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Voltage- and Time-dependent Actions of Piperocaine on the Ion Channel of the Acetylcholine Receptor

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SUMMARY

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The effects of the local anesthetic piperocaine were investigated on the endplate current (EPC) of frog sartorius muscles and on the binding of ligands to the acetylcholine (ACh) receptor and its ion channel in membranes from the electric organ of *Torpedo ocellata*. Piperocaine (10-100 μM) did not prevent action potential activity in nerve or muscle. However, these concentrations of piperocaine depressed reversibly the peak amplitude of EPCs in a dose-dependent manner without altering the EPC reversal potential. The current-voltage relationship obtained with short conditioning voltage durations preceding the EPC remained approximately linear at the piperocaine concentrations used. When the time during which the membrane potential was maintained preceding the EPC was lengthened from 10 to 500 msec in presence of piperocaine, the current-voltage relationship became markedly nonlinear, thus suggesting that there was more binding of the drug to the ACh-receptor ion channel complex. Both drug concentration and increasingly negative membrane potential augmented this time-dependent effect. At negative membrane potentials piperocaine also reversibly accelerated the rise and decay times of the EPC, while the EPC falling phase remained a single exponential function of time. The relationship between log of EPC decay time constant (τ) and membrane potential was linear in presence of piperocaine, and the slope progressively decreased and reversed its direction as piperocaine concentration was increased, with the maximum observed acceleration of τ being at 75 μM of drug. The effect of piperocaine on τ was voltage dependent but time independent. Piperocaine inhibited competitively [^{3}H]perhydrohistrionicotoxin binding to the electric organ membranes, with an inhibition constant (K_i) of 0.44 μM; and noncompetitively [^{3}H]ACh binding to its ACh-receptor with a K_i of 12.0 μM. These findings suggest that piperocaine has at least two separate actions at the ACh-receptor-ion channel complex. One is binding to open channels which causes concentration and voltage-dependent alteration of EPC time course and decreased EPC amplitude and voltage sensitivity of the EPC falling phase. Another is binding to a less well-defined site

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on the ACh receptor ion channel complex, an action that leads to a further depression of the peak amplitude of the EPC which is concentration, voltage and also time-dependent.

INTRODUCTION

At the mammalian or frog neuromuscular junction, the molecular reaction of ACh[3] with its recognition sites causes a rapid increase in ion conductance of the postsynaptic membrane, which can lead to endplate potentials (EPP) and miniature endplate potentials (MEPP) associated with synaptic transmission. Under voltage clamp conditions, the decay phase of the endplate current (EPC) represents the rate of closing of ion channels opened as the result of ACh reaction with its recognition site on the receptor macromolecule (1–3). Many drugs and toxins affect these events. Some react preferentially with ACh-receptor binding sites (e.g., α-bungarotoxin), causing at certain concentrations a reduction in the peak amplitude of the EPC without inducing abnormal nonlinearity in the voltage-current relationship or changing EPC time course. Other agents and toxins react preferentially with the sites of the ion channel of the ACh-receptor (e.g., histrionicotoxin (HTX) (4, 5), amantadine (6, 7), procaine (8), lidocaine derivatives (9, 10), atropine and scopolamine (3, 11) and barbiturates (12) leading to alteration of EPC time course. Yet other drugs (e.g., quinacrine (13) and tetraethylammonium (14), appear to react with both the receptor and its ion channel at similar concentrations. Biochemical studies of the nicotinic ACh-receptor and its ion channel have required the utilization of a tissue that has a higher concentration of them than skeletal muscle, namely, the electric organ of the electric ray, *Torpedo ocellata*. The ACh-receptor sites are identified *in vitro* by their binding of [^{125}I]α-bungarotoxin or [^{3}H]ACh, while the ion channel sites are identified by their binding of [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX) (15–17).

Local anesthetics have been shown to interact with lipids (18, 19), axonal ion channels (20) and the ion channel associated with the ACh-receptor. This last action is suggested by the effect of local anesthetics on EPCs (8–10, 21, 22), and by their inhibition of [^{3}H]H_{12}-HTX binding (15, 16), and their allosteric potentiation of [^{3}H]ACh binding (23) to electric organ membranes (15, 16). Indeed, as more knowledge is gained about compounds which affect the kinetics of ion conductances, the structure-activity relationship may provide us with clues as to the molecular organization of the ion channel and its relationship to the ACh-receptor. Thus, the present study was initiated in order to explore the actions of a local anesthetic, piperocaine, at the endplate and in electric organ membranes, as well as to shed more light on the molecular events that occur as a result of the interaction of ACh with its receptor.

METHODS

Electrophysiological techniques. Experiments were performed at room temperature (20–22°) on sciatic nerve-sartorius muscle preparations from 2.5–3 inch frogs (*Rana pipiens*). The sartorius muscles were pinned slightly stretched to a Plexiglas plate having a plano-convex lens in the center, placed in a 25 ml recording chamber continuously perfused at 1–3 ml/min, and dissected free of extraneous connective tissue. Muscle membrane potentials were measured from surface fibers with 3 M KCl glass microelectrodes (5–10 megohms) and conventional intracellular recording technique (24, 25). Endplate regions were localized visually with a dissecting microscope and electrophysiologically by recording spontaneous MEPPs or evoked EPPs with rise time <1.0 msec.

The normal Ringer's solution used in all experiments contained (in mM concentrations): NaCl, 115.0; KCl, 2.0; $CaCl_2$, 1.8, Na_2HPO_4, 1.3; and NaH_2PO_4, 0.7. The pH of this solution was 7. All stock drug solutions were stored at 4°. The Ringer's solutions containing 10 nM to 75 μM piperocaine

[3] Abbreviations used: ACh, acetylcholine; H_{12}-HTX, perhydrohistrionicotoxin; EPP, endplate potential; MEPP, miniature endplate potential; EPC, endplate current; τ, EPC decay time constant.

(Lilly) were prepared by using a 10^{-2} M stock solution. One micromolar tetrodotoxin (Cal-Biochem) was prepared from a 300 μM stock solution.

In the experiments examining nerve-evoked EPC, muscle contraction was blocked by 90 min exposure to hypertonic Ringer's solution containing 600–675 mM glycerol (26) followed by vigorous rinsing with normal Ringer's solution for at least 30 min and until muscle contraction in response to nerve stimulation ceased. The nerve was stimulated with supramaximal pulses 0.05–0.1 msec in duration delivered by bipolar platinum electrodes and a stimulus isolation unit (Devices) triggered by a Digitimer stimulator.

The voltage clamp circuit and method used to record EPCs were similar to those described previously (27, 28). Resistances of the recording microelectrode were 5–10 megohms while the microelectrode delivering feedback current, also filled with 3 M KCl, had resistances of 2–5 megohms. Voltage control of the endplate region was considered satisfactory when clamp errors were less than 2% of the unclamped EPP.

Using the voltage clamp circuit, the membrane potential of the glycerol-treated preparations was set to −50 mV and changes in the membrane potential were made using DC pulses delivered by a stimulus-isolator controlled by a Digitimer stimulator. Four kinds of command potential changes were used to record nerve evoked EPCs from −160 to + 50 mV membrane potential (Fig. 1). Sequence A consisted of changing the membrane potential every 10 mV and holding it at the new potential for a period of 25 msec, stimulating the nerve such that the evoked EPC occurred 10 msec after the beginning of the step. Sequence B consisted of maintaining the membrane potential at each new voltage for a longer period of 1.0 sec and stimulating the nerve 500 msec after the beginning of the conditioning pulse. In sequences C and D the membrane potential was maintained for 500 msec. In sequence C, EPCs were recorded 10, 110, 210, 310 and 410 msec after the beginning of the pulse, while in sequence D, all EPCs were recorded at −50 mV and at time intervals of 10, 110, 210, 310, 410, 510 and 610 msec after the end of the 500 msec conditioning potential step. For sequences A and B, the membrane potential was incremented in steps of approximately 10 mV to record EPCs at membrane potentials from −160 to +160 mV. For sequences C and D, the membrane potential was incremented in three steps from 30 to 85 mV, the resulting membrane potential varying from −77 to −135 mV, to follow closely the influence of pulse duration and voltage on EPC parameters.

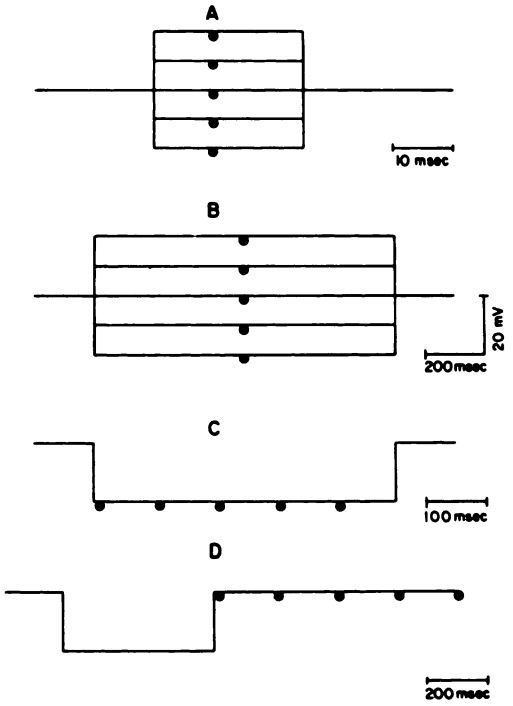

FIG. 1. *Sequences A, B, C and D used in voltage clamp experiments as described in detail under* METHODS

Each filled circle represents the time at which the EPC was recorded. Holding potential was −50 mV.

The nerve-evoked EPCs were measured and analyzed by DC recording of the output of a Tektronix oscilloscope which displayed the EPC and the membrane potential. The signal was sampled at a rate of 100 μsec/point using an analogue-to-digital converter and laboratory computer (PDP 1140, Digital equipment). During subsequent analysis of membrane potential, EPC amplitude, rise time, and decay time constant, the digitized points were displayed onto a Tektro-

nix storage oscilloscope for continuous monitoring of the measurements routines. The decay time constant (τ) of each EPC was determined by fitting the digitized points of the EPC between 20 and 80% decay of peak amplitude to an exponential regression curve.

Binding techniques. Membranes were prepared from the electric organ of *T. ocellata* (stored at −90°) by homogenization (20%, w/v) in ice-cold solution of 90 mM KCl, 10 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4), and centrifugation for 10 min at 1,000 × *g* followed by another centrifugation of the supernatant for 60 min at 30,000 × *g*. This pellet was resuspended in Krebs' original Ringer phosphate solution (mM): NaCl, 107; KCl, 4.8; $CaCl_2$, 0.65; $MgSO_4$, 1.2; and Na_2HPO_4, 15.7; pH 7.4. One milliliter 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.* (29), ranged from 1–2 mg/ml, and the maximum number of binding sites for [^{3}H]ACh and [^{3}H]H_{12}-HTX were 0.7 and 0.5 nmol/mg protein, respectively. Equilibrium dialysis was used to study the binding of [^{3}H]ACh (49.5 Ci/mole, New England Nuclear) to the electric organ membranes as described previously (16). One-half milliliter of membrane preparation in a dialysis bag was shaken for 4 hr at 21° in Krebs original Ringer phosphate (25 ml) containing [^{3}H]ACh in absence or presence of piperocaine. 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, diisopropylfluorophosphate was added to the membranes, at a final concentration of 1 mM 1 hr before the start of dialysis, and at 100 μM in the dialysis bath. Centrifugal assay was used to study the binding of [^{3}H]H_{12}-HTX to the electric organ membranes as previously described (15), so as to save on the radiolabeled toxin used. [^{3}H]H_{12}-HTX (21 Ci/mmole) was obtained by tritiation of isodihydro-histrionicotoxin and its activity tested on frog sartorius muscle (16). Samples (10 μl) of different concentrations of [^{3}H]H_{12}-HTX in ethanol were added to 1 ml samples of the *Torpedo* membrane preparation, mixed and incubated for 60 min at 21° in siliconized 1.5 ml polyethylene microcentrifuge tubes. Piperocaine (10 μl) was added to the membranes 30 min (at 21°) prior to the toxin, and the incubation mixture centrifuged at 30,000 × *g* for 60 min, then three samples (50 μl each) taken from the mixture before, as well as from the supernatant after, centrifugation. 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, which amounted to 10 ± 3% of control at 10 nM [^{3}H]H_{12}-HTX.

RESULTS

Effects of piperocaine on the action potentials, on resting membrane potentials, and on the amplitude of EPC. The local anesthetic piperocaine at concentrations of 10–100 μM did not prevent action potential activity in nerve or muscle. The membrane potential of the glycerol-treated muscle fibers prior to exposure to piperocaine varied from −40.1 to −75.6 mV with a mean of −43.7 ± 2.1 mV (n = 82 fibers in 17 muscles). The mean membrane potential in presence of piperocaine (10–100 μM) for periods of 90–120 min was −42.9 ± 0.85 mV (n = 70 fibers in 14 muscles). Piperocaine had a marked effect on the peak amplitude of the EPC, an effect that was apparent within 5 min after drug perfusion was started, reached steady levels by 15–25 min, and was completely reversible upon wash. The effect of piperocaine on the peak amplitude of the EPC was dependent upon the duration of the conditioning pulse preceding the EPC (Fig. 2). Figure 3 illustrates the current-voltage relationship from control fibers and in the presence of three concentrations of piperocaine using voltage sequence A (see METHODS, Fig. 1) in which the duration of the conditioning potential preceding the EPC was 10 msec. As previously observed (1, 2, 12–14, 28), the control current-voltage relationship was essentially linear between −100 and +50 mV, but at more negative membrane potentials a slight departure from linearity became apparent

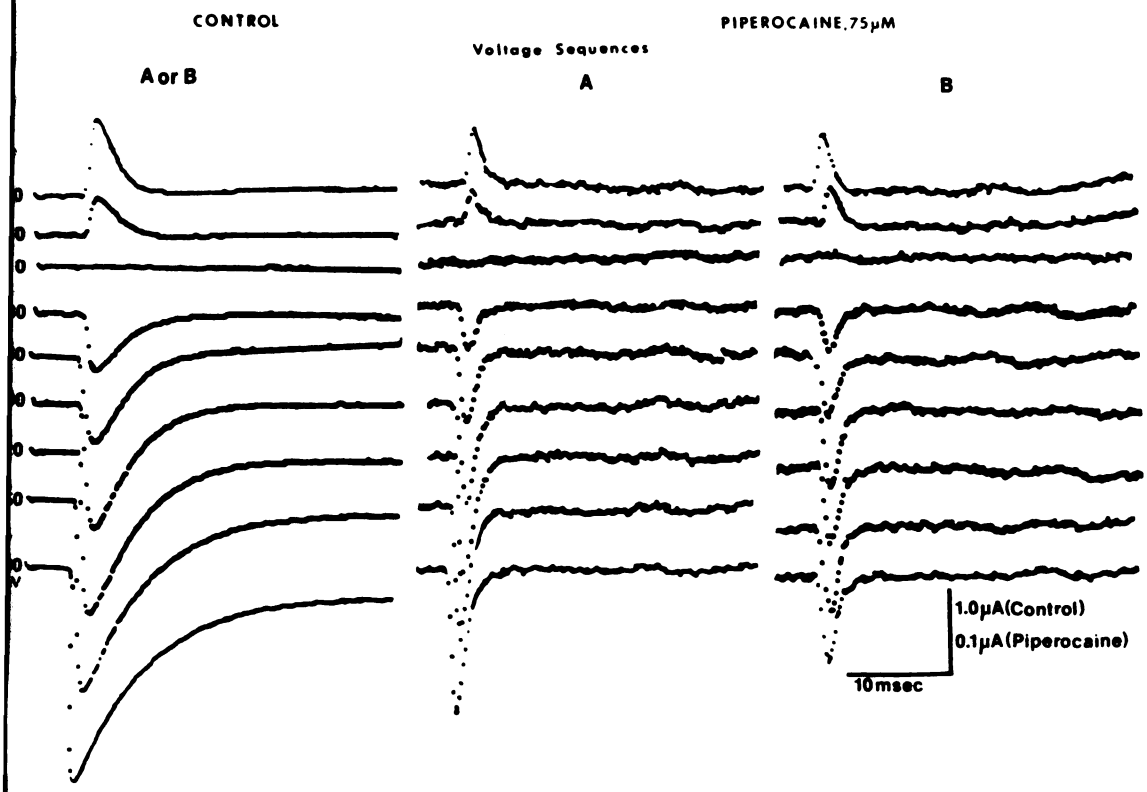

FIG. 2. *Digitized endplate currents at various membrane potentials (from −180 to +60 mV) in control and piperocaine-treated preparations using voltage sequences A and B as shown in Fig. 1.*

as was previously shown (30). In the presence of piperocaine there was a concentration-dependent depression of the current-voltage relationship (Fig. 3). The relationship between the percent control EPC response and membrane potential was a straight line having a slope of close to 0 at all concentrations of piperocaine examined.

Characteristics of voltage- and time-dependent actions of piperocaine on the peak amplitude of the EPC. Using voltage sequence A (Fig. 1), where a short conditioning pulse was applied, piperocaine caused a reduction in the EPC peak amplitude without altering the linearity of the current-voltage relationship (Figs. 2, 3). Sequence B (Fig. 1) was used to lengthen the time during which the membrane potential was maintained at the new voltage before the nerve was stimulated. No difference in linearity of current-voltage relationship of the EPC was observed when voltage sequences A or B were employed in either control conditions or in presence of piperocaine at a concentration of 10 μM (Fig. 4). However, using voltage sequence B at concentrations of piperocaine varying from 25 to 75 μM, a significant departure from linearity was observed in the current-voltage relationship at membrane potentials more negative than −70 mV. The dependence of EPC peak amplitude on membrane potential was substantially reduced in 25 μM piperocaine, almost eliminated in 50 μM, and even slightly reversed in 75 μM at membrane potentials more negative than −120 mV. In other words, nonlinearity in the current-voltage relationship appeared in the presence of 25–75 μM piperocaine when the membrane potential was maintained at each new voltage (i.e., conditioning duration) for 500 msec before the nerve was stimulated (sequence B), but not when it was maintained for only 10 msec. With conditioning durations longer than 500 msec, and up to 900 msec, no further in-

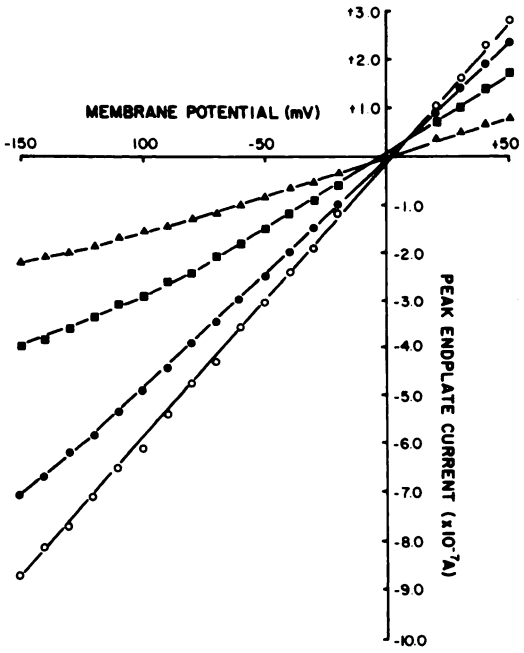

FIG. 3. *The current voltage relationship in the voltage clamped frog sciatic nerve-sartorius muscle preparation at 21° in absence* (○) *and presence of piperocaine at 10 μM* (○), *50 μM* (■) *or 75 μM* (▲)

Conditioning duration was 10 msec (i.e., sequence A).

crease in nonlinearity of the peak amplitude of the EPC was recorded.

In order to investigate this time-dependent effect of 25–75 μM piperocaine, which ranged from no apparent nonlinearity in the current-voltage relationship after 10 msec conditioning duration (sequence A) to marked nonlinearity after 500 msec, we used sequence C (Fig. 1). In each cell, the membrane was voltage clamped to −50 mV and subjected to three different holding potentials (−75, −100 and −130 mV) 500 msec in duration every 3 or 5 sec. The nerve was then stimulated with various latencies such that the evoked EPC occurred after conditioning durations that lasted 10, 110, 210, 310 and 410 msec. In the presence of 25–75 μM piperocaine a progressive reduction in EPC amplitude to an asymptotic value occurred with time (Fig. 5). This time-dependent effect was concentration- and voltage-dependent, increasing with higher concentrations and more negative membrane potentials (Fig. 5).

Recovery from the time-dependent effect of 50 μM piperocaine following the same three 500 msec hyperpolarizing steps was examined using sequence D (Fig. 1), which consisted of the conditioning step and EPCs elicited 10–610 msec later. As shown in Fig. 6, recovery from the time-dependent effect caused by piperocaine followed a similar time course and voltage-dependence as the onset.

Effect of piperocaine on the time course and reversal potential of the EPC. Piperocaine reversibly accelerated the rise time and decay time of the EPC in a dose-dependent manner (Table 1). The decay times of EPCs recorded at two holding potentials, −50 mV and −150 mV, in absence and presence of piperocaine (10–75 μM) are shown in Fig. 7. In agreement with previous studies (1, 28), the normal EPC decayed as a single exponential function of time over most of its falling phase. In the presence of piperocaine, the early nonexponential portion became progressively less apparent with increasing drug concentration. Over the rest of the falling phase, the decay phase remained a single exponential function of time even with doses of piperocaine that caused marked shortening of the EPC falling phase.

It is well known now that under normal conditions, the EPC decay time constant (τ) becomes progressively longer as the membrane potential is made more negative. In the present experiments the log τ-membrane potential relationship of control cells was linear and had a slope of -3.8 ± 0.2 msec/V (Fig. 8). In the presence of piperocaine, the relationship between τ and voltage remained a linear function, but the slope was progressively decreased as piperocaine concentration was increased from 10 to 50 μM (Fig. 8). At negative membrane potentials τ decreased in presence of piperocaine, but it increased at positive or slightly negative membrane potentials, depending on piperocaine concentration. In 50 μM piperocaine, τ was almost independent of membrane potential, whereas in 75 μM piperocaine the voltage sensitivity of τ was even slightly reversed (Fig. 8). The slopes of the log τ *vs.* membrane potential relationship in 10, 25, 50 and 75 μM pipero-

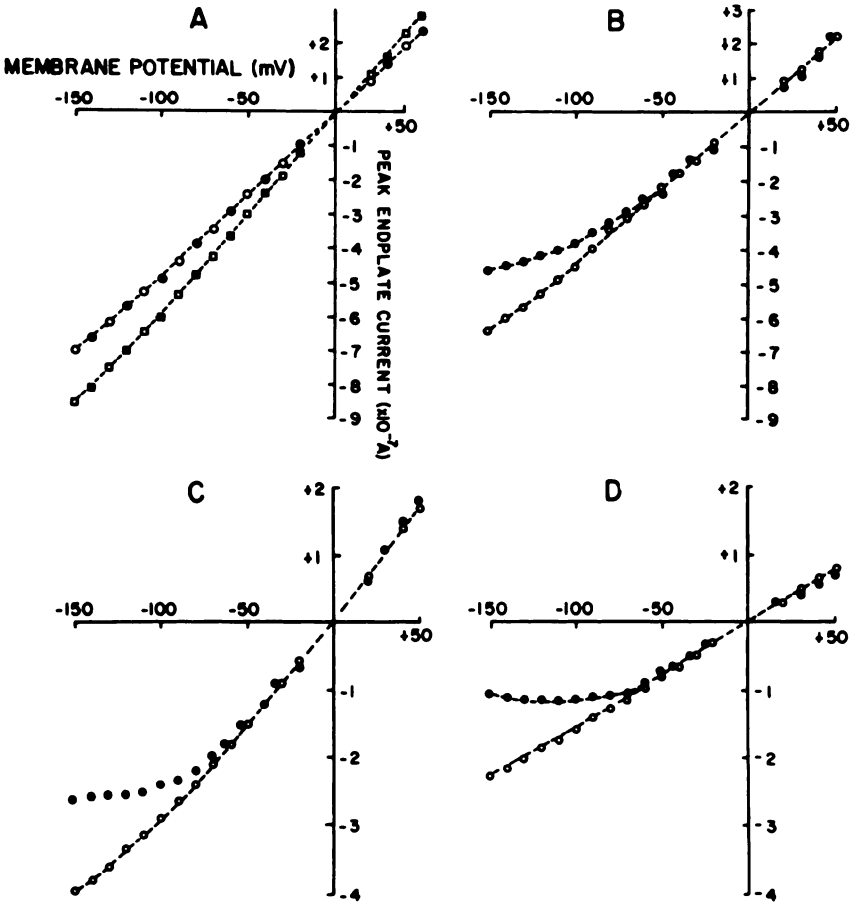

FIG. 4. *Current-voltage relationships of the EPC obtained in control and during the action of various concentrations of piperocaine*

Panel A. Using voltage sequence A (□, ○) and voltage seqeunce B (■, ●) alternately in control (□, ■) and in presence of 10 μM piperocaine (○, ●). In Panels B, C and D, voltage sequences A (○) and B (●) were used in presence of piperocaine, 25 μM in B, 50 μM in C and 75 μM in D. In D, use of 3 sec conditioning duration gave the same effect as the 500 msec conditioning used in voltage sequence B.

caine were, respectively: -1.6 ± 0.2 (n = 17), -1.0 ± 0.2 (n = 15), -0.15 ± 0.08 (n = 17) and $+0.16 \pm 0.06$ (n = 14) msec/V. This effect of piperocaine on τ was logarithmically related to membrane potential (Fig. 9), increasing with higher membrane polarity, positive or negative, and was voltage dependent.

The effects of piperocaine on EPC peak amplitude and τ were present without apparent alteration of the EPC reversal potential (Table 2), which suggests that the observed effects of piperocaine were not related to a selective alteration of the increase in either Na^+ or K^+ permeability resulting from transmitter action.

Although the effect of piperocaine on EPC peak amplitude was time-dependent in onset and recovery as shown above (Figs. 5, 6), the effect of piperocaine on τ was unaffected by both the various conditioning durations of sequences A, B and C, and the time after the conditioning pulse.

Effects of piperocaine on binding of ligands to the ACh-receptor and its ion channel. In order to establish whether piperocaine interacted with the ACh-receptor and/or its ion channel, we studied its effect on the binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX to *Torpedo* electric organ mem-

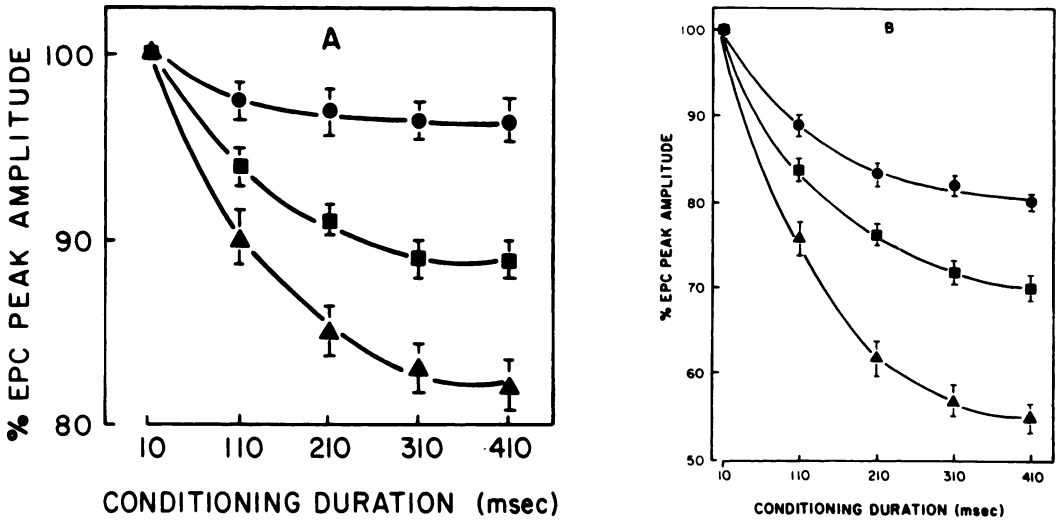

FIG. 5. *The effect of conditioning duration (sequence C) on % EPC peak amplitude at various holding potentials, −75 mV (●), −100 mV (■) and −130 mV (▲) in presence of A. 25 μM and B. 50 μM piperocaine*
Vertical bars represent ± standard error in 5 experiments.

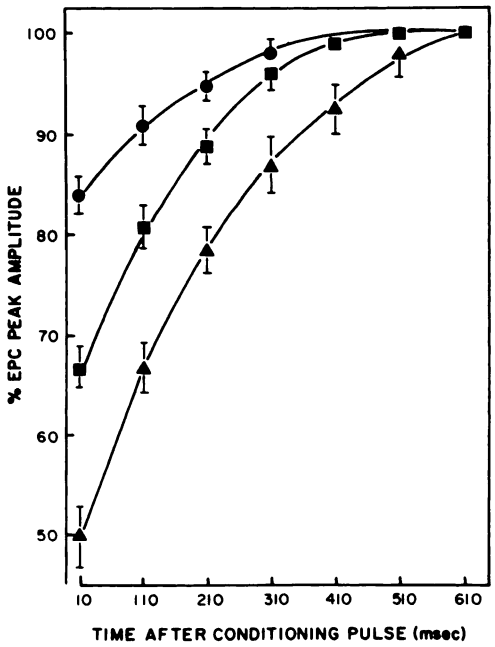

FIG. 6. *The recovery of peak EPC after conditioning pulse (sequence D) in presence of 50 μM piperocaine, at various holding potentials −75 mV (●), −100 mV (■) and −130 mV (▲)*
Bars represent ± standard error in 5 experiments.

branes, respectively. Binding of [^{3}H]H_{12}-HTX to the membrane bound ion channel was inhibited competitively by two concentrations of piperocaine, as judged by the common ordinate intercept in the double reciprocal plot of the binding data (Fig. 10). The inhibition constant (K_i) was calculated to be 0.44 μM. Binding of [^{3}H]ACh to membrane-bound *Torpedo* ACh-receptor was also inhibited by piperocaine (10 μM and 100 μM), but essentially noncompetitively (Fig. 11). The K_i was calculated to be 12.0 μM.

Previously we had found that the concentrations of TEA, amantadine or quinacrine that inhibited 50% of twitch tension in frog or rat skeletal muscles and the K_i values of the drugs in inhibiting [^{3}H]H_{12}-HTX binding to *Torpedo* membranes were within 3-fold for each of the three drugs (7, 13, 14). However, in the case of piperocaine, it is clear that the dose that inhibited 50% of EPC was approximately 100-fold higher than the K_i for its inhibition of [^{3}H]H_{12}-HTX (Figs. 3, 10). The reason for this discrepancy may be due to a change in the affinity of piperocaine to the H_{12}-HTX-binding site during receptor activation. Studies of the effect of piperocaine on the EPC revealed that the agent was reacting with an activated receptor-channel complex, while our biochemical studies showed that the displacement of [^{3}H]H_{12}-HTX occurred between piperocaine and non-activated complexes. We have now found that activation of the *Torpedo* receptor-channel

TABLE 1

Effects of piperocaine on the amplitude and the time course of EPC's recorded at −50 mV membrane potential

Condition	N[a]	Amplitude $\times 10^{-7}$ A	Rise time	Decay time constant (τ)
			msec	*msec*
Control	20	3.09 ± 0.37	0.77 ± 0.02	1.21 ± 0.05
Piperocaine				
10 μM	17	2.47 ± 0.40	0.69 ± 0.02	1.06 ± 0.04
25 μM	15	2.20 ± 0.33	0.63 ± 0.02	0.85 ± 0.08
50 μM	17	1.47 ± 0.30	0.58 ± 0.03	0.69 ± 0.03
75 μM	14	0.81 ± 0.25	0.50 ± 0.02	0.49 ± 0.07
Wash	10	2.80 ± 0.29[b]	0.75 ± 0.02[b]	1.14 ± 0.06[b]

[a] N, number of fibers tested.

[b] Values obtained after 30–45 min wash from muscles exposed to 50 μM piperocaine.

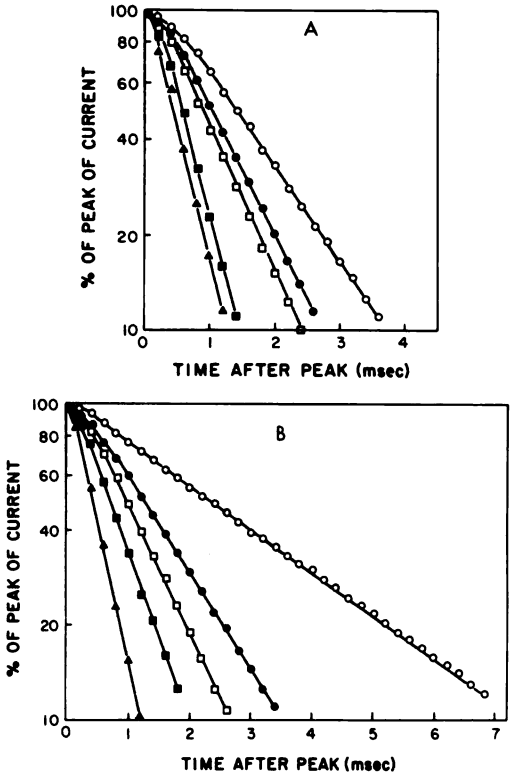

FIG. 7. *The effect of piperocaine on the decay phase of EPC at two holding potentials A. −50 mV and B. −150 mV*

Control (○) and in presence of piperocaine 10 μM (●), 25 μM (□), 50 μM (■) and 75 μM (▲). Standard error averaged 0.03%. No difference was observed between voltage sequences A and B.

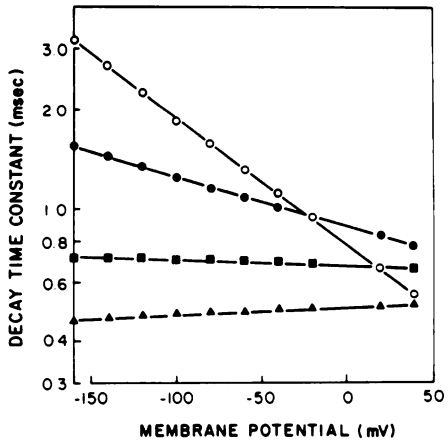

FIG. 8. *The relationship between decay time constant (τ) and membrane potential*

Control (○), and in presence of piperocaine, 25 μM (●), 50 μM (■) and 75 μM (▲). No difference was observed between voltage sequences A and B.

complex with carbamylcholine caused a strong allosteric shift in the affinity of piperocaine for the H_{12}-HTX-binding site so that the K_i for inhibition of toxin-binding approximated the range of concentrations which caused the voltage and time dependence changes of the EPC. Details of this allosteric effect resulting from receptor activation on the binding of [^{3}H]H_{12}-HTX and a number of ionic channel drugs will be published elsewhere.

DISCUSSION

Based on the electrophysiological and biochemical data, it is suggested that piperocaine has multiple actions. Piperocaine interacts with the ion channel of the ACh-receptor as judged by the following actions: (a) the nonlinearity it causes in the current-voltage relationship (Fig. 4), (b) the altera-

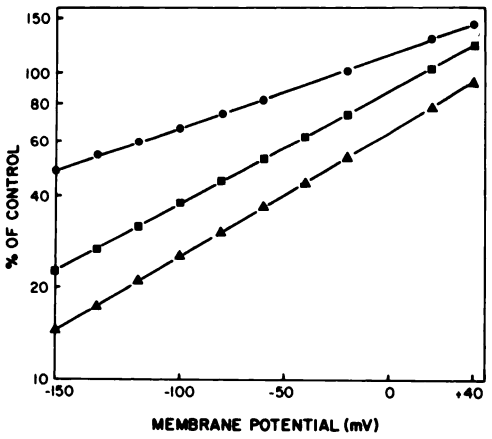

FIG. 9. *The relationship between log τ and membrane potential*

Piperocaine concentrations were 10 μM (●), 50 μM (■) and 75 μM (▲). No difference was observed between voltage sequences A and B.

tion it causes in rise time and decay time (τ) of the EPC (Table 1, Figs. 7, 8), and (c) by its inhibition of [^{3}H]H_{12}-HTX binding to the ion channel of *Torpedo* membranes (Fig. 10). Channel selectivity is apparently not altered since piperocaine causes no change in the EPC reversal potential. The reduction piperocaine causes in EPC decay time without changing its exponential nature is similar to the effects produced by drugs and toxins that have been shown to interact with the ion channel of the ACh-receptor, such as HTX (5, 16), amantadine (6, 7), quinacrine (13, 21) and tetraethylammonium (14). As suggested for other drugs and toxins that cause voltage-dependent changes in the decay time of the EPC (10, 13, 28), the effect of piperocaine on EPCs elicited after a short conditioning duration is suggested to be due to the interaction of piperocaine with open channels.

The alteration of EPC decay caused by piperocaine is voltage-dependent, with reduction in τ increasing with negative membrane polarity, and prolongation is evident with higher, positive membrane polarity at certain concentrations of the agent (Fig. 8). This action of piperocaine results in decreased voltage sensitivity of the EPC falling phase (Fig. 8). In the presence of 50 μM piperocaine, τ is almost independent of membrane potential, whereas in 75 μM, the voltage sensitivity is slightly reversed. These results suggest that as membrane polarity and piperocaine concentration increase, EPC decay becomes increasingly influenced by a mechanism(s) different from the normal voltage-dependent conversion of open channels to closed ones.

In addition to alteration of EPC time course, piperocaine also produces concentration-dependent changes in linearity of the current-voltage relationship when 500 msec conditioning durations are used. This nonlinearity caused by piperocaine is associated with a progressive depression of EPC amplitude as the conditioning duration is gradually increased (Fig. 5). Yet, the EPC decay time is independent of the conditioning duration or time after the conditioning pulse. It seems likely, therefore, that this time-dependent effect of piperocaine does not depend on piperocaine binding to open ion channels, an action that can explain the changes in EPCs elicited after 10 msec conditioning durations. This time-dependent effect does depend, however, on an action of piperocaine that is sensitive to the potential field across the membrane, but contrasts with the interaction of drugs with the ACh-receptor sites that are voltage-independent (6). It depends, thus, on drug concentration since with longer conditioning duration only higher piperocaine concentrations (25–75 μM) change the linearity of the current-voltage relationship, while 10 μM does not (Fig. 4). A differential effect between short and long conditioning dura-

TABLE 2

Effects of piperocaine on EPC reversal potential

Condition	N[a]	Reversal potential[b]
		mV
Control	20	+1.30 ± 0.07
Piperocaine		
10 μM	17	+1.14 ± 0.25
25 μM	15	+0.60 ± 0.13
50 μM	17	+0.71 ± 0.11
75 μM	14	+0.67 ± 0.10

[a] N, number of fibers tested.

[b] Values obtained 15 to 25 min after equilibration in the drug. Each point represents the mean ± SEM of 4 muscles.

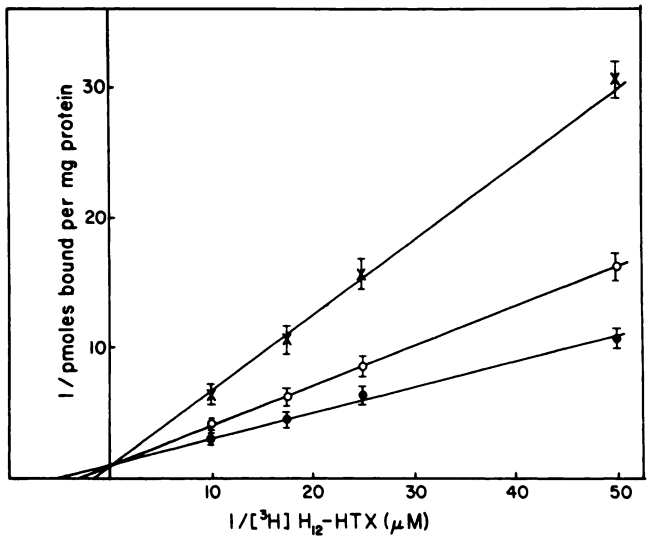

FIG. 10. *Double reciprocal plot of the binding of [³H]H₁₂-HTX to Torpedo electric organ membranes in absence* (●) *and presence of piperocaine, 0.5 μM* (○) *and 1 μM* (×)
Each bar represents ± standard deviation of 3 experiments.

tions has also been found for HTX, but the rates of onset and recovery from a time-dependent effect by HTX are 100-fold slower than those found for piperocaine in the present study (5).

It is not known whether the binding site for piperocaine that is responsible for its time-dependent action on the EPC peak amplitude is a site on the ACh-receptor or its ion channel or whether it involves alteration of the environment of the receptor-channel complex. If on the ACh-receptor, however, the site apparently is not the same site that binds α-bungarotoxin, because this antagonist has not been shown to have either voltage- or time-dependent actions. Piperocaine interacts with the ACh-receptor as judged by its inhibition of [³H]ACh binding to *Torpedo* electric organ membranes (Fig. 11), although it has a higher K_i value (12 μM) than on [³H]H₁₂-HTX binding (K_i = 0.44 μM). This action of piperocaine on ACh-receptor binding may contribute to the reduction in the EPC peak amplitude caused by 25–75 μM piperocaine (Fig. 2).

The effects of piperocaine on the EPC occur without alteration of the normal exponential decay of the EPC decay phase (Fig. 7). The action of piperocaine contrasts, therefore, with the effects of other local anesthetics and scopolamine on the ACh-receptor associated ion channel; these other agents cause multiexponential EPC decays (3, 9, 10, 21, 22). Kinetic models describing the action of these drugs suggest that the slow terminal component of the

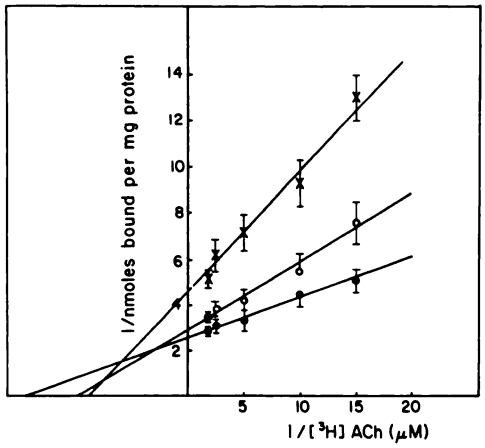

FIG. 11. *Double reciprocal plot of the binding of [³H]ACh to Torpedo electric organ membranes in absence* (●) *and presence of piperocaine, 10 μM* (○) *and 100 μM* (×)
Each bar represents ± standard deviation of 3 experiments.

multiexponential decay caused by these drugs is related to the secondary accumulation of open ion channels after drug molecules dissociate from the open channels during the EPC decay (3, 9, 22). Apparently, therefore, the rate of dissociation of the proposed piperocaine binding to open channels is sufficiently slow to prevent such a secondary accumulation of open channels during the time course of the altered EPC. In this respect, piperocaine action resembles that of atropine (3). Another effect not observed in the presence of piperocaine is hysteresis, or looping, of the current-voltage relationship at hyperpolarized membrane potentials. Such an effect has been shown with HTX, however, to be related to the relatively slow recovery from the time-dependent action of HTX to depress EPC amplitude (5). It is possible, therefore, that the faster rates of recovery from the time-dependent action caused by piperocaine observed in the present study (Fig. 6) prevent the development of hysteresis.

In summary, piperocaine appears to have multiple actions at the ACh-receptor-ion-channel complex. It interacts with the ACh-receptor, inhibiting its binding of [^{3}H]ACh and depressing the peak amplitude of the EPC. It also appears to bind to open ion channels, inhibiting competitively its [^{3}H]-H_{12}-HTX binding and causing dose- and voltage-dependent alteration of EPC time course and decreased EPC amplitude, as well as decreased voltage sensitivity of the EPC falling phase. Finally, the further depression of EPC amplitude that is concentration-, voltage-, *and* time-dependent may be due to the interaction of piperocaine with these sites just described, or possibly with a new class of sites that may be on the receptor or its ion channel.

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