50 mV) blockade of peak amplitude (Fig. 2). Considered together these findings suggest that TEA alters amplitude and time-course by separate mechanisms.

Effect of TEA on ACh Noise

The alterations produced by TEA on the EPC time-course and current-voltage relationship suggest that the drug acts on the endplate channel. This suggestion was pursued further by studying the possible interaction of TEA with endplate channels by ACh noise analysis. The use of the endplate current fluctuation analysis is particularly important in view of the presynaptic effects of TEA (Koketsu, 1958; Benoit and Mambrini, 1973). Any alterations in the ACh noise would necessarily be due to postsynaptic effects of the drug, and a good agreement between the results of the ACh noise analysis and the EPC would indicate that the effects on the EPC thus far studied are indeed due to postsynaptic, rather than presynaptic effects of TEA. Samples of the power spectra recorded at −65 and −105 mV in the presence of TEA (250 μM) are shown in Fig. 10. The control channel lifetime (τ_l) increased with hyperpolarization as first described by Anderson and Stevens (1973). In the present case, τ_l increased from 1.2 ms at −65 mV to 1.9 ms at −105 mV paralleling the changes described for τ_{EPC} (Fig. 9). After addition of TEA, τ_l was reduced, and became relatively voltage-independent. In the presence of 100 and 250 μM TEA, τ_l was shortened to ∼1 and 0.85 ms, respectively, at potentials more negative than −65 mV, in close agreement with the shortening in ν_{EPC} under comparable experimental conditions (Table I). These results strongly suggest that reductions in the average channel lifetime underlie the shortening in the EPC decay.

In addition to shortening τ_l, TEA also depressed the effective single channel conductance. Interestingly, channel conductances apparently became voltage-dependent in the presence of TEA, although it is well known that this parameter does not normally vary with membrane potential (Anderson and Stevens, 1973). Thus, γ became significantly depressed at hyperpolarized membrane potentials. At −65 mV, γ was depressed by < 5% at either 100 or 250 μM TEA, but at −105 the depression was ∼50% at both TEA concentrations.

Effects of TEA on Binding of Ligands to the ACh Receptor and Its Ionic Channel

To determine TEA's postjunctional binding sites the effect of the drug was studied on the binding of radiolabeled ligands to the ACh receptor and its ionic channel. Binding of [^{3}H]H$_{12}$-HTX (40 and 80 nM) to *Torpedo* electric organ
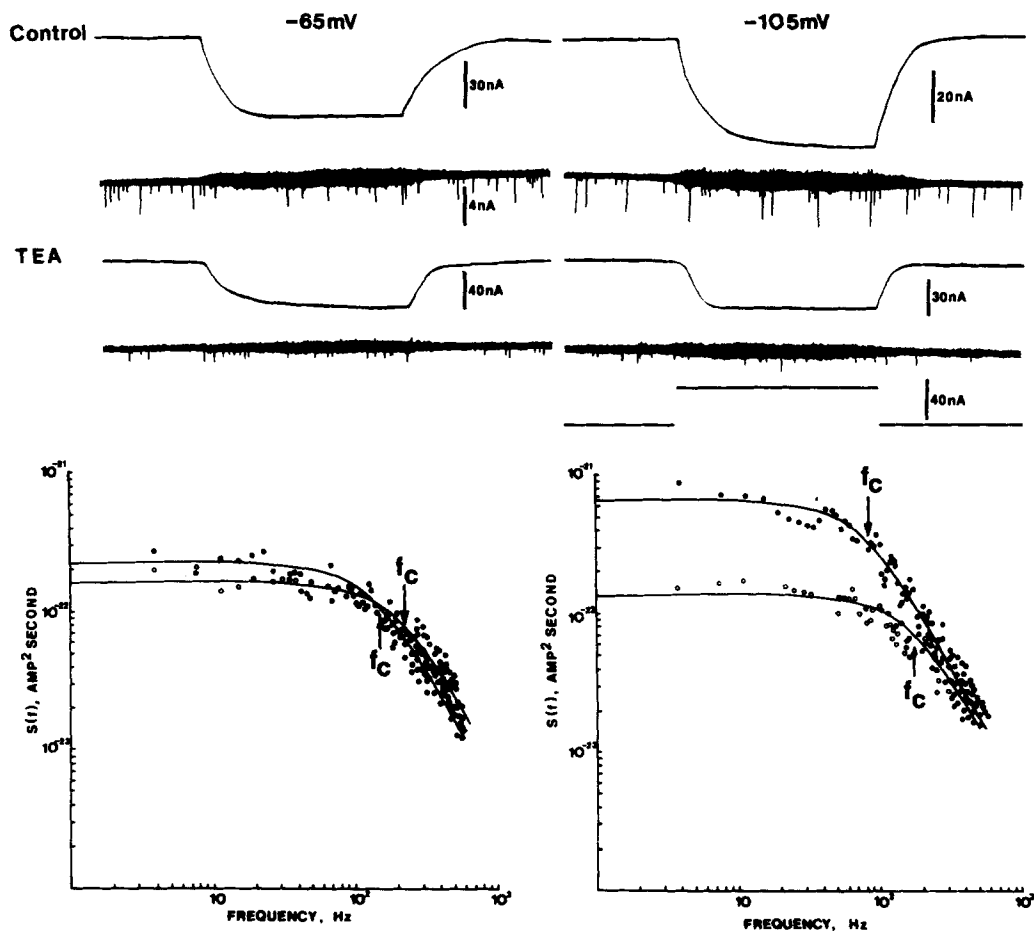
FIGURE 10. Experiments showing that TEA depresses single channel conductance and shortens channel lifetime in a voltage-dependent manner. At both holding potentials the pairs of records shown in control and in TEA solutions are endplate currents in the absence and presence of ACh, recorded on a low-gain, DC-coupled channel (upper trace) and a high-gain, AC-coupled channel (lower trace). The small downward deflections in the high gain trace are miniature endplate currents. The lowest trace in the column corresponding to the holding potential of -105 mV is the iontophoretic current. In the lower panels of the figure, power spectra obtained in control (●) and TEA (○) solutions, at two different holding potentials are depicted. The solid lines represent the least squares fit of the experimental points to a single Lorentzian. The half-power frequencies (f_c) are denoted by arrows, and at -65 mV they were 144 and 204 Hz, in control and in the presence of TEA, respectively, whereas at -105 mV the corresponding values were 82 and 182 Hz. These cut-off frequencies yielded channel lifetimes of 1.10 ms (control, -65 mV); 0.78 ms (TEA, -65 mV); 1.93 ms (control, -105 mV); and 0.87 ms (TEA, -105 mV). In the same respective situations, values for single channel conductance were: 16.4, 21.8, 20.4, and 9.9 pS. Temperature: 22°C. The cutoff frequency of the filter was 800 Hz.

membranes in the presence and absence of different concentrations of TEA is presented in a Dixon plot (Fig. 11). This graphical method provides a direct estimate of K_i. The value on the abscissa that corresponds to the point of intersection of the two lines equals $-K_i$. Competitive inhibition is indicated by the lines intersecting above the abscissa, while noncompetitive inhibition is disclosed by the point of intersection occurring on the abscissa (Dixon and Webb, 1964). The plots obtained in the presence of TEA (100–600 μM) show that it inhibited [^{3}H]H$_{12}$-HTX binding competitively with K_i of 280 μM. Binding of [^{3}H]ACh (at 0.2 and 1 μM) was also inhibited by TEA competitively with a K_i of 200 μM (Fig. 11).

Effect of TEA on Receptor-Activated ^{22}Na Efflux

The ACh receptor-activated ^{22}Na$^+$ efflux from microsacs, formed from electric organ membranes, has been shown to be inhibited by drugs and toxins that

TABLE I

EFFECT OF TEA ON SINGLE CHANNEL CONDUCTANCE (γ_S AND γ_V) AND LIFETIME (τ_l) IN THE FROG ENDPLATE

Condition	Holding potential	γ_s	γ_v	τ_l
	mV	pS	pS	ms
Control	−65	19.4±1.4 (10)	19.9±2.7 (10)	1.19±0.03 (10)
	−85	20.5±1.5 (8)	22.1±1.5 (8)	1.21±0.10 (8)
	−105	20.9±1.5 (4)	22.6±0.6 (4)	1.92±0.22 (4)
TEA, 100 μM	−65	19.6±1.6 (3)	18.8±2.9 (3)	1.08±0.11 (3)
	−85	15.9±0.6 (8)	16.1±1.3 (8)	1.05±0.08 (8)
	−105	12.1±0.6 (5)	12.3±1.0 (5)	1.00±0.09 (5)
TEA, 250 μM	−65	18.4±2.4 (4)	20.2±4.5 (4)	0.84±0.06 (4)
	−85	17.6±1.2 (4)	19.1±2.0 (4)	0.82±0.03 (4)
	−105	11.1±1.8 (4)	9.6±1.0 (4)	0.91±0.08 (4)

The numbers in parentheses represent the number of spectra obtained in at least three different preparations.

inhibit the ACh receptor and/or its ionic channel (Hess et al., 1975; Popot et al., 1976; Eldefrawi et al., 1978; Tsai et al., 1978). Therefore, if TEA inhibits neuromuscular transmission by interacting with postsynaptic macromolecules, it is expected to inhibit such specific ^{22}Na$^+$ efflux. Indeed, TEA inhibited significantly the carbamylcholine-induced ^{22}Na$^+$ efflux in a dose-dependent manner (Fig. 12).

DISCUSSION

The present findings demonstrate that TEA interacts with and inhibits the function of the ionic channel of the nicotinic ACh receptor as evidenced by the marked nonlinearity in the EPC current-voltage relationship (Fig. 2), the shortening in the EPC decay (Fig. 9), the reduction in single channel conductance and lifetime (Fig. 10), as well as the inhibition of [^{3}H]H$_{12}$–HTX binding (Fig. 11), and ACh receptor-activated ^{22}Na$^+$ efflux from *Torpedo* microsacs (Fig.
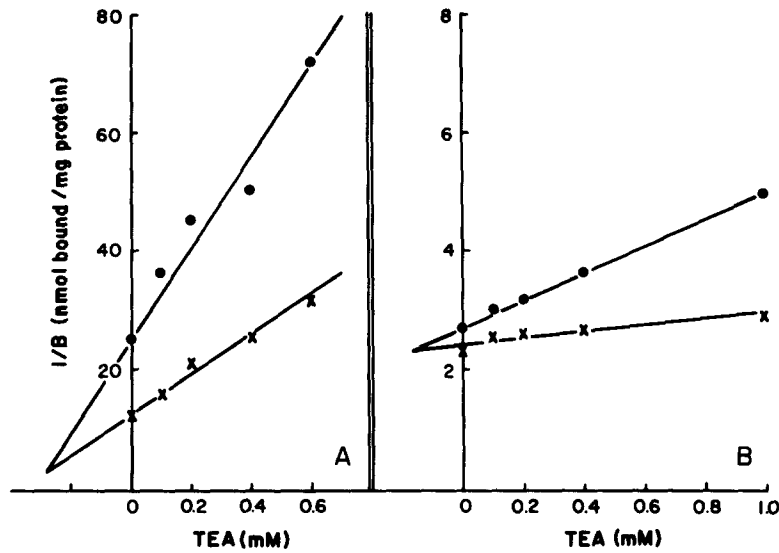
FIGURE 11.   Dixon plots of the effect of TEA on the binding of (A) [^{3}H]H$_{12}$—HTX at 40 nM (●) and 80 nM (×), and (B) [^{3}H] ACh at 0.2 μM (●) and 1 μM (×) to *Torpedo* electric organ membranes. Symbols represent mean values from three experiments.
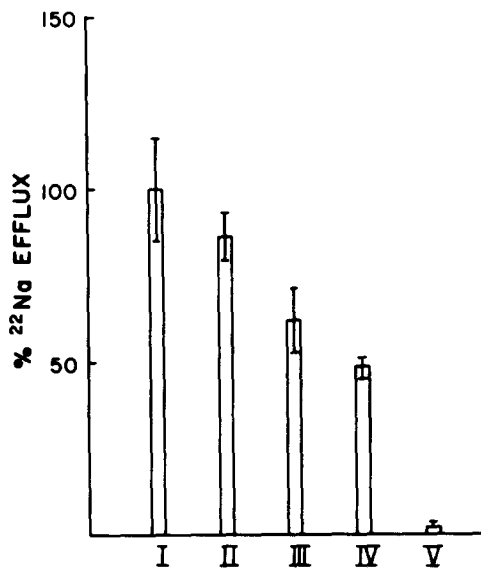

FIGURE 12.   Histogram of ^{22}Na$^+$ efflux from *Torpedo* microsacs induced by 100 μM carbamylcholine (I) alone and (II) in presence of 1,000 μM TEA, (III) 2,000 μM TEA and (IV) 4,000 μM TEA, as well as (V) in the absence of carbamycholine or TEA. Vertical bars represent ± standard deviation of three experiments. TEA appears to be less potent in blocking ^{22}Na$^+$ efflux than in inhibiting radioactive ligand binding because of the relatively high carbamylcholine concentration used to induce maximum ^{22}Na$^+$ efflux.

12). Although binding studies with *Torpedo* membranes indicate that TEA also reacts with the ACh receptor (Fig. 11), it is unlikely that the voltage-dependent effects observed are mediated by receptor blockade. Agent which act predominantly on the ACh receptor such as *d*-tubocurarine do not generally exhibit voltage dependence in their action (Albuquerque et al., 1978), except at very high concentration (Manalis, 1977; Katz and Miledi, 1978).

Receptor blockade may contribute an additional voltage-independent fraction to the total depression of EPC amplitudes, but its contribution in the frog muscle appears to be less than in the electroplax membranes. Although the current-voltage relationship is markedly nonlinear in the presence of 250 μM TEA (Fig. 2), this drug concentration has no significant effect on EPC amplitudes between 0 and +50 mV. If receptor blockade contributes appreciably to TEA's action *in situ*, there should have been depression in the EPC amplitude at positive membrane potentials with TEA concentrations between 50 and 250 μM. It is possible that the depression in the EPC amplitude at positive membrane potentials may have been masked by TEA's residual presynaptic facilitation. Arguing against this possibility was the finding that the EPP amplitude became depressed to the same extent as the MEPP amplitude when sufficient time was allotted for the facilitation to subside (Fig. 1).

In the presence of most TEA concentrations, the current-voltage relationship consists of an initial linear segment, an intermediate nonlinear segment, and a region of negative conductance. One possible explanation for such nonlinear current-voltage profiles is that at hyperpolarized potentials the TEA-altered endplate channel either opens more slowly or closes more rapidly than normal. The former has been suggested to underlie the slight upward curvature in the current-voltage relationship of control endplates (Dionne and Stevens, 1975), whereas the latter has been proposed for the nonlinearity observed in the presence of the belladonna alkaloids (Adler et al., 1978). Since TEA increases the EPC decay rate at hyperpolarized potentials (Fig. 9), it is possible that this effect, alone, or coupled with changes in the opening rate of the ionic channel, may be responsible for the alterations in the current-voltage relationship. However, two lines of evidence render this unlikely. First, concentrations of TEA between 10 and 50 μM produced significant shortening of τ_{EPC} but had little effect on the EPC amplitude. Second, mechanisms in which nonlinearity arises from changes in channel kinetics predict linear current-voltage relationships for instantaneous shifts in membrane potential (Magleby and Stevens, 1972 *b*). But, as shown in Fig. 7, the instantaneous current-voltage relationship is also nonlinear in the presence of TEA. It is therefore unlikely that changes in channel kinetics can account for the TEA-induced alterations in the current-voltage relationship. An alternative possibility is that TEA depresses peak conductance by reducing the fraction of endplate channels available for transporting ions. Accordingly, it is possible that TEA binds to the endplate channel and produces a complex which is only partially conducting or nonconducting. Some indication for the former comes from findings that the effective single channel conductance is reduced by TEA (Fig. 10, Table I). If it is assumed that TEA binding is voltage-dependent, this mechanism can account for striking nonlinearities in the EPC current-voltage relationship. Inasmuch as the [^{3}H]H$_{12}$–

HTX binding studies were done in the absence of ACh, it appears that depression in EPC amplitude involves an action of TEA on the closed conformation of the endplate channel.

The production of nonlinear current-voltage relationships appears to be a common feature of agents which act on the endplate channel. Current-voltage profiles similar to those described here have been reported for HTX and its derivatives (Albuquerque et al., 1974; Eldefrawi et al., 1977; Masukawa and Albuquerque, 1978, amantadine (Albuquerque et al., 1978), and piperocaine.[1] Their actions on the current-voltage relationship differ from one another primarily in the wide range of relaxation times following perturbations in membrane potential. HTX and TEA appear to be limiting cases: for a step change from -50 to -150 mV, HTX requires 8 s to equilibrate to 63% of its final value (Masukawa and Albuquerque, 1978), whereas a comparable voltage shift with piperocaine is complete by 400 ms, and with TEA by 50 μs. As a consequence of its rapid equilibration, the nonlinearity produced by TEA is independent of the sequence of potential changes. Moreover, if the time between successive nerve impulses is sufficient to prevent presynaptic depression (Koketsu, 1958), the nonlinearity is also independent of the interval between potential steps.

The nonlinearity encountered with HTX, by contrast, is markedly time-dependent, being influenced by both the sequence of potential changes and the interval between steps. The time-dependent action of HTX has been suggested as the basis for hysteresis in the current-voltage relationship observed with this toxin (Albuquerque et al., 1974; Masukawa and Albuquerque, 1978).

The other major effect of TEA is on the EPC decay. In the presence of TEA, EPC decays are abbreviated at hyperpolarized potentials but essentially unchanged at depolarized potentials (Fig. 9). The shortening in the EPC decay is accompanied by a corresponding shift in the cutoff frequency (f_c) of the power spectrum, indicating that both are the result of reductions in the single channel lifetime.
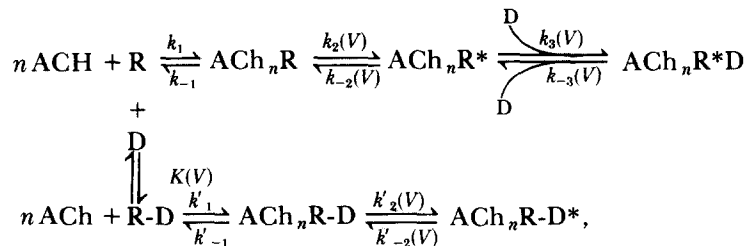
Unlike many local anesthetics (Steinbach, 1968; Beam, 1976; Ruff, 1977), the decay in the presence of TEA remains a single exponential function (Fig. 8). Moreover, τ values never approach the very brief 0.2-0.25 ms reported for decays in the presence of local anesthetics (Beam, 1976), octanol (Gage et al., 1974), and the belladonna alkaloids (Katz and Miledi, 1973; Feltz et al., 1977; Adler et al., 1978).

The effect of TEA on the EPC decay appears to involve sites distinct from those mediating the production of nonlinear current-voltage relationships. This is based on the finding that the effect on τ_{EPC} can be elicited at very low TEA concentrations and saturates at concentrations between 100 and 250 μM TEA (Fig. 9). One possible explanation for the different effects of TEA on the EPC amplitude and decay is that the former represents blockade of closed endplate channels, while the latter arises from blockade of open channels, both reactions being voltage sensitive.

Although blockade of the open channel can, in itself, produce depression of

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[1] Tiedt, T. N., and E. X. Albuquerque. Unpublished results.

the EPC amplitude (Adler et al., 1978), to account for the striking nonlinearity by this mechanism, the τ_{EPC} would have to become shorter with hyperpolarization over the range where the experimental findings indicate that it becomes concentration- and voltage-insensitive (Fig. 9). Therefore, it appears necessary to assume that TEA binds to both the open and closed conformation of the endplate channel. This suggestion is incorporated in the following kinetic model:

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

$$n\,\text{ACh} + \text{R-D} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} \text{ACh}_n\text{R-D} \underset{k'_{-2}(V)}{\overset{k'_2(V)}{\rightleftharpoons}} \text{ACh}_n\text{R-D*},$$

where n is the number of ACh molecules (assumed to be 1 or 2), D stands for TEA, R is the normal receptor-channel complex, R-D is the analogous TEA-altered complex (blocked in closed conformation), ACh_nR and $\text{ACh}_n\text{R-D}$ are the intermediate forms of the normal and altered activated receptors, respectively; $\text{ACh}_n\text{R*}$ and $\text{ACh}_n\text{R-D*}$ are the normal and altered "open" conformations, respectively. $\text{ACh}_n\text{R*D}$ is the low conducting species which results from the binding of TEA to $\text{ACh}_n\text{R*}$. The $k_1, k_{-1}, k_2, k_{-2}, k_3$, and k_{-3} are rate constants for the indicated reactions; those followed by (V) are assumed to be voltage-sensitive. $K(V)$ is the voltage-dependent equilibrium constant which controls the distribution of receptor between R and R-D. The anti-ACh-receptor effect of TEA revealed by the binding studies on *Torpedo* membranes (Fig. 11) has not been incorporated in the model since it was not possible to determine its contribution at the frog endplate.

For simplicity, it is assumed that k'_1 and k'_{-1} are identical to k_1 and k_{-1}, that k'_2 and k'_{-2} are identical to k_2 and k_{-2}, and that $\text{ACh}_n\text{R-D*}$ undergoes no further reaction with TEA. The reduction in the effective single channel conductance obtained with noise analysis (Table I, Fig. 10) indicates that one or both of the drug-altered species has a lower than normal conductance. Since there are two such forms, it is not possible at this time to determine their relative conductances. However, the model will account for the main features of the EPC alterations regardless of whether a small, but finite, or zero conductance is assumed for either $\text{ACh}_n\text{R*D}$ or $\text{ACh}_n\text{R-D*}$. Our preliminary digital computer simulations predict that the nonlinearity in the current-voltage relationship will be enhanced by increases in TEA and that, at a given drug concentration, will be influenced by the membrane potential. If the voltage dependence of k_3 is sufficiently steep, and if only a small fraction of R is converted to $\text{ACh}_n\text{R*D}$, the model will also predict that the relationship between τ_{EPC} and membrane potential will have a normal component at positive potentials, a relatively voltage-insensitive component at hyperpolarized potentials and a reduced dose dependence at high TEA concentration. Retention of single exponential decay requires that k_{-3} and the conductance of $\text{ACh}_n\text{R*D}$ be rather small at all membrane potentials. Increases in either parameter causes the appearance of

multiexponential decays. Although somewhat speculative the model provides a plausible interpretation for the actions of TEA on endplate conductance and elementary events and serves as a viable working hypothesis.

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