Voltage- and Time-Dependent Action of Histrionicotoxin on the Endplate Current of the Frog Muscle

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ABSTRACT Histrionicotoxin, a toxin isolated from skin secretions of a Colombian arrow poison frog, Dendrobates histrionicus, decreased the amplitude and time-course of the endplate current, and altered the voltage dependence of the half-decay time. In addition, the toxin produced a characteristic nonlinearity in the current-voltage relationship of the endplate current when 3-s voltage conditioning steps were used. Reduction in time of the conditioning steps to 10 ms made the current-voltage relationship linear. The decrease in peak amplitude of the endplate current (epc) produced by histrionicotoxin measured during long hyperpolarizing conditioning steps was fitted by a single exponential function. The calculated rate constants ranged from 0.03 to 0.14 s\(^{-1}\) and varied with membrane potential at hyperpolarizing levels. The voltage- and time-dependent action of histrionicotoxin does not require an initial activation of receptors by acetylcholine (ACh). The characteristic of the current-voltage relationship can be accounted for by the observed voltage and time dependency of the attenuation of the endplate current amplitude in the presence of histrionicotoxin during long conditioning steps. These effects of histrionicotoxin on the peak amplitude, and on the voltage and time dependence of the epc were concentration-dependent and slowly reversible upon washing out the toxin. Thus, the voltage- and time-dependent action of histrionicotoxin at the endplate is related to an increase in the affinity between the toxin and the ACh receptor-ionic channel complex. This increase in affinity is postulated to be due to a conformational change of the macromolecule in the presence of histrionicotoxin which is demonstrated to be relatively slow, i.e., on the order of tens of seconds.

INTRODUCTION

In previous reports the actions of histrionicotoxin (HTX) and its analogues, isolated from the skin secretions of a Colombian arrow poison frog, Dendrobates histrionicus were described (Daly et al., 1971; Albuquerque et al., 1973a, b; Albuquerque et al., 1974; Lapa et al., 1975; Kato and Changeux, 1976; Burgermeister et al., 1977; Dolly et al., 1977). HTX decreased the amplitude and the time-course of endplate current (epc), and altered the voltage dependency of its half-decay time. Analysis of the current-voltage relationship under control conditions and in the presence of other drugs disclosed nonlinearity in
the hyperpolarized region (Kördas, 1969, 1970; Deguchi and Narahashi, 1971; Magleby and Stevens, 1972a; Dionne and Stevens, 1975; Adler and Albuquerque, 1976). This nonlinearity in the current-voltage relationship has been attributed to the voltage dependence of opening and closing of unit channels (Magleby and Stevens, 1972b; Dionne and Stevens, 1975). It has been proposed that the potential field across a biological membrane could have a major influence on membrane macromolecules involved in ionic conductance changes (Magleby and Stevens, 1972a, b; Anderson and Stevens, 1973). Thus, it was considered worthwhile to study in detail the effects of HTX on the epc and in particular on the current-voltage relationship.

It was found that the alteration in amplitude of the epc produced by HTX was due to a voltage- and time-dependent action of the toxin on the acetylcholine (ACh) receptor-ionic channel complex leading to a suppression of channel conductance, rather than to a change in the time-course of the opening and closing of single channels. The findings reported herein support the view that the voltage- and time-dependent action of HTX may occur as a result of an increased affinity of the toxin for the ACh receptor or its channel in the closed conformation in the presence of a hyperpolarizing field. The time-dependent action is explained in terms of a slow transition in the conformational states of this macromolecule. The action of HTX was found to be concentration dependent and reversible upon washing.

MATERIALS AND METHODS

The experiments were performed at room temperature (20–23°C) on the surface fibers at the endplate region of the sartorius nerve-muscle preparations of Rana pipiens between October and May. The sartorius muscles were treated for approximately 60 min with normal frog Ringer solution containing 600 mM glycerol (Fujino et al., 1961; Gage and Eisenberg, 1967). At least 30-min exposure to normal Ringer solution was required before neurally evoked contractions ceased.

Normal Ringer solution used in these experiments had the following composition (in millimolar): NaCl, 115; KCl, 2.0; CaCl₂, 1.8; Na₂HPO₄, 1.3; and NaH₂PO₄, 0.7. The pH was 6.9–7.1. HTX (mol wt 283, pKa ~ 8.0) was prepared as a stock solution in 80% ethanol at a concentration of 10 mg/ml. Aliquots from this stock solution were added to the Ringer solution to produce a final concentration of 30 or 10 μM. Constant perfusion of the recording chamber was maintained when normal Ringer solution was used, but because of limited quantities of the toxin the chamber fluid was replaced with fresh solution every 15 min during exposure to HTX.

The sartorius muscle was pinned slightly stretched over a plano-convex lens embedded in Sylgard resin (Dow Corning Corp., Midland, Mich.). Microelectrodes were visually positioned at the endplate regions using a stereo, dissecting microscope with a maximum magnification of ×100. Optimal placement of microelectrodes at the endplate required mapping the regions of fine nerve twigs, observing endplate potentials that rose to peak amplitude within a minimum time and presence of miniature endplate potentials. 3 M KCl-filled microelectrodes of 2–5 MΩ resistance were used to pass current, and 5–10 MΩ resistance electrodes were used to record voltage.

The voltage clamp circuit used was similar to that previously described (Adler and Albuquerque, 1976; Kuba et al., 1974; Takeuchi and Takeuchi, 1959). Voltage clamp errors were < 5% of the unclamped endplate potential. Changes in membrane potential
from the holding potential were evoked either from a DC source under manual control or a stimulus pulse unit controlled by a programmable digital timer. Wave forms were recorded on film from the oscilloscope for analysis.

To study the voltage- and time-dependent changes of the epc wave forms, conditioning steps of varied duration were used. Voltage sequence A was similar to that previously used for examining HTX-treated fibers (Albuquerque et al., 1974). The sequence was made up of successive 3-s long, 10-mV steps starting from a holding potential of -50 mV. Conditioning steps were made sequentially in the depolarizing and then the hyperpolarizing direction between the voltage extremes of +80 and -180 mV. Immediately after the periodic generation of an epc, the conditioning potential was manually stepped with a decade potentiometer to a new level. Therefore the epc occurred approximately 0.5 s before the end of each step. A 3-s conditioning step was used here for two reasons. It was previously shown that when fibers were exposed to 35 μM HTX, epc amplitudes attenuated rapidly when they were generated at a high frequency, 20 Hz (Albuquerque et al., 1974). In addition, long steps minimized to a certain degree the error in the manually controlled conditioning duration.

Voltage sequence B was used to test the influence of conditioning step length on the relationship between epscs and the membrane potential. This sequence required that the endplate be clamped for relatively short periods of 20 ms every 3 s. The pulses were presented consecutively in the depolarizing and then the hyperpolarizing direction as they were in voltage sequence A. Epcs were evoked automatically 10 ms after the beginning of the conditioning pulse.

Voltage sequence C was used to study the actual time-dependent changes in the epscs during long hyperpolarizing steps, which lasted for at least 20 s. During and after the conditioning step, epcs were evoked every 3 s. Voltage sequence D was identical to voltage sequence C except that epscs were evoked before and immediately (within 1.5 s) after the termination of the conditioning step. However, epscs were not evoked during the hyperpolarizing step.

The analysis of the data was performed using Student's t test. P values < 0.01 were considered statistically significant. All values are expressed as the mean ± SEM. Using nonlinear regression analysis, the peak amplitudes of epscs from treated and untreated fibers were fitted by a single exponential function of the form

\[ \text{epc}_p(t) = ce^{-at} + b, \]

where epcp(t) is the peak endplate current as a function of time, c + b is the intercept, b is the asymptote, and a is the rate constant.

RESULTS

Effect of HTX on the Amplitude and Time-Course of the Endplate Current

The amplitude and time-course of the epc were significantly altered after exposure of the sartorius nerve-muscle preparation to HTX (30 μM) for 0.5 h. The peak amplitude was decreased by 60.6 ± 5.3%; the time to peak was reduced by 7.8 ± 1.60%; and the half-decay time was shortened by 41.2 ± 1.86% (n = 34). Resting membrane potentials of the glycerol-treated muscle fibers before exposure to the toxin were between -40 and -80 mV with a mean value of -55.6 ± 1.06 mV (n = 170 fibers in 20 muscles). The mean resting membrane potential of HTX-treated muscles was -51.1 ± 1.61 mV (n = 59).
The Nature of the Current-Voltage Relationship in the Presence of Histrionicotoxin

Several voltage sequences were used to study the voltage- and time-dependent effects of HTX under voltage clamp. These sequences were explained in detail in Materials and Methods and will be referred to here by their sequence letters. Voltage sequence A was used to confirm the earlier voltage clamp studies with HTX (Albuquerque et al., 1974). HTX (30 nM) had a voltage- and time-dependent effect and caused a significant departure from linearity in the current-voltage relationship of the epc. The conductance change seen at each conditioning voltage was less during depolarizing steps from -180 to -50 mV, than the initial hyperpolarizing steps from -50 to -180 mV. These changes produced a characteristic loop configuration in the third quadrant of the current-voltage plot (Fig. 1, inset). The current-voltage relationship in untreated fibers did not show such a marked departure from linearity when voltage sequence A (or B) was used. However, under control conditions, a slight nonlinearity was observed at potentials > -100 mV as was reported by others (Kördas, 1969; Magleby and Stevens, 1972a,b; Kuba et al., 1974; Dionne and Stevens, 1975). Studies of the effect on the current-voltage relationship of the epc by procaine, atropine, and scopolamine (Kördas, 1969, 1970; Deguchi and Narahashi, 1971; Adler and Albuquerque, 1976) revealed changes in the time-course and amplitude of epcs. Although these drug effects on the epc wave form and nonlinearity were commonly observed, no asymmetry as observed in Fig. 1 (inset) has been reported. These effects of HTX indicate an influence of the history of the endplate on the epc amplitude. This influence may depend on previous activation of ACh receptors or opening of the channel to make sites available for interaction with HTX. The following experiments were performed to test these possibilities and to investigate the probable site of action of HTX.

Voltage sequence B was used to determine whether this nonlinearity and voltage pathway sensitivity observed with the toxin were dependent on the duration of potential conditioning or dependent only on the potential sequence and activation of ACh receptors. As in voltage sequence A, epcs occurred at intervals of 3 s and changes in membrane potential were in the depolarizing and then hyperpolarizing direction. An illustration of the effect on the current-voltage relationship of decreasing the conditioning pulse to 20 ms is shown in Fig. 1. The current-voltage relationship using voltage sequence B is reasonably linear. Data in Fig. 1 (and inset) were taken from the same cell in which voltage sequence B was presented before A to avoid residual effects due to slow recovery from 3-s hyperpolarizing potential steps. The voltage-dependent effect on the epc caused by HTX was not observed using voltage sequence B. Thus, the process leading to voltage pathway sensitivity requires a conditioning pulse longer than 20 ms in duration and does not appear to be dependent on previous activation of ACh receptors. In addition, the factors leading to nonlinearity and asymmetry in the current-voltage relationship are mediated by a similar mechanism, as they are both absent in Fig. 1 when voltage sequence B is used. This time dependence in the shape of the current-voltage relationship was seen in all cells studied.

Because relatively long clamp durations were used during voltage sequence A
to make the voltage and time dependence evident, the possibility existed that we might be observing an indirect effect on the epc due to time-dependent changes in ion concentrations. Thus, reversal potentials of the epc of cells treated with HTX (15–40 μM) using voltage sequence A and B were compared after the entire series of epcs were evoked under each condition. The reversal potentials measured using the two voltage sequences were not significantly different ($P < 0.001$) from each other. The reversal potentials were +7.4 ± 3.73 (n = 10, voltage sequence B) and +5.9 ± 1.65 mV (n = 22, voltage sequence A). The reversal potential for control cells was +3.8 ± 0.96 mV (n = 54).

**Figure 1.** Current-voltage relationship of peak endplate current in the presence of histrionicotoxin using 20-ms conditioning steps in the form of voltage sequence B (see Methods). Voltage sequence B was presented to this cell before voltage sequence A. Inset: Significant nonlinearity in the current-voltage relationship of peak endplate currents in the presence of histrionicotoxin. The endplate region of a representative fiber was conditioned for 3 s at each potential level by voltage clamp using voltage sequence A. Endplate currents were evoked by nerve stimulation at the end of each step. The sequence of steps began at a holding potential of −50 mV and progressed first toward positive values following the direction of the arrows. Inward current is denoted by negative amperes.
In addition, untreated endplates were presented with voltage sequence A and B to determine whether long clamp periods or long conditioning steps alone would alter the current-voltage relationship. There do not appear to be significant ion concentration shifts nor time-dependent changes as revealed by the almost identical current-voltage relationship of the epcs using voltage sequence A or B.

**Voltage- and Time-Dependent Action of HTX on the Endplate Current Amplitude**

To investigate in more detail the voltage and time dependence of the epc amplitude in the presence of HTX, epcs were evoked every 3 s, during the long potential steps using voltage sequence C. Due to the slow reversal of the voltage-dependent attenuation of the epc, only one potential step per cell was made. Fig. 2 illustrates the changes in the endplate current from cells during 30-s potential steps from a holding level of −50 mV to a conditioning step of −90 or −150 mV. Untreated cells did not show consistent attenuation of peak amplitudes with steps up to −150 mV (Fig. 2, top row). Using nonlinear regression analysis (see Eq. 1), the peak amplitudes from epcs were fitted by a single exponential function. The rate constant, \( a \), at −90 mV (Fig. 2, middle row) and at −150 mV (Fig. 2, bottom row) in the presence of 30 M HTX was 0.05 and 0.12 s\(^{-1}\), respectively. Therefore a new equilibrium appeared to be established which

![Figure 2](image-url)
at \(-90\) mV required approximately 33 s to reach 67% of its final value and at \(-150\) mV required only about 8 s. It should be noted, however, that the half-decay times of epcs were not altered by potential conditioning although the amplitudes were significantly decreased.

The epc amplitudes did not completely recover to their previous levels when the membrane potential was returned to \(-50\) mV after the endplate was conditioned for 30-90 s at more hyperpolarized levels. Though the peak amplitude reached a plateau level with an exponential time-course within approximately 20 s after the return to \(-50\) mV, it was below the peak amplitude before the step (i.e., about 50% of the previous value). This suggests that the toxin may have two apparent sites of action, one readily reversible and the other nearly irreversible. However, at this stage the data do not permit one to assess whether or not the rate constant of recovery is potential dependent. In addition to the relatively slow rate of equilibration at hyperpolarizing levels, the slow recovery must also contribute to the marked nonlinearity of the current-voltage relationship observed in the presence of HTX. The effects of HTX on the current-voltage relationship were reversible after washing the muscles with normal Ringers. Indeed, after 1-2 h of washing the current-voltage relationship was linear and no longer showed looping in the 3rd quadrant when voltage sequence A was used. The slowness of this recovery is most likely related to a relatively high affinity of HTX for its recognition sites at the ionic channel combined with the lipophilic nature of the toxin which facilitates its penetration into the endplate membrane and intracellular compartments of the muscle fiber.

The decay rate constants of the epc amplitude for intermediate potential levels varied directly with voltage as illustrated in Fig. 3. The magnitude of the decay rate constants agreed well with the conditioning time intervals that produced the curves shown in Fig. 1. The relatively slow rates account for the lag in the response of epc amplitudes to the voltage- and time-dependent effect and also account for its absence when short, 20-ms, pulses were used.

The results obtained thus far indicated that the nonlinearity in the current-voltage relationship was not dependent on repeated activation of ACh receptors or opening of channels. It was necessary, however, to obtain more direct evidence of the nature and possible site of voltage-dependent action of HTX because the membrane potential in voltage sequence B was returned to the holding level between pulses, and there was some recovery when the membrane potential was returned to \(-50\) mV after a long hyperpolarizing step. Both of these conditions could account for the lack of nonlinearity and pathway sensitivity using voltage sequence B. To test further the potential-dependent action of HTX on the peak amplitude of the epc, voltage sequence D was used. Fig. 4 illustrates that voltage-dependent reaction of HTX can occur without the activation of ACh receptors as long as the membrane has been conditioned by a hyperpolarizing step. The membrane potential was stepped from \(-50\) to \(-150\) mV for 30 s. Endplate currents were evoked at \(-50\) mV immediately before and after the step, but not during the step. When the attenuation of these epcs was compared to that of epcs recorded during the step at the beginning and end of the 30-s hyperpolarization (with eight intervening epcs) they were similar in
magnitude (see legend of Fig. 4 for more details). Thus, the site of action of HTX appeared to be available before the activation of receptors by ACh as long as a hyperpolarizing potential was present and would, therefore, make the blocking of open channels by HTX improbable as a mode of action in the reduction of endplate current during hyperpolarization.

Inasmuch as a relationship exists between the amplitude and half-decay time of the epc, so that a decrease in half-decay time can lead to a reduction of the amplitude, an analysis of these two parameters, which were markedly changed in the presence of HTX, was made. The semilogarithmic plots in Fig. 5 illustrate the voltage dependence of the half-decay times of epcs whose ampli-

![Graph](image)

**Figure 3.** The voltage dependence of the calculated decay constants of endplate current amplitudes of fibers treated with histrionicotoxin. The decay constants were derived by fitting the exponential function (Eq. 1) to the peak amplitude of endplate currents using nonlinear regression analysis. The endplate currents used were from cells treated with histrionicotoxin and hyperpolarized for 30 s using voltage sequence C. Each point represents a mean ± SD from at least three cells.

Effect of a Lower Concentration of HTX on the Endplate Current

Exposure of the sartorius muscles to 10 µM HTX for 30 min caused a 47.4 ± 6.5\% decrease of the endplate current amplitude, 27.4 ± 4.3\% shortening of the half-decay time, and a 4.4 ± 5.5\% increase in the time to peak. These values

1Masukawa, L. M., S. L. Stewart, and E. X. Albuquerque. Unpublished observations from computer simulations.
obtained in 24 endplates from four muscles were found to be significantly different from those seen with 30 μM HTX. The current-voltage plot using voltage sequence A showed very little looping in the 3rd quadrant when compared to plots of 30 μM toxin-exposed muscles (Fig. 6). The means of normalized data are plotted in Fig. 6 for comparison purposes, and all values are normalized with respect to the epc amplitude at −50 mV. Nonlinearity did not occur until the −100 mV step was reached in 10 μM HTX, whereas nonlinearity usually began at the −80 mV step in 30 μM HTX. Short steps by voltage sequence B also prevented looping in 10 μM HTX as it did for endplates exposed to 30 μM toxin. The decrease in epc amplitude during hyperpolarizing steps was altered along with the relative changes in the current-voltage plots in a dose-dependent manner. After steps from −50 to −130 mV, the average decrease of epc peak amplitude was 21 and 50% for 10 and 30 μM toxin,

![Figure 4](image-url)

**Figure 4.** Evidence that the potential dependent action of histrionicotoxin occurs in the absence of acetylcholine activation of receptors. The top row shows two endplate currents of a control surface fiber during voltage sequence C at the beginning (a) and at the end (b) of the series of endplate currents during a 30-s step from −50 to −130 mV. In the middle row also using voltage sequence C, endplate currents are compared in the presence of histrionicotoxin at the beginning (a) and end (b) of the step. In the bottom row of endplate currents, using voltage sequence D, the endplate currents were recorded before (a) and after (b) the conditioning step in which the membrane potential was hyperpolarized from −50 to −130 mV. During this conditioning step no endplate currents were evoked. The percentage decrease in the epc amplitude with and without activation of ACh receptors during steps to −130 mV were 39.5±7.5% (n = 4) and 29.4±6.2% (n = 5), respectively. There is no significant change in the endplate current amplitude of control cells during steps to −130 mV.
respectively (Fig. 7). A larger step to \(-150\) mV produced a decrease of epc amplitude that continued to be related to concentration. The decreases were by 24 and 69% for 10 and 30 \(\mu\)M HTX, respectively. In toxin-free endplates application of voltage sequence C, where the potential was shifted from \(-50\) to \(-130\) mV, caused in all cases studied a negligible increase in epc amplitude. The data from treated cells were not corrected for this slight increase.

The voltage dependence of the decay rate constant was similar to that found for 30 \(\mu\)M toxin. The decay rate constant for 10 \(\mu\)M HTX was 0.039 s\(^{-1}\) and 0.170 s\(^{-1}\) for \(-130\) mV and \(-150\) mV steps, respectively. Recovery at \(-50\) mV was fairly complete for both steps, i.e., the epc amplitudes returned to 90% of the original amplitude of the epc immediately before the step.

**DISCUSSION**

The present investigation discloses a voltage- and time-dependent attenuation of the peak epc amplitude and a reduction of the half-decay time of the epc in...
FIGURE 6. The effects of 30 and 10 μM HTX on the shape of the current-voltage relationship of the endplate current using 3-s conditioning steps by voltage sequence A. The data from each endplate were expressed as the percent amplitude compared to the endplate current peak amplitude recorded at -50 mV. The results were plotted as the means and SEMs of the percent epc at each voltage step for (a) six cells in the 30 μM HTX and (b) seven cells in the 10 μM HTX. Bars for SEMs are drawn for errors >5%. Arrows indicate the sequence of steps as explained in Methods. The ordinates are percent endplate current amplitude for inward and outward current.

the presence of HTX. The nonlinearity and voltage-pathway-sensitivity of the current-voltage relationship of the epc appear to originate from the interaction of the toxin with a site sensitive to the potential field across the membrane. Two sites of action are possible to account for the effects of HTX— the ACh receptor and the ionic channel. Previous investigations (Albuquerque et al., 1974; Lapa et al., 1975; Eldefrawi et al., 1977)² support the notion that HTX reacts with one

² Eldefrawi, M. E., A. T. Eldefrawi, N. A. Mansour, J. W. Daly, B. Witkop, and E. X. Albuquerque. The acetylcholine receptor and ion conductance modulator of Torpedo electroplax: binding of perhydrohistrionicotoxin to membrane and solubilized preparations. Biochemistry. In press.
or more sites, because the toxin produces shortening of the half-decay time as well as a reduction of the peak amplitude of the epc. The effect on the time-course of the epc has been explained in terms of the voltage dependence of the transformation of the ACh receptor-channel complex from the open to the closed conformation (Magleby and Stevens, 1972a, b). According to this model, interaction of HTX with the ionic channel would produce a change in the rate of transition of the ionic channel macromolecule from the opened to the closed conformation and a resultant decrease in the time-course of the epc. The reaction of HTX with a second site located on the ACh receptor-ionic channel complex would cause the voltage- and time-dependent effect on the epc by depressing, partially or completely, the unit conductance of the channel without significantly affecting the time-course of the channel. The activity of HTX in the nonlinear segments of the current-voltage relationship is due to a large extent to this second site available in hyperpolarized membranes even before the activation of ACh receptors. This site of action is best described as being the channel in its resting (closed) conformation. The second site of HTX binding may only be revealed in intact membranes where a potential separation can be established. Thus, a single dissociation constant for HTX binding may be due

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**Figure 7.** The concentration-dependent decrease in peak endplate current amplitude during hyperpolarizing steps in the presence and absence of HTX. Endplate currents were evoked once every 3 s after the beginning of a step. The means and SEM are plotted as filled circles and bars. The data were normalized to the first endplate current of each step for each cell. SEM bars were drawn only when they were > 1% in magnitude. The curves drawn through the data points were generated by the nonlinear regression fit to the mean values for each condition. The constants for the fitted curves (see Eq. 1) are: 10 μM HTX, -150 mV, a = 0.039, b = 78.8%, c = 21.0% (n = 5); 10 μM HTX, -150 mV, a = 0.170, b = 75.8%, c = 30.0% (n = 3); 30 μM HTX, -150 mV, a = 0.095, b = 50.2%, c = 52.7% (n = 4); 30 μM HTX, -150 mV, a = 0.141, b = 51.3%, c = 80.4% (n = 3).
to disruption of the normal state of the postsynaptic membrane and a loss of the voltage-dependent affinity.

Inasmuch as it has been demonstrated that HTX is a noncompetitive inhibitor of the ACh response (Kato and Changeux, 1976; Dolly et al., 1977), it is concluded that HTX is not reacting with the ACh recognition site. The depression of the peak amplitude of the epc at hyperpolarized levels and during repetitive stimulations (Albuquerque et al., 1973b, 1974) could be related to the desensitization of the ACh receptor (Katz and Thesleff, 1957; Burgermeister et al., 1977). The calculated rate constants (see Fig. 3) which account for the decrease of the epc amplitude disclose a marked action of the toxin at hyperpolarized potentials, thus raising the possibility that HTX has caused desensitization of the ACh receptor or that HTX has an increased affinity for desensitized receptors. However, the experiment shown in Fig. 4 indicates that the depression of the epc is most likely unrelated to an increase in affinity to desensitized ACh receptors, because, in the absence of intervening stimulation of the ACh receptor, the decrease in the epc amplitude still occurs.

Recently (Katz and Miledi, 1977), it has been demonstrated that there is a low level depolarization (~40 μV) of the endplate which can be blocked by iontophoretically applied d-tubocurarine. Such an observation indicates that ACh is being released, or "leaked," at a constant slow rate. This slow release, which is occurring in the absence of nerve stimulation, may be responsible for the action of HTX when voltage step D is used, by producing desensitized receptor-ionic channel complexes with which HTX may react. According to Katz and Miledi (1977), the concentration of ACh due to leakage is 10^-8 M. A quantum of ACh on the other hand produces a concentration of 3 x 10^-4 M. If one assumes that there are 200 quanta producing an epc, the concentration is 6 x 10^-2 M if all ACh is restricted in a small space. This would be the highest possible concentration amounting to about 6 x 10^6 times more activated ACh receptors present for reaction with HTX during nerve stimulation than due to nerve leakage. Therefore, it would seem unlikely that such a low level of receptor activation generated by nerve-terminal leakage would be an adequate driving force for HTX binding as compared to the extremely high concentrations present during evoked release.

Furthermore, if the toxin induced ACh receptor desensitization, this effect would be most likely due to an indirect action via a "metaphillic effect" involving the ionic channel of the receptor. Recent biochemical studies (Eldefrawi, 1977, and footnote 2) showed two independent protein entities isolated from the electric organ of the electric ray, *Torpedo marmorata*, one entity which specifically bound ACh and the other which bound HTX. The binding of HTX to the latter entity was inhibited by drugs and toxins at concentrations that did not inhibit ACh or carbamylcholine binding to its receptor. Thus, the possibility that HTX is acting essentially like an agonist in causing ACh-receptor desensitization seems most unlikely.

The interaction of HTX with its second site is relatively slow, having a rate constant on the order of 0.03–0.14s⁻¹. It has been demonstrated that this reaction can be fitted by a single exponential function and that the calculated rate constant is voltage dependent at hyperpolarized levels. The rate constants
thus obtained are in agreement with the duration of the conditioning potential steps used. The nature of these slowly occurring events can be discussed in terms of the kinetics describing the interactions of HTX with its site of action. A relatively slow rate of conformational change appears to be a likely model to explain hysteretic metabolic enzyme systems in which half-lives on the order of seconds to minutes have been found (Frieden, 1970; Kurganov et al., 1976). It is of interest to note that there is an increase in the ability of procaine to block channels associated with the ACh receptors when the surrounding membrane is hyperpolarized, but this effect has a much faster time-course than the action of HTX that we see here (Adams, 1977). By analogy, a kinetic model can be developed to explain the slow changes in epc amplitude during hyperpolarizing steps based on slow conformational changes of the ACh receptor-ionic channel complex in the presence of HTX. The single exponential time-course of the decrease in epc amplitude during 30-s potential steps can be described by a pseudo-first order reaction, where HTX is assumed to be in excess.

\[
\text{HTX} + S \xleftrightarrow{K_1, K_2} \text{HTXS}
\]  

(2)

In the reaction, S is equivalent to the site of reaction of HTX which is voltage sensitive; \(k_1\) is the association rate constant; and \(k_2\) the dissociation rate constant. When site S is in its free form, ACh molecules can activate their receptors to produce a full conductance increase. If S is complexed with HTX to form HTXS, it is assumed that full unit conductance increases do not occur after binding of ACh to its recognition site. Thus, the attenuation of the epc is directly related to the number of HTX-complexed sites. It is the rate constant, \(k_1\), which appears to change with membrane potential by increasing with hyperpolarization. This follows from the solution of the differential equation which describes the reaction in Eq. 2. The form of this solution is

\[
S(t) = \frac{(S_T) k_2}{k_1 \text{HTX} + k_2} + c e^{-(k_1 \text{HTX} + k_2)t},
\]  

(3)

where \(S(t)\) is the voltage- and time-dependent concentration of the receptor-ionic channel complex; \(S_T\) is the total concentration at time zero; and

\[
c = \frac{(S_T) k_1 \text{HTX}}{k_1 \text{HTX} + k_2}.
\]  

(4)

The decay rate constant, \(a\), in Eq. 1 is equivalent to \((k_1 \text{HTX} + k_2)\). Because HTX concentration is assumed to remain constant during voltage steps, the voltage dependency of the decay rate constant appears to be due to changes in \(k_1\) or the rate constant controlling the transition between bound and unbound receptor S. It must also be assumed that at depolarizing potentials above \(-50\) mV, \(k_1\) does not change significantly because nonlinearity is not evident in this region (see Fig. 2, inset). Because HTX is an alkaloid with \(pK_a\) of \(\sim 8.0\), a major fraction of the toxin is positively charged in physiological saline. The possibility
that the local concentration of HTX around the site of action is increased due to the charged state of the toxin in the hyperpolarized membrane has not been ruled out.

By increasing the toxin concentration, there is an increase in the maximal blocking of the endplate current amplitude at two different voltage steps. When the toxin concentration was increased three times, the equilibrium epc amplitude decreased by a factor of 2.3–2.9 during steps to −130 and −150 mV, respectively. Because these values were normalized, the percent change was independent of the degree of initial block at −50 mV. The voltage dependency of the decay rate constant of the epc was retained under the two toxin concentrations, but because of the relative values of the constants it is not possible at this time to make a quantitative evaluation of the basic model. Although we cannot make quantitative conclusions concerning the kinetic scheme, it is obvious that the form of the current-voltage relationship is concentration dependent both in terms of voltage sensitivity and rates of action (see Fig. 6a,b). The total reaction scheme of HTX and the receptor-ion channel complex must be more elaborate than what we have described in Eq. 2 as indicated by the data from the two toxin concentrations.

There are two distinct physical bases for the voltage dependency of the decay rate constant, either it is due to a voltage-dependent binding of HTX or a voltage-dependent rate of conformational transition of the macromolecular complex in which one conformation has a higher affinity for HTX and is relatively more stable in a hyperpolarizing field. The overall effect of HTX can be explained at the present moment as an initial block of conductance units and a change in time-course of units that are not blocked. Upon an increase in the potential across the endplate membrane in the hyperpolarizing direction, the conductance units (ACh receptor-ionic channel complexes) change their conformation at a slow voltage-dependent rate. The conductance units in their new conformation have a higher affinity for HTX, and a new equilibrium is attained in which more conductance units are blocked. Upon returning the membrane to the holding potential there is a slow unblocking of conductance units, and upon removal of toxin by washing, all slowly developing voltage dependence in the epc peak amplitude is lost.

The results emphasize the importance of the potential field across the membrane as an influential factor during drug-receptor interaction. This would not be an improbable condition because channel gating mechanisms are voltage dependent (Bezanilla and Armstrong, 1977; Armstrong and Bezanilla, 1977) and gating currents are thought to be a reflection of fast conformational change of membrane-bound molecules.

In conclusion, the voltage- and time-dependent action of HTX at the endplate can be explained by an increase in the rate constant controlling the conformational changes of the receptor-channel macromolecule during hyperpolarization and an increase in the affinity of the toxin for the macromolecule in its resting state in the hyperpolarized membranes before activation. The conformational change of the macromolecule in the presence of HTX is postulated as being relatively slow, i.e., on the order of tens of seconds. It is this slow conformational
change, along with a slow recovery, that leads to voltage- and time-dependent action of HTX.

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REFERENCES

ADAMS, P. R. 1977. Voltage jump analysis of procaine action at frog endplate. *J. Physiol.* (Lond.) 268:291-318.

ADLER, M., and E. X. ALBUQUERQUE. 1976. An analysis of the action of atropine and scopolamine on the endplate current of the frog sartorius muscle. *J. Pharmacol. Exp. Ther.* 196:360-372.

ALBUQUERQUE, E. X., E. A. BARNARD, T. H. CHIU, A. J. LAPA, J. O. DOLLY, S. E. JANSSON, J. DALY, and B. WITKOP. 1973a. Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction: evidence from specific toxin reactions. *Proc. Natl. Acad. Sci. U. S. A.* 70:949-953.

ALBUQUERQUE, E. X., K. KUBA, A. J., LAVA, J. W. DALY, and B. WITKOP 1973b. Acetylcholine receptor and ionic conductance modulator of innervated and denervated muscle membranes. Effect of histrionicotoxins. In *Exploratory Concepts in Muscular Dystrophy Vol. II*, A. T., MILHORAT, editor Excerpta Medica, Amsterdam. 585-600.

ALBUQUERQUE, E. X., K. KUBA, and J. DALY. 1974. Effect of histrionicotoxin on the ionic conductance modulator of the cholinergic receptor: a quantitative analysis of the endplate current. *J. Pharmacol. Exp. Ther.* 189:513-524.

ANDERSON, C. R., and C. F. STEVENS. 1973. Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.* (Lond.) 235:655-691.

ARMSTRONG, C. M., and F. BEZANILLA. 1977. Inactivation of the sodium channel. II. Gating current experiments. *J. Gen. Physiol.* 70:567-590.

BEZANILLA, F., and C. M. ARMSTRONG. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol.* 70:549-566.

BURGERMEISTER, W., W. A. CATTERALL, and B. WITKOP. 1977. Histrionicotoxin enhances agonist-induced desensitization of acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* 74(12):5754-5758.

DALY, J. W., I. KARLE, C. W. MYERS, T. TOKUYAMA, J. A. WATERS, and B. WITKOP. 1971. Histrionicotoxins: Roentgen-ray analysis of the novel allenic and spiroalkaloid isolated from a Colombian frog, *Dendrobates histrionicus*. *Proc. Natl. Acad. Sci. U. S. A.* 68:1870-1875.

DEGUCHI, T., and T. NARAHASHI. 1971. Effects of procaine on ionic conductances of endplate membranes. *J. Pharmacol. Exp. Ther.* 176:423-433.

DIONNE, V. E., and C. F. STEVENS. 1975. Voltage dependence of antagonist effectiveness at the frog neuromuscular junction: resolution of a paradox. *J. Physiol.* (Lond.). 251:245-270.

DOLLY, J. O., E. X. ALBUQUERQUE, J. M. SARVEY, B. MALLICK, and E. A. BARNARD. 1977. Binding of perhydro-histrionicotoxin to the postsynaptic membrane of skeletal
muscle in relation to its blockade of acetylcholine-induced depolarization. *Mol. Pharmacol.* **13:**1-14.

Eldefrawi, A. T., M. E., Eldefrawi, E. X. Albuquerque, A. C. Oliveira, N. Mansour, M. Adler, J. Daly, G. B. Brown, W. Burgermeister, and B. Witkop. 1977. Perhydrohistrionicotoxin: a potential ligand for the ion conductance modulator of the acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **74:**2172–2176.

Fujino, M., T. Yamaguchi, and K. Suzuki. 1961. 'Glycerol effect' and the mechanism linking excitation of the plasma membrane with contraction. *Nature (Lond.)* **192:**1159–1161.

Frieden, C. 1970. Kinetic aspects of regulation of metabolic processes: The hysteretic enzyme concept. *J. Biol. Chem.* **345:**5788–5799.

Gage, P. W., and R. S. Eisenberg. 1967. Action potentials without contraction in frog skeletal muscle fibers with disrupted transverse tubules. *Science (Wash. D.C.)* **158:**1702–1703.

Katz, B., and R. Miledi. 1977. Transmitter leakage from motor nerve endings. *Proc. R. Soc. Lond. B. Biol. Sci.* **196:**59–72.

Katz, B., and S. Thesleff. 1957. A study of desensitization produced by acetylcholine at the motor endplate. *J. Physiol. (Lond.)* **138:**63–80.

Kato, G., and J. P. Changeux. 1976. Studies on the effect of histrionicotoxin on the monocellular electroplaque from *Electrophorus electricus* and on the binding of [3H]acetylcholine to membrane fragments from *Torpedo marmorata*. *Mol. Pharmacol.* **12:**92–100.

Kördas, M. 1969. The effect of membrane polarization on the time course of the endplate current in the frog sartorius muscle. *J. Physiol. (Lond.)* **204:**493–502.

Kördas, M. 1970. The effect of procaine on neuromuscular transmission. *J. Physiol. (Lond.)* **209:**589–699.

Kuba, K., E. X. Albuquerque, J. Daly, and E. A. Barnard. 1974. Study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate, on time course of endplate currents in frog sartorius muscle. *J. Pharmacol. Exp. Ther.* **189:**499–512.

Kurganov, B. I., A. I. Dorozhko, Z. S. Kagan, and V. A. Yakovlev. 1976. The theoretical analysis of kinetic behaviour of “hysteretic” allosteric enzymes. I. The kinetic manifestations of slow conformational change of an oligomeric enzyme in the Monod, Wyman and Changeux model. *J. Theor. Biol.* **60:**247–269.

Lapa, A. J., E. X. Albuquerque, J. M. Sarvey, J. Daly, and B. Witkop. 1975. Effects of histrionicotoxin on the chemosensitive and electrical properties of skeletal muscle. *Exp. Neurol.* **47:**558–580.

Magleby, K. L., and C. F. Stevens. 1972a. The effect of voltage on the time course of endplate currents. *J. Physiol. (Lond.)* **223:**151–171.

Magleby, K. L., and C. F. Stevens. 1972b. A quantitative description of endplate currents. *J. Physiol. (Lond.)* **223:**173–197.

Takeuchi, A., and N. Takeuchi. 1959. Active phase of frog’s end-plate potential. *J. Neurophysiol.* **22:**395–411.