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Binding of Perhydro-histrionicotoxin to the Postsynaptic Membrane of Skeletal Muscle in Relation to Its Blockade of Acetylcholine-Induced Depolarization

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SUMMARY

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Perhydro-histrionicotoxin (H_{12}-HTX), a potent neuromuscular blocking agent, was investigated as a potential probe for conductance systems associated with the acetylcholine receptor. In rat muscles, H_{12}-HTX blocked the end plate potential and also the extrajunctional acetylcholine sensitivity, with half-maximal effects seen at about 8.4 μM and 2.4 μM, respectively. The retardation of α-bungarotoxin binding to denervated muscle membranes by increasing concentrations of H_{12}-HTX showed a noncompetitive type of behavior, with the half-maximal effect at concentrations of H_{12}-HTX much higher than needed to block the acetylcholine response. The binding of H_{12}-HTX, as detected by this protection from α-bungarotoxin, was essentially the same in the membrane and in the pure, soluble receptor. H_{12}-HTX at sufficient concentrations could block 90–100% of the bungarotoxin binding sites; the same was true for *d*-tubocurarine, confirming that these are the receptor sites, in both innervated and denervated muscle preparations. Measurements of the affinity of *d*-tubocurarine, by blockade of electrophysiological response, gave values for the apparent dissociation constant in rat extensor digitorum longus muscle (innervated) of 0.039 μM, and in rat soleus (denervated) of 0.8 μM, at 23°. The results of these studies support an earlier proposal that H_{12}-HTX blocks neuromuscular transmission by acting at a site other than the acetylcholine recognition site of the receptor.

INTRODUCTION

There has recently been considerable progress in the isolation and characterization of the nicotinic acetylcholine binding receptor protein from fish electric organs (for recent reviews, see refs. 1 and 2) and from mammalian muscle (3–5). It has therefore become of more immediate importance to find a specific probe for the

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component that controls the ionic translocations linked to that ACh[4] binding, in the system as it exists *in vitro* in the postsynaptic membrane. The latter component has been termed (6) (making no assumptions as to its macromolecular identity) the ionic conductance modulator. An agent which binds to an ionic conductance modulator site and blocks the conductance change without directly blocking ACh binding would be a powerful tool in analyzing this system.

Indications were obtained (6–8) that H_{12}-HTX may have an action of this type. This is a derivative of the alkaloid histrionicotoxin, which was isolated from the Colombian arrow poison frog *Dendrobates histrionicus* and characterized by Daly *et al.* (9). The perhydro derivative, H_{12}-HTX, like the parent HTX but somewhat more potently, reversibly blocked the EPP of the innervated skeletal muscle and also the ACh-induced extrajunctional depolarization of denervated muscle (6). Its efficacy increased progressively during successive transient applications of ACh (6, 10, 11). Similar effects were seen in the end plate current upon successive stimulations in the presence of HTX (7). This, and the finding (7) that HTX shortens the falling phase of the end plate current, suggested that H_{12}-HTX and HTX, in their actions at the postsynaptic membrane, affect the ionic conductance modulator site. Two other reports have appeared on HTX action—by Glavinovic *et al.* (11) and (since the completion of this work) by Kato and Changeux (33)—on other tissues, suggesting that it may act like procaine and thus may be an allosteric ligand of the ACh receptor.

The suggestion of ionic conductance modulator blockade was further tested by studying the effects of H_{12}-HTX on the reaction of muscle end plate receptors with BuTX, which is a specific and virtually irreversible blocker of the muscle ACh receptor (5, 12). Electrophysiological studies showed that, in contrast to *d*-TC, the presence of a concentration of H_{12}-HTX sufficient to block the EPP did not decrease the irreversible blockade of the EPP by BuTX (6). This again suggests that the ACh recognition site of the receptor was not directly affected by H_{12}-HTX at concentrations that block transmission. However, binding of the [^{3}H]acetylated derivative of BuTX to mouse diaphragm end plates was partially inhibited by H_{12}-HTX (6). A further investigation was therefore initiated, since only fixed times of incubation with BuTX or with [^{3}H]BuTX were used in this initial examination, and kinetic series are needed to distinguish in detail between the behavior of H_{12}-HTX and that of *d*-TC in their binding at the receptor complex. These studies, using both binding and potential measurements to analyze the postsynaptic action of H_{12}-HTX, show that the toxin interacts with the ACh receptor–ICM complex at a site other than the ACh binding site. A brief preliminary account of these findings has been given (13).

MATERIALS AND METHODS

Toxins. BuTX was isolated chromatographically from the venom of *Bungarus multicinctus* (14) and was shown to be homogeneous by polyacrylamide gel electrophoresis at pH 7. [^{3}H]Triacetylated BuTX, at a specific activity of 4.5 Ci/mmole, was prepared as described elsewhere (15). H_{12}-HTX was prepared by catalytic reduction of HTX, with chemical criteria for its purity as given elsewhere (6, 9). Stock solutions of H_{12}-HTX (1–2 mM) were made up in 80% ethanol, stored at 4°, and diluted immediately before use in appropriate aqueous medium to the final concentration employed. Control experiments, both in EPP or ACh potential measurements and in [^{3}H]BuTX binding measurements, showed that ethanol alone at the final concentrations employed had no effect.

Reaction of innervated muscle with [^{3}H]BuTX in the presence of H_{12}-HTX. Diaphragm muscles were removed immediately after decapitation of mice (RR, 18–22-g males) or rats (Wistar, 180–250-g males), keeping the rib cage attached and

[4] The abbreviations used are: ACh, acetylcholine; HTX, histrionicotoxin; H_{12}-HTX, perhydro-histrionicotoxin; EPP, end plate potential; BuTX, α-bungarotoxin; [^{3}H]BuTX, [^{3}H]triacetyl-α-bungarotoxin; *d*-TC, *d*-tubocurarine; ICM, ionic conductance modulator.

without cutting any muscle fibers. Oxygenated Tyrode's solution was used as the medium in this and all subsequent treatments. The diaphragm muscles were incubated on a shaking water bath with 0.25 μM [^{3}H]BuTX, then rinsed rapidly in 100 μM *d*-TC and (in fresh vials) washed for 1 hr in a solution containing unlabeled BuTX (6.25 μM) plus *d*-TC (100 μM), all at 25°. They were then washed with 100 μM *d*-TC solution (about six changes), with shaking at 4°, for 4–14 hr. Subsequent concentration and counting of these wash solutions showed that significant radioactivity was eluted initially but not at the final stages. A sector containing the entire end plate zone was then microdissected from each diaphragm muscle and an adjacent equivalent non-end plate sector; each was dried by blotting, weighed, and dissolved in Soluene 100 at 60°, for 3 hr.

The radioactivity was counted in a toluene medium containing 2,5-diphenyloxazole (0.7%) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.06%), in a Packard (model 3385) counter at 8° at about 35% efficiency. For each experimental point recorded, the mean counts for two to four hemidiaphragms were used after subtraction of the counts due to an equal weight of non-end plate muscle from the same specimens. In the protection experiments a preliminary 30-min incubation in H_{12}-HTX solution was carried out, and the samples were then labeled and washed as described above.

Reaction of membrane-bound receptor with [^{3}H]BuTX in the presence of H_{12}-HTX. Muscle membranes were prepared as described in detail elsewhere.[5] Briefly, denervated leg muscles of cats were homogenized in 0.25 M sucrose medium, and the membrane-containing pellet was extracted with 0.8 M LiBr for 12 hr; a resulting membrane-rich fraction was extracted in 0.6 M KCl and then in water, and finally a membrane-rich fraction was obtained by differential centrifugation, all at 4°. The specific activity of the membranes used was not below 5 nmoles of receptor per gram of protein. Crude membrane homogenates (volumes containing the equivalent of 50–125 mg of muscle, wet weight) were incubated in 70 mM NaCl–morpholinopropanesulfonate buffer, pH 7.5 (final volumes, 1.0–2.5 ml), at 25° or 37° on a shaking water bath. Semipurified[5] membrane suspensions (volumes containing the equivalent of 20–40 mg of muscle, wet weight) were incubated similarly in 10 mM morpholinopropanesulfonate, pH 7.5 (final volume, 1.0 ml), or other medium where noted. The reaction was initiated by adding [^{3}H]BuTX (final concentration, 40 nM). Assay of receptor in these membranes was performed as described elsewhere.[5] The H_{12}-HTX was present for 15 min prior to the addition of [^{3}H]BuTX.

Reaction of purified ACh receptor with [^{3}H]BuTX in the presence of H_{12}-HTX. ACh receptor protein was isolated from chronically denervated cat lower leg muscles according to the techniques described by Dolly and Barnard (3). The muscles used as source were fresh (not frozen), and the isolated receptor preparation was used within a few days. Partly purified receptor (at 15 nmoles/g of protein) was obtained after the initial gel filtration stage of the purification. The pure receptor material obtained from the final stage of the purification procedure was shown to be a homogeneous protein and to possess the properties of the nicotinic ACh receptor (3, 4). When used here, it had a specific activity of about 3500 nmoles of BuTX binding sites per gram of protein.

The receptor (2 nM) in 0.2% Triton X-100 was incubated at 25° in 10 mM potassium phosphate buffer, pH 7, with [^{3}H]BuTX (9 nM). H_{12}-HTX at the stated concentration was present for 30 min prior to the toxin addition. At intervals, aliquots were assayed by the DEAE-cellulose disc method for muscle receptor (15). The rate of loss of receptor activity was measured when necessary by second-order logarithmic plots to determine the initial rate of reaction. All other details were the same as specified elsewhere for the kinetic analyses of soluble receptor (15).

Recording of indirectly elicited twitch tension. Diaphragm muscles with intact phrenic nerves were removed from male RR strain mice (20–25 g) or male Wistar

[5] J. O. Dolly, V. J. Coates, and E. A. Barnard, manuscript in preparation.

rats (180–250 g) after cervical dislocation under ether anesthesia. The cardiac region of the left hemidiaphragm was mounted in a 15-ml twitch bath with the ribs secured to a stationary support and the central tendon attached by a thread to a Grass FT-03 strain gauge. Isometric twitch tension was recorded on a Grass model 5 polygraph. The nerve was stimulated via an agar salt bridge in contact with a silver–silver chloride wire. Supramaximal 0.1-msec stimuli were delivered from a Grass S4 or S88 stimulator at the rate of 0.1 Hz. Ringer's solution was constantly bubbled with 95% O_2 and 5% CO_2 in the bath.

Intracellular recording of EPPs and action potentials. EPPs and indirectly elicited action potentials were recorded with glass microelectrodes filled with 3 M KCl as previously described (16, 17). EPPs were recorded in the presence of 12 mM $MgCl_2$ and 3 mM $CaCl_2$. The microelectrode was considered to be placed focally (i.e., at the end plate) if miniature end plate potentials or EPPs having a rise time of 1 msec or less could be recorded. When the EPPs were completely blocked by a drug, end plates were visually located and multiple impalements were made to ensure that at least a few recordings were made precisely at the end plate. The amplitude of the EPP was taken as the mean of the last 20 from a train of 30 EPPs elicited at 0.5 Hz (for the *d*-TC experiments) or 0.2 Hz (for the H_{12}-HTX experiments). The means from three surface fibers per muscle from at least 10 control muscles were pooled and assigned the value of 100%. When H_{12}-HTX or d-TC was present the recordings then were usually made from six surface fibers in each of two muscles, at each drug concentration used. The slow stimulus frequency (0.2 Hz) in the H_{12}-HTX experiments was employed to prevent the progressive decline in EPP amplitude seen at higher frequencies in the presence of H_{12}-HTX (6, 8).

Recording of extrajunctional sensitivity to microiontophoretically applied ACh. The soleus muscles of male Wistar rats (180–250 g) or male mice (RR, 20–25 g) were denervated as described elsewhere (17); 10–15 days later they were removed under ether anesthesia and pinned to paraffin-covered Perspex plates. Membrane potential was recorded by conventional microelectrode techniques, while a second electrode, filled with 2 M ACh, was positioned on the surface of the muscle fiber within 50 μm of the recording electrode (17). ACh was ejected onto the surface of the membrane by passing a 1–10-msec current pulse through the ACh pipette. ACh sensitivity is expressed as millivolts of depolarization per nanocoulomb of charge passed.

Focal depolarization of nerve terminal. For the studies involving focal depolarization of the nerve terminal of the rat diaphragm muscle, an external glass microelectrode filled with 2 M NaCl and having a resistance of 3 MΩ was used. This method is essentially similar to that used by Katz and Miledi (18–20). The brief pulses used to evoke the EPP varied between 0.5 and 40 μamp in intensity and from 0.5 to 6 msec in duration. In these experiments tetrodotoxin (3.13 μM) was used to block sodium conductance, thus preventing action potentials, together with neostigmine (0.3 μM) to allow maximal increase in amplitude of the EPP. The nerve terminal was located by trial and error; focal location at the nerve terminal region was indicated by the graded responses that were recorded at the recording electrode located nearby. Under these conditions a progressive increase in current intensity gave a measurable progressive increase in the EPP.

Solutions. Ringer's solution contained NaCl, 135 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 15 mM; Na_2HPO_4, 1 mM; $CaCl_2$, 2 mM; and glucose, 11 mM, pH 7.2–7.4. To record EPPs, the preparations were bathed in Ringer's solution containing 12 mM $MgCl_2$, 3 mM $CaCl_2$, and 118 mM NaCl. All solutions were continuously bubbled with 95% O_2 and 5% CO_2. *d*-Tubocurarine was obtained from K & K Laboratories; a fresh solution was prepared for each experiment.

RESULTS

Blockade by BuTX and [^{3}H]BuTX of EPP and extrajunctional ACh sensitivity. Previous investigations (5, 16) showed that

the monoacetyl derivative of BuTX has the same affinity for the receptor sites and ability to block EPP as native BuTX. However, the triacetyl derivative, used here in the binding studies to achieve higher radioactivity incorporation, is known to have a lower reaction rate at the receptor than native BuTX (15, 21), and it was necessary to confirm that its potency in blockade of the EPP was adequate under the conditions used in the protection studies here.

When BuTX was applied to mouse diaphragm muscles, the degree of blockade of the twitch and EPP responses after 30 or 60 min increased progressively with toxin concentration, but about 1 μM BuTX was required to block these responses completely within such a period (Table 1). With [^{3}H]BuTX, each of these responses declined at about half the rate indicated for BuTX, in that about twice the concentration of [^{3}H]BuTX was needed to reach a given effect (Table 1). The triacetyl-toxin nevertheless showed an identical, nearly irreversible effect upon prolonged washing. As noted previously (16, 17), it was confirmed here with each toxin that washing the BuTX-inhibited preparation with Ringer's solution for 2–4 hr resulted in the return of a very small EPP (0.5–1 mV); washing for longer periods did not further increase the EPP amplitude.

The extrajunctional ACh sensitivity of the chronically denervated diaphragm muscles of the mouse was abolished by BuTX (Table 1). Observations suggesting a similar effect were made previously (16) in the chronically denervated mouse soleus muscle. To measure the rate of this response of the denervated diaphragm to BuTX would have required such low toxin concentrations that errors due to adsorption of the toxin might have become serious, and a comparison of the reaction rates of the two forms of toxin was not attempted in this system.

Suppression of [^{3}H]BuTX binding by saturating concentrations of d-TC. It was previously reported (6, 16, 22–24) that a maximum of 65% protection against radioactive BuTX reaction at end plates was found with up to 100 μM *d*-TC; the possibility was thus raised that a class of sites is associated with the receptor that is reactive to both [^{3}H]BuTX and H_{12}-HTX but not to *d*-TC. This has since been investigated in detail. When the *d*-TC protection experiments with [^{3}H]BuTX in diaphragm muscles were repeated with the exceptionally lengthy washout time of BuTX from muscle taken into account, i.e., with the protecting *d*-TC present throughout post-labeling overnight washes, different results were obtained (Table 2).[6] The same was also true for cholinergic agonists as protectors. The difference is ascribed to the presence of a slowly diffusing pool of free [^{3}H]BuTX in the treated muscle; when the exchange treatment used previously (washing for 10–30 min with excess unlabeled BuTX in the absence of *d*-TC) was given, followed by a wash without *d*-TC, not all of that pool was removed. Thus hitherto protected receptor sites became labeled unless 100 μM *d*-TC was maintained in the washing solution for much longer periods. In end plates, extrajunctional receptors, muscle membrane preparations, and purified soluble ACh receptor from muscle, 90–100% of the [^{3}H]BuTX sites were sensitive to *d*-TC (Table 2). Hence we can regard those sites as located in the active center of the receptor component that binds ACh.

Effect of d-TC on action of BuTX at end plate of rat diaphragm muscle. A similar high level of protection by *d*-TC against blockade by BuTX was observed in previous electrophysiological studies (6, 16, 17). However, it is recognized that recovery of the action potential and muscle twitch after simultaneous exposure to *d*-TC and BuTX for fixed periods of time is not a sufficient criterion for full protection of the receptor sites, since considerably less than 100% of the receptor population should be able to generate an EPP large

[6] It is difficult to demonstrate, by measurement of ACh sensitivity of denervated muscle fibers, protection by *d*-TC against BuTX blockade (16, 17); this is now thought to be due to (a) the fast rate of BuTX reaction on these fibers compared with innervated fibers, necessitating different levels of applied toxin in the two cases, and (b) the use of higher *d*-TC levels on account of its lower affinity for extrajunctional receptors.

TABLE 1

Effects of BuTX and [^{3}H]BuTX on innervated and chronically denervated muscles

The twitch and EPP experiments were done at room temperature (23°) on mouse diaphragm. EPPs were recorded from surface fibers, and extrajunctional ACh sensitivity was recorded from 10–15-day denervated soleus muscles of the mouse. For details of the electrophysiological recording, see MATERIALS AND METHODS. Values use means ± standard deviations of the numbers of muscles or fibers shown in parentheses.

Toxin	Concentration	Innervated muscle				Chronically denervated muscle: extrajunctional ACh sensitivity[b]	
		Twitch[a]		EPP[b]			
		30 min	60 min	30 min	60 min	30 min	60 min
	μM	*% control*		*mV*	*mV*	*mV/nanocoulomb*	
	0	100	100	10.0 ± 2.1 (10)	10.0 ± 1.9 (12)	120 (50)	110 (30)
Native BuTX	0.03					0.8 (3)	0.07 (3)
	0.06					0.4 (3)	0.04 (3)
	0.13	75 ± 3.2 (5)	40 ± 2.3 (5)	7.5 ± 1.0 (4)	3.5 ± 1.1 (4)	0.2 (5)	0.03 (4)
	0.25	60 ± 2.6 (5)	30 ± 1.7 (5)	4.5 ± 0.7 (4)	2.0 ± 0.6 (3)	0.2 (4)	<0.001 (4)
	0.63	5 ± 0.7 (4)	0 ± 0 (4)	0 ± 0	0 ± 0	<0.001 (3)	<0.001 (3)
	1.25	0 ± 0 (3)	0 ± 0 (3)	0 ± 0	0 ± 0	<0.001 (3)	<0.001 (3)
[^{3}H]BuTX	0.13	88 (1)	66 (1)	8.8 ± 2.0 (5)	5.8 ± 1.3 (3)		
	0.25	72–74 (2)	40–42 (2)	6.6 ± 1.1 (4)	4.0 ± 0.8 (3)		
	0.63	30–32 (2)	9–11 (2)	3.0 ± 0.7 (3)	0 ± 0		
	1.25	0 ± 0	0 ± 0	0 ± 0	0 ± 0		

[a] Numbers in parentheses in these columns refer to the number of muscles studied.

[b] Numbers in parentheses in these columns refer to the number of surface fibers studied.

TABLE 2

Maximum inhibition by cholinergic ligands of [³H]BuTX binding to muscle and to a purified receptor preparation

The preparations were incubated at 25° for 1 hr with [³H]BuTX in the presence and absence of the appropriate concentration of ligand. The amount of toxin bound was determined as described in MATERIALS AND METHODS. Intact muscle, the membrane fraction, and the purified receptor preparation were incubated with 250, 50, and 9 nM [³H]BuTX, respectively. Numbers in parentheses show, first, the number of separate experiments and, second, the range, if any, of the percentage values found.

Preparation	Inhibition	
	d-TC (0.3–1.0 mM)	ACh[a] (0.1–1.0 mM)
	%	%
Intact diaphragm (mouse)		
Normal	90 (4; 85–95)	
Denervated	90 (2)	
Muscle membrane fraction (denervated, cat leg)	95 (7; 87–100)	100 (4; 98–100)
Isolated receptor (denervated, cat leg)	100 (5; 96–100)	100 (2)

[a] The preparations had been treated with 10 μM diisopropyl fluorophosphate.

enough to elicit an action potential and a subsequent twitch. In order to measure any blockade of the EPP that might remain, we took advantage of a system established by previous investigators (18–20), in which an EPP is generated by focally depolarizing the presynaptic nerve terminal by brief depolarizing pulses applied through an external microelectrode in the presence of tetrodotoxin. This electrophysiological method is limited by one's inability to obtain adequate information regarding the amplitude of the presynaptic potential change. Initially we determined the viability of the nerve-diaphragm muscle preparations employed by stimulating the nerve and obtaining an action potential and twitch. The preparation was then exposed to tetrodotoxin and neostigmine, and graded current pulses were applied through a microelectrode containing 2 M NaCl, positioned over the nerve terminal. The relationship between the amplitude of the EPP and the intensity of the current applied in the presence of tetrodotoxin and neostigmine after a 2-hr washout of *d*-TC is shown in Fig. 1. Our control values are similar to those reported by Kuba and Tomita (25). The current-voltage curve has a sigmoid shape and tends to approach an asymptote at about 30 μamp. With this control situation established, we examined the effect of BuTX in the presence of *d*-TC. Following incubation for 30 min with *d*-TC (28 μM), the preparation was incubated with BuTX (0.62 μM) and *d*-TC (28 μM) for another 30 min, subsequently washed with *d*-TC (140 μM) alone for 1 hr, and finally washed for 3 hr in normal Ringer's solution. When the nerve was subsequently stimulated, an action potential and twitch were observed, indicating full recovery of neuromuscular transmission, as observed previously (6, 16, 17). The preparation was then exposed to a combination of tetrodotoxin and neostigmine at the concentrations used above, and the voltage-current relationship was again determined. The results obtained (Fig. 1) may suggest that the protection afforded by *d*-TC at this concentration was essentially complete, since an EPP similar to that obtained under control conditions was recovered; i.e., the response was not shifted along the current axis, and the maximum depolarization was virtually unchanged.

Concentration dependence of d-TC blockade of ACh-induced depolarization. In view of the results above, it became of interest to determine the apparent affinity of *d*-TC for the receptor sites, in both chronically denervated and innervated muscle, from its blockade of ACh-induced depolarization. While such values have

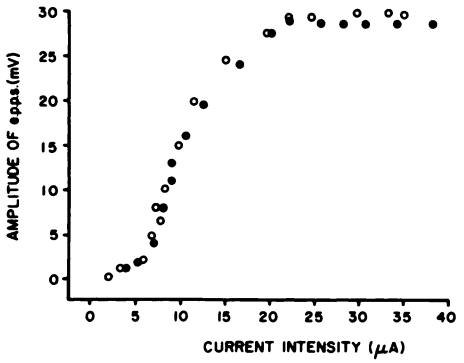

FIG. 1. *Current-voltage relationship for end plates of rat diaphragm, obtained in the presence of tetrodotoxin (3.13 μM) and neostigmine (0.3 μM)*

The diaphragm muscle was initially exposed to *d*-TC (28 μM) and subsequently washed for 2 hr, as a control; ○, curve then obtained for EPP magnitude as a function of the depolarizing current applied to the nerve terminal. In another set of experiments the following sequence of drug treatments was given: initial exposure to *d*-TC (28 μM), followed by a combination of *d*-TC (28 μM) and BuTX (0.62 μM) for another 30 min, with subsequent removal of BuTX and exposure only to *d*-TC (140 μM) for 1 hr, followed by washing of the preparation for 3 hr. ●, recordings finally made on that preparation (after its subsequent exposure to tetrodotoxin and neostigmine for 30 min). There was no significant difference between the data recorded after this treatment with *d*-TC and BuTX and those from the control preparation.

been reported previously by others (26–28), some are for frog muscles, and they vary among different authors. Mammalian extensor digitorum longus and soleus muscles were used here. With indirect stimulation of the muscle, in medium containing 12 mM $MgCl_2$ to reduce the EPP amplitude below the action potential threshold, recordings were made with a series of concentrations of *d*-TC present. The mean amplitude A of the EPP (obtained from at least 12 fibers in every case), together with that (A_0) from the control case in the absence of *d*-TC, gave the fractional blockade of the EPP by *d*-TC, $B = (A_0 - A)/A_0$. For accuracy in determining the concentration of *d*-TC giving 50% blockade of the EPP (K_{app}), the dose-blockade curve was linearized by employing a Hill plot (Fig. 2). This allowed a K_{app} value of 39 nM to be determined.

In a parallel experiment, the extrajunctional sensitivity to microiontophoretically applied ACh was determined on denervated soleus muscles of the rat, in media containing various concentrations of *d*-TC. The fractional blockade by *d*-TC of this response was expressed as before, and a Hill plot was again constructed (Fig. 3), allowing K_{app} = 800 nM to be determined. It appears that the end plates of inner-

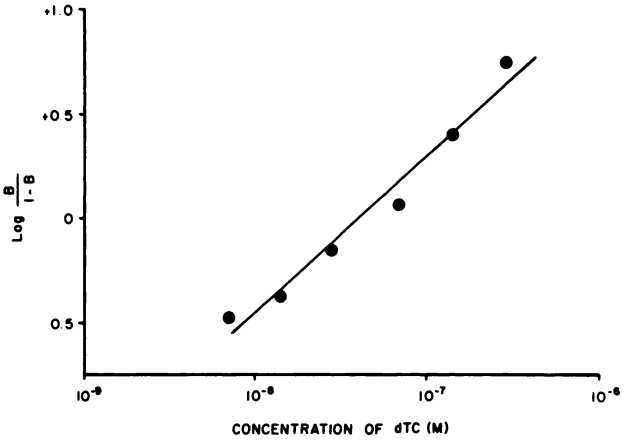

FIG. 2. *Effect of d-TC on EPP amplitude in rat extensor digitorum longus muscle*

Muscles were bathed in Ringer's solution containing 12 mM $MgCl_2$ and 3 mM $CaCl_2$ at 23°; stimulus frequency, 0.5 Hz. Each point employs the mean EPP amplitude from at least six fibers, in each of two muscles, after exposure to each *d*-TC concentration for 30–60 min. B is the fractional blockade by *d*-TC of the EPP response; the Hill plot was fitted by a least-squares line.

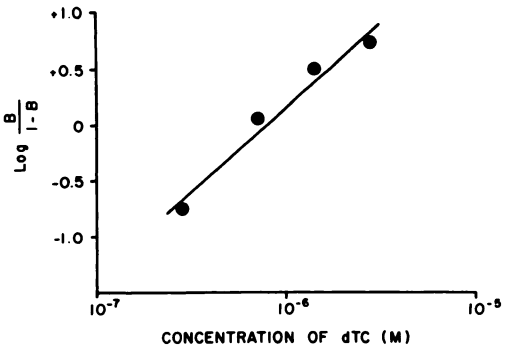

FIG. 3. *Effect of d-TC on ACh sensitivity in denervated rat soleus muscle*

B is the fractional reduction, as a result of the *d*-TC present, in the sensitivity to microiontophoretically applied ACh; the Hill plot was fitted by a least-squares line. ACh pulse duration was 1–5 msec. Each point represents the mean for at least six fibers from at least two muscles, at 23°.

vated rat muscles are about 20 times more sensitive to *d*-TC than the extrajunctional region of denervated rat muscle. In the partially purified receptor from cat denervated leg muscle, K_p = 330 nM (15).

The Hill plots obtained are linear, but have different slopes for the two cases. It is known that depolarizations of nicotinic junctions produced by agonists fit Hill plots with slopes differing in different cases and greater than unity (29, 30). The present plots, based on membrane depolarizations, are only empirical expressions for interpolating the value of $B = 0.5$.

Actually, the value of K_{app} is a much better estimate of K_D than would be expected on the basis of the classical treatment, in which both agonist and antagonist are assumed to reach equilibrium with the receptors. All known evidence supports the view that ACh is present in appreciable quantities in the synaptic cleft for less than 1 msec (31). By comparison, the dissociation of *d*-TC from the receptor has a time constant of the order of 1 sec (32). Thus an insignificant fraction of receptors bound to *d*-TC would become available during the transient presence of ACh, and a concentration of *d*-TC which binds to 50% of the receptors (K_D) should reduce the response to ACh by 50% (K_{app}).

Blockade by H_{12}-HTX of EPP of junctional, and of ACh sensitivity of extrajunctional, receptors. Previously (6) an action of H_{12}-HTX in antagonizing the activation of ACh of junctional and extrajunctional receptors was demonstrated. These effects were investigated quantitatively here in innervated and denervated muscles. Various concentrations of H_{12}-HTX were applied to the extensor digitorum longus muscle of the rat, and the EPPs were recorded as described above. At each concentration from 3.5 to 70 μM H_{12}-HTX (after a 30-min equilibration), depression of the EPP was produced (Fig. 4A). The data obtained for the dose-blockade curve were linearized by a Hill plot as before (Fig. 4B, *I*), yielding K_{app} = 8.4 μM for H_{12}-HTX on the innervated muscle.

When a similar study on the chronically denervated rat soleus muscles was performed (Figs. 4B and 5) an approximately 4-fold lower concentration (2.4 μM) of H_{12}-HTX was shown to be able to block 50% of the ACh sensitivity.

Blockade by H_{12}-HTX of reactions of [^{3}H]BuTX at muscle end plates and denervated muscle membranes. Mouse diaphragm muscle was labeled at 25° with [^{3}H]BuTX at a concentration (0.25 μM) previously shown (16) to give specific end plate labeling. This labeling was completely prevented by a prior treatment with unlabeled BuTX (1.2 μM, 2 hr, 24°).

When labeling was conducted in the presence of H_{12}-HTX, retardation was exhibited (Fig. 6). At the highest concentrations of H_{12}-HTX used, more than 90% retardation of the initial rate of [^{3}H]BuTX binding was obtained, showing that all the latter sites were saturated by H_{12}-HTX. A detailed kinetic study was not made for protection in whole muscle, owing to the known difficulties arising from diffusion limitation of BuTX entry into intact muscle specimens.[5]

A membrane fraction rich in ACh receptors was prepared from denervated cat leg muscles,[5] and the rate of [^{3}H]BuTX reaction therein was determined. In the presence of H_{12}-HTX (100 μM), this reaction was significantly retarded (Fig. 7). Such retardations were measured over a wide range of H_{12}-HTX concentrations (Fig. 5)

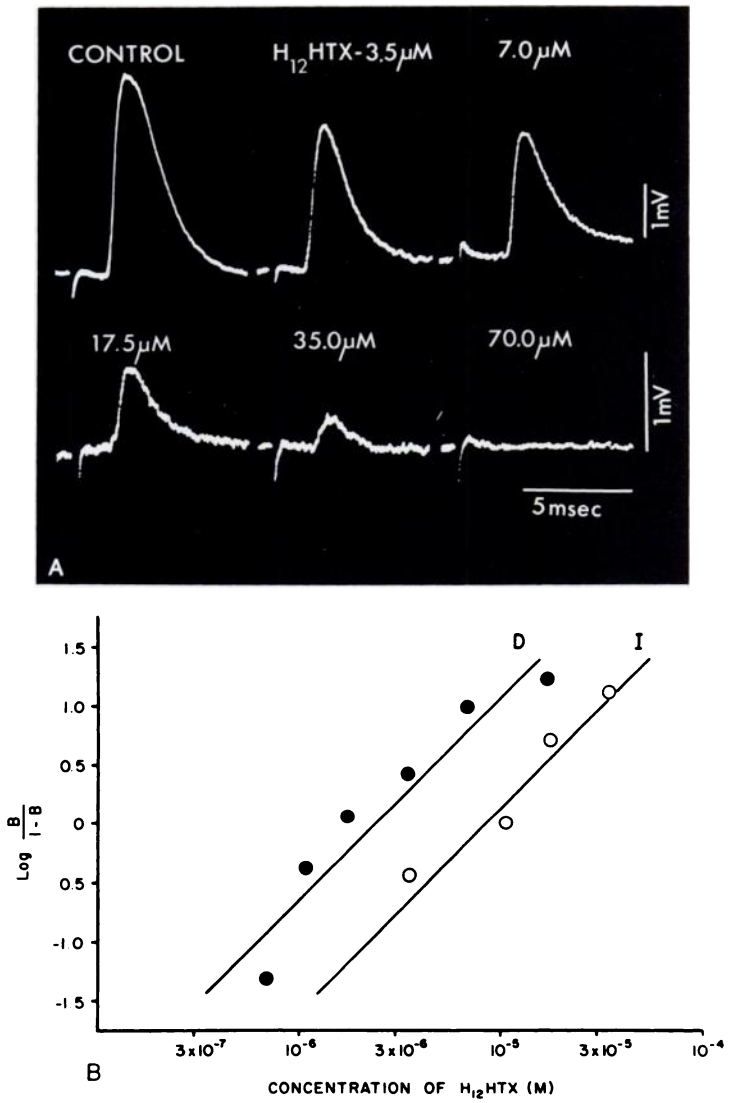

FIG. 4. *Effect of H_{12}-HTX on the end plate potential of innervated extensor digitorum longus muscle and extrajunctional ACh sensitivity of the denervated soleus muscle.*

A. Typical oscilloscope records showing the effect of H_{12}-HTX on the EPP amplitude of innervated rat extensor digitorum longus muscle; the concentration of H_{12}-HTX used is noted over each recording. B. Data of the type shown in Fig. 4A for innervated (*I*) muscle, expressed as a percentage of the control (○), and also the effect of H_{12}-HTX on the ACh sensitivity (●) of denervated (*D*) rat soleus muscle, shown as Hill plots. Conditions and plots are the same as in Figs. 2 and 3. The muscles were exposed to each H_{12}-HTX concentration for 30–60 min prior to the recording. The stimulus frequency was 0.2 Hz; temperature, 23°.

when it was seen that simple competitive behavior was not exhibited. The concentration for half-maximal effect, taken as the apparent protection constant K_p, was 800 μM.

Blockade by H_{12}-HTX of reaction of [³H]BuTX with soluble ACh receptor from denervated cat leg muscles. The kinetics of [³H]BuTX reaction with the soluble ACh receptor was studied as described previously (15). H_{12}-HTX gave significant protection from the [³H]BuTX labeling, of the

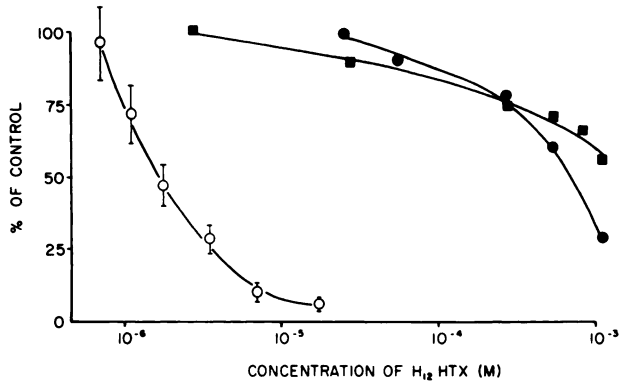

FIG. 5. *Comparison of concentration dependence of H_{12}-HTX in blocking response to ACh and preventing binding of BuTX in denervated mammalian muscle*

O, ACh sensitivity of rat soleus in the presence of H_{12}-HTX, as a percentage of that in the absence of H_{12}-HTX. Values are means ± standard errors for at least 4 muscles; the data are the same as in Fig. 4B. Retardation by H_{12}-HTX of [^{3}H]BuTX (50 nM) uptake in cat leg muscle membranes (cf. Fig. 7) was measured after 7 min at 37° (●) or after 6 min at 25° (■); the former points are only approximate, since the [^{3}H]BuTX reaction was so fast at 37° that it was not entirely linear over 7 min at the lower H_{12}-HTX concentrations. Each point (● and ■) represents activity as a percentage of activity in the absence of H_{12}-HTX and is the mean of values from two experiments, which agreed closely.

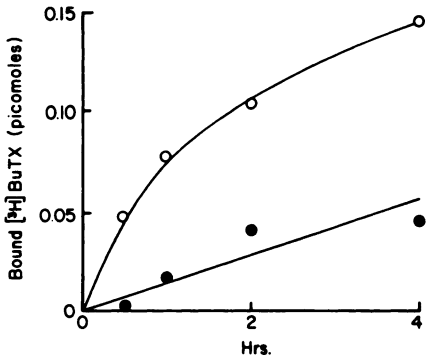

FIG. 6. *Typical plots of binding of [^{3}H]BuTX (0.25 μM) at 25° to end plate region of mouse diaphragm muscle, alone* (O) *or in the presence of 270 μM H_{12}-HTX* (●)

After the appropriate incubation period the samples were washed with an excess of unlabeled BuTX and *d*-TC, dissolved, and counted as described in MATERIALS AND METHODS. The ordinate represents the mean quantity of [^{3}H]BuTX bound (at the end plates) per hemidiaphragm (see the text).

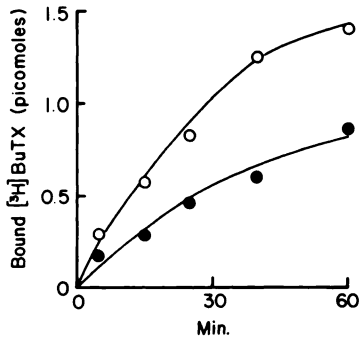

FIG. 7. *Typical plot of binding of [^{3}H]BuTX (50 nM) at 25° to a crude membrane preparation of denervated leg muscles, alone* (O) *or in the presence of 100 μM H_{12}-HTX* (●)

Aliquots of the incubation mixture were removed after various times, the membranes were washed by filtration, and the amount of [^{3}H]BuTX bound was measured by counting the filtrates, with details as described elsewhere.[5] The ordinate represents the amount of bound [^{3}H]BuTX in the membrane suspension.

type already seen with membrane-bound receptor. This reaction was performed both on an impure preparation at the gel filtration stage of isolation (15) and on the completely pure receptor protein (3). In all cases the same degree of retardation at a given concentration (140 μM) of H_{12}-HTX was found. An apparent protection constant K_p for H_{12}-HTX at the receptor was estimated from the equation (15)

$$\frac{v_i}{v} = \frac{K_p}{K_p + [L]} \qquad (1)$$

where v_i and v are the initial velocities in the presence of H_{12}-HTX at concentration $[L]$ and in its absence, respectively. This treatment was also applied in the case of the protection experiments with the membranes (as in Fig. 7), taking a fixed concentration of 140 μM HTX; the apparent K_p values obtained in the various cases are shown in Table 3. While these values do not represent actual binding constants, owing to the form of the concentration dependence shown in Fig. 5, they were used to show that H_{12}-HTX binds (in this concentration region) equally to both membrane-bound and soluble receptors. Table 3 shows that H_{12}-HTX is much more effective in inhibiting the physiological responses to ACh than in preventing BuTX binding (cf. Fig. 5).

DISCUSSION

H_{12}-HTX has been seen to inhibit the transient membrane depolarization by ACh at both synaptic and extrajunctional receptor sites. The mechanism of this effect warrants further study. One useful approach to this problem would appear to be the comparison of H_{12}-HTX effects on electrophysiological measurements and on molecular parameters such as the specific binding of BuTX, a probe for the ACh recognition site of the receptor. This comparison was made here with the receptor in several types of situations (Table 3), and in each case a distinct difference was found between the two classes of effect just mentioned. H_{12}-HTX inhibits BuTX binding [extending a brief study of this phenomenon that we previously reported (6)], but at much greater concentrations than those required to block ACh depolarization. The curve for H_{12}-HTX antagonism of BuTX binding does not follow the normal titration curve expected for competition at a single site.

Previous electrophysiological evidence (6–8) led to the suggestion, regarding a possible mechanism of action for HTX and H_{12}-HTX, that this type of drug interacts at a site other than the ACh recognition site, this being a site controlling ion translocation when the receptor is activated [ICM site (6)]. The present evidence supports the above suggestion that the action is not exerted at the ACh recognition site itself, since the concentration dependencies seen here are quite different. Partial similarities can be noted between the effects of HTX and a class of local anesthetics [e.g., prilocaine, procaine, or dimethisoquin, although there are quantitative differences between the effects of the latter set]. The similarities in question are the shortening of the end plate current (7, 34, 35) and the mode of inhibition (which occurs at higher local anesthetic concentrations) of ACh or α-neurotoxin binding seen by workers with electroplax membranes (33, 36–38), which may be compared with the inhibition of BuTX binding seen here. Possibly, therefore, HTX, its derivatives, and the local anesthetics exert their actions on the end plate current as seen here, by binding to a common site (11, 33). This site may be on the ICM, or, in view of the wide range of drug structures that are efficacious, may be located at an interface with the lipid membrane that affects the ion channel (39).

It should be borne in mind, however,

TABLE 3

Apparent dissociation constants of H_{12}-HTX for ACh receptor-ICM complex of muscle in membrane-bound and purified forms

The protection constant, K_p, was determined from the inhibition of [^{3}H]BuTX binding (see MATERIALS AND METHODS) using Eq. 1, except for the value in parentheses, which is the concentration giving half-maximal inhibition of the [^{3}H]BuTX binding rate (see Fig. 5). Duplicate values did not differ by more than 10%. The apparent dissociation constant, K_{app} (means ± standard errors), was determined by the concentration giving half-maximal inhibition of ACh-induced depolarization (see Fig. 4).

Preparation	K_p	K_{app}
	μM	μM
Rat innervated extensor digitorum longus		8.4 ± 1.14
Cat denervated leg muscles		
Membrane fraction	120 (800)	
Partially purified receptor	100	
Homogeneous receptor	200	
Rat denervated soleus		2.4 ± 1.16

that subtle but significant differences exist between the actions of H_{12}-HTX and of such compounds as procaine. For example, H_{12}-HTX shortens the falling phase of the end plate current much as atropine does (39), while several of the local anesthetics, which also produce an initial shortening, introduce a biphasic falling phase into the end plate current (34, 35).

The blockade of BuTX binding is observed with higher concentrations of H_{12}-HTX than those that block ACh-induced depolarization (Table 3). This could be due to a second, weaker binding site for H_{12}-HTX in the region of the ACh recognition site, or to some allosteric interaction elsewhere. The difference (Table 3) between the K_p value taken from the half-maximal effect and that derived from Eq. 1 is to be expected, since the BuTX-blocking effect of H_{12}-HTX does not follow a simple binding curve (Fig. 5) whereas Eq. 1 assumes simple mutual exclusion. These effects must arise on the receptor protein itself and not on the membrane, since they are observed at the purified receptor level as well as in membrane fragments (Table 3).

This rather low affinity of H_{12}-HTX in affecting the BuTX binding site explains the paradoxical effect discussed previously (6, 7, 40): H_{12}-HTX, at a concentration (140 μM) chosen for its effectiveness in abolishing neuromuscular transmission, was unable to afford protection against the irreversible blockade of transmission by relatively high concentrations of BuTX. In contrast to the previous observation, we have been able to obtain, under appropriate conditions, essentially complete saturation of the BuTX binding sites by either *d*-TC or H_{12}-HTX. Hence the near additivity of protection of BuTX binding seen with a mixture of the two drugs, used at a subsaturating concentration, is to be expected.

Some apparent differences between the ACh receptor systems of innervated and denervated muscle are indicated by the present results, but their basis needs further study. Thus BuTX blocks at denervated muscle receptors much faster than at end plates (Table 1). Almon *et al.* (41) reported a 10-fold higher affinity for BuTX in crude extracts of denervated compared with innervated muscle, but this disappeared after 8 days in solution at 4°, while a much smaller difference between the two types was found by Brockes and Hall (42). We do not interpret the data of Table 1 as necessarily showing an intrinsic difference in reactivity, since the barriers to diffusion of BuTX into the intact end plate structures in normal muscle are probably much greater than those on the surface of the sarcolemma of denervated muscle. Another apparent difference between denervated and normal rat muscle receptors is a low affinity for H_{12}-HTX (Table 3) in the former case. A third such difference is the lower (by 10–20-fold) affinity, as measured electrophysiologically, of *d*-TC for the extrajunctional receptors. This difference was noted previously by Beranek and Vyskocil (27), Chiu *et al.* (16), and Lapa *et al.* (17). It should not necessarily be taken to mean that at the molecular level the affinity of *d*-TC for the ACh receptor differs to this extent between junctional and extrajunctional sites. Although accurate analysis can be made of the blocking effects of *d*-TC on the EPP (see Fig. 1), or of its blocking effects on ACh sensitivity (see Fig. 3), comparisons may not necessarily be made between the two systems, since the two types of depolarization induced by ACh follow different time courses. The relationships between potential change and current change are not identical in the two systems. For these reasons, the values of K_{app} for an antagonist may vary systematically. Likewise, other reports, noted above, suggesting a difference between the extrajunctional receptors of denervated muscles and the receptors at the end plate region, do not establish that this exists at the level of the ACh receptor molecule itself. The much lower density of the receptors at the denervated muscle membrane compared with the end plate postsynaptic membrane (16) may, with the operation of factors just noted, be sufficient to lead to an apparent greater ef-

fectiveness of *d*-TC at the much more densely packed end plate receptors.

The findings reported here for the receptor affinity for *d*-TC agree with previous electrophysiological findings (6, 17, 27) on the effect of *d*-TC on intact rat diaphragm (see also Fig. 1), and also with results obtained on extracts of rat diaphragm (38, 43), although not in every case (44). It is noteworthy that the apparent affinity for *d*-TC, as measured here on denervated muscles by its blockade of the response to ACh, is the same as that determined (3) with the purified soluble receptor from denervated muscle.

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