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Studies on the Effect of Histrionicotoxin on the Monocellular Electroplax from *Electrophorus electricus* and on the Binding of [^{3}H]Acetylcholine to Membrane Fragments from *Torpedo marmorata*

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SUMMARY

KATO, G. & CHANGEUX, J.-P. (1976) Studies on the effect of histrionicotoxin on the monocellular electroplax from *Electrophorus electricus* and on the binding of [^{3}H]acetylcholine to membrane fragments from *Torpedo marmorata*. *Mol. Pharmacol.*, 12, 92-100.

The effects of histrionicotoxin (HTX) and dihydroisohistrionicotoxin (DHTX) on the depolarization produced by carbamylcholine on the monocellular electroplax preparation of *Electrophorus electricus* and on binding of [^{3}H]acetylcholine to membrane fragments rich in acetylcholine receptor protein from *Torpedo marmorata* are described. HTX and DHTX at concentrations of 1.0 μM reversibly and noncompetitively blocked the steady-state depolarization produced by carbamylcholine on the monocellular electroplax preparation. On the other hand, neither HTX or DHTX blocked the binding of [^{3}H]acetylcholine to membrane fragments rich in acetylcholine receptors, even at concentrations of 500 μM. Rather, both toxins increased the affinity of [^{3}H]acetylcholine for the receptor protein in its membrane environment at concentrations which blocked the carbamylcholine effect on the electroplax. The similarity between the effects of histrionicotoxin and those of certain local anesthetics suggests that at the receptor level histrionicotoxin acts as a powerful local anesthetic.

INTRODUCTION

Histrionicotoxin is an alkaloid present in the skin of the Colombian arrow poison frog, *Dendrobates histrionicus*. Its isolation and chemical characterization were first described by Daly *et al.* (1), and some of its pharmacological properties have recently been studied by Albuquerque and coworkers (2-5). Although HTX[1] and its analogues dihydroisohistrionicotoxin (Fig. 1) and perhydroisohistrionicotoxin are potent neuromuscular blockers, little is known about their mechanism of action, at least in the case of the electroplax.

Studies on the rat diaphragm showed a reversible blockade of contractions, miniature end plate potentials, and depolarization by acetylcholine (2, 6). Paralysis of neuromuscular transmission, however, did not occur by a curare-like antagonism of acetylcholine receptors, but was interpreted on the basis of a blockade of the cholinergic ionophore or "ion conductance modulator" (2). Histrionicotoxin and its analogues DHTX and PHTX also prolong the falling phase of the action potential (2) and have minimal effects on its rising phase (5). From these results, Albuquerque *et al.*

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[1] The abbreviations used are: HTX, histrionicotoxin; DHTX, dihydroisohistrionicotoxin; PHTX, perhydroisohistrionicotoxin.

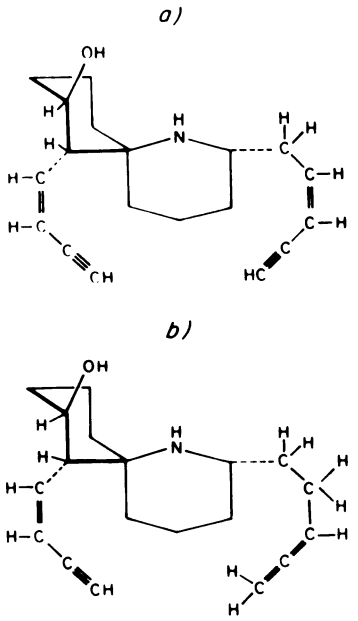

FIG. 1. *Chemical structures of histrionicotoxin (a) and dihydroisohistrionicotoxin (b)*

(2) concluded that HTX affects primarily potassium conductance and, to a lesser extent, sodium conductance.

The effects of HTX have also been evaluated at the frog neuromuscular junction and have been shown to reduce markedly the response to repeated application of acetylcholine, suggesting either accentuation of a desensitization phenomenon or blockade of the ionophore when in the open state (2). Glavinović *et al.* (6) showed that microiontophoretic applications of HTX or procaine to cells of the central nervous system of anesthetized cats blocked unspecifically the excitation of cholinoceptive cortical neurons by acetylcholine or glutamate. The similarity between the effects of HTX and procaine suggested a common mechanism of action. However, studies on the end plate currents in frog sartorius muscles revealed differences between the action of HTX (3) and that of procaine and other local anesthetics (7, 8).

In the present study we describe the effects of HTX and DHTX on the depolarization produced by carbamylcholine on the monocellular electroplax of *Electrophorus electricus* and on the binding of [^{3}H]acetylcholine to membrane fragments rich in acetylcholine receptor protein isolated from electric organs of *Torpedo marmorata*. With the electroplax preparation, the effect of drugs is evaluated on the functional properties of the acetylcholine receptor; binding studies at the membrane level give more direct information about the effect of drugs on the receptor site. A satisfactory monocellular electroplax preparation has not yet been developed for *Torpedo*. This tissue, however, has a high density of innervation and an exceptionally high content of cholinergic receptor; it was therefore used as a source of material in the present biochemical studies.

MATERIALS AND METHODS

Isolation of HTX and DHTX. One thousand twenty frogs were collected near Espriella, a village 35 km southeast of Tumaco, Department of Narino, Colombia. They were killed in the filed with enflurane, and the skins were immediately cut into small pieces and placed in methanol in plastic bottles, in which they were transported to Montreal within 1 week. The histrionicotoxins were extracted by the procedure described in Daly *et al.* (1). The following yields were obtained: histrinoicotoxin, 75 mg; dihydroisohistrionicotoxin, 110 mg; minor alkaloids, 60 mg. Thin-layer chromatography on silica gel GF, using methanol–chloroform–aqueous ammonia (100:10:1), revealed a single spot for both HTX and DHTX, with R_f values of 0.82 and 0.85, respectively. Ultraviolet spectra showed maxima of 224 nm (HTX) and 226 nm (DHTX). The samples were slightly colored in the visible region.

Several tests were performed on the rat phrenic nerve diaphragm preparation to ascertain the potency of our toxins (6). HTX and DHTX in concentrations of 10–30 μM at 22° blocked contractions and miniature end plate potentials within 30–40 min after application. A slow recovery was seen after washing for 1.5–2 hr. Conduction in frog sciatic nerves was blocked only little after 3 hr of exposure to a concentration of 500 μM. Further details of these experiments are given in ref. 6 and are largely in agreement with those of Albuquerque *et al.* (2, 3).

Electrophysiological experiments on Electrophorus electroplax. The monocellu-

lar electroplax isolated from the Sachs organ of large eels, *E. electricus*, was mounted as described (9). The membrane potential was monitored with intracellular glass microelectrodes, with carbamylcholine and HTX or DHTX applied in the solution bathing the innervated face. HTX and DHTX were dissolved in 95% ethanol at concentrations of 0.1–10 mM and were later diluted in *Electrophorus* Ringer's solution prior to bath application. The composition of *Electrophorus* Ringer's solution is 160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3 mM NaH_2PO_4, and 1.2 mM Na_2HPO_4.

Preparation of receptor-rich membrane fragments from T. marmorata. The membrane fragments were prepared from electric organs of live *T. marmorata* as described by Cohen *et al.* (10), with the following modifications. After homogenization and low-speed centrifugation, 25 ml of the supernatant were layered on 6 ml of 1.2 M sucrose–0.02% NaN_3 and centrifuged in an SW 27 rotor for 90 min at 24,000 rpm. The loose pellet was resuspended in a small volume of the above sucrose solution. The concentrations of [^{3}H]α-toxin and acetylcholinesterase catalytic sites were 600 ± 400 and 12 ± 5 nmoles/g of membrane protein, respectively.

Assays. Proteins were determined by the method of Lowry *et al.* (11), using bovine serum albumin as the standard. Acetylcholinesterase activity was measured by the method of Ellman *et al.* (12), using acetylthiocholine as substrate. The concentration of toxin binding sites was estimated by titrating a specific concentration of [^{3}H]α-toxin of *Naja nigricollis* (13) with increasing amounts of membrane suspension.

Binding of [^{3}H]acetylcholine to membrane fragments of T. marmorata. The binding at equilibrium of [^{3}H]acetylcholine was determined by a centrifugation assay described by Weber and Changeux (13). The membrane suspension was first incubated for 30 min with 0.1 mM Tetram, a potent acetylcholinesterase inhibitor (14). The membrane suspension was then diluted in Torpedo physiological solution before the addition of ligand. The composition of *Torpedo* physiological solution is 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0.

When the effect of HTX or DHTX was studied, the diluted membrane suspension was incubated for 30 min with the desired concentration of the toxin before addition of [^{3}H]acetylcholine. Binding studies were done at 20°. The radioactivities of the media before and after centrifugation at 100,000 × *g* for 90 min were counted on 400-μl samples in 10 ml of Bray's solution in an Intertechnique scintillation counter. The efficiency of counting was 30%.

Chemicals. The purified α-toxin of *N. nigricollis* was a gift of Dr. P. Boquet, and [^{3}H]α-toxin, a gift of Drs. A. Menez, J. L. Morgat, and P. Fromageot. Prilocaine and dimethisoquin were gifts of Laboratoire Roger Bellon, Neuilly, France. [*N-methyl*-^{3}H]Acetylcholine chloride (120 Ci/mole) was obtained from the Radiochemical Centre.

RESULTS

Effects of HTX and DHTX on response of Electrophorus electroplax to carbamylcholine. Since the samples of HTX or DHTX in *Electrophorus* Ringer's solution also contained small quantities of ethanol (maximum volume, 5 μl/5 ml of Ringer's solution), controls were done to test the effects of ethanol on the depolarization by carbamylcholine. Exposure of the innervated face of the electroplax to minute quantities of ethanol had little if any effect on the membrane potential or on the response to carbamylcholine (Fig. 2A). In the presence of 1 μM HTX, however, the maximum response was reduced by more than half (Fig. 2B). Then, if the cell was rinsed with *Electrophorus* Ringer's solution, the membrane potential slowly recovered in 15 min to within 10–20% of normal (60–80 mV). Bath application of 1–10 μM HTX or DHTX to the innervated face did not itself produce any depolarization or hyperpolarization.

We next examined the time course of inhibition by DHTX. Maximum blockade by 1 μM DHTX in the presence of 15 μM carbamylcholine occurred in 15–25 min. Figure 3 shows that recovery of the carbamylcholine response followed approxi-

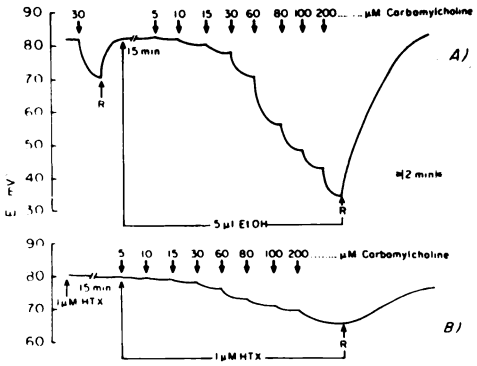

FIG. 2. *Effect of histrionicotoxin on response of Electrophorus electroplax to carbamylcholine*

A. Control experiment. After the first response to 30 μM carbamylcholine, the innervated face of the cell was incubated for 15 min with 5 μl of absolute ethanol, and this concentration was present in all subsequent solutions. R, *Electrophorus* Ringer's solution (see MATERIALS AND METHODS for composition).

B. Blockade by 1 μM HTX of the response to carbamycholine. The cell was first incubated for 15 min with 1 μM HTX; then carbamylcholine was added in the presence of 1 μM HTX. Temperature, 22°. *E* is the membrane potential.

mately the same time course (15–25 min), although the amplitude of the response never returned to its initial value. A similar time course was observed for the blocking action of HTX. All studies dealing with the effects of HTX or DHTX on the monocellular electroplax were therefore done with preliminary incubation times of 15–25 min.

Figures 4 and 5 show that HTX and DHTX (1 μM) decreased the response to carbamylcholine in a noncompetitive manner. HTX and DHTX (1 μM each) reduced the maximum response to carbamylcholine (100 μM) by 70% and 50%, respectively. The Hill coefficient n_H was reduced from 1.9 to 1.4 and from 1.8 to 1.3 by 1 μM HTX and DHTX, respectively.

The effect of increasing concentrations of DHTX on the response of the electroplax to carbamylcholine (15 μM) is shown in Fig. 6: 0.8 μM DHTX caused 50% blockade. At 20 μM DHTX, the response to 15 μM carbamylcholine was completely abolished and only partially (30%) recovered after 1–2 hr of repeated washing with *Electrophorus* Ringer's solution.

Effects of HTX and DHTX on binding of [^{3}H]acetylcholine to receptor-rich membrane fragments from T. marmorata. Binding of [^{3}H]acetylcholine to purified membrane fragments from *Torpedo* was measured at 20° in *Torpedo* physiological solution in the presence of 0.1 mM Tetram, which at this concentration does not interfere with [^{3}H]acetylcholine binding (13, 15). Figure 7 shows the binding curve of [^{3}H]acetylcholine in the presence and ab-

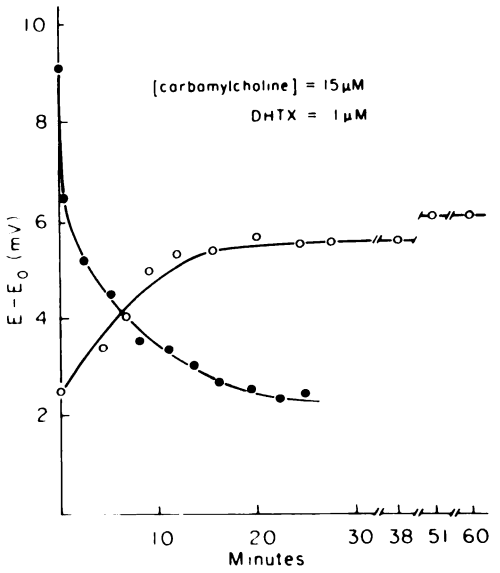

FIG. 3. *Time course of inhibition by DHTX*

DHTX (1 μM, dissolved in *Electrophorus* Ringer's solution) was injected into the bath (facing the innervated face of the cell) simultaneously with 15 μM carbamylcholine. One minute later the cell was washed with 1 μM DHTX (5 ml) until the membrane potential returned to a steady level. A second dose of carbamylcholine (15 μM) was then injected in the presence of 1 μM DHTX. One minute later the cell was washed with 1 μM DHTX. This procedure was repreated until the size of the response to carbamylcholine did not change. The cell was then washed with *Electrophorous* Ringer's solution at the times indicated (○), and the recovery of the carbamylcholine response was measured. The electroplax exposed to the same treatment schedule, but without DHTX, was capable of producing the same depolarization response to carbamylcholine after 2 hr as at the beginning of the experiment. ●, curve for inhibition of the carbamylcholine response in the presence of DHTX; ○, recovery of the carbamylcholine response without DHTX in the bath. Temperature, 22°. E_0 is the membrane potential with no drug present; E is the potential in the presence of carbamylcholine.

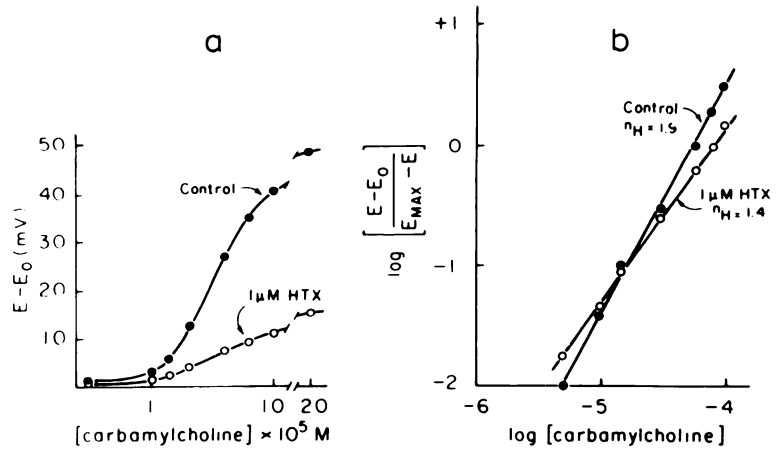

FIG. 4. *Pharmacological action of HTX*

a. Effect of HTX (1 μM) on the response of *Electrophorus* isolated electroplax to increased concentrations of carbamylcholine. The ordinate shows steady-state membrane depolarization recorded in the presence of a given concentration of carbamylcholine; E_0, resting potential (−80 mV). The bath solution was *Electrophorus* Ringer's solution (160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1.5 mM sodium phosphate, pH 7). E_0 is the membrane potential with no drug present; E is the potential in the presence of the depolarizing drug; E_{MAX} is the potential in the presence of a large excess of the drug. Temperature, 22°.

b. Hill plot of the same data.

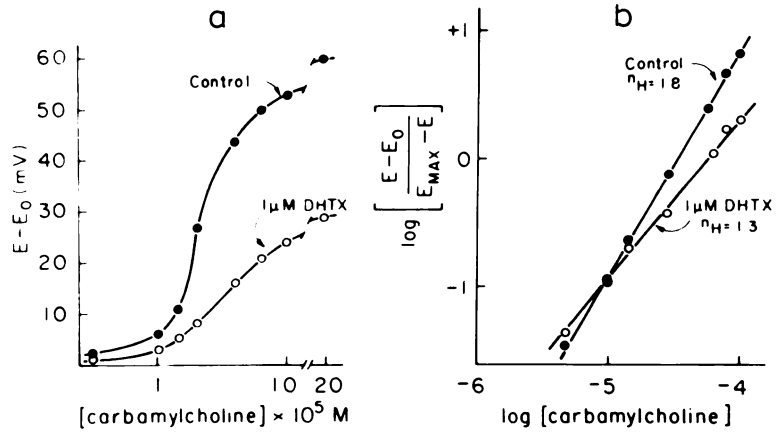

FIG. 5. *Pharmacological action of DHTX*

a. Effect of DHTX (1 μM) on the response of *Electrophorus* isolated electroplax to increased concentrations of carbamylcholine. Details are given in Fig. 4; E_0 was −75 mV. Temperature, 22%.

b. Hill plot of the same data.

sence of 8 μM DHTX to membrane fragments containing 1200 nmoles of [^{3}H]α-toxin binding sites per gram of membrane protein. The binding curve of [^{3}H]acetylcholine was sigmoidal, whereas that in the presence of DHTX appeared hyperbolic. A Hill plot of the same data (Fig. 7) shows that the Hill coefficients n_H in the presence and absence of 8 μM DHTX are 1.05 and 1.40, respectively. Moreover, DHTX increased the apparent affinity of [^{3}H]acetylcholine for the acetylcholine receptor site. The dissociation constants K_D for [^{3}H]acetylcholine, calculated from double-reciprocal plots, were 8.0 and 16 nM in the presence and absence of DHTX. The assumption that [^{3}H]acetylcholine binds to the acetylcholine receptor site was confirmed, since α-toxin (1.0 μM) from *N. nigricollis* completely (100%) inhibited the

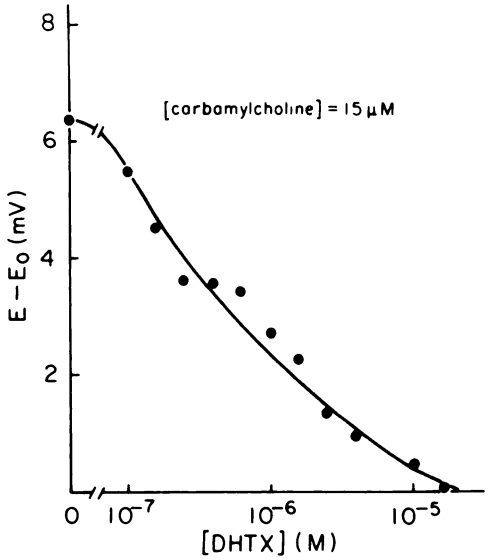

FIG. 6. *Effect of increasing DHTX concentration on steady-state membrane depolarization caused by fixed concentrations of carbamylcholine*

The innervated face of the electroplax was exposed to a fixed concentration of DHTX for 15 min, and the depolarization produced by 15 μM carbamylcholine (in the presence of the same concentration of DHTX) was measured. After a steady-state depolarization was reached, the bath was rinsed with eel Ringer's solution until steady membrane potential was attained. The above procedure was repeated, using increasing concentration of toxin. Without DHTX, the electroplax exposed to the same treatment was capable of producing the same depolarization in response to carbamylcholine.

binding of [^{3}H]acetylcholine (39 nM) (Fig. 7).

We observed that the binding curve of [^{3}H]acetylcholine and the effects of HTX and DHTX on this curve were variable in different membrane preparations. In some instances the binding curve was sigmoidal whereas in others it was not. This phenomenon has been described previously (13) and will not be discussed here in detail. This variability is summarized in Table 1, using three different membrane preparations. We also include in this table the concentrations ($F_{\frac{1}{2}}$) of free [^{3}H]acetylcholine giving half-saturation of receptor sites with and without HTX and DHTX (see ref. 13).

In preparation A, which had the highest specific activity of [^{3}H]α-toxin binding sites (1200 nmoles/g), the concentration of [^{3}H]acetylcholine required for half-saturation ($F_{\frac{1}{2}}$) was 16.0 and 6.8 in the absence and presence of 8 μM HTX, respectively; the Hill coefficient n_H in the same preparation changed from 1.40 to 1.07. Using DHTX, $F_{\frac{1}{2}}$ changed from 16.0 to 12.4 and 8.0, and n_H, from 1.40 to 1.20 and 1.05, when the membranes were incubated in the absence and presence of 2 and 8 μM DHTX, respectively.

In the preparation exhibiting the lowest specific activity of [^{3}H]α-toxin binding sites (450 nmoles/g), the presence of 8 μM DHTX resulted in changes in $F_{\frac{1}{2}}$ and n_H from 21.3 to 16.4 and from 1.10 to 1.08, respectively.

Figure 8 shows that HTX and DHTX increase the binding of [^{3}H]acetylcholine to about the same extent in the range of concentrations in which they block the response of *Electrophorus* electroplax to carbamylcholine. At higher concentrations (50–1000 times their apparent dissociation constants *in vivo*) a decrease in [^{3}H]acetylcholine binding takes place (see Figs. 8 and 11).

Prilocaine and dimethisoquin, two local anesthetics, also increased the binding of [^{3}H]acetylcholine to membrane fragments. Maximum potentiation occurred at concentrations of 1.0 and 0.01 mM prilocaine and dimethisoquin, respectively. These experiments confirm those of Cohen *et al.* (15) and are shown here in Figs. 9 and 10. These two local anesthetics also blocked the half-maximal response to carbamylcholine on *Electrophorus* electroplax at these concentrations.

DHTX was then added in the presence of 1 mM prilocaine or 0.01 mM dimethisoquin, local anesthetic concentrations which give maximal potentiations of [^{3}H]acetylcholine binding. Figures 9 and 10 show that no further increase of [^{3}H]acetylcholine binding took place. On the other hand, a decrease was found in the same range of DHTX concentration (above 10 μM) as in the absence of local anesthetics.

DISCUSSION

Histrionicotoxin (0.8 μM) and dihydroisohistrionicotoxin (1.0 μM) inhibit noncompetitively the response of *Electrophorus* elec-

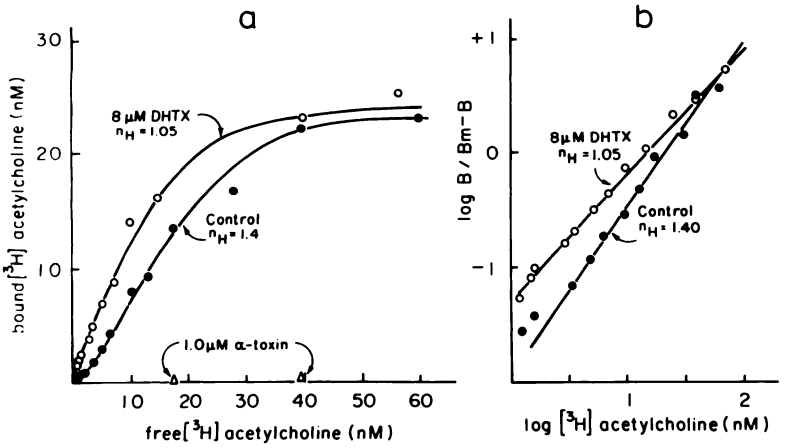

FIG. 7. *Binding of [³H]acetylcholine to membrane fragments: effect of DHTX*

a. ●——●, *Torpedo* membrane fragments (10 μM *Naja* α-toxin binding sites, 5 g of protein per liter) were diluted 500-fold in *Torpedo* physiological solution plus 0.1 mM Tetram. ○——○, dilution in the same medium plus 1 μM DHTX; a double-reciprocal plot of the data gave a value of 30 nM for the total number of [³H]acetycholine binding sites. The concentration of [³H]α-toxin binding sites was 20 nM. *B* is the concentration of bound [³H]acetylcholine, and *Bm*, the total concentration of its binding sites determined by extrapolation of a double-reciprocal plot.

b. Hill plot of the data shown in Fig. 7a.

troplax to carbamylcholine. At similar concentrations these toxins do not inhibit the binding of [³H]acetylcholine to *Torpedo* membrane fragments, but rather augment its affinity for the receptor site. At much higher concentrations HTX and DHTX partially inhibit [³H]acetylcholine binding to the membrane fragments. A comparison of the effect of DHTX on the response to carbamylcholine of the elctroplax preparation and on [³H]acetylcholine binding to the membrane fragments is shown in Fig. 11. DHTX (0.8 μM) reduced the response to carbamylcholine by 50%, the half-maximal potentiation of [³H]acetylcholine binding occurring at 2 μM DHTX. Although those two phenomena (i.e., inhibition of the response to carbamylcholine and potentiation of [³H]acetylcholine binding) were studied using electric tissue from different sources (*E. electricus* and *T. marmorata*), the effects produced by these alkaloid toxins occurred at similar although not identical concentrations. This small discrepancy may be due to the different tissues used.

The marked resemblance between the effects of HTX and DHTX and those found by Cohen *et al.* (15) for prilocaine and dimethisoquin seems significant. Both groups of compounds noncompetitively block the response to carbamylcholine, and both augment [³H]acetylcholine binding to membrane fragments rich in receptors. The concentration required to pro-

TABLE 1

Effect of HTX and DHTX on binding of [³H]acetylcholine to three different membrane preparations

$F_{1/2}$ is the concentration of acetylcholine at half-saturation, and n_H is the Hill coefficient.

Preparation	[³H]α-Toxin binding sites	No toxin		Histrionicotoxin				Dihydroisohistrionicotoxin			
				2 μM		8 μM		2 μM		8 μM	
		$F_{1/2}$	n_H	$F_{1/2}$	n_H	$F_{1/2}$	n_H	$F_{1/2}$	n_H	$F_{1/2}$	n_H
	nmoles/g	*nM*		*nM*		*nM*		*nM*		*nM*	
A	1200	16.0	1.40	11.8	1.17	6.8	1.07	12.4	1.20	8.0	1.05
B	800	18.1	1.25	16.4	1.05	8.4	1.00				
C	450	21.3	1.10					19.0	1.25	16.4	1.08

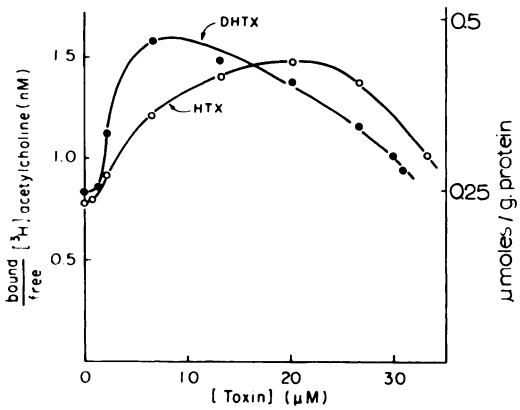

FIG. 8. *Effect of concentration of HTX or DHTX on binding of [³H]acetylcholine to receptor-rich membrane fragments*

A membrane suspension (3 μM *Naja* α-toxin binding sites; 6 g of protein per liter was diluted 155-fold in *Torpedo* physiological solution plus 0.1 mM Tetram and the indicated concentrations of HTX or DHTX. Binding was measured in the presence of 20 nM total [³H]acetylcholine. In the absence of HTX or DHTX the concentration of bound [³H]acetylcholine was 12 nM. The concentration of [³H]α-toxin binding sites was 20 nM.

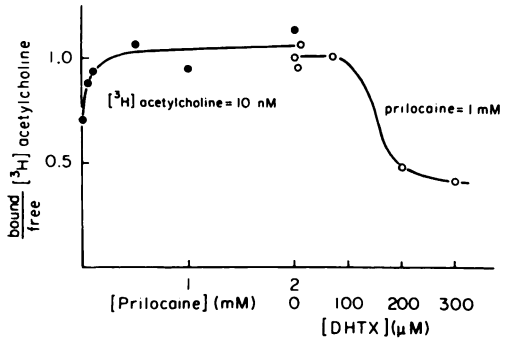

FIG. 9. *Effect of DHTX concentration on potentiation of [³H]acetylcholine binding to membrane fragments from Torpedo by prilocaine*

Left: A membrane suspension (4.5 μM *Naja* α-toxin binding sites, 4.7 g of protein per liter) was diluted 225-fold in *Torpedo* physiological solution plus 0.1 mM Tetram and the indicated concentrations of prilocaine. Binding was measured in the presence of 10 nM total [³H]acetylcholine. In the absence of prilocaine the concentration of bound [³H]acetylcholine was 8.2 nM. The concentration of [³H]α-toxin binding sites was 20 nM.

Right: The above membrane suspension contained 10 nM [³H]acetylcholine and 1 mM prilocaine, and the concentration of DHTX was varied as indicated.

duce 50% inhibition of the carbamylcholine response or an augmentation of [³H]acetylcholine binding are approximately 1000, 10, and 2 μM for prilocaine, dimethisoquin, and HTX or DHTX, respectively. We did not detect any significant differences in potency between HTX and DHTX on the electroplax and membrane fragments.

Although precise information on the action on electroplax is lacking, in muscle, procaine reduces peak sodium and potassium end plate currents (7, 8, 16); the action of HTX on muscle appears very similar (2, 3). The similarity between the effects of HTX, prilocaine, and dimethisoquin, as well as procaine (6), may indicate a common mode of action.

Albuquerque and coworkers (2–5) have suggested that HTX block specifically the ion conductance modulator, i.e., the acetylcholine ionophore. The action of HTX is not specific to the cholinergic system, however, since it also inhibits glutamate-evoked spikes in the cat central nervous system (6).

In electroplax tissue HTX may bind to the acetylcholine receptor protein or to the phospholipids surrounding the receptor in the membrane structure and thus alter the affinity of acetylcholine to the receptor site.

The conversion of the binding curve of

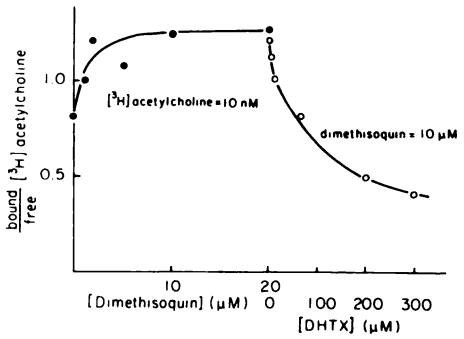

FIG. 10. *Effect of DHTX concentration on potentiation of [³H]acetylcholine binding to membrane fragments from Torpedo by dimethisoquin*

Left: Conditions were the same as in Fig. 9, except that dimethisoquin was used instead of prilocaine.

Right: The concentrations of dimethisoquin and [³H]acetylcholine were 10 μM and 10 nM, respectively. The concentration of DHTX was varied as indicated.

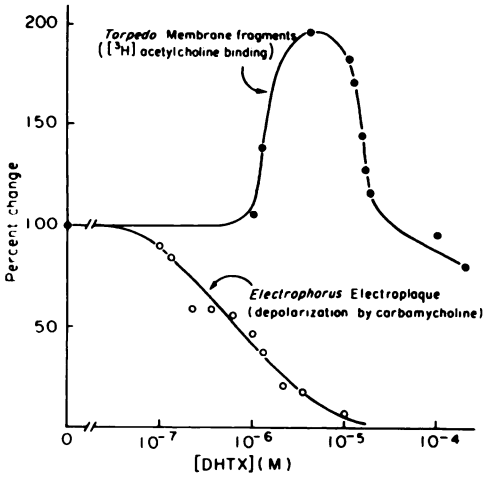

FIG. 11. *Comparison of effect of DHTX concentrations on carbamylcholine response of electroplax and on binding of [³H]acetylcholine to Torpedo membrane fragments*

Data in the lower graph were taken from Fig. 6, and in the upper graph, from Fig. 8, and are plotted as percentage change. Temperature, 22°.

acetylcholine from an S-shape (with a Hill coefficient of 1.4) to a hyperbolic one (with a Hill coefficient of 1.0) in the presence of HTX or DHTX is reminiscent of a characteristic property of regulatory enzymes (17). Thus one might conjecture that HTX and certain local anesthetics act as allosteric ligands of the cholinergic receptor protein (13).

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