Binding of Perhydrohistrionicotoxin to Intact and Detergent-Solubilized Membranes Enriched in Nicotinic Acetylcholine Receptor†

Janet Elliott and Michael A. Raftery*

ABSTRACT: A tritiated derivative of perhydrohistrionicotoxin was prepared and characterized and used in direct binding studies to membrane fragments from *Torpedo californica* electroplax. Perhydrohistrionicotoxin bound to a site distinct from the α-bungarotoxin and agonist binding sites in a ratio of one perhydrohistrionicotoxin bound per four α-bungarotoxin sites. In the presence of 10 μM carbamylcholine, the dissociation constant for perhydrohistrionicotoxin was lowered from 0.57 to 0.27 μM in *Torpedo* Ringer's solution and from 0.9 to 0.5 μM in the absence of divalent cations. Perhydrohistrionicotoxin binding activity was solubilized from the membranes by the anionic detergent sodium cholate but was sensitive to detergent concentration. Binding activity was lost reversibly at cholate concentrations above 0.6%, and the addition of Triton X-100 resulted in loss of binding activity at extremely low concentrations. The perhydrohistrionicotoxin and agonist binding sites, which are conformationally linked in the membranes, are also closely associated in cholate extracts. The ratio of perhydrohistrionicotoxin binding sites to α-bungarotoxin binding sites remained constant upon solubilization. Treatment of the extract with a resin made by coupling α-bungarotoxin to Sepharose 2B resulted in the removal of α-bungarotoxin and perhydrohistrionicotoxin binding activities. The fluorescent probe of receptor function, ethidium bromide, was used to measure perhydrohistrionicotoxin binding to membrane fragments and to cholate extracts and yielded I_{50} values for the quenching of specific fluorescence which were comparable to the dissociation constants obtained by direct binding.

The apparatus in the postsynaptic membrane of the neuromuscular junction which is responsible for acetylcholine-mediated depolarization is thought to consist of at least two functionalities: (1) an AcCh[1] recognition site which binds agonists such as AcCh or Carb and antagonists such as curare and α-BuTx and (2) a site responsible for ion translocation. Histrionicotoxin (HTX), an alkaloid isolated from skin extracts of the Columbian arrow poison frog *Dendrobates histrionicus*, and its fully saturated derivative perhydrohistrionicotoxin (H_{12}-HTX) are thought to interact with or exert control on this later component.

Electrophysiological evidence demonstrated that HTX and H_{12}-HTX reversibly blocked neuromuscular transmission, acting only in the presence of agonists (Albuquerque et al., 1973a,b), and blocked the steady-state depolarization produced by Carb on the isolated *Electrophorus electricus* electroplax (Kato & Changeux, 1976). Biochemical studies have shown that HTX is not competitive with α-BuTx or agonists (Dolly et al., 1977; Kato & Changeux 1976). We have shown previously that [^{3}H]H_{12}-HTX binds to AcChR-enriched *Torpedo californica* electroplax membrane fragments in a ratio of one [^{3}H]H_{12}-HTX bound per four [^{125}I]-α-BuTx sites, with a dissociation constant of 0.3-0.5 μM (Elliott & Raftery, 1977). This site, although distinct, is conformationally linked with the AcCh recognition site, since HTX has been reported to increase the affinity of membrane-bound AcChR for [^{3}H]AcCh (Kato & Changeux, 1976) and the presence of Carb increased the affinity of membranes for [^{3}H]H_{12}-HTX (Elliott & Raftery, 1977). It has been proposed that this affinity change may be the pharmacologic mode of action of HTX (Kato & Changeux, 1976; Burgermeister et al., 1977). However, we found in vitro, using AcChR-enriched membrane fragments from *T. californica*, that H_{12}-HTX did not affect the rates of interconversion between the conformational states of the membrane-bound AcChR that have high affinity and low affinity for agonists. Another proposed mode of action is actual blockage of the ion "channel" when the "channel" is in the conducting state (Albuquerque et al., 1973a,b).

The AcChR from *T. californica* has been purified in Triton X-100 (Schmidt & Raftery, 1973b), and the purified protein has been shown to bind AcCh and a variety of other cholinergic ligands (Moody et al., 1973; Martinez-Carrion & Raftery, 1973; Raftery et al., 1975). However, specific [^{3}H]H_{12}-HTX binding could not be detected in Triton extracts of membrane fragments (Elliott & Raftery, 1977). Sobel et al. (1978) have reported that HTX binding could be recovered in the precipitate accompanying Triton X-100/Berol 043 extraction of membrane fragments. A different result yet was reported by Eldefrawi et al. (1977) who chromatographed a Triton extract of *Torpedo ocellata* membranes on a Sephadex G-200 column and reported [^{3}H]H_{12}-HTX binding activity which separated from [^{3}H]AcCh binding activity. However, this solubilized binding component was not shown to be a protein nor was the observed binding demonstrated to be specific.

It is our purpose here to report work further characterizing the properties of the HTX binding site in membrane fragments from *T. californica* electroplax and in detergent solutions obtained from such membranes with direct binding of a radiolabeled derivative. In addition, the effects of H_{12}-HTX on a fluorescent probe of receptor function, ethidium bromide, are discussed.

Experimental Section

Materials. AcChR-enriched membrane fragments were prepared as previously described (Duguid & Raftery, 1973; Reed et al., 1975) from *T. californica* electroplax obtained from freshly killed animals or from electric organs stored at

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received July 11, 1978; revised manuscript received December 15, 1978.* Contribution No. 5818. Supported by U.S. Public Health Service Grants NS-10294, GM-16424, and NS-10218, by a grant from the Muscular Dystrophy Association of America, and by a National Institutes of Health Predoctoral Fellowship (to J.E.)

[1] Abbreviations used: AcCh, acetylcholine; Carb, carbamylcholine; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; H_8-HTX, octahydrohistrionicotoxin; α-BuTx, α-bungarotoxin; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate.

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−90 °C. The buffer used throughout the preparation was 10 mM sodium phosphate, 400 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4. The concentration of [^{125}I]-α-BuTx binding sites was determined by DEAE-cellulose disc assay (Schmidt & Raftery, 1973a) with [^{125}I]-α-BuTx prepared from *Bungarus multicinctus* venom (Miami Serpentarium) by the procedure of Clark et al. (1972). [^{3}H]Carb was a generous gift of Dr. Yuan Chao. Detergent extracts of membrane fragments were prepared by stirring the membranes with detergent for approximately 0.5 h at 2 °C, followed by centrifugation for 1 h at 100000*g*.

H_8-HTX and H_{12}-HTX were the kind of gifts of Dr. Y. Kishi, who produced them synthetically (Aratani et al., 1975; Fukuyama et al., 1975). [^{3}H]H_{12}-HTX was prepared at ICN Pharmaceuticals, Irvine, CA, by the catalytic reduction of H_8-HTX with carrier-free $^3H_2$ over Pd/C as previously described (Tokuyama et al., 1974). The product was purified by preparative thin-layer chromatography on silica gel in 9:1 chloroform–methanol. The [^{3}H]H_{12}-HTX was routinely checked for purity by analytical thin-layer chromatography and was found to be stable for extended periods up to 1 year following initial purification.

Methods. [^{3}H]H_{12}-HTX binding to AcChR-enriched membrane fragments was measured by a centrifugation assay and by equilibrium dialysis. In the centrifugation assay, 150-μL samples containing membrane fragments and [^{3}H]-H_{12}-HTX were incubated for 20 min at room temperature. The membranes (in *Torpedo* Ringer's solution) were then pelleted by centrifugation for 20 min at 30 psi (g_{av} = 132000) in a Beckman airfuge. Samples (10 μL) from the top of each centrifuge tube (in triplicate) were counted *before and after centrifugation* in 5 mL of Triton–toluene scintillation fluid (in a Packard Model 3375 scintillation spectrometer). Binding to detergent extracts was measured by equilibrium dialysis. Sample (0.5 mL) plus [^{3}H]H_{12}-HTX was dialyzed against 1.5 mL of Ringer's solution plus [^{3}H]H_{12}-HTX for 16 h at 4 °C with gentle shaking. Results obtained for membrane fragments in the centrifugation assay were the same as those obtained by equilibrium dialysis. Unless otherwise stated, all binding experiments were performed in *Torpedo* Ringer's solution (250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 20 mM Hepes, pH 7.4, and 0.02% NaN_3). Scatchard plots (Scatchard, 1949) of binding data were fit with an unweighted linear, least-squares fit.

Fluorescence measurements were made in a Perkin-Elmer MPF-4 spectrofluorimeter thermostatted to 25 ± 1 °C with an excitation wavelength of 483 nm and with emission monitoring at 610 nm. Ethidium bromide was obtained from Calbiochem. $NaDodSO_4$–polyacrylamide gels were run according to the method of Laemmli (1970).

Results

Characterization of [^{3}H]H_{12}-HTX. After purification, the [^{3}H]H_{12}-HTX was homogeneous by thin-layer chromatography and cochromatographed with unlabeled H_{12}-HTX (Figure 1). The purified product bound to membrane fragments and was displaced by increasing amounts of cold H_{12}-HTX with I_{50} equal to approximately 0.5 μM (Figure 2). For use in binding assays the [^{3}H]H_{12}-HTX was isotopically diluted approximately 100-fold with unlabeled H_{12}-HTX, and the specific activity was therefore defined by the known concentration of the unlabeled toxin. Two dilutions were used for the work reported here with specific activities of 1.03 and 1.38 Ci/mmol. The dissociation constants for binding to *T. californica* membrane fragments obtained with the two dilutions were the same within experimental error (K_d = 0.57 ± 0.06 μM). In order to verify the specific activity of the [^{3}H]H_{12}-HTX dilutions, we measured inhibition of binding by two concentrations of unlabeled H_{12}-HTX (Figure 3). This experiment yielded a K_d for [^{3}H]H_{12}-HTX of 0.2 μM and K_I for unlabeled H_{12}-HTX of 0.3 μM.

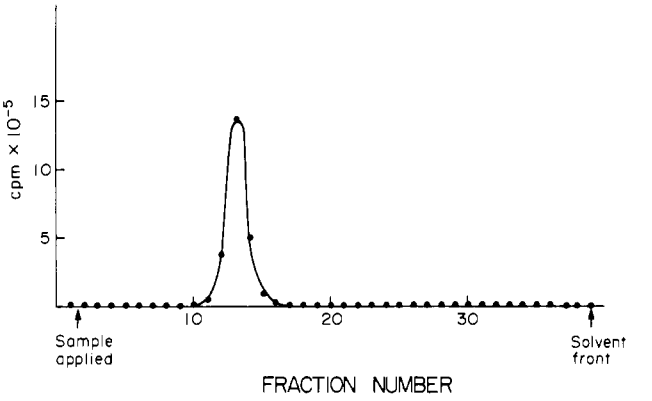

FIGURE 1: Thin-layer chromatography of purified [^{3}H]H_{12}-HTX in 9:1 chloroform–methanol on a silica plate. The plate was cut into pieces approximately 1/8-in. wide, which were counted in 5 mL of scintillation fluid. The [^{3}H]H_{12}-HTX comigrated with authentic H_{12}-HTX which was detected by staining the plate with iodine.

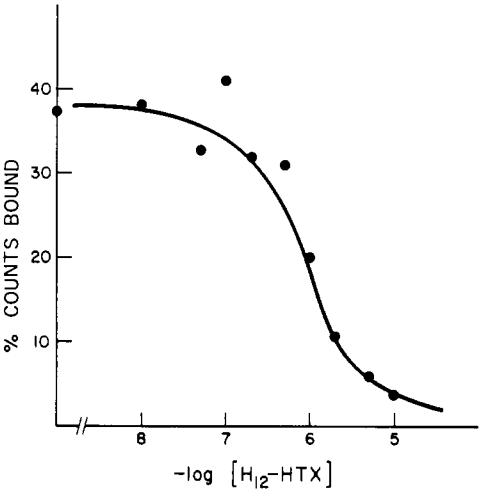

FIGURE 2: Displacement of undiluted [^{3}H]H_{12}-HTX by cold H_{12}-HTX. Binding to membrane fragments, having a concentration of 0.5 μM in [^{125}I]-α-BuTx sites, was measured by centrifugation assay in order to show that all [^{3}H]H_{12}-HTX binding could be displaced by cold H_{12}-HTX. The concentration of the [^{3}H]H_{12}-HTX before isotopic dilution was not determined.

Binding to AcChR-Enriched Membrane Fragments. As has been previously reported (Elliott & Raftery, 1977), the binding of [^{3}H]H_{12}-HTX to membrane fragments could be resolved into two components, a hyperbolic saturable binding isotherm superimposed on a linear, nonsaturable uptake. The saturable component was completely inhibited by competition with 40 μM unlabeled H_{12}-HTX and in this way the linear component was determined and subtracted from the total binding to yield the saturable component alone. This component followed a normal hyperbolic curve characteristic of a single class of noninteracting binding sites (see Figure 7A). The number of binding sites was found to equal one-fourth of the number of [^{125}I]-α-BuTx binding sites present in the preparation.

[^{3}H]H_{12}-HTX bound with slightly lower affinity in the absence of divalent cations than in *Torpedo* Ringer's solution. In both cases the addition of 10 μM Carb resulted in an approximately twofold lowering of the dissociation constant without affecting the total number of HTX binding sites (Figure 4A,B and Table I). On the other hand, the presence

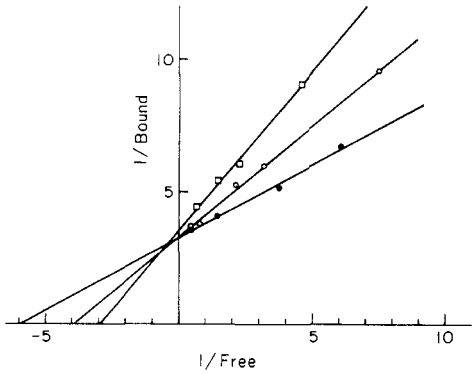

FIGURE 3: Inhibition of the binding of the isotopically diluted [^{3}H]H_{12}-HTX by cold H_{12}-HTX. This experiment was a check on the specific activity of the isotopically diluted [^{3}H]H_{12}-HTX which was used for all further binding experiments. The concentration of the membrane fragments was 1.19 μM in [^{125}I]-α-BuTx sites. (●) Concentration of cold H_{12}-HTX was zero. (○) Concentration of cold H_{12}-HTX was 0.22 μM. (□) Concentration of cold H_{12}-HTX was 0.44 μM. The cold H_{12}-HTX concentrations were corrected for nonspecific binding. The units of bound and free ligand are micromolar.

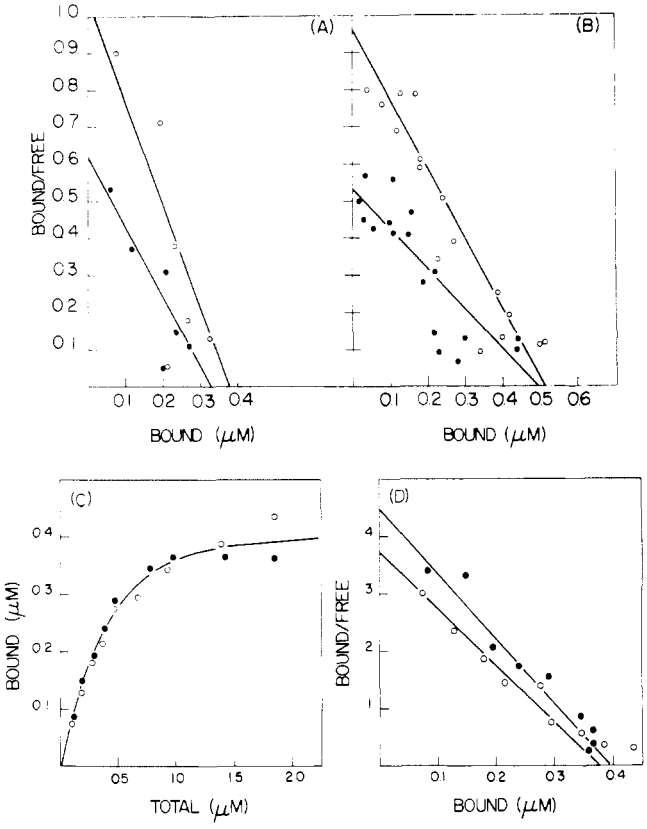

FIGURE 4: (A) Effect of Carb on [^{3}H]H_{12}-HTX binding to membrane fragments. The concentration of [^{125}I]-α-BuTx sites was 1.3 μM. The buffer was *Torpedo* Ringer's solution. (●) No Carb present. (○) Carb concentration was 10 μM. (B) [^{3}H]H_{12}-HTX binding to membrane fragments in the presence of 2 mM EDTA. The concentration of [^{125}I]-α-BuTx sites was 1.84 μM. The buffer was 250 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4, 2 mM EDTA, and 0.02% NaN_3. (●) No carb present. (○) Carb concentration was 10 μM. (C) Effect of H_{12}-HTX on [^{3}H]Carb binding to membrane fragments. The membranes were 0.83 μM in [^{125}I]-α-BuTx sites. (●) No H_{12}-HTX present. (○) H_{12}-HTX concentration was 5 μM. (D) Scatchard plot of [^{3}H]Carb binding data. (●) No H_{12}-HTX present. (○) H_{12}-HTX concentration was 5 μM.

of 5 μM H_{12}-HTX did not significantly affect [^{3}H]Carb binding to membrane fragments (Figure 4C,D). The dissociation constant for [^{3}H]Carb was 0.09 ± 0.01 μM in the absence of H_{12}-HTX; in the presence of H_{12}-HTX the value

Table I

buffer	concn of BuTx sites (μM)	K_d in absence of carb (μM)	K_d in presence of 10 μM carb (μM)	n^a in absence of carb	n^a in presence of 10 μM carb
b	1.30	0.5	0.3	0.27	0.29
b	1.84	0.6	0.3	0.22	0.17
b	1.45	0.6	0.2	0.26	0.22
c	1.84	0.9	0.5	0.27	0.28

[a] n denotes the number of specific [^{3}H]H_{12}-HTX binding sites compared with those for [^{125}I]-α-BuTx. [b] Buffer: 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 20 mM Hepes, pH 7.4, and 0.02% NaN_3. [c] Buffer: 250 mM NaCl, 5 mM KCl, 2 mM EDTA, 20 mM Hepes, pH 7.4, and 0.02% NaN_3.

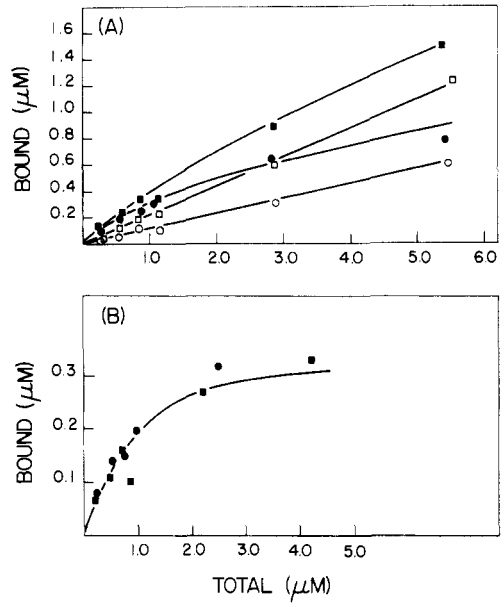

FIGURE 5: Effect of saturating α-BuTx on [^{3}H]H_{12}-HTX binding to membrane fragments. The concentration of the membrane fragments was 1.15 μM in [^{125}I]-α-BuTx sites. (A) (●) No α-BuTx present, total binding. (○) No α-BuTx present, binding in the presence of 40 μM H_{12}-HTX; (■) Membranes which been incubated with 2.3 μM α-BuTx, total binding. (□) α-BuTx (2.3 μM), binding in the presence of 40 μM H_{12}-HTX. (B) Specific component of binding only. (●) No α-BuTx. (■) α-BuTx (2.3 μM).

obtained was 0.10 ± 0.01 μM. The number of [^{3}H]Carb sites in the first case was 0.48 of the α-BuTx sites and 0.46 of the α-BuTx sites in the second experiment.

It has previously been shown that α-BuTx binds to a site distinct from the H_{12}-HTX binding site (Dolly et al., 1977; Elliott & Raftery, 1977). However, the presence of saturating amounts of α-BuTx had an interesting effect on the observed binding of [^{3}H]H_{12}-HTX: the dissociation constant and number of binding sites were unaffected but the amount of nonspecific binding was very nearly doubled (Figure 5).

Solubilization of an [^{3}H]H_{12}-HTX Binding Component. A specific [^{3}H]H_{12}-HTX binding component(s) could be solubilized from membrane fragments by the anionic detergent sodium cholate as distinct from Triton X-100 (Elliott & Raftery, 1977). Above 0.6% cholate, which is near the critical micelle concentration of the detergent, specific binding rapidly fell off (Figure 6A). This loss of binding activity was reversible since a 2% cholate extract could be diluted to 0.5% cholate with restoration of binding activity to the level of one-fourth of the [^{125}I]-α-BuTx sites present.

This reconstitution of [^{3}H]H_{12}-HTX binding activity was accompanied by aggregation, which was manifested by a large

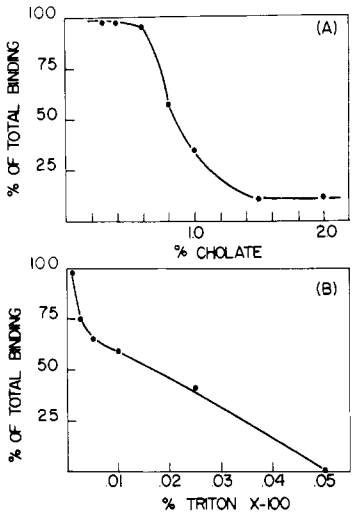

FIGURE 6: Effect of detergents on $[^3H]H_{12}$-HTX binding. (A) A 2% cholate extract was diluted to various final cholate concentrations. The concentration of $[^{125}I]$-α-BuTx sites was constant at 1.07 μM in one experiment and 0.8 μM in another, and results were combined and normalized. The percent of the total binding was plotted as a function of cholate concentration. (B) A 2% cholate extract was diluted to a final cholate concentration of 0.5% cholate, and small amounts of Triton X-100 were added. The concentration of $[^{125}I]$-α-BuTx sites was 0.81 μM. Percent of total binding was plotted as a function of Triton concentration.

increase in light scattering of the sample. If a 2% cholate extract was diluted to 0.5% cholate and again centrifuged at 100000g for 1 h, approximately half the protein and $[^{125}I]$-α-BuTx binding activity were lost in the pellet.

The residue, which was not solubilized upon treatment of membrane fragments with 2% cholate, was washed several times by resuspension in cholate-free buffer and was recovered by recentrifugation. Specific $[^3H]H_{12}$-HTX binding to this material could be measured by airfuge assay. Figure 7 shows a comparison of $[^3H]H_{12}$-HTX binding to *T. californica* membrane fragments, to a 2% cholate extract diluted to 0.4% cholate, and to the cholate-insoluble pellet. The partitioning of binding into specific and nonspecific components was similar in each of these preparations. The ratio of $[^3H]H_{12}$-HTX binding sites to $[^{125}I]$-α-BuTx sites did not change significantly, while the dissociation constant was higher for the extract and residue; the K_d for the membranes in this experiment was 0.56 μM, for the extract it was 1.6 μM, and for the residue it was 0.86 μM.

We showed previously that no specific $[^3H]H_{12}$-HTX binding could be demonstrated in Triton X-100 extracts of membrane fragments (Elliott & Raftery, 1977). This result was confirmed in the studies reported here in that the addition of small amounts of Triton X-100 to a diluted cholate extract (final cholate concentration 0.5%) obliterated the specific component of binding (Figure 6B). It is worth noting also that no visible precipitate was observed in the samples to which Triton X-100 had been added.

Ethidium Bromide Fluorescence. Ethidium bromide has been shown to bind to *T. californica* AcChR-enriched membrane fragments (Schimerlik & Raftery, 1976) and to respond to cholinergic ligand binding by a change in quantum yield of the bound dye (Schimerlik et al., 1979a). Specific ethidium bromide fluorescence, defined as the difference in fluorescence in the presence and absence of α-BuTx (Schimerlik & Raftery, 1976), was quenched by H_{12}-HTX in experiments with both membrane fragments and 2% cholate extracts diluted to 0.4% cholate (Figure 8A and 8B). The I_{50} values obtained for HTX were 0.25 μM for membrane fragments and 0.45 μM for cholate extracts under the experimental conditions described in the figure legend. The plot of specific fluorescence vs. H_{12}-HTX concentration was a simple titration curve for membrane fragments, whereas for the cholate extract it was steeper and did not level off at zero specific fluorescence. In four experiments with cholate extracts, the amount of specific fluorescence quenched ranged from 69 to 90% and averaged 78% of the total specific fluorescence. In the presence of 40 μM Carb, the I_{50} for HTX binding membrane fragments was 0.36 μM and was 0.71 μM for the cholate extract (Figure 8A and 8B), both determined by the fluorescence method.

It is unlikely that the quenching of ethidium fluorescence involved an actual displacement, since H_{12}-HTX did not displace $[^3H]$ethidium at concentrations up to 30 μM (Schimerlik et al., 1979a) and $[^3H]H_{12}$-HTX was displaced

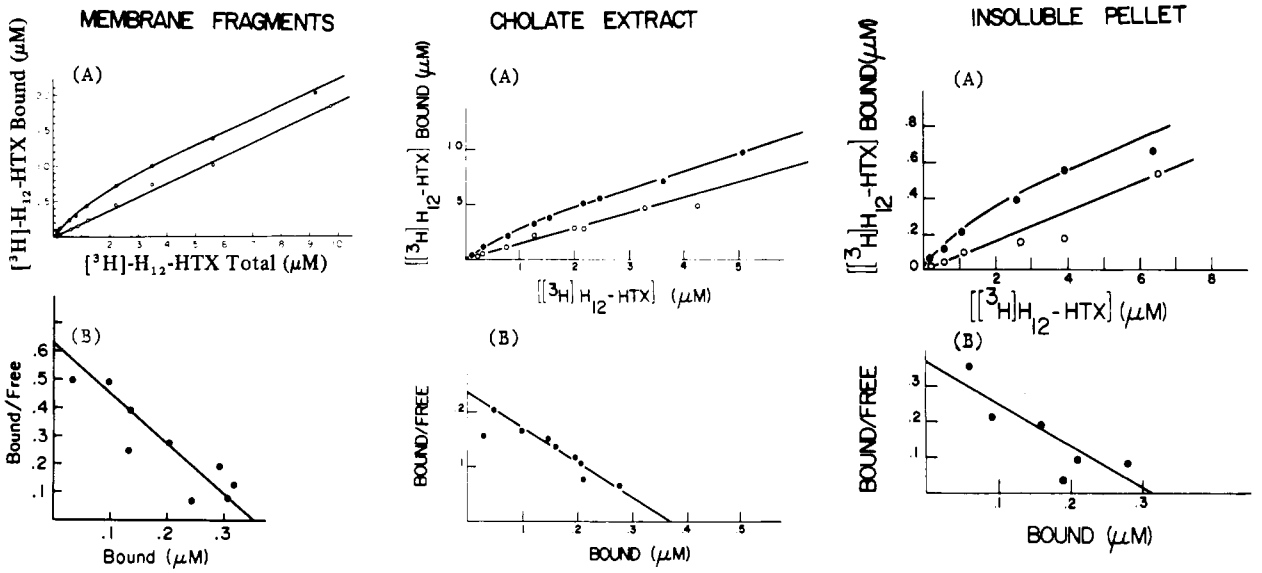

FIGURE 7: $[^3H]H_{12}$-HTX binding to membrane fragments, cholate extract, and the insoluble residue. Membrane fragment concentration was 1.45 μM in $[^{125}I]$-α-BuTx sites. Cholate extract concentration was 1.38 μM in $[^{125}I]$-α-BuTx sites. Insoluble residue was resuspended in *Torpedo* Ringer's solution to a concentration of 1.18 μM in $[^{125}I]$-α-BuTx sites. (A) Total binding in the absence (●) and presence (○) of 40 μM H_{12}-HTX. (B) Scatchard plot of the specific component of binding.

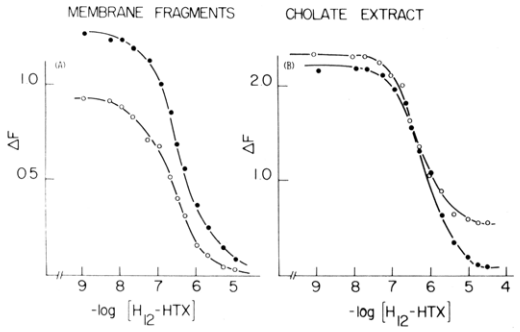

FIGURE 8: Ethidium bromide fluorescence. The difference in fluorescence in the presence and absence of a fivefold molar excess of α-BuTx is plotted vs. the H_{12}-HTX concentration. (A) Membrane fragments having 0.31 μM in [^{125}I]-α-BuTx sites. Ethidium concentration was 0.5 μM. (O) No carb present. (●) Carb concentration was 40 μM. (B) Cholate extract with final cholate concentration of 0.4% and final concentration of [^{125}I]-α-BuTx sites of 1.1 μM. Ethidium concentration was 1.16 μM. (O) No carb present. (●) Carb concentration was 40 μM.

by ethidium only at high ethidium concentrations. In experiments not shown, ethidium displaced [^{3}H]H_{12}-HTX binding to membrane fragments with an apparent K_I of 25 μM, whereas K_d determined directly for [^{3}H]ethidium was 3.2 μM (Schimerlik et al., 1979a).

Binding to α-BuTx–Sepharose 2B. Treatment of a 2% cholate extract with a resin consisting of α-BuTx coupled to Sepharose 2B resulted in loss of specific [^{3}H]H_{12}-HTX binding as measured by equilibrium dialysis following dilution of the extract to 0.5% cholate as well as loss of [^{125}I]-α-BuTx binding activity (Figure 9A). Most of the protein, however, was not bound to the resin. After rinsing the resin with the cholate buffer used in the experiment (2% cholate, 250 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4, and 0.02% NaN_3) or with cholate-free *Torpedo* Ringer's solution, we treated the resin with 8 M urea, 1% $NaDodSO_4$, and 0.05 M Tris, pH 8.8. The material thus removed from the resin was analyzed by $NaDodSO_4$ gel electrophoreses (Figure 9B). The four bands identified as constituent polypeptides of the purified AcChR of molecular weight 40 000, 50 000, 60 000, and 65 000 (Raftery et al., 1974, 1975; Weill et al., 1974; Karlin et al., 1975; Vandlen et al., 1976; Lindstrom et al., 1978) were the major components present in the material that bound strongly to the resin (Figure 9B, left). Figure 9B, right, shows material that was bound to the resin after the milder treatment with *Torpedo* Ringer's solution containing 2% cholate. Most of the 43 000-dalton band frequently observed in *Torpedo* membrane preparations (Sobel et al., 1978; Witzemann & Raftery, 1978) appeared in the supernatants which did not bind to the resin in either experiment.

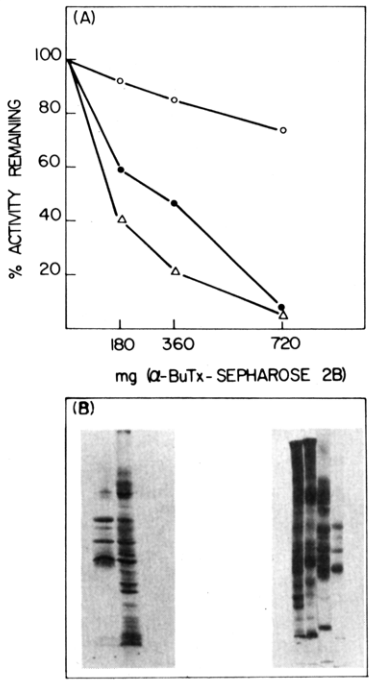

FIGURE 9: (A) Effects of treatment of a 2% cholate extract with α-BuTx–Sepharose 2B. Weighed amounts of α-BuTx–Sepharose 2B were added to 1 mL of a cholate extract initially containing 1.9 μM in α-BuTx sites, 2.6 mg/mL of protein, and binding 0.18 μM [^{3}H]H_{12}-HTX after fourfold dilution to 0.5% cholate. After gentle shaking at 4 °C for 48 h, we pelleted the resin by centrifugation, and the supernatant was assayed. Both α-BuTx binding (Δ) and [^{3}H]H_{12}-HTX binding (●) were removed more rapidly than bulk protein (O). (B) $NaDodSO_4$ gels of the material that remained bound to α-BuTx–Sepharose 2B after rinsing with buffer. (Left panel) Left, material remaining bound to the resin after extensive rinsing with cholate buffer (2% cholate, 250 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4, and 0.02% NaN_3); right, the membrane fragments used in this experiment. (Right panel) Left to right, membrane fragments, the supernatant of the cholate extract which did not bind to the resin (this contains the bulk of the material migrating as the 43 000-dalton protein), and the material which remained bound to the resin after rinsing with 100 mL of *Torpedo* Ringer's solution containing 2% cholate, purified AcChR standard.

Discussion

We have previously shown that the presence of Carb lowered the dissociation constant for the binding of [^{3}H]H_{12}-HTX to *T. californica* AcChR-enriched membrane fragments (Elliott & Raftery, 1977) and therefore that the binding sites for the ligands are distinct. This same result was also obtained in the absence of divalent cations (Figure 4). Furthermore, the numbers of HTX and Carb binding sites relative to α-BuTx binding sites are 1:2:4 (Raftery et al., 1975; Elliott & Raftery, 1977). Our lowest value for *T. californica* AcChR molecular weight is 270 000 ± 30 000 (Martinez-Carrion et al., 1976), and our most recent estimates of specific activity (Vandlen et al., 1978) for α-BuTx binding indicate that two toxin molecules bind per AcChR monomer [S value ~ 9; Raftery et al. (1972)]. Recent evidence also indicates that in membrane preparations the AcChR of *T. californica* occurs mainly as a dimer [S value ~ 13.5; Raftery et al. (1972)] specifically linked by means of a disulfide bond(s) involving 65 000-dalton subunits (Hucho et al., 1978; Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978). This membrane-bound dimeric form would therefore bind four [^{125}I]-α-BuTx molecules, two Carb molecules with high affinity, and one [^{3}H]H_{12}-HTX molecule. This alteration of the $K_{d_{app}}$ for HTX in the presence of Carb clearly shows that the binding sites for these ligands are discrete and in addition that the sites are capable of interacting.

Ethidium bromide provides a probe of both cholinergic ligand– and HTX–receptor interactions (Schimerlik & Raftery, 1976; Schimerlik et al., 1979a). The enhanced fluorescence of ethidium due to its association with membrane-bound AcChR is reduced by virtue of its displacement by α-BuTx (Schimerlik et al., 1979a). The fact that the same amount of ethidium fluorescence which is quenched by α-BuTx (by displacement) can also be quenched by H_{12}-HTX [without displacement; Schimerlik et al. (1979a)] yields further evidence for conformational linkage of the cholinergic ligand and α-BuTx binding sites with the H_{12}-HTX site in the membrane-bound receptor. As a fluorescence probe of receptor properties, ethidium has been shown to be an excellent monitor of HTX–AcChR interaction and has been used to determine a kinetic mechanism (Schimerlik et al., 1979b).

The membrane-bound AcChR has been shown to exist in states having high and low affinity for agonists (Weber et al., 1975; Weiland et al., 1976, 1977; Barrantes, 1976; Colquhoun & Rang, 1976; Quast et al., 1978), and the conformational change from the low affinity to high affinity form(s) has been compared with the desensitization process observed by electrophysiologists (Katz & Thesleff, 1957; Rang & Ritter, 1970a,b). It has been proposed that HTX and its derivatives may block neuromuscular transmission by stabilizing the high affinity nonconducting ("desensitized") state of the AcChR (Kato & Changeux, 1976; Burgermeister et al., 1977). As shown here, we found no increase in the affinity of the membranes for [^{3}H]Carb in the presence of H_{12}-HTX; however, since Carb is a strong effector the fraction of ligand bound at equilibrium and the degree of desensitization are very nearly the same (Lee et al., 1977; Quast et al., 1978) so that any change in equilibria between states induced by HTX would only be detected at extremely low [^{3}H]Carb concentration. That this effect would probably be small can also be inferred from the finding that 10 μM Carb, which would have completely converted the membranes rapidly to the high affinity state with $t_{1/2} \sim 50$ s (Lee et al., 1977), caused only a twofold increase in affinity for [^{3}H]H_{12}-HTX. Any effect of 3 μM H_{12}-HTX on conversion to a high affinity state toward Carb was too small to be detected by an assay which involved monitoring the affinity for Carb by its inhibition of the rate of [^{125}I]-α-BuTx binding of AcChR with and without preincubation (Elliott & Raftery, 1977). Burgermeister et al. (1977) recently reported that HTX induced conversion of an AcChR preparation to a state of high ligand affinity. However, the embryonic chick muscle cell preparation used may not be comparable to *Torpedo* electroplax membrane preparations since it seems to have very different affinity for agonists; 20 μM Carb was reported not to cause desensitization, whereas this concentration would have completely converted *Torpedo* electroplax membrane AcChR extremely rapidly to the high affinity state ($t_{1/2} < 30$ s) [see Lee et al. (1977)]. Thus, with the in vitro *Torpedo* AcChR membrane system, there is no conclusive evidence that the mechanism of HTX receptor blockage is by conversion of the system to the high affinity state induced by agonists. Furthermore, kinetic evidence (Schimerlik et al., 1979b) supports the notion that the final receptor conformations induced by cholinergic agonists on the one hand and by HTX on the other are indeed different.

The [^{3}H]H_{12}-HTX binding protein has also been reported to be a component separable in Triton solution from the AcCh "recognition protein" (Eldefrawi et al., 1977) or a protein of molecular weight 43 000 not solubilized at all by Triton (Sobel et al., 1978). The results reported here suggest that even low levels of Triton may mask or denature the [^{3}H]H_{12}-HTX binding site. If it were the case that the [^{3}H]H_{12}-HTX binding protein is distinct from the classical AcChR, it should exist in very close association with it, as evidenced by the constancy of the ratio of [^{3}H]H_{12}-HTX sites to [^{125}I]-α-BuTx sites upon solubilization with 2% cholate and in the insoluble residue. Since both [^{3}H]H_{12}-HTX and [^{125}I]-α-BuTx are removed from the cholate extract by the α-BuTx–Sepharose 2B resin, [^{3}H]H_{12}-HTX binding activity is most likely associated with one or more of the AcChR subunits rather than the 43 000-dalton component.

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