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Nereistoxin Interaction with the Acetylcholine Receptor–Ionic Channel Complex

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SUMMARY

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The effect of nereistoxin (NTX) was studied, by electrophysiological methods on neuromuscular transmission in frog sartorius and rat diaphragm muscles, and by biochemical methods on binding of ligands to the acetylcholine (ACh) receptor and its ionic channel in membranes from *Torpedo* electroplax. NTX blocked the indirectly elicited twitch tension but not the directly elicited ones, and did not affect action potential, quantal content and frequency of the spontaneous miniature endplate potentials. The postsynaptic inhibition by NTX was evident from the reduction it caused in the amplitudes of the endplate potential and endplate current as well as the extrajunctional ACh sensitivity of denervated rat soleus muscle, and its inhibition of binding of [^{3}H]ACh and [^{125}I]α-bungarotoxin to *Torpedo* ACh receptors. In addition, NTX caused initial postsynaptic depolarization and potentiation of the indirectly elicited twitch tension. Although NTX by itself activated receptor-induced ^{22}Na influx in *Torpedo* microsacs to a small degree, it also inhibited the carbamylcholine-activated ^{22}Na influx. Since NTX did not inhibit binding of [^{3}H]perhydrohistrionicotoxin to *Torpedo* membranes and did not alter the linearity of the current voltage relationship, nor the time course of endplate current in frog sartorius muscle, we suggested that its inhibition of neuromuscular transmission was due to its inhibition of the ACh-receptor sites and not the ionic channel sites. Also, NTX acted as a partial agonist since it could activate the ACh receptor although its major action was that of an antagonist.

INTRODUCTION

Detailed studies of the actions of agonists and antagonists at the neuromuscular junction have provided definite clues for the understanding of the properties of the acetylcholine (ACh)[1] receptor and its ionic channel. A few agents have been identified that are specific probes for the postsynaptic macromolecules at the endplate. For example, α-bungarotoxin (α-BGT) reacts selectively with the ACh receptor sites (1), while histrionicotoxin (2) and amantadine (3) are specific for the receptor's ionic channel sites.

Nereistoxin (NTX) is a toxin isolated from the marine segmented worm, *Lumbriconereis heteropoda*, which has been identified as 4-*N*,*N*-dimethylamino-1,2-dithiolane (4). In 1966, Sakai (5) showed that NTX suppressed the frog rectus abdominis muscle contraction evoked by nerve stimulation, but had no effect on the response of the muscle to direct stimulation. Thus, he concluded that the action of NTX in suppressing muscle contraction was due to blockade of the ACh receptor. In 1971, Deguchi *et al.* (6) also found that NTX blocked neuromuscular transmission in frog sartorius muscle preparation without causing membrane depolarization. They reported decreased frequency and amplitude of the miniature endplate potential (MEPP), ACh sensitivity and the Na^+ and K^+ components of the endplate currents. Finally, these investigators concluded that the neuromuscular blockade induced by the toxin was due primarily to inhibition of the mechanism whereby the Na^+ and K^+ conductances of the endplate current were increased. Many questions remained to be answered regarding the precise mechanism and site of action of this toxin.

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[1] The abbreviations used are: NTX, nereistoxin; ACh, acetylcholine; α-BGT, α-bungarotoxin; H_{12}-HTX perhydrohistrionicotoxin; EPP, endplate potential, MEPP, miniature endplate potential; EPC, endplate current; DTT, dithiothreitol.

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The present investigation was aimed at reevaluating the mode of action of NTX on the neuromuscular junction and establishing its molecular site of action, using both biophysical and biochemical techniques. The effect of NTX on neuromuscular transmission was studied in innervated and denervated amphibian and mammalian muscles. On the other hand, since biochemical investigations are facilitated by the use of a tissue that has high concentrations of ACh receptors, the electric organ of the electric ray, *Torpedo ocellata*, was used. The ACh receptors in both tissues have similar drug specificities (7). Our studies disclosed that NTX bound to the ACh receptor and not its ionic channel sites, and that the interaction was complex, combining both agonist and antagonist actions.

MATERIALS AND METHODS

A. Electrophysiological Techniques

Animals and preparations. Experiments were carried out at room temperature (20–23°) on sartorius muscle of the frog, *Rana pipiens*, and soleus and diaphragm muscles of the rat (Wistar females, 180–200 g).

Solutions. During dissection and throughout the electrophysiological experiment the frog muscles were bathed in a physiological solution having the following composition (mM concentrations): NaCl 115.5, KCl 2.0, $CaCl_2$ 1.8, Na_2HPO_4 1.3, NaH_2PO_4 0.7, for frog muscles, and NaCl 135.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 1.0, and glucose 11.0, for mammalian muscles. For frog muscles the pH of the solution was 6.9–7.1, and they were bubbled with 100% O_2. Mammalian physiological solutions were continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 which gave them a pH of 7.1–7.3. In voltage clamp experiments, the muscles were treated with a hyperosmotic solution of glycerol in normal frog physiological solution until the indirect elicited twitches were blocked (8). The muscles were pretreated with 400–700 mM glycerol to disconnect functionally the sarcotubular system from the sarcolemmal membrane and allow the superimposition of several action potentials without causing contraction of the muscle fibers.

Twitch tension. The nerves supplying the sartorius muscles and phrenic nerve of diaphragm muscles were stimulated with supramaximal pulses having a duration of 0.05 msec and frequency of 0.1 Hz via a Ag–AgCl bridge electrode connected to a wet electrode (indirect stimulation) (9). Direct stimulation of the muscle was obtained by applying supramaximal rectangular pulses of 1.0–2.0 msec duration at a rate of 0.1 Hz through a bipolar platinum electrode placed at the base of the diaphragm muscles near the intercostal muscles or at the base of the sartorius muscles. The twitch tension was recorded by attaching the muscles to a Grass FT 03 force displacement transducer and by displaying the twitch on a grass polygraph model 5E. At least 30 min was allowed for the muscles to stabilize in the chamber.

Intracellular recordings. The muscles were stretched slightly beyond their length in a Plexiglas plate which had a planoconvex lens at its center and were immersed in a 15-ml bath. Nerve stimulation was delivered by two platinum electrodes. All recordings were made from surface fibers using glass microelectrodes filled with 3 M KCl (2–15 MΩ). Membrane excitability was studied in the frog with standard microelectrode techniques (17). The maximum rate of rise of the action potential (dV/dt) was measured by means of a RC circuit (100 k Ω–100 pF).

Endplate potentials. Endplate potentials (EPPs) were monitored on a Tektronix 565 oscilloscope and sampled at 100-μsec intervals by a PDP 11/40 computer. Care was taken to see that electrical artifacts or noise points were not mistaken by the computer for the peak of the potential. The first 10 potentials were discarded and the next nine groups of 20 potentials were used to obtain estimates of the quantal content and size as described previously (10). The size of each EPP was corrected for nonlinearity of the endplate response. The voltage clamp circuit used was similar to that previously described (11). The voltage clamp error was <3% of the unclamped EPP and the time constant of the clamping cirucit (10–90%) with 3–8 MΩ microelectrodes was less than 20 μsec. Changes in membrane potentials from the holding potentials were evoked from a DC source under manual control. The waveforms were recorded on film from the display of a 565 Tektronix oscilloscope or sampled by the PDP 11/40 computer.

ACh sensitivity. The extrajunctional membrane of denervated rat soleus muscle was assayed by microiontophoretic application of ACh as previously described (12). Acetylcholine pipets were filled with a 2 M solution of ACh and delivered by current pulses of 1-msec duration, with diffusion from the tip of the pipet prevented by a steady dc braking current. Acetylcholine sensitivity was expressed as the amplitude of the transient membrane depolarization per unit charge passed through the ACh pipet, i.e., millivolts of depolarization per nanocoulomb (nC) of charge. A correction was made for the differences in the level of the resting membrane potential of muscle fibers as previously described (13). The distance between the recording and ACh pipets was < 50 μm.

B. Biochemical Techniques

Membrane preparation. Membranes were prepared from the electric organ of *T. ocellata* (stored at −90°) by homogenization (20%, w/v) in an ice-cold solution of 90 mM KCl, 10 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4), and centrifugation of the supernatant of a 5000g × 10 min spin for 60 min at 30,000 g. This pellet was resuspended in Krebs original Ringer phosphate solution (mM): NaCl 107, KCl 4.8, $CaCl_2$ 0.65, $MgSO_4$ 1.2, and Na_2HPO_4 15.7, pH 7.4. One milliliter represented 1 g of the electric organ, and the membranes formed microsacs. The final protein concentration, as determined by the method of Lowry *et al.* (14), ranged from 1 to 2 mg/ml, and the maximum numbers of binding sites for [^{3}H]ACh and [^{3}H]H_{12}-HTX were 0.7 and 0.5 nmole/mg protein, respectively.

Equilibrium dialysis was used to study the binding of [^{3}H]ACh (49.5 mCi/mmole, from New England Nuclear) to the electric organ membranes as previously described (15). One-half milliliter of membrane preparation in a dialysis bag was shaken for 4 hr at 21° in Krebs original Ringer phosphate (25 ml) containing [^{3}H]ACh in the absence or presence of NTX. Triplicate samples of 50 μl

were then taken from each dialysis bag and bath, the excess radioactivity in the former representing the amount of ligand bound. In order to inhibit all cholinesterases without affecting the binding of ACh to its receptor, diisopropylfluorophosphate was added to the membranes, at a final concentration of 1 mM, 1 hr before the start of dialysis, and was present at 100 μM during dialysis.

A previously described *filter assay* was used to study the binding of ^{125}I-labeled α-BGT (130 Ci/mmole, from New England) using Whatman GF/C glass fiber filters (16). To 1 ml tissue mixture, typically containing 10 μg protein/ml, ^{125}I-labeled α-BGT was added to reach a final concentration of 10 nM. After exposure of microsacs to toxin for various lengths of time at 21°, unlabeled α-BGT (final concentration 1 μM) was added to stop the reaction. To reduce nonspecific ^{125}I-labeled α-BGT binding to the glass filters, each 200-μl sample was mixed with an equal volume of normal rabbit serum, the mixture filtered, and the filter washed with 20 ml cold Ringer's solution. Filters were counted in a Packard 5230 auto-gamma scintillation spectrometer. Specific binding of ^{125}I-labeled α-BGT was taken as the binding inhibited by 100 μM *d*-tubocurarine. Each experiment was repeated three times using triplicate samples.

Centrifugal assay was used to study the binding of [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX; 21 Ci/mmole) to the electric organ membranes. Samples (10 μl) of different concentrations of [^{3}H]H_{12}-HTX in ethanol were added to 1-ml samples of the *Torpedo* membrane preparation, mixed and incubated for 60 min at 21° in siliconized 1.5 ml polyethylene microcentrifuge tubes. NTX was added to the membranes 30 min (at 21°) prior to addition of [^{3}H]H_{12}-HTX. The incubation mixture was then centrifuged at 30,000*g* for 60 min, and three samples (50 μl each) were taken from the mixture before, as well as from the supernatant after centrifugation, and the radioactivity counted. Excess radioactivity in the former represented the bound [^{3}H]H_{12}-HTX. Binding to the ionic channel was obtained after subtraction of nonspecific binding to membranes immersed in boiling water for 30 min, which bound 10 ± 3% of control at 10 nM [^{3}H]-H_{12}-HTX. We had previously shown that nonspecific binding to the boiled membranes was linear and parallel to the total binding to native membranes at high [^{3}H]-H_{12}-HTX concentrations (15).

^{22}Na flux measurements. The method was that essentially used by Epstein and Racker (17) on reconstituted membranes, with some modifications. Dowex 50 W-X8 (100–200 mesh, hydrogen form, Bio-Rad) was converted to the Tris form, pH 7 (18), and a 2-ml suspension equilibrated in 0.2 M sucrose and 10 mM Tris, pH 7, then packed into disposable pipet droppers (Dynatech Labs, Inc.). The microsacs (200 μl) were rapidly added to 200 μl of 1 μCi/ml of ^{22}Na (carrier free, from New England Nuclear) in 20 μl original buffer and 50 μM carbamylcholine. At 0.8 min, 300 μl of the mixture was added to the Dowex column, washed with 2 ml of 0.2 M sucrose and 10 mM Tris, pH 7. The effluent was collected and counted in a Packard auto-gamma scintillation spectrometer, and represented ^{22}Na inside microsacs. Air pressure was used to speed the passage of sample and buffer.

RESULTS

Effect of NTX on muscle twitch and resting membrane potential. At concentrations ranging from 10 μM to 5 mM, NTX caused a partial to complete block of neuromuscular transmission of the rat diaphragm muscle (Fig. 1). Even at the highest concentrations used, (i.e., 5 mM), NTX did not affect the directly elicited muscle twitch (Fig. 2). The inhibition of the indirectly elicited twitch was reversible, and twitch tension was completely recovered to control level 5 min after repetitive washing. At the concentrations used, the toxin also induced a potentiation of the indirectly elicited twitch tension, which was observed at the initial phase just prior to beginning the neuromuscular blockade. On frog sartorius muscle, NTX at 0.4 and 7 μM produced 40 and 75% depression of the indirectly elicited twitch of the sartorius muscle, respectively (Fig. 3). The depression of the muscle twitch and its blockage were concentration dependent so that at the concentration of 50 μM the toxin also caused blockade of neuromuscular transmission in about

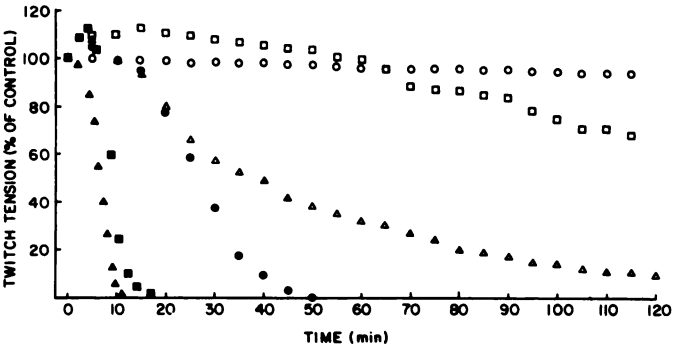

FIG. 1. *Indirectly elicited isometric twitch tension of the rat diaphragm muscle, expressed as percentage of control tension, is plotted against time in minutes*

Each point is the mean ± SEM of at least three preparations. The concentrations of NTX are: 10 μM (○); 800 μM (□); 1000 μM (△); 1500 μM (●); 3000 μM (■); 5000 μM (▲). Temperature is 23°, and simulation frequency is 0.1 Hz. Note a slight potentiation of the twitch tension which is attributable to the membrane depolarization induced by the toxin.

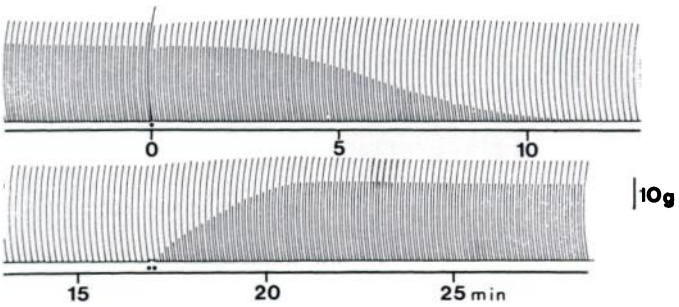

FIG. 2. *Indirectly and directly elicited isometric twitch tension of the rat diaphragm muscle*

The dot indicates the addition of NTX (5 mM) to the experimental chamber containing one muscle fiber and the double dots the moment when the toxin is removed from the muscle bath and the washout phase begun. Note that only the indirect twitch (small deflections) was blocked while the direct twitch remained unaltered.

70 min. However, at higher concentrations, such as 1 mM, blockade of the indirectly elicited twitch occurred within 5 min, followed by a partial recovery, and finally succeeded by a further blockade that was maximal at 20 min. This raised the possibility that blockade of neuromuscular transmission by higher concentrations of NTX might involve partial depolarization of the endplate region.

Measurement of the membrane potential at the junctional region disclosed a membrane depolarization of about 12–20 mV which occurred at the endplate region of the diaphragm muscle during the first 15 min of application of the toxin (Fig. 4A). This depolarization was gradually recovered and by 25 min the membrane potential regained control levels. Similarly, at the frog endplate, NTX (100 μM) caused a significant membrane depolarization followed by a phase of repolarization (Fig. 4B). However, since complete depolarization of the endplate occurred at a time when indirectly elicited muscle twitch remained partially depressed but not blocked, as seen in lower concentrations of NTX, it was assumed that a given population of ACh receptor remained in a desensitized form. Measurements at the extrajunctional membrane of the innervated muscle disclosed no membrane depolarization, suggesting that the membrane depolarization induced by NTX was mostly related to marked activation of the junctional ACh receptor.

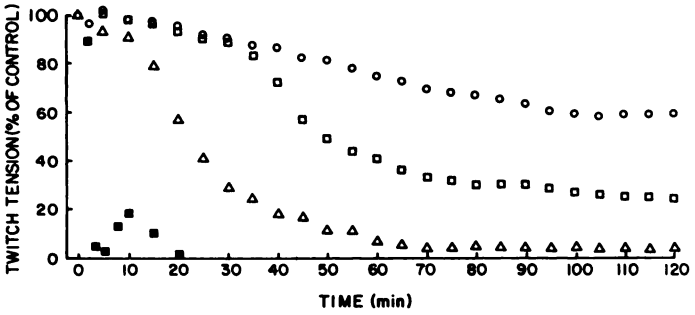

FIG. 3. *The legend for this figure is identical to that of Fig. 1, except that the preparation used is the frog sartorius muscle*

The concentrations of NTX are: 0.1 μM (○); 1 μM (□); 10 μM (△); 1000 μM (■). Note that the endplate region of the frog sartorius muscles is more sensitive to the actions of NTX than that of the rat diaphragm muscle.

Effect of NTX on the junctional and extrajunctional ACh sensitivity. In order to eliminate contributions from any possible presynaptic effects of NTX and to determine its postsynaptic effects, we studied the action of NTX on the ACh sensitivity of the muscle of the rat chronically denervated for 7 days. When the muscle was exposed to NTX (10 μM) for a period of 60 min, the ACh sensitivity was reduced from 945 ± 185 mV/nC (m = 15) to 37 ± 16 mV/nC (m = 43). An example of this response can be seen in Fig. 5. Similar to the effect at the junctional region, there was an initial membrane depolarization from an initial membrane potential of −58 ± 3 to −48 mV in 5 to 10 min followed by a recovery to normal level. At the junctional region, the endplate potential was also affected by NTX. Indeed the agent produced an initial potentiation followed by a marked depression and subsequent block of the endplate potential (Fig. 6).

Presynaptic effects of NTX. The possibility that NTX could produce this effect as a consequence of presynaptic action has been considered. However, as shown in Table 1, the quantal content was not affected and predominantly only the quantal size was decreased, thus indicating that the agent might not have an effect on the storage and release of ACh. In none of the endplates studied did we observe significant increase in MEPP frequency. This suggested that the membrane depolarization recorded at the junctional region might be the result of an initial activation of the ACh receptor followed by block of the receptor in a partially desensitized conformation.

Effect of NTX on the endplate current. After equilibration of the sartorius muscles with Ringer's solution containing NTX (50–100 μM) for 15–30 min the amplitude of the endplate current (EPC) was decreased in a concentration-dependent manner. The peak amplitude of the EPC recorded at membrane potential of −90 mV was decreased to 85 and 32% at toxin concentrations of 50 and 100 μM, respectively (Fig. 7). Although NTX caused a significant decrease in the EPC amplitude, no departure from linearity was observed in the relationship between peak EPC amplitude and membrane potential. Two hours of intensive washing with Ringer's solution produced only a partial recovery of the peak amplitude of the EPC (Table 2). In all concentrations studied, NTX did not cause alterations of the reversal potential (Fig. 7). NTX showed no significant effect on the decay phase

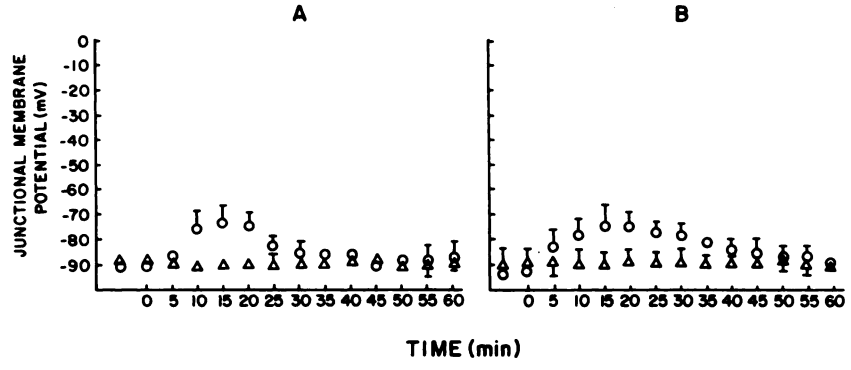

FIG. 4. *Resting membrane potentials of (A) the endplate of rat diaphragm muscle and (B) frog sartorius muscle*

In control (△) and in the presence of 100 μM NTX (○), which is added at zero time. Each point is the mean of three preparations, and the vertical bar is SEM. If no error bars are shown, the SEM is smaller than the symbols.

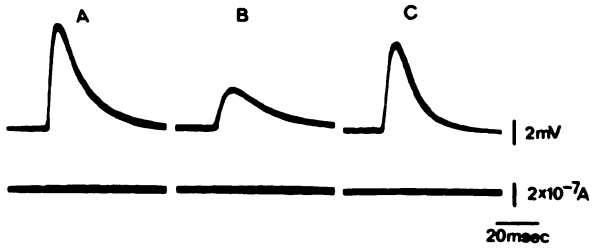

FIG. 5. *Effect of NTX on the extrajunctional sensitivity to ACh of the 7 days denervated rat soleus muscle*

Upper traces are membrane potential; lower traces are current passed through the ACh pipet. Membrane potentials are −48 mV at 90 min after application of 10 μM NTX (B) and −53 mV after washing with Ringer's solution 60 min. The duration of the pulse applied to the ACh pipet is 2 msec. The current pulses are slightly retouched to allow better visualization.

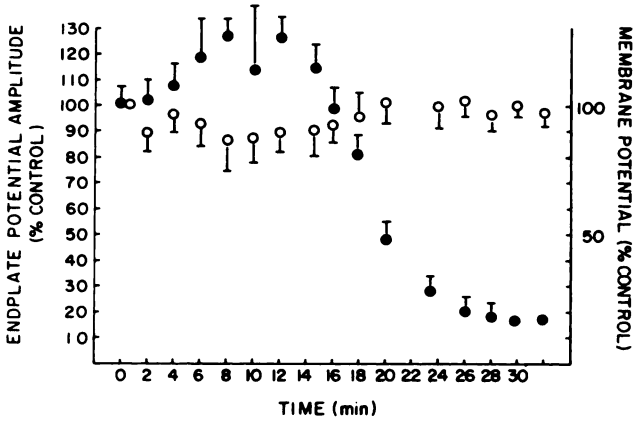

FIG. 6. *Effect of NTX (100 μM) on EPP amplitude* (●) *and junctional membrane potential* (○) *of frog sartorius muscle*

Each point is the mean of 10–15 endplate regions from four muscles. Vertical bars represent standard deviations.

of the EPC recorded at various membrane potentials (Fig. 8). Indeed, this relationship remained linear and disclosed a slope of −3.3 msec/V for control condition and −3.1 and 3.4 msec/V at 50 and 100 μM NTX, respectively (Fig. 8).

Effect of NTX on evoked action potential of sartorius muscle of the frog. When the action potential was recorded at the surface fiber of the frog sartorius muscle under control conditions and 60 min after exposure to 100 μM NTX, no significant difference was observed between the two potentials (Fig. 9).

Effect of NTX on binding of the ACh receptor and its ionic channel. To determine whether the postsynaptic effects of NTX had to do with its interactions with the ACh receptor or its ionic channel, its effects were studied on binding of ligands to *Torpedo* membranes. NTX, at 10 μM and higher concentrations, inhibited binding of [^{3}H]ACh to the ACh receptor, while up to 300 μM NTX had no effect on the binding of [^{3}H]H_{12}-HTX to the ionic channel (Fig. 10). Only at 1 mM, did NTX cause slight inhibition (7%). In a double reciprocal plot (Fig. 11) the inhibition of [^{3}H]ACh binding to the ACh receptor was shown to be competitive as judged by the common intercept on the ordinate. The K_i was calculated to be 0.15 ± 0.02 mM. Not only did NTX inhibit ACh binding to the ACh receptor, but it also inhibited binding of ^{125}-labeled α-BGT to the ACh receptor (Fig. 12).

Effect of NTX on the uptake of ^{22}Na into receptor enriched Torpedo microsacs. To further evaluate whether NTX activates ACh receptor, its effect on the uptake of ^{22}Na by receptor-enriched microsacs made from *Torpedo* electric organ membranes was studied. Concentrations from 0.05 to 5 mM of NTX caused ^{22}Na influx into microsacs ranging from 11 to 23% the amount induced by 0.05 mM carbamylcholine (Fig. 13). However, the level of ^{22}Na influx did not significantly differ between 0.05 and 5 mM NTX. As to the effect of NTX in inhibiting carbamylcholine-induced ^{22}Na influx, there was no inhibition with 0.1 mM NTX. But higher concentrations of 0.5, 1 and 5 mM inhibited 11, 14 and 58%, respectively. The discrepancy between the finding that 0.1 mM NTX inhibited [^{3}H]ACh binding (Fig. 11), but not receptor-induced ^{22}Na influx, was cleared when we found that the effect of NTX was time dependent. Although 1 mM NTX inhibited only 14% of the carbamylcholine-induced ^{22}Na influx by simultaneous addition of toxin and carbamylcholine and exposure for only one minute, the toxin inhibited 100% (Fig. 14) of the flux when preincubated with *Torpedo* microsacs for 10 min prior to the addition of carbamylcholine ^{22}Na.

DISCUSSION

It is evident that NTX blocks neuromuscular transmission as judged by its inhibition of the indirectly elicited twitch tension of both rat diaphragm muscle and frog sartorius muscle. The toxin blocks the indirectly elicited twitch tension following an initial slight potentiation (Fig. 1), this effect occurring without altering the action potential generating mechanism (Fig. 9). Although our preliminary experiments pointed to some presynaptic effects for NTX (19), in agreement with an earlier report (6), the quantitative analysis reported herein clearly indicates that NTX has little if any presynaptic action as shown by the absence of effect on quantal content (Table 1) and on frequency of the spontaneous MEPPs. The decrease in quantal size is mostly due to the postjunctional action of NTX. This action of the toxin is not due to inhibition of ACh-esterase because NTX has a low affinity for the esterase (20). Thus, the blockade of neuromuscular transmission by NTX must be related to the postsynaptic action of NTX, which has also been previously reported in frog and rat muscles (5, 6) and in the cockroach sixth abdominal ganglion (21, 22). The biophysical and biochemical data gathered in the present study disclose that the toxin attenuates the amplitudes

TABLE 1

Effect of NTX on the quantal content and size of the EPP of frog sartorius muscles[a]

	Quantal content	Quantal size (mv)
Control	32.1 ± 2.4	0.62 ± 0.06
NTX (50 μM)	34.1 ± 0.1	0.25 ± 0.05[b]
Washing for 1–2 hr	42.7 ± 10.7	0.24 ± 0.03[b]

[a] Endplate potentials were recorded in the presence of frog Ringer solution containing 10 mM $MgCl_2$.

[b] $P < 0.01$ with respect to control.

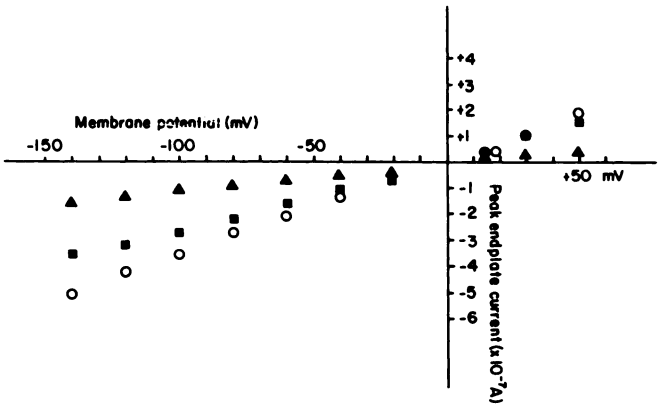

FIG. 7. *The relationship between current and peak amplitude of the EPC in frog sartorius muscle in control* (○) *and in the presence of 50 μM* (■) *and 100 μM* (▲) *NTX*

of the EPP and EPC of frog sartorius muscle (Figs. 6, 7) as well as the extrajunctional ACh sensitivity of the denervated rat soleus muscle (Fig. 5) and also inhibits the ACh receptor-induced ^{22}Na influx into *Torpedo* microsacs (Fig. 13). These inhibitions could be due to blockade of the binding sites of either the ACh receptor or its ionic channel. Several of our results eliminate the second possibility and clearly demonstrate that the action of NTX is indeed on the ACh receptor and not its ion channel sites. First, the toxin does not alter the linearity of the relationship between membrane potential and peak EPC amplitude (Fig. 7). In addition, NTX does not affect the time constant of EPC decay (Fig. 8, Table 2), thus disclosing a slope for the relation of the time constant of the EPC decay and membrane potential which is not significantly different from control conditions. Second, NTX blocks the binding of [^{3}H]ACh and ^{125}I-labeled α-BGT to the ACh receptor (Figs. 10, 11, 12), but not [^{3}H]H_{12}-HTX to the ionic channel sites (Fig. 10) in *Torpedo* membranes. Histrionicotoxin has been shown to block postsynaptic conductances at the endplate by interacting with the ionic channel in its open as well as closed conformation (23). Whether the ionic channel is part of the ACh-receptor macromolecule or is a separate protein has not yet been resolved.

A characteristic of NTX that deserves special attention is its dual action on ACh receptors. NTX depolarizes the muscle membrane, though to a lesser extent than does ACh, and thus may be considered as an agonist. This agonistic effect of NTX is supported by the finding that the agent causes a potentiation of twitch tension (Fig. 1) and endplate potential (EPP) (Fig. 6), and induces some influx of ^{22}Na when applied to *Torpedo* microsacs rich in ACh receptors (Fig. 13). The initial action on the ACh receptor causes a local junctional membrane depolarization followed by a recovery of the potential as shown in Figs. 4A and B. However, neither this membrane depolarization nor the degree of ^{22}Na influx is as marked as that seen using a classic agonist such as carbamylcholine (Fig. 13). The initial junctional depolarization was not observed previously (6) possibly because recording was not precisely inside the junctional region. The second action of the toxin is related to an antagonist-type reaction where the toxin is able to displace ACh and block the receptor as revealed by the repolarization of membrane potential (Fig. 4), and the inhibitions of the twitch tension (Figs. 1–3), the EPC (Fig. 7) as well as the carbamylcholine-induced ^{22}Na influx into *Torpedo* microsacs (Figs. 13, 14).

It should be noted that the inhibition of ACh or α-BGT binding observed can be caused by both receptor agonists and antagonists (Figs. 10, 11). The inhibition of ACh binding is competitive (Fig. 12) with a K_i of 0.15 mM. This makes NTX almost three orders of magnitude less potent on the receptor site than *d*-tubocurarine (24), and should be considered as only a weak antagonist on the ACh receptor. The stronger inhibition by NTX of neuromuscular transmission than of ACh binding to *Torpedo* receptors may be explained by a possible protective effect of the presence of an agonist. This is evident from the data on the effect of NTX on ^{22}Na uptake by *Torpedo* microsacs, where the longer preexposure of the membranes to NTX the stronger is the inhibition of the carbamylcholine-induced ^{22}Na influx (Fig. 14). In addition, the presence of ACh has previously been shown to protect binding of [^{3}H]ACh to *Torpedo* receptors against the inhibiting effect of 1,4-dithiothreitol (DTT) (25). In the binding studies, the membranes are exposed to both NTX and ACh simultaneously, while in the electrophysiological ones, the muscles are exposed to NTX before nerve stimulation takes place.

One possibility for the molecular basis of the dual action of NTX is that it may bind to the same site on the ACh receptor as does ACh (shown by the common intersect of the *Y*-axis in Fig. 11), but the bonds involved may differ such that NTX–ACh receptor opens the ionic channel to a lesser degree than occurs with ACh. This would be followed by very slow transition of the receptor into a less active form. Since inhibition of the ACh receptor is the major action of NTX (Figs. 5, 6), one has to assume that the toxin binds to the same recognition site for ACh. Another possibility is that the sites that bind agonists on the ACh receptors are indeed different from the ones that bind antagonists as proposed earlier (16, 25–27); NTX may bind to both kinds of sites.

Although the same concentration of NTX produces similar depolarization of the junctional membrane in the rat and frog muscles (Fig. 4), NTX is much more effective in inhibiting frog sartorius than rat diaphragm muscles, since a 100-fold higher concentration is needed to produce similar inhibition in the latter (Figs. 1, 3). In addition, the complete and fast recovery of twitch tension of

TABLE 2

Effect of NTX on the amplitude and time course of endplate current in frog sartorius muscle recorded at −90 mV

	Amplitude (nA)	Time constant EPC decay (msec)	Rise time (msec)
Control	308 ± 48 (10)[a]	1.71 ± 0.09	0.99 ± 0.02
NTX (50 μM)	262 ± 60 (8)	1.53 ± 0.04	1.02 ± 0.02
NTX (100 μM)	98 ± 25 (11)[b]	1.63 ± 0.14	1.04 ± 0.02
Washing[c]	161 ± 30 (12)	1.64 ± 0.10	1.00 ± 0.04

[a] The values refer to the mean ± SEM. The number in parentheses refers to the number of endplates sampled in at least three muscles.

[b] $P < 0.01$ with respect to control.

[c] Values refer only to muscles previously exposed to 10 μM NTX for 1 hr and then washing for 2 hr.

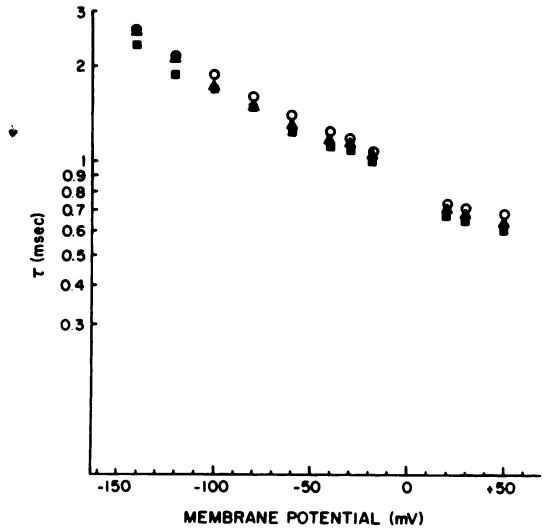

FIG. 8. *The relation of the decay time constant of EPC decay in frog sartorius muscle and membrane potential in control* (○) *and in the presence of 50 μM* (■), *and 100 μM* (▲) *NTX*

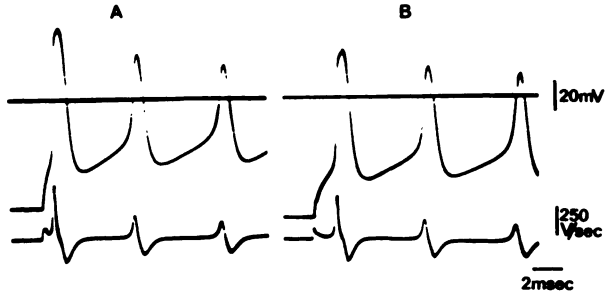

FIG. 9. *Effect of NTX on the action potential (upper trace) and their first derivatives (lower trace) obtained from a single surface fiber of frog sartorius muscle, before (A) and 60 min after (B) treatment with 10 μM NTX*

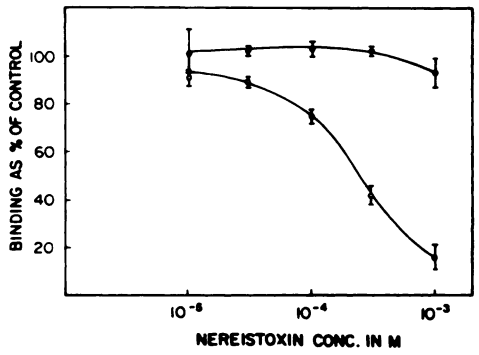

FIG. 10. *The effect of increasing concentrations of NTX on the binding of 0.1 μM [³H] ACh* (○) *and 1 nM [³H]H₁₂-HTX* (●) *to Torpedo electric organ membrane*

Each point and vertical bar represents the mean ± SD of three experiments.

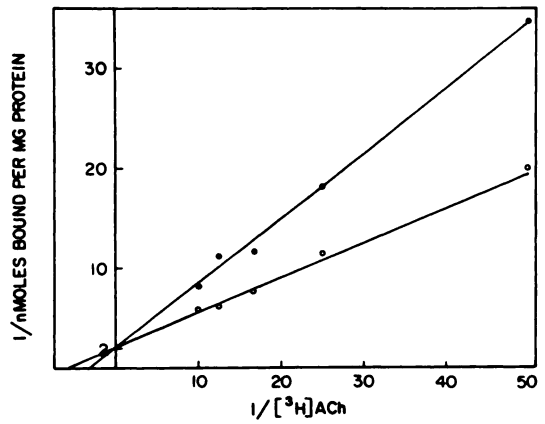

FIG. 11. *Double reciprocal plot of the binding of [³H]ACh to Torpedo electric organ membranes in the absence* (○) *and presence* (●) *of 0.1 mM NTX*

Concentration of [³H]ACh is in μM. Each point is the mean of three experiments. The K_i value, calculated by weighted regression analysis, is 0.15 ± 0.02 mM.

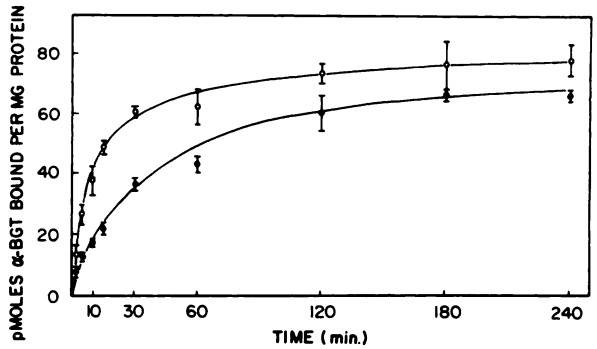

FIG. 12. *Specific binding of 10 nM bungarotoxin (α-BGT) to Torpedo electric organ membranes alone* (○) *and in the presence of 0.1 mM NTX* (●)

Each symbol and vertical bar is the mean ± SD of three experiments.

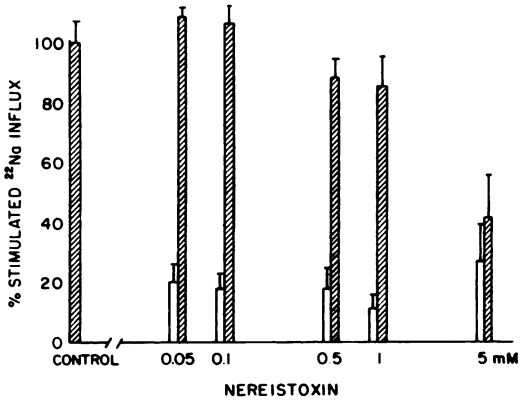

FIG. 13. *Histogram of the effect of different concentrations of NTX on ²²Na influx into microsacs made from Torpedo electric organ (clear columns) and on the carbamylcholine-induced ²²Na influx (dashed columns)*

First column on left represents influx due to carbamylcholine alone and is considered as 100%. Each bar represents SD of three experiments.

the rat diaphragm after 5 mM NTX treatment (Fig. 2) suggests that the toxin may react noncovalently with the receptor. This is in contrast to the partial and slow recovery of EPP amplitude of frog sartorius muscle (Table 2) which suggests that a different mode of binding may take place to cause inhibition in the two animals or muscles. Since NTX is a dithiolane compound that forms a heterocyclic ring structure through a disulfide bond between the two sulfhydryl groups, it may be converted by reduction to a dithiol capable of reacting with the

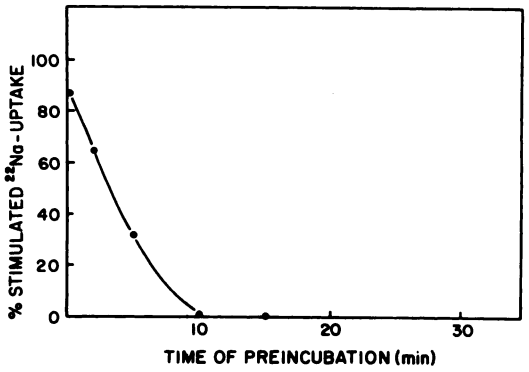

FIG. 14. *The effect of time of preincubation to 1 mM NTX on the inhibition by NTX of the carbamylcholine-induced ^{22}Na influx*

ACh receptor covalently. Thus NTX may be reacting with the ACh receptor covalently in a manner similar to that of the reaction of DTT. Prior treatment of *Electrophorus* electroplax with DTT was found to cause reduction of the depolarization caused by ACh and carbamylcholine (28). It also changed the effect of hexamethonium from an inhibitor into an activator of depolarization (29). Thus one may suggest that in the presence of frog muscle, the toxin may be reduced to the dithiol structure, and by virtue of its ability to bind to the receptor site is brought into the vicinity of a crucial disulfide bond. Covalent reaction with this bond would increase its binding affinity and potency as an inhibitor.

In conclusion, NTX blocks neuromuscular transmission without affecting presynaptic events or inhibiting ACh-esterase activity. Its molecular target is the ACh receptor with which it interacts in a dual manner. Although NTX activates the ACh receptor slightly, its major effect is inhibition of the receptor action without affecting the ionic channel sites. Both biophysical and biochemical data suggest that NTX acts as a partial agonist or antagonist of the ACh receptor.

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