Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4639–4643, July 1981
Neurobiology

Anatoxin-a interactions with cholinergic synaptic molecules

(acetylcholine receptors/ion channels/histrionicotoxin/phencyclidine)

ROBERT S. ARONSTAM* AND BERNHARD WITKOP†

*Department of Pharmacology, Medical College of Georgia, Augusta, Georgia 30912; and †Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Contributed by Bernhard Witkop, April 16, 1981

ABSTRACT **Anatoxin-a, a bicyclic amine isolated from blue-green alga, binds to the nicotinic acetylcholine receptor of *Torpedo* electric tissue, thereby inducing conformational changes in the postsynaptic receptor–ion channel complex as evidenced by alterations in the binding of radiolabeled ligands to the complex. Anatoxin-a binds to the acetylcholine recognition site (K_d = 0.1–0.2 μM) as indicated by its competitive inhibition of specific [^{3}H]acetylcholine and *d*-[^{3}H]tubocurarine binding. Anatoxin-a stimulates the binding of three physiologically identified "ion channel blockers," [^{3}H]perhydrohistrionicotoxin, [^{3}H]phencyclidine, and [^{3}H]phencyclidine methiodide. The 50% effective doses for these effects range from 0.14 to 0.28 μM. Incubation of *Torpedo* membranes with anatoxin-a before addition of a radiolabeled channel probe produces a time- and concentration-dependent attenuation of the binding compared to the situation in which anatoxin-a and the probe are added simultaneously. The time course for the elaboration of this decrease corresponds to electrophysiological measurements of anatoxin-a-induced desensitization of neuromuscular junction responses. In these nicotinic actions, anatoxin-a is about as potent as acetylcholine. Anatoxin-a has relatively low affinity for the muscarinic acetylcholine receptors of rat brain, inhibiting 3-[^{3}H]quinuclidinyl benzilate binding (10^{-10} M) by 50% at concentrations between 10 and 20 μM. In contrast to classical muscarinic agonists, anatoxin-a displays little regional selectivity in its binding, and its receptor affinity is unaltered by alkylation of the neural membranes with *N*-ethylmaleimide.**

Biochemical investigation of the interaction of a cholinergic agonist with the postsynaptic macromolecules involved in neurotransmission includes a consideration of occupancy by the agonist of the acetylcholine (AcCho) binding site (referred to here as the AcCho receptor, AcChoR) and the ability of the agonist to induce conformational changes in the receptor–ion channel complex subsequent to this binding. At nicotinic synapses, these conformational changes can be detected by alterations in the binding kinetics of a number of compounds that block neuromuscular transmission without interacting with the receptor (i.e., AcCho binding site) *per se* (1). These compounds influence the time course of single ion channels as revealed by synaptic noise analysis and have been termed "channel blockers," although the actual site of their interactions has not been precisely determined (2). Receptor agonists have been found to stimulate the binding of channel blockers, such as tritiated perhydrohistrionicotoxin (H_{12}-HTX) and phencyclidine (PCP), to the receptor–ion channel complex by up to several hundredfold. The amount of this stimulation is decreased substantially when membranes containing the postsynaptic complex are incubated with the agonist before channel binding is measured (1). Thus, different patterns of channel probe binding are seen under conditions in which one would expect to have a preponderance of resting, activated (i.e., open or conducting), or desensitized receptor–channel complexes.

Anatoxin-a (AnTX-a) is a bicyclic amine isolated from the blue-green alga *Anabaena flos-aquae* (3–5). It is of interest because of its high affinity and intrinsic activity at nicotinic synapses (3). Moreover, the time course and conductances of neuromuscular ion channels are the same when activated by AcCho or AnTX-a (6). AnTX-a does not possess an ester linkage which might be subject to enzymatic hydrolysis and is a semirigid molecule insofar as its conformation is restricted to the *s-cis* and the *s-trans* rotamers of the planar conjugated system O=C—C=C (see Fig. 1) (7). These properties makes it useful in the elucidation of the molecular nature of nicotinic AcChoR through structure–activity relationship studies.

In the present study, details of AnTX-a actions at nicotinic synapses of *Torpedo* electric organs and muscarinic receptors of rat brain are described, extending preliminary observations made in collaboration with other researchers (8).

METHODS

Tissue Preparations. Electric organs of *Torpedo ocellata* (provided by M. Eldefrawi) which had been stored frozen at 70°C were homogenized in 10 mM Tris·HCl, pH 7.4/1 mM Na_2EDTA/100 μM diisopropyl fluorophosphate and spun for 10 min at 5000 × *g*; the resulting supernatant was spun at 30,000 × *g* for 60 min. This pellet was rinsed with glass-distilled water, lyophilized, and stored for up to 3 months at 20°C. The material was suspended at 2–3 mg/ml in 50 mM Tris·HCl. The protein content of this suspension (0.8–1.3 mg/ml) was determined by the method of Lowry *et al.* (9) with bovine serum albumin as the standard. Lyophilization denatured a substantial fraction of the channel probe binding sites, although the properties of the surviving sites were identical to those measured in membranes from nonlyophilized electric organ.

Neural membranes were prepared by removing the cerebral cortex, hippocampus, or brainstem (medulla, pons, and midbrain) from adult male Wistar rats and homogenizing in 10 vol of 50 mM Tris·HCl at pH 7.4. The homogenate was spun at 1000 × *g* for 5 min to remove nuclei, large membrane fragments, and tissue debris. The supernatants were then spun at 40,000 × *g* for 20 min and the pellets were resuspended in fresh buffer.

Binding Assays. [^{3}H]AcCho (250 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and *l*-quinuclidinyl [*phenyl*-4(n)-^{3}H]benzilate ([^{3}H]QNB; 44 Ci/mmol) were purchased from Amersham. [*Piperidyl*-3,4-^{3}H(N)]phencyclidine ([^{3}H]PCP; 48 Ci/mmol), *dextro*-[13^1-^{3}H(N)]tubocurarine chloride ([^{3}H]dTC; 26 Ci/mmol), and [^{3}H]methyl iodide (94 Ci/mmol) were purchased from New

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; AnTX-a, anatoxin-a; dTC, *d*-tubocurarine; H_{12}-HTX, perhydrohistrionicotoxin; PCP, phencyclidine; PCP-MeI, phencyclidine methiodide; QNB, 3-quinuclidinyl benzilate; ED_{50}, dose required for 50% of maximal effect.

England Nuclear. [*N-methyl*-^{3}H]Phencyclidine methyl iodide ([^{3}H]PCP-MeI) was prepared by treating PCP free base with a 10% molar excess of [^{3}H]methyl iodide in a minimal volume of acetone. After 4 days at room temperature, the quaternized product crystallized to yield pure [^{3}H]PCP-MeI.

The binding of [^{3}H]AcCho and [^{3}H]dTC to the AcChoR was determined by equilibrium dialysis. An aliquot of tissue (0.8–1.3 mg of protein per ml) was placed in dialysis tubing that had been extensively washed with ethanol, EDTA, and deionized distilled water to remove contaminants, placed in a bath containing 100 mM Tris·HCl at pH 7.4 and 0.1 μM [^{3}H]AcCho or [^{3}H]dTC and any competing ligands, and shaken for 4 hr at room temperature. The difference in radioactivity content of the bags and bath reflected the amount of binding. Nonspecific binding was that binding that was not blocked by inclusion of 100 μM nicotine in the bath; it was negligible in the case of [^{3}H]AcCho and <5% of the total binding with [^{3}H]dTC.

The binding of [^{3}H]QNB to rat brain muscarinic receptors and of [^{3}H]PCP, [^{3}H]PCP-MeI, and [^{3}H]H_{12}-HTX to electric organ tissue was determined by a filtration procedure using Whatman GF/B glass fiber filters. A suspension of brain or *Torpedo* membranes was incubated for an appropriate time in the presence of the radiolabeled ligand and 50 mM Tris·HCl in 1 ml before being filtered. The concentrations of channel probes used were 1 nM [^{3}H]H_{12}-HTX, 2 nM [^{3}H]PCP, and 50 nM [^{3}H]PCP-MeI; [^{3}H]QNB was used at 0.1 nM. The pH of the incubation medium was 7.4 in the cases of [^{3}H]QNB and [^{3}H]H_{12}-HTX and 8.0 with [^{3}H]PCP and [^{3}H]PCP-MeI. The radioactivity content of the filters was determined by scintillation counting (10).

The binding isotherms and exponential decay curves were determined by computer nonlinear regression analyses using the MLAB system developed at the National Institutes of Health.

RESULTS

Interaction of AnTX-a with the Nicotinic AcChoR. AnTX-a inhibits the specific binding of 0.1 μM[^{3}H]AcCho and [^{3}H]dTC to the nicotinic AcChoR in *Torpedo* electroplax with mean (±SD) doses required to achieve 50% of maximal effect (ED_{50}) of 0.18 ± 0.03 and 0.12 ± 0.03 μM (n = 3), respectively (Fig. 1; Table 1). Hill coefficients associated with the interaction of AnTx-a with the AcCho and dTC binding sites were 1.5 ± 0.1 and 1.7 ± 0.1, respectively, and maxima were observed in Scatchard plots of the data at AnTX-a concentrations at which about 15% of the sites are occupied by AnTX-a (not shown). AnTx-a produced a parallel shift in [^{3}H]AcCho and [^{3}H]dTC binding curves (measured from 0.01 to 0.32 μM), suggesting a competitive interaction.

Influence of AnTX-a on the Binding of Ion Channel Probes. In the presence of AnTX-a, the binding of 1 nM [^{3}H]H_{12}-HTX and 2 nM [^{3}H]PCP to sites on the ion channel was enhanced in a concentration-dependent fashion (Fig. 2). With both probes, the initial rates of binding were accelerated, as was that of [^{3}H]PCP-MeI (not shown). With [^{3}H]PCP the total amount of binding did not increase significantly after the first 5 min, and equilibrium levels of binding were quite different in the presence of different concentrations of AnTX-a. On the other hand, [^{3}H]H_{12}-HTX binding equilibria were not established for at least 20 min (not shown) and the final levels of binding differed by less than 20% no matter how much AnTX-a, if any, was present. The ability of AnTX-a to enhance the binding of each channel probe was inhibited about 50% by 1 μM dTC, gallamine, or benzoquinonium, although alone these can stimulate the binding of the ion channel probes to a modest extent (1).

AnTX-a and one of the radiolabeled channel probes were added simultaneously to a membrane suspension, and the amount of binding was determined after 30 sec (Fig. 2*B*). The amount of channel binding after a 30-sec incubation was negligible in the absence of AnTX-a; all of the binding in excess of background levels [determined in the presence of 1 mM amantadine (11)] represented AnTX-a-stimulated binding. AnTX-a was equally effective in stimulating the binding of [^{3}H]PCP,

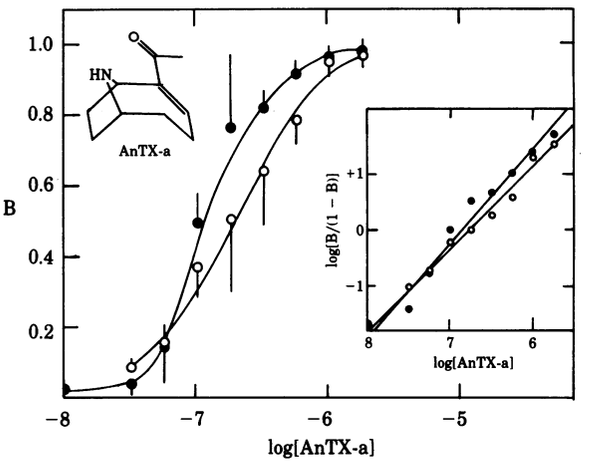

FIG. 1. Binding of AnTX-a to the nicotinic AcChoR, inferred from its inhibition of the binding of 0.1 M [^{3}H]AcCho (○) or [^{3}H]dTC (●). (*Inset*) Hill plots of the same data. *B*, fractional receptor occupancy by AnTX-a. Each point represents the mean (±SD) of three independent assays.

Table 1. Kinetic constants associated with interaction of AnTX-a and postsynaptic cholinergic receptor–ion channel complex of *Torpedo* electric organ and rat brain muscarinic receptors

Ligand	Interaction variable: ED_{50},* μM	K_d,† μM	n_h‡	K_i§
Nicotinic receptor:				
[^{3}H]AcCho	0.18±0.03	0.2±0.1	1.5±0.1	0.26±0.08
[^{3}H]dTC	0.12±0.05	0.1±0.1	1.7±0.1	0.22±0.07
Nicotinic ion channel:				
[^{3}H]PCP	0.28±0.07	0.3±0.1	1.16±0.03	
[^{3}H]PCP-MeI	0.28±0.05	0.3±0.1	1.41±0.04	
[^{3}H]H_{12}-HTX	0.22±0.08	0.2±0.1	1.5 ±0.1	
AcCho/[^{3}H]H_{12}-HTX¶	0.14±0.08	0.1±0.1	1.4 ±0.2	
Muscarinic receptor:				
[^{3}H]QNB				
Brainstem	18 ±2			
Hippocampus	10 ±1			
Cortex	15 ±5			

Data are shown as mean ± SD.

* AnTX-a concentration at which the specific binding of 0.1 μM [^{3}H]AcCho or [^{3}H]dTC to the nicotinic receptor of *Torpedo* electric organ or 0.1 nM [^{3}H]QNB to muscarinic receptors from the indicated areas of rat brain is inhibited by 50%. In the case of nicotinic ion channels, the ED_{50} value refers to the AnTX-a concentration at which the binding of [^{3}H]PCP, [^{3}H]PCP-MeI, or [^{3}H]H_{12}-HTX to the "ion channel" associated with the nicotinic AcChoR of *Torpedo* electric organs is stimulated half-maximally.

† Dissociation constant determined by nonlinear regression fit to a mass action expression for a single population of interaction sites, $B = B_{max}C/(C + K_d)$, in which B is the fractional response (i.e., receptor occupancy or stimulation of "ion channel" binding), B_{max} = 1, C is the concentration of AnTX-a, and K_d is the dissociation constant for the interaction.

‡ Hill coefficients as determined by linear least-squares regression analyses.

§ Inhibition constants were obtained from the slopes of double-reciprocal plots of ligand binding curves in the presence of various concentrations of AnTX-a.

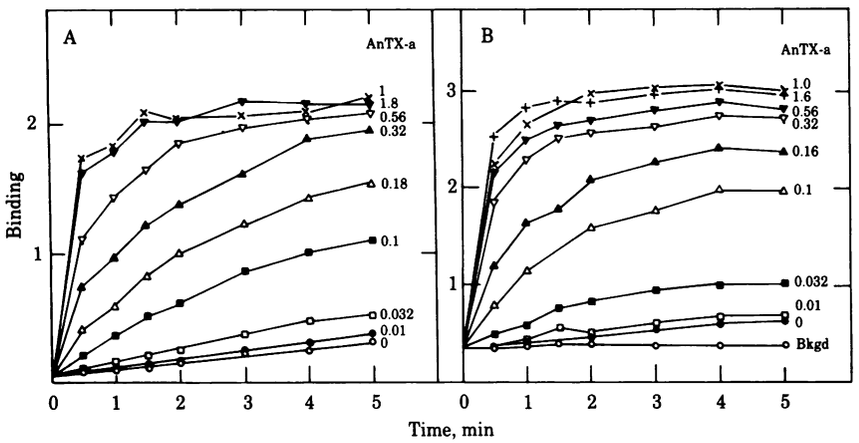

FIG. 2. Influence of AnTX-a on the binding of [^{3}H]H_{12}-HTX (*A*) and [^{3}H]PCP (*B*) to the ion channel associated with the nicotinic AcChoR. Suspensions of *Torpedo* membranes (final concentration, 0.2 mg of protein per ml) were added to medium containing 1 nM [^{3}H]H_{12}-HTX or 2 nM [^{3}H]PCP and the indicated concentration (in μM) of AnTX-a. At the time indicated, binding was determined by rapid filtration through glass fiber filters and measurement of the radioactivity associated with the tissue trapped on the filters. Nonspecific channel binding, determined in the presence of 1 mM amantadine, did not vary with the time of incubation (e.g., see "Bkgd" curve in *B*) and the *y*-axis intercept represents this value.

[^{3}H]PCP-MeI, and [^{3}H]H_{12}-HTX (Fig.3); a curve depicting AcCho stimulation of [^{3}H]H_{12}-HTX binding is included for comparison. AcCho was slightly more effective than AnTX-a in enhancing channel binding, although the difference was significant only when compared with AnTX-a stimulation of [^{3}H]PCP binding. The ED_{50} values for AnTX-a stimulation of [^{3}H]PCP, [^{3}H]PCP-MeI, and [^{3}H]H_{12}-HTX binding are 0.28 ± 0.07, 0.28 ± 0.05, and 0.22 ± 0.08 μM (n = 3), respectively; the ED_{50} value for AcCho stimulation of [^{3}H]H_{12}-HTX binding is 0.14 ± 0.08 μM (Table 1).

Scatchard and Hill treatments of the data for AnTX-a stimulation of [^{3}H]PCP, [^{3}H]PCP-MeI, and [^{3}H]H_{12}-HTX binding and AcCho stimulation of [^{3}H]H_{12}-HTX binding are presented in Fig. 4. Maxima are seen in the Scatchard plots of AnTX-a stimulated [^{3}H]PCP-MeI and [^{3}H]H_{12}-HTX, but not [^{3}H]PCP, binding at the level at which about 20% of the maximal response is obtained. Hill coefficients vary from 1.2 to 1.5. Binding constants and Hill coefficients for the three channel probes are summarized in Table 1. The degree of stimulation of channel probe binding is closely related to the extent of AcChoR occupancy by AnTX-a.

When the electric organ tissue suspension was incubated with AnTX-a before initiating a channel binding reaction by addition of the radiolabeled channel probe, the amount of channel probe bound after 30 sec was decreased compared to the situation in which the probe and AnTX-a were added simultaneously (Fig. 5). The extent of this decrease was dependent upon the concentration of AnTX-a and the length of the preincubation, being virtually complete within 1 min. Rate constants for elicitation of this effect ranged from 0.046 to 0.03 sec^{-1}. Although a significant amount of the stimulation of ion channel binding was observed in the presence of 0.1 and 0.15 μM AnTX-a, a decrease in this stimulation was not observed subsequent to preincubation of the membranes at these AnTX-a concentrations. Stimulation of channel binding cannot be totally suppressed by preincubation with AnTX-a, no matter how high the concentration or how long the preincubation period.

Interaction of AnTX-a with Neural Muscarinic Receptors. AnTX-a inhibited the binding of [^{3}H]QNB to brain muscarinic receptors with ED_{50} values in the range 10–20 μM (Fig. 6). The affinities of AnTX-a for muscarinic receptors from different brain areas were similar and treatment of neural membranes with 1 mM *N*-ethylmaleimide for 20 min at 37°C had little influence on the ability of AnTX-a to inhibit [^{3}H]QNB binding.

DISCUSSION

The present results indicate that AnTX-a is a cholinergic agonist with 100-fold selectivity for nicotinic compared to muscarinic receptors. AnTX-a binds to the nicotinic AcChoR of *Torpedo* electric organ, thereby inducing conformational changes in the receptor–ion channel complex such that the kinetics of binding of certain ligands to the complex are altered. In these nicotinic actions, AnTX-a is as potent as AcCho.

Drugs that inhibit neurotransmission at the neuromuscular junction may do so through interactions with the receptor (i.e., the AcCho binding site) or with the ion channel associated with the receptor (2). The distinction between drugs that interact at the two sites is seen most clearly in biophysical studies. Effects associated with ion channel blockade include a nonlinearity of the end-plate current–membrane potential relationship and an alteration in the lifetime of the individual ion channels. In such studies, drugs that interact with the channel and not the receptor have been identified, and a number of these have proven useful in radiolabeled forms as direct biochemical probes of the channel (e.g., [^{3}H]H_{12}-HTX and [^{3}H]PCP). Although these compounds have been termed "channel probes," the precise relationship of their binding sites to the ion channel has not been established. There is no clear biochemical or biophysical evi-

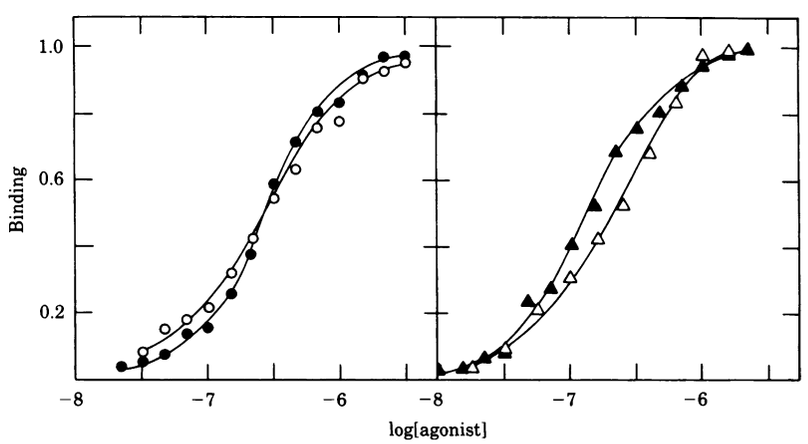

FIG. 3. Stimulation of channel probe binding by AnTX-a and AcCho. A suspension of *Torpedo* membranes was incubated with 1 nM [^{3}H]H_{12}-HTX (△, ▲), 2 nM [^{3}H]PCP (○), or 50 nM [^{3}H]PCP-MeI (●) and the indicated concentration of AnTX-a (○, ●, △) or AcCho (▲) as agonist. After 30 sec the reaction was quenched by filtration. The degree of stimulated binding is expressed as the fraction of maximal binding stimulation. Each point represents the mean from three or four separate determinations which varied by less than 5%.

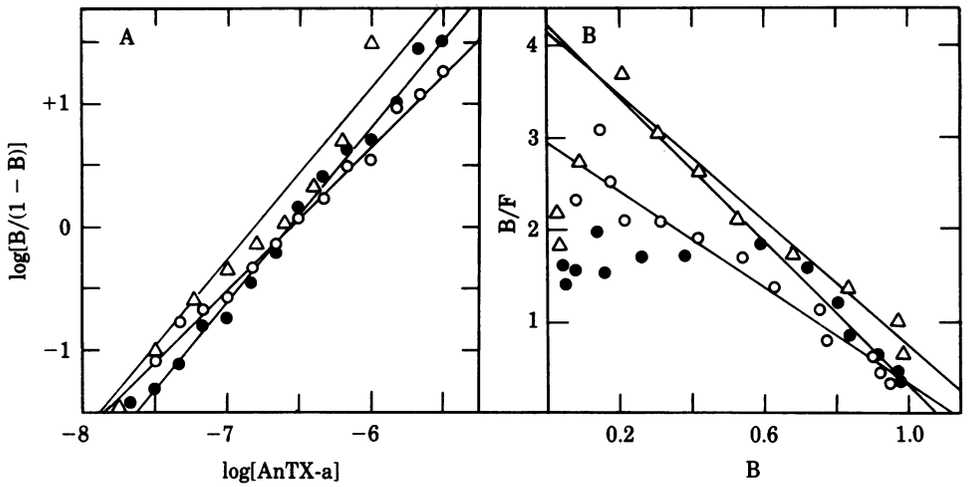

FIG. 4. Hill (*A*) and Scatchard (*B*) plots describing the concentration dependency for stimulation of ion channel binding by AnTX-a are presented. The channel probes were 1 nM [^{3}H]H_{12}-HTX (△), 2 nM [^{3}H]PCP (○), or 50 nM [^{3}H]PCP-MeI (●). Lines are drawn according to linear least-squares regression analyses, and the constants associated with these analyses are presented in Table 1. Certain points are off the scale used in the Hill plots but were included in the mathematical analyses. Each data point represents the mean binding at a given AnTX-a concentration of data pooled from three or four separate experiments. The Scatchard relationship was not linear over the entire response range; points representing values of B < 0.2 were eliminated from the Scatchard regression analysis of the effect of AnTX-a on [^{3}H]H_{12}-HTX binding; values of B < 0.37 were omitted from the corresponding analysis for AnTX-a stimulation of [^{3}H]PCP-MeI binding. B, fractional enhancement of specific ion channel binding induced by various concentrations of AnTX-a; F, concentration of AnTX-a in μM.

dence that these probes bind directly to the ion channel rather than to a site adjacent to the channel. Preliminary evidence suggests that different channel probes do not bind to identical sites on the receptor–channel complex (unpublished results).

Cholinergic ligands have been shown to affect the equilibrium binding of the channel probe [^{3}H]H_{12}-HTX and vice versa, although the magnitude of these effects is not great (12). The kinetics of [^{3}H]H_{12}-HTX binding were then shown to be altered to a much greater extent, such that the initial rate of [^{3}H]H_{12}-HTX binding can be accelerated several hundredfold (1) and therefore can serve as a more sensitive (as well as more easily monitored) probe of postsynaptic molecular events. The significance of these observations is not yet clear; however, it was initially suggested that [^{3}H]H_{12}-HTX binds only to sites associated with the ion channel that are exposed upon activation (i.e., opening) of the channel, a process under the control of receptor agonists. The extremely slow approach to equilibrium suggested that the availability of binding sites is rate limiting and that agonists accelerate the binding by exposing binding sites. However, AcChoR antagonists also accelerate (albeit weakly) [^{3}H]H_{12}-HTX and [^{3}H]PCP binding, indicating that the conformational changes responsible for the exposure of channel probe binding sites are not identical to those movements involved in the channel opening. Moreover, [^{3}H]H_{12}-HTX appears to bind to the same population of sites in the presence and in the absence of receptor ligands, although spontaneous opening of the ion channel is not observed in biophysical studies. In addition, certain aspects of H_{12}-HTX blockade of end-plate currents are elucidated in the absence of receptor stimulation (and, presumably, channel opening) (2).

The influences of receptor ligands on a second channel blocker/probe, [^{3}H]PCP, are somewhat different. The initial rate of [^{3}H]PCP binding is increased only 2- to 3-fold by receptor agonists although the equilibrium position of [^{3}H]PCP binding is directly related to the concentration of agonist. On the other hand, [^{3}H]H_{12}-HTX binding rates may be accelerated several hundredfold while equilibrium binding levels remain independent of the concentration, or even the presence, of receptor ligands. Thus, [^{3}H]PCP binding sites do not appear to be spontaneously "exposed" as are those of [^{3}H]H_{12}-HTX but rather require the action of a receptor ligand.

AnTX-a has the pharmacological properties of a depolarizing neuromuscular blocker (3). In the present work we demonstrate that AnTX-a occupies [^{3}H]AcCho and [^{3}H]dTC binding sites on the nicotinic receptor at the same concentrations at which it induces conformational changes in the postsynaptic receptor–ion channel complex, as signaled by alterations in the binding kinetics of radiolabeled ion channel probes. Moreover, this induction of conformational changes can be lessened by compounds that would prevent the occupancy of receptor AcCho

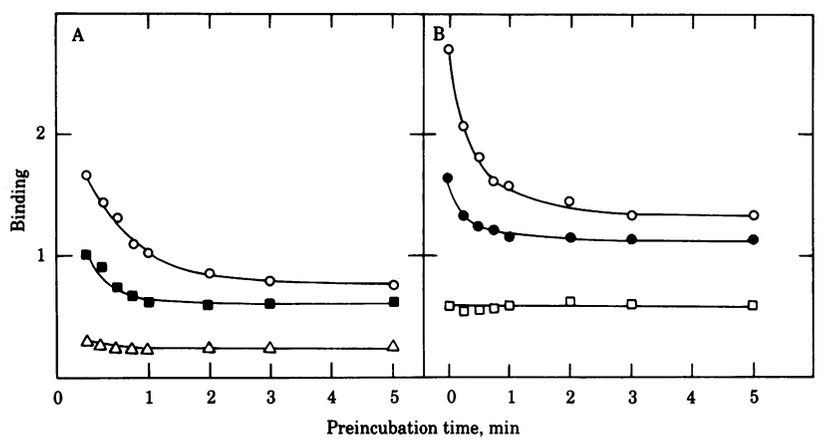

FIG. 5. Effect of preincubation with AnTX-a on the binding of [^{3}H]H_{12}-HTX and [^{3}H]PCP to electric organ tissue. *Torpedo* membranes were incubated in medium containing 2.2 (○), 0.5 (●), 0.46 (■), 0.15 (□), or 0.1 (△) μM AnTX-a for the times indicated on the abscissa before an ion channel binding reaction was initiated by the addition of 1 nM [^{3}H]H_{12}-HTX (*A*) or 2 nM [^{3}H]PCP (*B*). Binding was measured by filtration after a 30-sec incubation. Nonspecific binding has been subtracted.

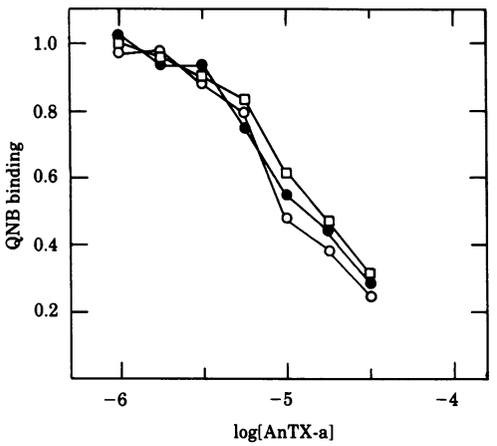

FIG. 6. Influence of AnTX-a on muscarinic AcChoR. The binding of 0.1 nM [^{3}H]QNB to muscarinic receptors in membranes isolated from the hippocampus (○), cerebral cortex (●), or brainstem (□) of rat brains in the presence of AnTX-a is illustrated. Binding, expressed as the fraction of total specific binding, was 2.5 times greater in the cortex and hippocampus than in the brainstem. Nonspecific binding was determined in the presence of 1 μM atropine. Each point represents the mean of three determinations.

binding sites by AnTX-a. The Hill coefficients for the interaction by which AnTX-a occupies the AcCho binding site and the reaction by which AnTX-a stimulates channel binding are greater than unity (n_h = 1.2–1.7). AnTX-a binds to the nicotinic AcChoR and, as a consequence of this binding, induces conformational changes in postsynaptic membrane proteins. Although the concentrations of AnTX-a at which receptor occupancy and conformational changes are half-maximal are in agreement, there are substantial deviations in the observed concentration–response relationships from simple mass action expressions. Obviously, a number of processes are occurring simultaneously, such as ion channel activation and desensitization, processes not well understood. By the nature of the assay methods used, we are sampling at any one time the average distribution of receptor–channel complexes between a number of different states [e.g., resting, "activated," "desensitized," and other(s)].

There is substantial agreement between the present biochemical results and those obtained by biophysical measurements of synaptic transmission at the neuromuscular junction (6). In particular, AnTX-a activates nicotinic AcChoRs in the same concentration ranges and with the same potency as AcCho, and there is no evidence from either approach to indicate a direct interaction of AnTX-a with the ion channel (6). There is also a degree of agreement in the rate of onset of desensitization, although the rates of desensitization determined in the present study (k_1 = 0.046–0.03 sec^{-1}) are somewhat slower than those determined electrophysiologically (0.084 sec^{-1}) (6). The reasons for this discrepancy are not obvious but, in light of the different preparations and techniques used in the two approaches, they are hardly surprising.

There is a heterogeneity of muscarinic AcCho receptors in brain that has been attributed to the presence of multiple receptor populations which differ in their affinity for agonists but not antagonists (13). Receptors in membranes isolated from different areas of the brain differ greatly in their affinity for agonists, and these differences are consistent with differing distributions between forms with high and low affinity for agonists (14, 15). For example, 70% of muscarinic receptors in membranes isolated from the brainstem appear to be in high-affinity form(s), whereas only 30% of cortical receptors display high-affinity agonist binding. A conversion of receptors from low- to high-affinity form by reductive alkylation with *N*-ethylmaleimide has also been demonstrated (16). Thus, one would expect muscarinic agonists to inhibit the binding of [^{3}H]QNB (an antagonist) to brainstem more strongly than to cortex receptors and to inhibit [^{3}H]QNB binding more strongly to *N*-ethylmaleimide-treated than to untreated cortical receptors. In both of these, AnTX-a fails to behave as a typical agonist; its weak inhibition of [^{3}H]QNB binding is largely unaffected by tissue source or sulfhydryl group alkylation. AnTX-a's affinity for muscarinic receptors is comparable to that of local anesthetics and a number of hydrophobic amines that are nonspecific inhibitors of muscarinic binding.

We thank Dr. Edson X. Albuquerque (University of Maryland) for guidance during the design and interpretation of these experiments. This work was supported in part by grants from the National Institutes of Health (DA 02834) and the Pharmaceutical Manufacturers Association Foundation.

1. Eldefrawi, M. E., Aronstam, R. S., Bakry, N. M., Eldefrawi A. T. & Albuquerque, E. X. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2309–2313.
2. Albuquerque, E. X. & Oliveira, A. C. (1979) in *Advances in Cytopharmacology*, eds. Ceccarelli, B. & Clementi, F. (Raven, New York), Vol. 3, pp. 197–211.
3. Carmichael, W. W., Briggs, D. F. & Gorham, P. R. (1975) *Science* **187**, 542–544.
4. Carmichael, W. W., Briggs, D. F. & Peterson, M. A. (1979) *Toxicon* **17**, 229–236.
5. Devlin, J. P., Edwards, E. O., Gorham, P. R., Hunter, N. R. Pike, R. K. & Stavric, B. (1972) *Can. J. Chem.* **55**, 1367–1371.
6. Spivak, C. E., Witkop, B. & Albuquerque, E. X. (1980) *Mol. Pharmacol.* **18**, 384–394.
7. Huber, C. S. (1972) *Acta Crystallogr. Ser. B* **28**, 2577–2582.
8. Spivak, C. E., Albuquerque, E. X., Aronstam, R. S. & Eldefrawi, M. E. (1979) *Soc. Neurosci. Abstr.* **5**, 489.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
10. Aronstam, R. S., Triggle, D. J. & Eldefrawi, M. E. (1979) *Mol. Pharmacol.* **15**, 227–235.
11. Tsai, M.-C., Mansour, N. A., Eldefrawi, A. T., Eldefrawi, M. E. & Albuquerque, E. X. (1978) *Mol. Pharmacol.* **14**, 787–803.
12. Elliott, J. & Raftery, M. A. (1979) *Biochemistry* **18**, 1868–1874.
13. Birdsall, N. J. M., Burgen, A. S. V. & Hulme, E. C. (1978) *Mol. Pharmacol.* **14**, 723–736.
14. Aronstam, R. S., Abood, L. G. & Hoss, W. (1978) *Mol. Pharmacol.* **14**, 575–586.
15. Aronstam, R. S., Kellogg, C. & Abood, L. G. (1979) *Brain Res.* **162**, 231–241.
16. Aronstam, R. S., Hoss, W. & Abood, L. G. (1977) *Eur. J. Pharmacol.* **46**, 279–282.