Acetylcholine Receptor and Ion Conductance Modulator Sites at the Murine Neuromuscular Junction: Evidence from Specific Toxin Reactions

(binding sites/perhydrohistrionicotoxin/a-bungarotoxin/d-tubocurarine/sodium-potassium conductance/muscle action potential/endplate potential)

EDISON X. ALBUQUERQUE*, ERIC A. BARNARD†, TIEH H. CHIU†, ANTONIO J. LAPA*, J. OLIVER DOLLY†, STEN-ERIK JANSSON*, JOHN DALY‡, AND BERNHARD WITKOP‡

Departments of Pharmacology and Biochemistry, State University of New York at Buffalo, Buffalo, N.Y. 14214; Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT The perhydro derivative of histrionicotoxin reversibly blocks the excitatory ionic transduction system in the synaptically and sarcolemmal membranes of mammalian skeletal muscle cells. The efficacy of perhydrohistrionicotoxin as an antagonist at the postsynaptic membrane is increased by the transient presence of acetylcholine in the endplate of innervated muscles and at extrajunctional receptors in denervated muscles. α-Bungarotoxin and [3H]monoacetyl-o-bungarotoxin block the endplate acetylcholine receptors, each binding to the same extent. The effect of bungarotoxin is partially reversible. These electrophysiological results, together with the effects of perhydrohistrionicotoxin and/or d-tubocurarine on the binding of [3H]monoacetyl-o-bungarotoxin at endplates of murine diaphragm muscle and on the bungarotoxin-elicited irreversible blockade of neuromuscular transmission, suggest that at least two types of sites participate in the synaptic excitation by acetylcholine. One site, competitively blocked by bungarotoxin and by curare, is presumably the acetylcholine receptor. Binding of bungarotoxin at this site is responsible for an irreversible blockade of neuromuscular transmission. The second site, competitively blocked by bungarotoxin and perhydrohistrionicotoxin, is proposed to be part of the cholinergic ion conductance modulator. Binding of bungarotoxin to this site does not result in an irreversible blockade.

The excitatory action of acetylcholine at postsynaptic membranes clearly involves at least two stages: first, the combination of the transmitter with a receptor site, and second, the induced increase in Na+ and K+ conductances that cause the transient depolarization of the membrane. Little is known about the molecular basis for the coupling between these two steps. Hypotheses (1-6) include (a) topographically separate ACh receptors and ion conductance modulators§ (ICM); (b) a cooperative complex consisting of receptor and ICM subunits; and (c) one macromolecule, serving as both receptor and ICM. α-Bungarotoxin (BuTX) has been proposed as a specific reagent for the acetylcholine receptor site in vertebrate skeletal muscle synapse (7-10). No such specific probe for the postsynaptic ICM has been described. The results reported herein suggest that the perhydroderivative (H₂HTX) of histrionicotoxin, itself an active principle, isolated from the Colombian arrow-poison frog Dendrobates histrionicus (11), is a specific probe for the ICM.

MATERIALS AND METHODS

Male albino mice, RR strain, weighing 19-21 g, were used. When toxicity of BuTX was measured by injection into the caudal vein, replicates of survival times agreed within 5%. Crude Bungarus multicinctus venom was from the Miami Serpentarium. d-Tubocurarine chloride pentahydrate was from Sigma. Radioactivity was measured in a dioxane-based fluid (12) or a toluen medium containing 7 g/liter of 2,5-diphenylloxazole and 0.6 g/liter of 1,4-bis-2-4-methyl-5-phenylloxazolylbenzene for aqueous and Soluene (Packard Instrument Co.) samples, respectively. Pure BuTX was prepared as described (9), except that further chromatography on carboxymethyl cellulose was performed in order to obtain a homogeneous protein. After [3H]acetylation (9), a major monoacetylated species ([3H]BuTX) was isolated by repeated chromatography. The isolation and characterization of this product will be the subject of a future report. Concentrations of BuTX and [3H]BuTX were determined with pure BuTX as a standard by A280nm or by the Lowry et al. method (13). Histionicotoxin (11) was reduced in tetrahydrofuran with hydrogen gas and a palladium on charcoal catalyst to the dodecahydro derivative, H₁₂-HTX (Fig. 1), homogeneous on thin-layer chromatography and pure by mass spectral analy-

Abbreviations: BuTX, a-bungarotoxin; [3H]BuTX, [3H]monoacetyl-a-bungarotoxin; H₁₂-HTX, perhydrohistrionicotoxin; ICM, ion conductance modulator.

§ The term, ion conductance modulator, is proposed as a general descriptive designation for the macromolecular entities that are involved in regulation of ion conductance in membranes. Specific examples would include the cholinergic ICM and the action potential ICM. An ICM could regulate conductance of a particular ion or could have a broader specificity. We prefer ICM to the term "ionophore," which is commonly used to mean an ion-transporting molecule; an ICM may or may not comprise such an ion-transporting molecule.

Fig. 1. Perhydrohistrionicotoxin (H₁₂-HTX).
sis, with a molecular ion at m/e 295. Reduction of histrionicotoxin with tritium gas gave $^3$H-labeled H$_{12}$-HTX. Histrionicotoxin has pharmacological activities similar to those of H$_{12}$-HTX (unpublished results).

**Electrophysiological Recordings.** The techniques for preparation of nerve diaphragms and denervated soleus muscles from mice, and for recording of endplate potentials, action potentials, delayed rectification, and microiontophoresis of acetylcholine were essentially as described (14-18). Experiments were done at 30°.

**Binding of $^3$H]BuTX to Diaphragm Endplates.** The diaphragm, removed with ribs attached and washed, was shaken continuously at 27° for 1 hr with $^3$H]BuTX (1 $\mu$g/ml). The muscle was then briefly washed, shaken for 10 min with unlabeled BuTX (20 $\mu$g/ml), and finally washed 10 times (5 min for each wash). All washings and incubations were in Krebs-Ringer solution. The labeled muscles were dissected into endplate and nonendplate regions of equal weight. These samples (2 diaphragms, where the uptake was low) were dissolved in Soluene at 60° and counted. The mean binding for the endplate was calculated by subtraction of the nonendplate component of the labeling, assumed to be the same in both endplate-free and endplate-containing halves and averaging 15% of total binding for the endplate-containing half. When in a few tests the time for the final 10 washings was extended to 14 hr, the results were unchanged. After in vitro labeling, the same procedure was followed, except for omission of the incubation with $^3$H]BuTX.

**RESULTS**

**Comparison of $^3$H]BuTX with Native BuTX.** The reactivity and specificity of the $^3$H]monoacetyl derivative, $^3$H]BuTX, at the receptor was identical to that of BuTX by several criteria: (i) the mean survival time of mice injected at 1.0 $\mu$g/g was the same, 4 min, 10 sec; (ii) the two compounds at 10 $\mu$g/ml had identical blocking potency in the murine phrenic-diaphragm preparation, $t_{1/2} = 139$ sec; and (iii) the binding of the two was identical at the endplate, as shown by isotope dilution experiments; i.e., after intravenous injection into mice of 1.0 $\mu$g of total toxin per g, the net binding of $^3$H]BuTX at the diaphragm endplates was reduced to 45% if the $^3$H]BuTX had been diluted to half the specific activity with unlabeled BuTX. Similar results were obtained in vitro.

**Effect of H$_{12}$-HTX on Diaphragm Muscle.** When a diaphragm was bathed in Krebs-Ringer solution containing H$_{12}$-HTX (20 $\mu$g/ml), there was a slight potentiation of the indirectly- and directly-elicited twitch, and a subsequent blockade of the indirectly-elicited twitch (Fig. 2). Exposure of the muscle to H$_{12}$-HTX (20 $\mu$g/ml) resulted in a 2-4 mV hyperpolarization of the muscle fiber in 3-5 min, while only a 10-20% increase was observed in the input resistance and membrane resistance of a unit area. When the directly- or indirectly-elicited action potentials (0.1/sec) were recorded at intervals of 1 min in the presence of 10 $\mu$g/ml of H$_{12}$-HTX, the amplitude of the action potential was decreased somewhat after 10 min. Simultaneously, the rising phase of the spike was slightly increased and the falling phase greatly prolonged. The onset of the action potential was highly dependent upon the frequency of nerve stimulation, so that when repetitive stimulation was given at a rate of 1 pulse per sec, the same effects now occurred within 2 min. When the toxin was applied at a low concentration of 5 $\mu$g/ml, the falling phase of the action potential, measured 10 min after the application of the toxin, was greatly prolonged, while the rising phase was much less affected (Fig. 3). These results indicate that H$_{12}$-HTX affects primarily potassium conductance and, to a less extent, sodium conductance (Lapa, Albuquerque, Daly and Witkop, unpublished work). Delayed rectification has been shown

**Fig. 3.** Ten superimposed records of the action potential (upper trace) and its first derivative (dV/dt; lower trace) obtained from single fibers of glycerol-treated diaphragm muscles, before and during treatment with H$_{12}$-HTX (5 $\mu$g/ml) for 10 min. The toxin-induced prolongation of the falling phase occurs with little change in the rising phase of the action potential. The resting membrane potentials of these cells were -70 and -75 mV, respectively. The horizontal line is the zero potential.

**Fig. 4.** Electrotonic potentials produced by anodal hyperpolarization and cathodal depolarization in diaphragm muscle fibers. In the control condition, the preparation was first treated with glycerol (16), and subsequently with 1 $\mu$g/ml of tetrodotoxin to block sodium conductance. The preparation was then treated with tetrodotoxin and H$_{12}$-HTX (20 $\mu$g/ml). Within a few minutes, complete blockade of the delayed rectification was observed.
Fig. 5. Voltage–current relations of the diaphragm muscle. The delayed rectification remains unaltered after treatment with 1 μg/ml of tetrodotoxin (O—O) for 15 min, and is blocked when the preparation is treated with 1 μg/ml of tetrodotoxin plus 20 μg/ml of H₁₂-HTX (△...△).

(17–19) to be an indicator of the increase in potassium conductance that occurs, with a predictable delay, upon depolarization of the electrogenic membrane. Delayed rectification was completely blocked within a few minutes when H₁₂-HTX was applied at 20 μg/ml (Figs. 4 and 5). All the effects of this toxin were reversible within 2–4 hr upon washing.

Fig. 6. Response of 10-days chronically denervated soleus muscle to acetylcholine applied microiontophoretically to the extra-junctional membrane of surface fibers. Zero time refers to control potentials obtained before exposure of the preparation to either H₁₂-HTX (5 μg/ml) or d-tubocurarine (d-TC, 2.5 μg/ml). The upper trace is membrane response to microiontophoretically applied acetylcholine, and the lower trace is the current applied through the acetylcholine pipette.

Table 1. Effect of d-tubocurarine (d-TC) and H₁₂-HTX on the binding of [³H]BuTX at diaphragm endplates

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Conditions</th>
<th>Binding (% of control)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>In vitro, [³H]BuTX (1.0) 30 min</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>90 min</td>
<td>100†</td>
</tr>
<tr>
<td>2</td>
<td>(a) BuTX + d-TC (100) → wash → [³H]BuTX</td>
<td>62 (70)*</td>
</tr>
<tr>
<td></td>
<td>(b) BuTX + d-TC (10) → wash → [³H]BuTX</td>
<td>34 (50)*</td>
</tr>
<tr>
<td>3</td>
<td>d-TC (100) → wash → [³H]BuTX</td>
<td>93</td>
</tr>
<tr>
<td>4</td>
<td>H₁₂-HTX (15) → wash → [³H]BuTX</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>BuTX + H₁₂-HTX (15) → wash → [³H]BuTX</td>
<td>9 (36)*</td>
</tr>
<tr>
<td>6</td>
<td>(a) d-TC (10) + H₁₂-HTX (30) + [³H]BuTX</td>
<td>23 (1)*</td>
</tr>
<tr>
<td></td>
<td>(b) d-TC (10) + H₁₂-HTX (40) + [³H]BuTX</td>
<td>9 (0)*</td>
</tr>
</tbody>
</table>

All treatments with [³H]BuTX or BuTX were in vitro at 1.0 μg/ml and 27° for 60 min except in Exp. 1. The terms in parentheses after drugs are the concentrations in μg/ml. Binding is expressed in every case as a percentage of that in a parallel control diaphragm treated with 1 μg/ml of [³H]BuTX for 60 min, and processed identically; the standard deviation of the mean for endplate binding in control diaphragms was 3.2% (n = 4). In the protection experiments, the diaphragms were treated with d-tubocurarine or H₁₂-HTX both for 30 min before incubation with BuTX (Exp. 2 and 5), or for 60 min before incubation with [³H]BuTX (Exp. 6). Incubation with d-tubocurarine and H₁₂-HTX in Exp. 3 and 4 was for 30 min. The wash period before labeling with [³H]BuTX was 3 hr, with 15 min per washing.

*Values in parentheses are the percentage predicted, assuming additivity in the effect of each agent when used alone, based upon the data of Fig. 7.
†This is 91% of the saturation value for the [³H]BuTX binding at these endplates, as measured after in vivo injection of different doses of [³H]BuTX (21).

Dependence of the Action of H₁₂-HTX upon the Presence of Acetylcholine. Exposure to 5 μg/ml of H₁₂-HTX did not block the transient depolarization induced by microiontophoretically applied acetylcholine at the extra-junctional region of the denervated soleus muscle during 30 min. However, when acetylcholine was applied microiontophoretically (pulse duration, 1–10 msec) at a rate of 1/sec, the transient potential rapidly decreased in amplitude, and, after the fifth stimulus, complete blockade was observed (Fig. 6). Upon interruption of acetylcholine application for about 30 sec, the initial sensitivity returned; reapplication of acetylcholine at the same frequency then produced a similar set of responses. As the time of incubation with the toxin progressed further, only the first potential occurred; the subsequent potentials were completely absent. No such effects were observed with d-tubocurarine (Fig. 6). The sensitivity of the extra-junctional membrane to acetylcholine in the presence of H₁₂-HTX decreased more rapidly when the duration of the pulse was increased. Under these conditions, the first potential was longer in duration. These results indicate that the presence of acetylcholine markedly potentiates the effect of H₁₂-HTX. However, this toxin, at concentrations of 40 μg/ml, completely blocked in 30 min both the extra-junctional sensitivity to acetylcholine of the chronically denervated soleus muscles.
end and the endplate potential of normal soleus muscle. In concentrations of 5-20 μg/ml, H12-HTX blocked the spontaneous transmitter release in the diaphragm (control miniature endplate potentials: 5 sec⁻¹) and decreased the amplitude and half-decay time of the endplate current (Kuba, Albuquerque, Daly and Witkop, unpublished observations). H12-HTX had similar effects on the endplate potential to those observed on the acetylcholine sensitivity of the denervated muscle; i.e., repetitive stimulation resulted in blockade of the endplate potential. Intervals of 10-20 sec between trains of impulses allowed the reappearance of the endplate potential, with subsequent blockade occurring by the end of the 20th pulse of the train. No such effect was observed when d-tubocurarine was used at a concentration that caused an initial decrease in amplitude similar to that produced by H12-HTX. All the effects of H12-HTX and d-tubocurarine were reversible when the preparation was washed for 2-4 hr. Similar results were obtained with histrionicotoxin (Albuquerque, Lapa, Daly and Witkop, unpublished work).

Effect of d-Tubocurarine and H12-HTX on Binding of [3H]BuTX. The sites of binding of H12-HTX and curare at the neuromuscular junctions were investigated through their inhibitory effects upon binding of [3H]BuTX at the diaphragm endplates. In vitro, binding of [3H]BuTX reached a near-saturation value at the endplates after 60 min of reaction at 27°C (Exp. 1, Table 1). When d-tubocurarine was present before and during the reaction, binding of [3H]BuTX was markedly decreased (Fig. 7). The form of the concentration dependence curve suggests the presence of at least two classes of binding sites. Binding of [3H]BuTX was not decreased in endplates that had been exposed to d-tubocurarine and then washed (Exp. 3, Table 1). When endplates were first incubated with d-tubocurarine and unlabeled BuTX, followed by washing and incubation with [3H]BuTX, the extent of binding of the radioactive compound (Exp. 2, Table 1) was in good agreement with that predicted from the data of Fig. 7. When H12-HTX was present before and during the labeling reaction, binding of [3H]BuTX was decreased and the curve for concentration dependence appeared biphasic (Fig. 7). Binding of [3H]BuTX was not decreased in endplates that had been exposed to H12-HTX and then washed (Exp. 4, Table 1). Labeling with [3H]BuTX after incubation of endplates with H12-HTX and BuTX afforded higher than expected binding of [3H]BuTX (Exp. 5, Table 1). When both curare and H12-HTX were present together, the combined
antagonism of $^{3}H$BuTX binding was less than the sum of their individual effects (Exp. 6, Table 1). The results of electrophysiological measurements suggest that the efficacy of H$_{12}$-HTX as an antagonist of $^{3}H$BuTX binding would be enhanced in the presence of acetylcholine. However, antagonism of BuTX binding by acetylcholine (20) or by a cholinergic agonist would probably render such an experiment inconclusive.

When $^{3}H$-labeled H$_{12}$-HTX was applied to diaphragms, the uptake was high in both the endplate and nonendplate areas, and did not show signs of saturation up to 40 $\mu$g/ml. The same result was found in diaphragms after in vivo injection of H$_{12}$-HTX (up to 10 $\mu$g/g). The $^{3}H$-labeled H$_{12}$-HTX could be slowly washed out of the preparations. H$_{12}$-HTX thus appears to enter the muscle cells rather freely, in addition to binding at specific sites.

Effect of H$_{12}$-HTX and of d-Tubocurarine on BuTX-Elicited Blockade of Endplate Potential. BuTX (5 $\mu$g/ml) completely blocked the endplate potential in diaphragm endplates within 30 min, and the acetylcholine sensitivity of the denervated soleus muscle in 15 min. After washing (2-3 hr), a maximum recovery of 0.5-1.0 mV endplate potential was consistently recorded in most of the surface fibers (Fig. 8). The endplate potential observed after wash-out of BuTX was highly sensitive to curare, i.e., 0.5 $\mu$g/ml of d-tubocurarine blocked this potential in less than 0.4 min. Thus, a small fraction of the endplate receptors appear to bind BuTX reversibly.

Either d-tubocurarine (100 $\mu$g/ml) or H$_{12}$-HTX (40 $\mu$g/ml) completely blocked the endplate potential of the innervated diaphragm (Fig. 8). These effects were reversible within 2-4 hr upon washing. When d-tubocurarine (100 $\mu$g/ml) and BuTX were applied together for 1 hr under conditions where 65% of the BuTX binding sites are protected (Fig. 7), an endplate potential, sufficient in magnitude to initiate an action potential and subsequent muscle twitch, appeared 2-4 hr after washing (Fig. 9). In fact, after incubation with BuTX and d-tubocurarine, the tubocurarine at concentrations (5-10 $\mu$g/ml) that protected only 40-50% of BuTX binding sites, an action potential could still be indirectly elicited 2-4 hr after washing. In experiments performed with BuTX and H$_{12}$-HTX, the latter at a concentration (80 $\mu$g/ml) that protects about 65% of the BuTX binding sites, no recovery of twitch or of an endplate potential of sufficient magnitude to initiate spike activity was observed after washing (Fig. 9); in fact, the preparation behaved as if it had been treated with BuTX alone (Fig. 8). When H$_{12}$-HTX and d-tubocurarine were present together in the BuTX-containing bath, and the preparation was again washed, the full response was recovered (Fig. 9). In a similar experiment in which binding of $^{3}H$-BuTX was measured in the presence of both H$_{12}$-HTX and curare (Exp. 6b, Table 1), nearly complete protection of BuTX binding sites was manifest.

DISCUSSION

The results of the present study of H$_{12}$-HTX, d-tubocurarine, and BuTX may be interpreted as follows: the BuTX binding sites at the endplate of the murine diaphragm (9) are not homogenous, since although separately curare or H$_{12}$-HTX block binding of $^{3}H$BuTX at only a fraction of the total sites, in combination, these compounds completely block binding of $^{3}H$BuTX at the endplate. The ability of curare to antagonize only a part of the BuTX binding has been confirmed by autoradiographic determination directly at the endplates (21). Some weak binding of either curare or H$_{12}$-HTX at the alternate site may occur (Fig. 7). The site that is occupied preferentially by curare has the properties of the acetylcholine receptor; i.e., protection of this site from binding with BuTX allows complete recovery of acetylcholine sensitivity of the endplates. The site occupied preferentially by H$_{12}$-HTX appears to be different from that occupied by curare. Thus, when only these sites are protected by H$_{12}$-HTX from the binding of BuTX, no recovery of acetylcholine sensitivity is observed in the endplate upon washing. The site, which interacts with H$_{12}$-HTX, is proposed to be associated with the ICM moiety of the cholinergic receptor–ICM complex. This hypothesis is based primarily upon the H$_{12}$-HTX-induced failure of consecutive acetylcholine depolarizations (Fig. 6). The simplest interpretation of that novel phenomenon is that the action of H$_{12}$-HTX occurs more rapidly after an initial conformational change in the ICM is induced by binding of acetylcholine at the receptor. Evidence that H$_{12}$-HTX affects sodium and potassium permeability, rather than the acetylcholine binding, is afforded by its effect upon the action potential generation (Figs. 3–5) and by a decrease produced in the half-decay time of the endplate current (unpublished observations). The results indicate that both true acetylcholine receptor sites and a nearly equal number of receptor-associated sites exist at vertebrate neuromuscular junctions (Fig. 7), compatible with a 1:1 association of receptor and ICM moieties.