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Acetylcholine Receptor and Ionic Channel of *Torpedo* Electroplax: Binding of Perhydrohistrionicotoxin to Membrane and Solubilized Preparations†

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ABSTRACT: The electric organ of the ray, *Torpedo ocellata,* can serve as a source for both the acetylcholine (ACh) receptor and its ionic channel. The two entities were identified by their specific binding of [^{3}H]ACh and [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX), respectively. Binding of [^{3}H]H_{12}-HTX was inhibited by certain drugs and toxins, e.g., histrionicotoxin (HTX), amantadine, and tetraethylammonium (TEA) ions at concentrations that did not inhibit [^{3}H]ACh binding. However, the specific carbamoylcholine-induced ^{22}Na efflux from microsacs from the electric organ membranes was blocked by inhibitors of either the receptor or its ionic channel. The ionic channel had the properties of a protein as judged by heat sensitivity and the inhibition of [^{3}H]H_{12}-HTX binding, after incubation of the electric organ membranes with protein reagents such as *p*-chloromercuribenzenesulfonic acid (PCMBS) or *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). The "binding" of [^{3}H]H_{12}-HTX at 4×10^{-8} M to lipids in the microsacs was 12% of the total binding to intact microsacs and was nonsaturable and insensitive to heat or specific drugs. After solubilization with cholate, the [^{3}H]H_{12}-HTX binding subunits retained the same affinities for toxins and drugs. The K_d for [^{3}H]H_{12}-HTX was 3×10^{-7} M. The majority of the ionic channel could be separated from the ACh receptors in the cholate extract by incubation with ACh-receptor affinity gel and ACh-receptor antibodies. The ACh receptor purified by this affinity gel contained only a few active ionic channel units as judged by low levels of high affinity binding of [^{3}H]H_{12}-HTX. On the other hand, after solubilization with Triton X-100, all the ionic channel molecules were either separated or denatured so that the purified ACh receptor did not exhibit high affinity binding for [^{3}H]H_{12}-HTX.

A basic assumption in nicotinic neuromuscular transmission is that binding of acetylcholine (ACh)[1] to receptor sites induces a change in conformation of the receptor, which in turn causes a channel to open for an average duration of a millisecond (Katz & Miledi, 1972; Neher & Stevens, 1977). It is suggested that at the endplate, Na^+ and K^+ currents are simultaneously "gated", so that there is only one channel type for both cations (Dionne & Ruff, 1977). The terms "ionophore" (Bon &

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[1] Abbreviations used: ACh, acetylcholine; HTX, histrionicotoxin; H_2-HTX, dihydroisohistrionicotoxin; H_8-HTX, octahydrohistrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; TEA, tetraethylammonium; DTT, 1,4-dithiothreitol; PCMBS, *p*-chloromercuribenzenesulfonic acid; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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Changeux, 1975; Carpenter et al., 1977) and "ion conductance modulator" (Albuquerque et al., 1973) are also being used to describe this ionic channel of the ACh receptor.

It has been proposed that histrionicotoxin (HTX), an alkaloid isolated from the skin extracts of the Colombian frog, *Dendrobates histrionicus* (Daly et al., 1971), modifies responses to ACh in neuromuscular preparations by interaction with the ionic channel rather than with the recognition site of the ACh receptor (Albuquerque et al., 1973, 1974; Lapa et al., 1975; Dolly et al., 1977). HTX and its completely saturated analogue, perhydrohistrionicotoxin (H_{12}-HTX), reversibly block the endplate potential of murine phrenic nerve-diaphragm preparations and block the ACh-induced extrajunctional depolarization of denervated rat soleus muscle. In *Electrophorus* electroplax, HTX reversibly blocks the steady-state depolarization without inhibiting binding of ACh to the membrane bound receptor (Kato & Changeux, 1976). These toxins reduce the amplitude of the endplate current and shorten the half-decay time without altering the equilibrium potential in mammalian and amphibian nerve-muscle preparations. It is specifically this voltage-dependent effect on the time course of the endplate current that suggests that HTX and its analogues may be reacting with the ionic channel (Albuquerque et al., 1974).

Studies on the inhibiting effect of dihydroisohistrionicotoxin (H_2-HTX) on ACh-receptor-dependent $^{22}Na^+$ uptake by cultured chick embryo muscle cells have led to the suggestion that H_2-HTX inhibits the nicotinic receptor by causing an increase in the affinity of the desensitized form of the receptor for agonists and thereby stabilizing the desensitized state (Burgermeister et al., 1977). In addition, H_2-HTX has been found to inhibit the muscarinic receptor system; it, as well as tetracaine, inhibited noncompetitively [^{3}H]scopolamine binding to neuroblastoma glioma hybrid cells (Burgermeister et al., 1978).

Our preliminary study on the binding of [^{3}H]H_{12}-HTX to membrane preparations made from the electric organ of the electric ray, *Torpedo ocellata,* suggested that this toxin bound specifically to the ionic channel of the ACh receptor, and that the density of the binding sites was higher than the ACh-binding sites. On the other hand, there was no specific binding of [^{3}H]H_{12}-HTX to the ACh receptor that was purified by affinity chromatography from Triton X-100 extracts of the electric organ membranes (Eldefrawi et al., 1977). In addition, preliminary data on the removal of the ACh-binding sites from the Triton X-100 extract by means of adsorption onto a specific affinity gel or precipitation with antireceptor antibodies, while at the same time retaining the [^{3}H]H_{12}-HTX binding sites in solution, led us to suggest that the two binding sites might be on two separable macromolecules, the ACh receptor and its ionic channel.

The ionic channel of the ACh receptor has its own binding characteristics which are different from those of the ACh receptor. It has high affinities for natural and semisynthetic analogues of HTX (Albuquerque et al., 1973, 1974) and medium affinities for the antiviral and anti-Parkinson drug amantadine (Albuquerque et al., 1978; Tsai et al., 1978), the muscarinic drugs atropine and scopolamine (Adler & Albuquerque, 1976), diisopropyl fluorophosphate (Kuba et al., 1974), and some local anesthetics (Kato & Changeux, 1976; Cohen et al., 1974; Ruff, 1977). Although these agents at low concentrations do not inhibit the binding of ACh and agonists to the nicotinic ACh receptor, they do block neuromuscular transmission (Albuquerque et al., 1973; Eldefrawi et al., 1977; Kato & Changeux, 1976; Weber & Changeux, 1974). Thus, they are expected to inhibit the carbamoylcholine-induced ^{22}Na efflux from microsacs prepared from *Torpedo* electric organ membranes that are enriched in ACh receptors. The present research was initiated to investigate this possibility, to solubilize the ionic channel of the ACh receptor while retaining its affinity for drugs and toxins, to study the effects of detergents on separation of the ionic channel from the ACh receptor, and to characterize it further.

Materials and Methods

Chemicals. Isodihydrohistrionicotoxin (Daly et al., 1971) was reduced with $^3H_2$ to [^{3}H]H_{12}-HTX and purified as previously described (Eldefrawi et al., 1977). Its radiochemical purity was 90% as determined by scanning of a thin-layer chromatogram, its specific radioactivity was 48 Ci/mmol, and its effectiveness was routinely determined in blocking neuromuscular transmission in the frog sartorius and reducing the half-decay times and peak amplitudes of the endplate and miniature endplate currents (Eldefrawi et al., 1977). In some binding studies, [^{3}H]H_{12}-HTX was diluted with H_{12}-HTX to a specific activity of 4.8 Ci/mmol. [*acetyl*-^{3}H]Acetylcholine ([^{3}H]ACh) (49.5 mCi/mmol), ^{22}NaCl (carrier free), [^{3}H]-glucose (25 Ci/mmol), $^{35}SO_4^{2-}$ (carrier free), and $^{36}Cl^-$ (5 mCi/g of chlorine) were purchased from New England Nuclear.

Preparation of Microsacs and Purified ACh Receptor. The electric organ of *Torpedo ocellata,* obtained from Alexandria, Egypt, and stored frozen at −90 °C for not more than 6 months, was homogenized (20 g) in 200 mL of 90 mM KCl, 10 mM NaCl, and 1 mM NaH_2PO_4, pH 7.4, in a Waring blender at high speed for 5 min. The homogenate was filtered through two layers of cheesecloth, deaerated, then centrifuged at 5000*g* for 10 min. The supernatant was centrifuged at 30 000*g* for 60 min at 4 °C in a Sorvall centrifuge. The final pellet was suspended in a solution of 10 mM NaCl, 90 mM KCl, and 1 mM Na_2HPO_4, pH 7, and 0.02% NaN_3 at an average protein concentration of 1.5 mg/mL. The maximum number of binding sites for [^{3}H]ACh and [^{3}H]H_{12}-HTX was determined in each preparation by equilibrium dialysis as described below. This *Torpedo* microsac preparation was utilized in all experiments as a source of receptor protein, ionic channel, and membrane lipids.

The ACh receptor was purified from detergent extracts of these microsacs by affinity chromatography on a gel to which α-neurotoxin of the cobra *Naja naja siamensis* was covalently bound. Elution from the affinity column was with 1 M carbamoylcholine as previously described (Eldefrawi & Eldefrawi, 1973; Eldefrawi et al., 1975). Protein was assayed by the method of Lowry et al. (1951). The purified ACh receptor bound 10–12 nmol of ACh or α-bungarotoxin per mg of protein.

Efflux of ^{22}Na. The method used was that of Hess et al. (1975) with some modifications. It has the advantage over other methods (Kasai & Changeux, 1971a; Michaelson et al., 1974) of monitoring carbamoylcholine-induced $^{22}Na^+$ efflux without much interference from nonspecific efflux, since carbamoylcholine is not added until most of the nonspecific $^{22}Na^+$ efflux has ended and a steady state has been attained. To each 1 mL of microsac preparation, 25 μL of ^{22}NaCl was added and incubated at 1–2 °C for 36 h. A portion of 100 μL of the labeled microsac preparation was then diluted with 20 mL of an ice cold solution of 250 mM KCl, 5 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM Na_2HPO_4, pH 7. Duplicate 1-mL samples were taken at zero time and after 30, 35, 40, 42.5, 45, 50, 60, and 65 min. Each sample was filtered on a HAWP 0.45-μm Millipore filter and rinsed twice with 10 mL of ice cold solution of 5 mM KCl, 250 mM NaCl, 4 mM $CaCl_2$,

2 mM $MgCl_2$, and 5 mM Na_2HPO_4, pH 7. The radioactivity retained in the filters was counted in an Autogamma scintillation spectrometer (Packard 5230). After 35 min, the remaining preparation had been divided, one-half serving as control for this experiment having no drugs added, while the other half was exposed to various agents. This internal control for each run was necessary because of the variability that was found between different membrane preparations. After 39 min, carbamoylcholine was added to result in a final concentration of 100 μM. When the effect of other agents on the carbamoylcholine-induced $^{22}Na^+$ efflux was studied, such agents were added at 36 min (i.e., 3 min before the addition of carbamoylcholine).

Binding of Radioactive Ligands. Equilibrium dialysis served to determine the amount of [^{3}H]ACh or [^{3}H]H_{12}-HTX bound to the microsacs, detergent extracts, or purified ACh receptor. Dialysis was carried out in Krebs' original Ringer–phosphate solution (concentrations in mM: NaCl, 107; KCl, 4.8; $CaCl_2$, 0.65; $MgSO_4$, 1.23; Na_2HPO_4, 15.7; pH 7.4). One-half of a milliliter of receptor or ionic channel preparation was pipetted into dialysis tubing [5.6 mm, Union Carbide, pretreated to remove contaminants (McPhie, 1971)] which was then tied at both ends and placed in a flask containing 50 mL of dialysis solution and radioactive ligand and shaken for 4 h at 21 °C. Triplicate samples of 50 μL were taken from each dialysis bag and bath; each was placed in 5 mL of toluene scintillation solution (4.75 g of PPO, 0.32 g of dimethyl-POPOP, and 40 mL of Beckman BBS 3 solubilizer for every 3.8 L of toluene) and counted in a Beckman LS-3133 P liquid scintillation spectrometer. Excess radioactivity in the bag over bath samples represented bound ligand. When binding of [^{3}H]ACh was studied, diisopropyl fluorophosphate was added 1 h before start of dialysis to the membranes or their extracts and to the dialysis bath to result in a final concentration of 0.1 mM. This inhibited all ACh-esterase present without affecting [^{3}H]ACh-binding to its receptor. When the effects of drugs on binding of [^{3}H]-ACh or [^{3}H]H_{12}-HTX were determined, each drug was placed in one dialysis bath except for α-bungarotoxin, which was incubated with the sample 1 h prior to dialysis.

Binding of [^{3}H]H_{12}-HTX to *Torpedo* microsacs was also studied by two centrifugal assays, one using the Beckman airfuge and the other a Sorvall centrifuge. In both cases, 10-μL aliquots of different concentrations of [^{3}H]H_{12}-HTX in ethanol were added to 1-mL samples of the *Torpedo* microsac preparations, mixed, and incubated for 20 min at 21 °C. If the effect of an agent on this binding was studied, 10 μL of solution was mixed with the microsacs for 1 h at 21 °C prior to the addition of [^{3}H]H_{12}-HTX. Aliquots of 175 μL of the incubation mixture were centrifuged in cellulose nitrate tubes in the airfuge at 100 000g for 20 min at 21 °C, or the whole mixture was centrifuged in polyethylene 1.5-mL tubes in the Sorvall centrifuge at 30 000g for 60 min at 4 °C. Three samples of 50 μL were taken from the mixture before, as well as from the supernatant after, centrifugation. The radioactivity was counted as described above. The radioactivity in samples taken from the supernatant after centrifugation represented the free toxin concentration, and the difference in radioactivity between the samples taken before and after centrifugation represented the bound toxin.

Production of Antibodies against the ACh Receptor. Adult male white New Zealand rabbits were inoculated subcutaneously along the sides of the spine with 75 μg of ACh receptors in 0.5 mL of 5 mM Na_2HPO_4, pH 7.4, emulsified with an equal volume of Freund's complete adjuvant. The rabbits were boosted after 15 days. Blood was obtained after paralysis of the rabbit at day 21, and the sera were separated by centrifugation. The presence of antibodies was assayed by immunodiffusion in agar on glass slides against the pure receptor as described (Ouchterlony, 1948; Sanders et al., 1976). Immunoglobulins were isolated from rabbit antisera by three repeated precipitations with saturated ammonium sulfate, followed by centrifugation at 9000g in a Sorvall centrifuge for 15 min at 4 °C. The final reconstituted precipitate was dialyzed against 0.005 M Na_2HPO_4 and chromatographed on Sephadex G-200 with 0.005 M Na_2HPO_4 and 0.1 M NaCl as eluent. The peak which eluted at an average molecular weight of 150 000 was collected, lyophilized, and stored at −20 °C until use. This immunoglobulin fraction was used at 10 mg of protein/mL. When the effect of antibodies on the binding macromolecules was studied, the receptor antibodies were incubated, with the detergent extract of electric organ microsacs, for 16 h at 4 °C at a volume ratio of 1:1 in polyethylene microfuge tubes; then the precipitate was removed by centrifugation at 20 000g for 20 min, and binding to the supernatant was determined by equilibrium dialysis.

Extraction of Membrane Lipids. The lyophilized *Torpedo* electric organ membranes were homogenized in chloroform–methanol (2:1) (50 mg of membranes/5 mL). The homogenate was filtered on a Whatman no. 1 filter paper and the clear filtrate collected in a Pyrex test tube and evaporated to dryness under nitrogen. To the lipid residue was added 5 mL of Ringer solution and the contents of the tube sonicated for 15 min at 4 °C. This treatment resulted in formation of liposomes varying in size from 100 to 500 nm in diameter. This liposome preparation, which was predominantly lipids but contained 38 ± 5 μg of protein/mL (possibly proteolipids), was used to measure binding of [^{3}H]H_{12}-HTX to membrane lipids.

Results

$^{22}Na^+$ Efflux. To establish whether *Torpedo* microsacs formed from electric organ membranes contain a functional ACh receptor–channel system, we studied the carbamoylcholine-induced efflux of ^{22}NaCl from these microsacs. The increase of protein concentration from 10 to 15 mg/mL increased $^{22}Na^+$ efflux by 50%. The difference (Δcpm) in radioactive content of the microsacs before ($\simeq$7000 cpm in Figure 1) and after carbamoylcholine ($\simeq$5000 cpm in Figure 1) was taken to reflect the amount of carbamoylcholine-induced $^{22}Na^+$ efflux. The Δcpm ranged between 1500 and 2000 in the different preparations used. When NaCl was eliminated from the solution used for homogenization of the tissue, the $^{22}Na^+$ influx during incubation was increased from 1400 to 2500 cpm. Preparation of microsacs in 0.6 M sucrose as described by Popot et al. (1976) did not change the $^{22}Na^+$ influx or efflux, but increased the time for microsac preparation from 2 to 6 h. Equilibration of microsacs with $^{22}Na^+$ for 36 h, rather than overnight, produced a twofold increase in influx of $^{22}Na^+$. The concentration of NaCl in the dilution medium also affected $^{22}Na^+$ efflux. When no NaCl was present, Δcpm was 1500, compared with Δcpm of 1200 and 900 in the presence of 5 mM and 10 mM NaCl, respectively. Carbamoylcholine at 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M caused, respectively, the following $^{22}Na^+$ effluxes, presented as Δcpm: 428, 1138, 1985, and 2675. The flux measurements made were of extent of $^{22}Na^+$ efflux because of the limitation of the technique to measurements that occur within minutes or at best seconds. Rate measurements, that are relevant to physiologic events, should be made in the millisecond range because activation and inactivation of the ACh receptor–ionic channel complex occurs within 10 ms. The completion of the carbamoylcholine-induced $^{22}Na^+$ efflux within 1 min (Figure 1) does not preclude the

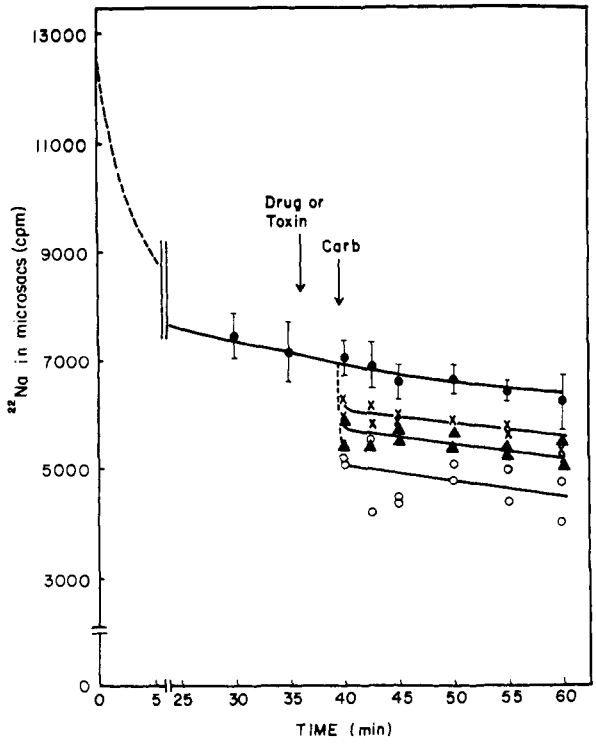

FIGURE 1: Efflux of $^{22}Na^+$ from microsacs from *Torpedo* electric organ and the effect of drugs and toxins. Zero time is when the $^{22}Na^+$ loaded microsacs are diluted 200-fold with buffer as described in text. Arrows point to the time at which the drug or toxin is added, which is 36 min after start of efflux, and carbamoylcholine (Carb) is added, at 39 min. No drugs or toxins are added to the control (●); 0.1 mM Carb added (○); 20 μM H_{12}-HTX + 0.1 mM Carb added (▲); 0.1 mM curare + 0.1 mM Carb added (X). Vertical bars represent SD of three experiments.

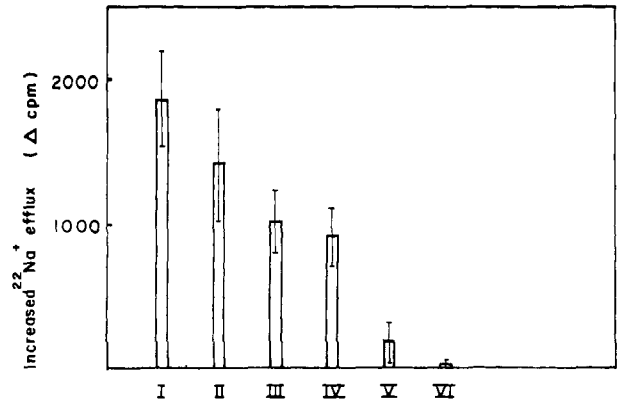

FIGURE 2: Histogram of the effect of drugs and toxins on the carbamoylcholine (Carb) induced $^{22}Na^+$ efflux from microsacs. Drug or toxin is added 36 min after start of $^{22}Na^+$ efflux and Carb is added at 39 min. Only 0.1 mM Carb (I); 0.1 mM TEA then 0.1 mM Carb (II); 20 μM H_{12}-HTX then 0.1 mM Carb (III); 0.1 mM curare then 0.1 mM Carb (IV); 0.1 mM tetracaine then 0.1 mM Carb (V); no drug or toxin added (VI).

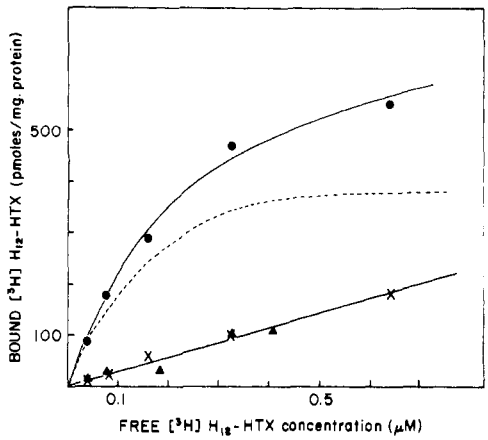

FIGURE 3: Binding of $[^3H]H_{12}$-HTX measured by equilibrium dialysis to microsacs (●), boiled microsacs (X), and chloroform–methanol extract of the microsacs (▲). The dashed line represents the difference in binding between the first binding and the last two and is considered as specific binding.

assumption that efflux was completed as early as seen during physiological events.

Control experiments were carried out with [^{3}H]glucose, $^{36}Cl^-$, and $^{35}SO_4^{2-}$ using the same experimental procedures and solutions to test for the possibility that the observed carbamoylcholine-induced increases in $^{22}Na^+$ efflux may be caused by lysis rather than a specific effect on the ACh receptor. The rate of efflux from zero time after dilution to 30 min was close for $^{22}Na^+$, $^{36}Cl^-$, and $^{35}SO_4^{2-}$, but the apparent efflux rate of [^{3}H]glucose was 2.5-fold slower. The addition of 10 mM carbamoylcholine at 39 min increased dramatically $^{22}Na^+$ efflux as shown in Figure 1, but had no effect on the rates of efflux of $^{36}Cl^-$, $^{35}SO_4^{2-}$, or [^{3}H]glucose. Therefore, the carbamoylcholine-induced $^{22}Na^+$ efflux was considered specific and not due to osmotic lysis of microsacs.

In order to assure that the carbamoylcholine-induced $^{22}Na^+$ efflux was due to ACh-receptor activation, the effects of drugs on this efflux were determined routinely after a 36-h incubation as described under Materials and Methods. The ACh-receptor inhibitor *d*-tubocurarine (0.1 mM) and the ionic channel inhibitor H_{12}-HTX (20 μM) both reduced the specific $^{22}Na^+$ efflux that was elicited by carbamoylcholine (Figure 1). When the $^{22}Na^+$ efflux induced by carbamoylcholine in the presence of a drug or toxin was compared to that produced by carbamoylcholine alone, the data could be presented as shown in Figure 2. Tetracaine (0.1 mM) and tetraethylammonium (TEA) (0.1 mM) also inhibited $^{22}Na^+$ efflux. The inhibitory effect was stronger at higher drug or toxin concentrations. If carbamoylcholine was absent, and only the steady-state $^{22}Na^+$ efflux was studied, H_{12}-HTX had no effect.

Binding of $[^3H]H_{12}$*-HTX to Torpedo Microsacs.* The binding of $[^3H]H_{12}$-HTX to *Torpedo* microsacs was measured by two assays, centrifugation and equilibrium dialysis. Centrifugal assays were used so as to compare the present findings on $[^3H]H_{12}$-HTX binding with those previously reported by Elliott & Raftery (1977). The cellulose nitrate tubes used in the airfuge, in control experiments with buffer alone, adsorbed more than 50% of the total amount of $[^3H]H_{12}$-HTX present in the solution at concentrations ranging from 0.1 to 25 nM. The polyethylene tubes used in the Sorvall adsorbed 20–50% of the $[^3H]H_{12}$-HTX present. Both types of centrifuge tubes were siliconized by first immersing in 2% NaOH, rinsing with glass-distilled water, immersing and shaking in 1% Siliclad (Clay Adams, Parsippany, N.J.), rinsing thoroughly, then drying at 100 °C for 10 min. Siliconization of the polyethylene tubes significantly reduced their adsorption of $[^3H]H_{12}$-HTX to 0.23% and 5.6% of the $[^3H]H_{12}$-HTX present at 0.1 and 25 nM, respectively. On the other hand, siliconization of the cellulose nitrate airfuge tubes did not significantly affect their adsorption of $[^3H]H_{12}$-HTX. The dissociation constant and saturation point of $[^3H]H_{12}$-HTX binding to *Torpedo* membranes, determined by a centrifugal assay using siliconized polyethylene tubes, were quite similar to those obtained by equilibrium dialysis.

Since $[^3H]H_{12}$-HTX is a lipophilic toxin, it was important

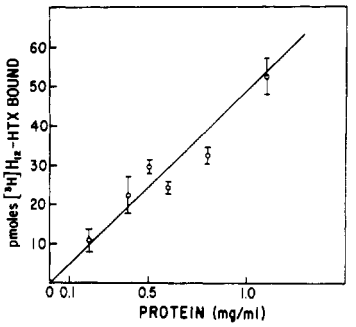

FIGURE 4: Binding of [^{3}H]H_{12}-HTX (40 nM) to *Torpedo* microsacs as a function of their protein concentration.

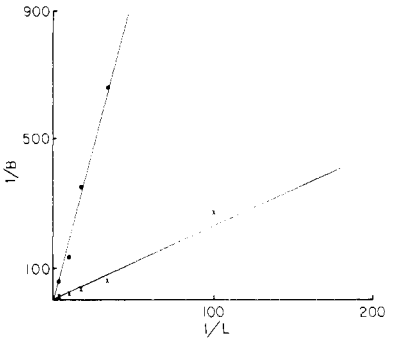

FIGURE 5: Lineweaver-Burk plot of the binding of [^{3}H]H_{12}-HTX to solutions of 0.1% (●) and 1% (X) Triton X-100.

to determine whether the toxin was bound to, or was adsorbed by, membrane lipids. Two preparations were made from the microsacs: The first was a chloroform–methanol extract of membrane lipids, and the second consisted of boiled membranes (100 °C for 30 min). Binding of [^{3}H]H_{12}-HTX to these preparations was studied by equilibrium dialysis at 21 °C for 4 h. The chloroform–methanol extract that was sonicated to produce liposomes, bound small but significant amounts of [^{3}H]H_{12}-HTX. This binding was nonsaturable and reached 12% and 30% of the total binding observed to the microsacs at 50 nM and 1 μM, respectively (Figure 3). Boiled membranes bound similar amounts. This nonspecific binding of H_{12}-HTX at 40 nM to liposomes prepared from chloroform–methanol extract or boiled membranes was not displaced by drugs and toxins that alter the time course of endplate currents, such as amantadine or tetracaine, at 400 μM. When the contribution of nonspecific "binding" was subtracted from total binding of [^{3}H]H_{12}-HTX to microsacs, binding was saturable (broken line in Figure 3), and the K_d for [^{3}H]H_{12}-HTX was calculated to range from 1 to 3×10^{-7} M in eight different preparations with a mean of 2×10^{-7} M. There was direct correlation between [^{3}H]H_{12}-HTX binding (40 nM) to *Torpedo* microsacs and their protein concentration (Figure 4). This binding was drug sensitive as shown below.

To determine the reversibility of the binding of [^{3}H]H_{12}-HTX to the microsacs, equilibrium dialysis was performed at 21 °C for 4 h at a toxin concentration of 4×10^{-8} M; then the dialysis bags with their contents were dialyzed again at 21 °C for 4 h two times in two changes of [^{3}H]H_{12}-HTX-free Ringer solution. The toxin concentration in the final dialysis bath was thus calculated to be 5×10^{-10} M, which suggests that 80% of the amount of toxin, bound to the microsacs, had dissociated. Most of the 20% irreversibly "bound" toxin may be related to the nonspecific binding or adsorbed component observed with boiled microsacs.

TABLE I: Binding of [^{3}H]ACh (1 μM) and [^{3}H]H_{12}-HTX (0.64 μM) to Three Different Membrane Preparations of *Torpedo* Electroplax.[a]

membrane prep	bound ligand (pmol/mg of protein) [^{3}H]ACh	[^{3}H]H_{12}-HTX[b]	[^{3}H]ACh/ [^{3}H]H_{12}-HTX
I	132 ± 47	101 ± 17	1.30 ± 0.5
II	472 ± 28	308 ± 9	1.53 ± 0.4
III	747 ± 13	567 ± 155	1.31 ± 0.5

[a] I: a resuspended pellet of 30 000*g*, 60 min of the total homogenate (protein content = 4 mg/mL). II: the total homogenate was centrifuged at 500*g*, 10 min, then the supernatant fraction centrifuged at 30 000*g*, 60 min, and the pellet resuspended (protein content = 1.4 mg/mL). III: the total homogenate was centrifuged at 10 000*g*, 10 min, then the supernatant fraction centrifuged at 30 000*g*, 60 min, and the pellet resuspended (protein content = 0.7 mg/mL). [b] The values reported have been corrected for nonspecific "binding" to membrane lipids.

The Ratio of Ionic Channel to ACh Receptor. If [^{3}H]-H_{12}-HTX binds to the ionic channel of the ACh receptor and this channel is a subunit of, or closely coupled to, the ACh receptor, then different membrane preparations may have an equal ratio of channel to ACh receptor. Three membrane preparations from the electric organ were studied, which varied 5.7-fold in their content of ACh receptors, and the purity of their ACh receptors ranged from 1.3 to 7.5% when compared with the pure receptor protein. The concentration of ACh receptors was determined by maximum binding of [^{3}H]ACh at 10^{-6} M and the concentration of ionic channel by the corrected binding of [^{3}H]H_{12}-HTX at 6.4×10^{-7} M. The ratio of the ACh to H_{12}-HTX binding sites obtained from four separate experiments varied from 1.3 ± 0.5 to 1.5 ± 0.4 in the three membrane preparations (Table I).

Chemical Nature of the Putative Ionic Channel. The effect of pretreatment of *Torpedo* microsacs with protein-modifying reagents on the binding of [^{3}H]H_{12}-HTX was investigated. The reagents were chloromercuribenzenesulfonic acid (PCMBS), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), and 1,4-dithiothreitol (DTT). Each fraction of 1 mL of *Torpedo* microsacs was incubated with the reagent (10^{-3} M) for 1 h at 21 °C before the start of equilibrium dialysis. Binding of [^{3}H]H_{12}-HTX (40 nM) and [^{3}H]ACh (1 μM) to the treated membranes was compared with that of the control. Treatment of the membranes with PCMBS and EEDQ reduced binding of [^{3}H]H_{12}-HTX to the ionic channel by 60 ± 4% and 28 ± 3%, respectively, and of [^{3}H]ACh to its receptor by 61 ± 5% and 76 ± 7%, respectively. On the other hand, DTT did not affect [^{3}H]H_{12}-HTX, but reduced binding of [^{3}H]ACh by 46 ± 7%.

Solubilization of the Putative Ionic Channel. In a preliminary study both [^{3}H]ACh and [^{3}H]H_{12}-HTX were found to bind to proteins present in 1% Triton X-100 extracts of *Torpedo* membranes. However, drugs which were capable of displacing [^{3}H]H_{12}-HTX from *Torpedo* membranes were less effective in displacing the toxin from the Triton-solubilized preparations (Eldefrawi et al., 1977). The binding of [^{3}H]-H_{12}-HTX at various concentrations to Triton X-100 in Krebs original Ringer phosphate solution (0.1 and 1%) was now investigated by equilibrium dialysis. Triton alone bound significant amounts of the toxin, but the binding was nonsaturable (Figure 5). Furthermore, the binding of toxin to Triton was not displaced by any of the drugs that displaced [^{3}H]H_{12}-HTX from its binding sites in *Torpedo* microsacs, such as HTX and amantadine (400 μM).

Of the other detergents known to solubilize the ACh-re-

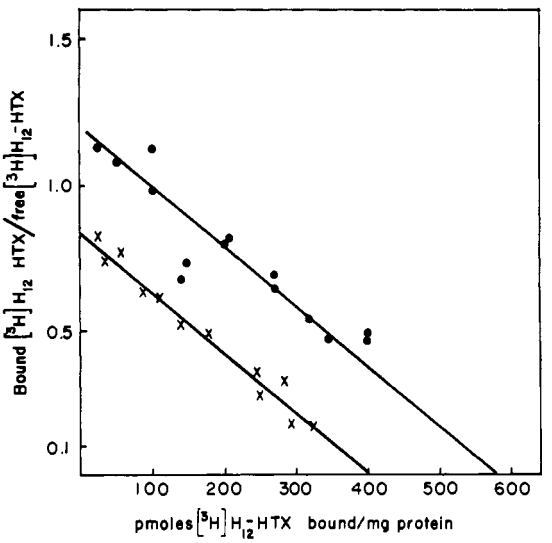

FIGURE 6: Scatchard plot of the binding of [^{3}H]H_{12}-HTX to 0.5% cholate extract of the electric organ membranes (●) and the ACh receptor preparation purified from this extract by affinity chromatography (X).

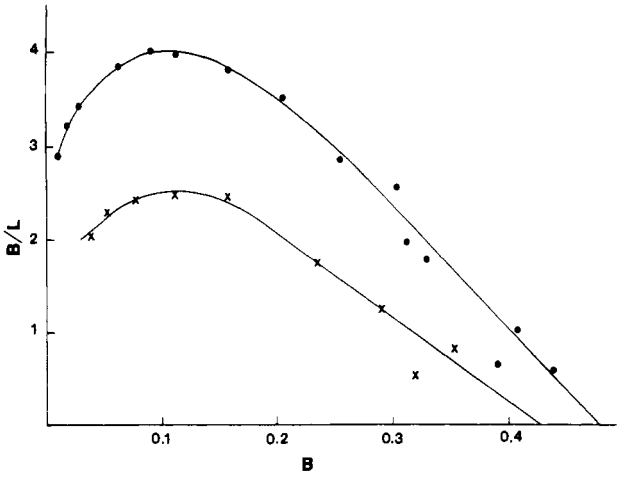

FIGURE 7: Scatchard plot of the binding of [^{3}H]ACh alone (●) and in presence of 10^{-4} M tetracaine (X) to *Torpedo* electric organ membranes. *B*, amount bound in nmol per mg of protein; *L*, concentration of free [^{3}H]ACh in μM.

ceptor protein from *Torpedo* membranes (Eldefrawi et al., 1972), 0.5% cholate efficiently solubilized ACh- and HTX-binding proteins and did not bind [^{3}H]H_{12}-HTX at concentrations of up to 1 μM. Accordingly, *Torpedo* microsacs were suspended in 0.5% cholate solution and shaken for 30 min at 4 °C; then the supernatant of 100 000*g*, 1-h centrifugation, was collected and used as a source for soluble ACh receptor as well as the putative ionic channel. These conditions were adequate, but not optimal, for solubilization. The mean recovery of HTX-binding sites for 12 experiments was 38 ± 10% with 55 ± 13% remaining in the pellet. Saturable binding of [^{3}H]H_{12}-HTX was observed in the cholate extracts (Figure 6). The maximum number of binding sites (uncorrected) was 580 pmol/mg of protein and the apparent K_d was 3×10^{-7} M, a value similar to that obtained for binding of the toxin to *Torpedo* microsacs obtained by equilibrium dialysis (Eldefrawi et al., 1977) and by centrifugal assay (see above).

The specificity of binding of [^{3}H]H_{12}-HTX to cholate extracts of *Torpedo* microsacs was determined by studying the ability of heat treatment and drugs and toxins to block this binding. Binding of [^{3}H]H_{12}-HTX to the membranes or cholate extract was blocked similarly by many agents (Table II). The most potent blockers were the histrionicotoxins, followed by several local anesthetics, such as tetracaine, piperocaine, and dibucaine; lidocaine and procaine were less effective blockers. TEA ions, scopolamine, and atropine blocked [^{3}H]H_{12}-HTX binding, particularly to the solubilized extract. However, toxins that reacted with the ACh receptor (e.g., α-bungarotoxin) or the axonal Na^+ channels (e.g., batrachotoxin and tetrodotoxin) did not. Furthermore, 70–80% of the binding observed in 0.5% cholate extracts was heat sensitive. The balance presumably represents binding to membrane lipids present in the cholate extract.

Effect of Tetracaine on the Binding of [^{3}H]ACh to Membrane-Bound and Purified Receptor Protein. Since tetracaine was shown to inhibit carbamoylcholine-elicited $^{22}Na^+$ efflux (Figure 1), and other local anesthetics have been shown to affect binding of receptor ligands to the membrane-bound ACh receptor (Cohen et al., 1974; Weber & Changeux, 1974), the effect of tetracaine on binding of [^{3}H]ACh to the ACh receptor, both native and solubilized, was investigated. Binding of [^{3}H]ACh to the native ACh receptor was inhibited by 0.1 mM tetracaine (Figure 7). However, after purification of the ACh receptor from Triton X-100 extracts with affinity chromatography (Eldefrawi & Eldefrawi, 1973), tetracaine at 0.5 mM or even 1 mM did not inhibit [^{3}H]ACh binding to the purified ACh receptor (Figure 8). Furthermore, the purified ACh receptor in Triton *X*-100 failed to bind [^{3}H]H_{12}-HTX at toxin concentrations below 1.0 μM, indicating that the high affinity binding of [^{3}H]H_{12}-HTX was absent in the pure receptor protein. The binding sites for [^{3}H]H_{12}-HTX and tetracaine must either have been denatured during purification, or were separable from the ACh receptor during purification.

TABLE II: Effect of Various Drugs and Toxins on Binding of [^{3}H]-H_{12}-HTX (40 nM) to *Torpedo* Membrane and Cholate Solubilized Preparations.

ligand	% blockade of binding[a]	
	membranes	solubilized
HTX (1 μM)	74 ± 18	60 ± 16
H_{12}-HTX (1 μM)	75 ± 7	82 ± 9
H_8-HTX (1 μM)	78 ± 10	83 ± 11
lidocaine (400 μM)	9 ± 5	38 ± 4
procaine (400 μM)	33 ± 11	49 ± 5
tetracaine (400 μM)	83 ± 4	94 ± 5
piperocaine (400 μM)	85 ± 3	99 ± 5
dibucaine (400 μM)	91 ± 5	102 ± 6
quinacrine (400 μM)	98 ± 5	103 ± 5
TEA (400 μM)	49 ± 15	67 ± 4
scopolamine (400 μM)	18 ± 10	48 ± 3
atropine (400 μM)	15 ± 8	49 ± 11
amantadine (400 μM)	90 ± 8	103 ± 5
α-bungarotoxin (1 μM)	0 ± 2	0 ± 3
batrachotoxin (1 μM)	2 ± 3	0 ± 8
tetrodotoxin (1 μM)	0 ± 12	2 ± 16

[a] Values represent the mean of three experiments ± SD. Total binding was corrected for nonspecific binding to membrane lipids. A zero value for % blockade was used when there was no inhibition or when there was increased binding.

Chromatography of Torpedo Cholate Extracts on Sephadex G-200. Five-milliliter aliquots of the 0.5% cholate extract of *Torpedo* microsacs were chromatographed on Sephadex G-200 packed in 2.5 × 45 cm glass columns using phosphate-buffered saline (pH 7.4), containing 0.5% cholate as the eluting agent, and 5-mL fractions collected at 4 °C. The fractions were

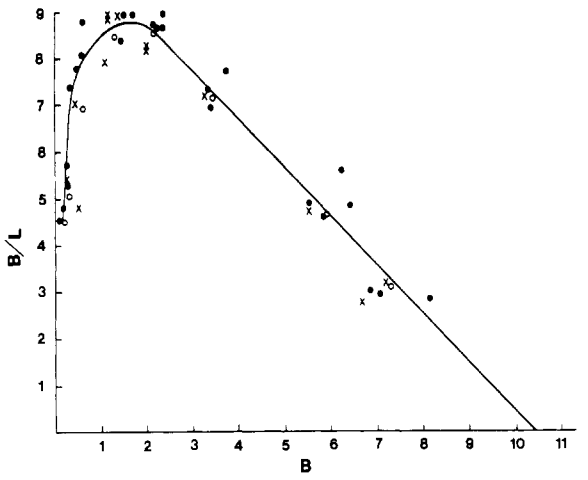

FIGURE 8: Scatchard plot of the binding of [^{3}H]ACh alone (●) and in presence of 5×10^{-4} M (○) and 10^{-3} M (X) tetracaine to ACh receptor purified from Triton extract of *Torpedo* electric organ. *B* and *L* are same as in Figure 7.

TABLE III: Separation of Binding Sites for [^{3}H]ACh (1 μM) from Binding Sites for [^{3}H]H_{12}-HTX (40 nM) by Cobra Toxin Affinity Gel.[a]

prep	affinity gel treatment	bound ligand (pmol/mL ± SD) [^{3}H]ACh	[^{3}H]H_{12}-HTX
Triton extract	−	677 ± 26[b]	52 ± 4[b]
	+	10 ± 11[b]	48 ± 4[b]
cholate extract	−	645 ± 17[c]	61 ± 3[c]
	+	17 ± 15[c]	27 ± 2[c]

[a] Detergent extracts were studied by equilibrium dialysis before (−) and after (+) incubation with the gel. [b] Calculated in pmol/mL from Δcpm values presented in Eldefrawi et al. (1977). [c] Binding of [^{3}H]ACh at 1 μM represents ≃90% saturation of the binding sites, while binding of [^{3}H]H_{12}-HTX at 40 nM represents only ≃15% saturation.

assayed for proteins and binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX. There was a single peak for binding of both ligands which appeared in the void volume. This suggested that either receptor and putative ionic channel proteins remained coupled in the cholate extract or that the two proteins were separate but formed fairly large aggregates of similar size and thus were eluted together. Attempts to separate the binding of the two ligands on Sepharose 6B failed.

Separation of the Receptor from the Putative Ionic Channel. Two approaches were used to investigate whether or not the putative ionic channel and the ACh receptor were separable or coupled in detergent extracts of *Torpedo* microsacs. In the first experiment, 1% Triton X-100 and 0.5% cholate extracts were incubated with cobra toxin affinity gel which had been used to purify receptor protein from Triton extracts (Eldefrawi & Eldefrawi, 1973). After incubation for 2 h with stirring at 21 °C, the incubation mixtures were passed through fritted glass filters and the binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX to the filtrates and the original extracts was determined (Table III). The data indicate that the ACh receptors (identified by their specific binding of [^{3}H]ACh) were totally removed from the Triton X-100 or cholate extracts by incubation with the affinity gel. Although [^{3}H]H_{12}-HTX binds to Triton X-100 as well as to the ionic channel, and there was no significant difference in [^{3}H]H_{12}-HTX binding to the Triton X-100 extract before and after exposure to the affinity gel, we may conclude that none of the putative ionic channel was adsorbed by the gel. On the other hand, 56% of [^{3}H]H_{12}-HTX binding molecules were removed by the affinity gel from the 0.5% cholate extract, which by itself does not bind [^{3}H]H_{12}-HTX significantly.

TABLE IV: Separation of Binding Sites for [^{3}H]ACh (1 μM) from Binding Sites for [^{3}H]H_{12}-HTX (40 nM) by Antireceptor Antibodies.[a]

prep	bound ligand (pmol/mL ± SD) [^{3}H]ACh	[^{3}H]H_{12}-HTX
Triton extract + Ringer	677 ± 25[b]	52 ± 4[b]
supernatant of Triton extract + antibodies	56 ± 7[b]	54 ± 1[b]
cholate extract + Ringer	380 ± 28[c]	39 ± 4[c]
supernatant of cholate extract + antibodies	8 ± 7	14 ± 1
antibodies + Ringer	21 ± 6	2 ± 1

[a] Detergent extracts were investigated by equilibrium dialysis before and after treatment with antibodies. [b] Calculated in pmol/mL from Δcpm values presented in Eldefrawi et al. (1977). [c] Binding of [^{3}H]ACh at 1 μM represents ≃90% saturation of the binding sites, while binding of [^{3}H]H_{12}-HTX at 40 nM represents only ≃15% saturation.

TABLE V: Binding of [^{3}H]H_{12}-HTX Binding (40 nM) to the Various Fractions during Purification of ACh Receptors from Cholate Extracts of *Torpedo* Microsacs.

purification steps	amount bound (pmol/mL ± SD)
1. original extract	61 ± 3
2. filtrate (after incubation with affinity gel)	27 ± 2
3. 1st buffer wash (with 0.1% cholate)	9 ± 3
4. 2nd buffer wash (with 0.1% cholate)	3 ± 1
5. 1st 1 M NaCl wash (with 0.1% cholate)	1[a] ± 1
6. 2nd 1 M NaCl wash (with 0.1% cholate)	1[a] ± 1
7. Carbamoylcholine desorbed then dialyzed purified receptor preparation	6 ± 1
% recovery[b]	73 ± 5

[a] Values were nonsignificant at $p < 5\%$, $n = 3$. [b] Incubation with 1 M carbamoylcholine for 4 h at 21 °C inhibited [^{3}H]H_{12}-HTX binding by the cholate extract by 60%. Also, 1 M NaCl inhibited binding by another 50%. Recovery of the total binding was 70%, based on the additive significant Δcpm obtained for the 5-mL fraction of steps 2–7 divided by the Δcpm of the 5 mL of original extract (step 1). If the inhibition in binding caused by 1 M carbamoylcholine and 1 M NaCl were taken into consideration, the purified receptor preparation would bind 32 instead of 6 pmol/mL, and recovery of binding by the purified protein, the filtrate, and buffer washes would be about 101% of the original extract.

In the second approach, detergent extracts were mixed with anti-receptor antibodies and incubated at 4 °C for 16 h in microfuge polyethylene tubes. After incubation, the tubes were centrifuged at 10 000*g* for 20 min and the supernatant layer tested for binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX. The data (Table IV) indicate that receptor antibodies precipitated the receptor protein almost totally from both detergent extracts. None of the [^{3}H]H_{12}-HTX binding present in Triton X-100 extracts was precipitated. On the other hand, 64% of the [^{3}H]H_{12}-HTX binding was precipitated from the cholate ex-

TABLE VI: Binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX to *Torpedo* ACh Receptors Solubilized with Two Detergents and Purified by the Same Affinity Gel.

[^{3}H]ACh (μM) (μM)	binding (pmol/mg of protein)		[^{3}H]H_{12}-HTX (μM)	binding (pmol/mg of protein)	
	1% Triton	0.5% cholate		1% Triton	0.5% cholate
0.1	3387.7	2325.6	0.04	0	67.3
0.2	3885.0	2665.6	0.08	0	98.4
0.5	4693.1	3439.3	0.16	0	145.9
1.0	6410.2	4001.0	0.32	0	183.8
2.0	6752.1	4897.7	0.64	0	296.5

tracts. Precipitation of the [^{3}H]H_{12}-HTX binding molecule by antibody does not necessarily imply that this molecule and the ACh receptor are in a complex, particularly if excess antibody was used.

The affinity and binding of [^{3}H]H_{12}-HTX to cholate extracts remaining after affinity column adsorption of all the ACh-receptor molecules was investigated. The K_d was 3×10^{-7} M, and this binding at 40 nM of [^{3}H]H_{12}-HTX was blocked by 1 μM octahydrohistrionicotoxin (H_8-HTX), 400 μM tetracaine, and 400 μM atropine by 36 ± 4%, 42 ± 6%, and 23 ± 4%, respectively. The blockade by these agents was only slightly lower than that obtained in the original cholate extract. Thus, it is suggested that most of the binding of [^{3}H]H_{12}-HTX, in preparations which did not contain any ACh receptors, appeared to involve the ionic channel.

The data obtained with the cholate extracts suggest the possibility that the putative ionic channel exists in the cholate extracts of *Torpedo* microsacs in two forms, one separable from (35%), and the other coupled to (65%), the ACh-receptor protein. Triton X-100 seems to dissociate and/or denature the putative ionic channel totally.

Purification of a Receptor–Channel Complex. The previous experiments suggested that a significant fraction of the putative ionic channel (i.e., [^{3}H]H_{12}-HTX-binding sites) present in 0.5% cholate extracts of *Torpedo* microsacs were still coupled to the receptor protein. Receptor protein was now purified from cholate extracts by *Naja naja siamensis* α-neurotoxin affinity chromatography as described previously (Eldefrawi & Eldefrawi, 1973). The binding of [^{3}H]H_{12}-HTX was determined at every step during purification of the ACh receptor from the cholate extract (Table V). About 54% of the toxin-binding molecules was adsorbed from the cholate extracts onto the affinity gel (Table III). However, about 35% of the adsorbed fraction was desorbed in the first buffer wash of the gel. Thus, only about 38% of the original [^{3}H]H_{12}-HTX-binding molecules remained tightly bound to the affinity gel. Very little was removed by the next three washes. The final protein fraction obtained by desorption with 1.0 M carbamoylcholine retained about 50% of the tightly adsorbed toxin-binding sites which represented only 10% of the total present in the original cholate extract.

When the same experiment was repeated with 1% Triton X-100 extract, instead of the 0.5% cholate, all the [^{3}H]H_{12}-HTX binding was retained in solution after removal of the receptor by the affinity gel, and the purified receptor protein finally obtained showed no binding for [^{3}H]H_{12}-HTX even though its ACh-binding activity was intact (Table VI). In one experiment the protein concentration obtained after receptor purification from Triton X-100 extracts was 0.053 mg/mL and from cholate extracts was 0.086 mg/mL. Both bound similar amounts of [^{3}H]ACh per mL which, when converted to pmol per mg of protein, resulted in lower specific binding of [^{3}H]-ACh for the receptor solubilized with, and purified from, cholate. Three out of 5 of the ACh-receptor preparations purified from cholate extracts bound significant amounts of [^{3}H]H_{12}-HTX. The best preparation bound a maximum of 0.4 nmol of the toxin per mg of protein with a K_d of 0.3 μM (Figure 6).

These results suggest that substitution of 0.5% cholate for 1% Triton X-100 in the protocol used for receptor purification from *Torpedo* electroplax results in the purification of a receptor–channel complex. However, the ratio of ACh- to H_{12}-HTX-binding sites in the best preparation, purified from cholate, was about 25 compared with a ratio of 1.38 ± 0.42 in the membranes. This change in ratio reflects a tremendous loss of H_{12}-HTX-binding sites during purification of the ACh receptor, which is probably due to dissociation of the ionic channel or to denaturation of the ionic channel which must be very labile in the solubilized form.

Discussion

In the membranes, the intact ACh receptor has two functions: binding of ACh which causes activation of ion transport. The reduction of carbamoylcholine-induced $^{22}Na^+$ efflux from *Torpedo* microsacs either by inhibitors of the ACh receptor, e.g., by *d*-turbocurarine, or the ionic channel, e.g., by H_{12}-HTX (Figures 1 and 2), suggests that these microsacs contain both receptor and ionic channel. This confirms earlier findings on the inhibition of $^{22}Na^+$ efflux from *Electrophorus* microsacs by *d*-tubocurarine and tetracaine (Kasai & Changeux, 1971b). It also suggests that the postsynaptic effects of H_{12}-HTX can be detected biochemically by such studies of $^{22}Na^+$ efflux. The two entities involved in responses to ACh in *Torpedo* microsacs are the ACh receptor, identified by its specific binding of [^{3}H]ACh (Table I; Eldefrawi et al., 1971), and the ionic channel, identified by its specific binding of [^{3}H]H_{12}-HTX (Table I; Eldefrawi et al., 1977). The specificity of the latter binding is demonstrated by its saturability (Figures 3 and 6), correlation with protein concentrations (Figure 4), high affinity ($K_d = 1\text{-}3 \times 10^{-7}$ M) that is comparable to the dose which inhibits carbamoylcholine-induced depolarization of *Electrophorus* electroplax (Kato & Changeux, 1976), sensitivity to heat, and inhibition by drugs and toxins that modulate end-plate currents (Table II). Such drugs and toxins, which are suggested to interact with the ionic channel, are HTX (Albuquerque et al., 1974; Lapa et al., 1975), amantadine (Albuquerque et al., 1978; Tsai et al., 1978), atropine and scopolamine (Adler & Albuquerque, 1976; Feltz et al., 1977), and several local anesthetics. Dose–response studies demonstrate that the binding of 10 nM [^{3}H]H_{12}-HTX to *Torpedo* microsacs is totally inhibited by 5 μM H_{12}-HTX (an extensive analysis of binding displacement by drugs and toxins will be published elsewhere). Though local anesthetics have multiple sites of action in excitable tissue (cf. Ritchie & Greengard, 1966; Papahadjopoulos, 1972), they have been shown to affect endplate currents (Katz & Miledi, 1975; Ruff, 1977; Beam, 1976). The ACh-receptor inhibitor, α-bungarotoxin, does not inhibit binding of [^{3}H]H_{12}-HTX to the ionic channel and

neither does batrachotoxin nor tetrodotoxin (Table II), the specific activator and inhibitor, respectively, of Na^+ channels in axons and muscles (Albuquerque & Daly, 1977; Narahashi et al., 1967; Henderson et al., 1974).

The nonspecific "binding" of [^{3}H]H_{12}-HTX observed herein as a component of the total binding to the microsacs is similar to that previously reported by Elliott & Raftery (1977). The higher percentage of nonspecific binding they reported compared with our present data may be due to differences in the two [^{3}H]H_{12}-HTX preparations used, ours being derived from the natural histrionicotoxin from frog skins, while theirs is derived from a synthetic compound. Also, the effect of our [^{3}H]H_{12}-HTX on end-plate currents was tested and shown to be active electrophysiologically (Eldefrawi et al., 1977) before use in binding experiments for in vitro identification of the ionic channel. In addition, the use of cellulose-nitrate tubes of the airfuge for centrifugal assay of binding of [^{3}H]H_{12}-HTX may affect the data because these tubes have a high capacity for adsorbing the toxin. The ratio of binding of [^{3}H]ACh and [^{3}H]H_{12}-HTX to *Torpedo* electric organ membranes averages 1.38 ± 0.42 in three membrane preparations representing different, though low, levels of enrichment in ACh receptors (Table I). This value, although larger than the ratio of 0.5 which we reported earlier (Eldefrawi et al., 1977) is obtained by correcting for the nonspecific binding. However, it is still much smaller than the value of 4 reported by Elliott & Raftery (1977) for the ratio of α-bungarotoxin to [^{3}H]H_{12}-HTX binding sites. The affinity of the toxin to the heat-sensitive component of *Torpedo* membranes is in line with that reported previously (Eldefrawi et al., 1977) and later confirmed (Elliott & Raftery, 1977).

Several findings suggest that the ionic channel is a protein. Pretreatment of the electric organ membranes with trypsin, chymotrypsin or pepsin reduces [^{3}H]H_{12}-HTX binding (Eldefrawi et al., 1977). Similar reduction is detected after pretreatment with PCMBS, a reagent reacting with sulfhydryl groups, and the carboxyl group reagent EEDQ. In addition, the lipids extracted from electric organ membranes bind only a small percentage of [^{3}H]H_{12}-HTX that the intact membranes bind. These data do not exclude the possibility that the ionic channel may be a lipoprotein or glycoprotein. The ACh receptor of *Torpedo* electric organ is a glycoprotein (Michaelson et al., 1974; Meunier et al., 1974) and resembles the [^{3}H]H_{12}-HTX binding protein in being sensitive to pretreatment with PCMB and EEDQ, but it differs in being greatly affected by DTT (Eldefrawi & Eldefrawi, 1972).

The fact that there are agents (e.g., *d*-tubocurarine, α-bungarotoxin) which, at certain concentrations, inhibit the specific [^{3}H]ACh binding and not [^{3}H]H_{12}-HTX binding to *Torpedo* electric organ membranes, while other agents (e.g., HTX, amantadine) inhibit only the [^{3}H]H_{12}-HTX binding, and the finding that these differences are reflected in electrophysiological studies (Albuquerque et al., 1973, 1978), indicate that [^{3}H]ACh binds to sites different from the ones binding [^{3}H]H_{12}-HTX. It is much more difficult to determine whether the ACh receptor and the ionic channel are subunits of a single molecule or whether the two are separate molecules that are coupled in the membrane and may dissociate during purification. Although our previous findings on the Triton X-100 extract of *Torpedo* electric organ suggested that ACh receptor and its ionic channel were separate molecules (Eldefrawi et al., 1977), partitioning of [^{3}H]H_{12}-HTX in, or adsorption to, Triton X-100 micelles weakened this conclusion. With 0.5% cholate the ionic channel could be solubilized with retention of affinity for [^{3}H]H_{12}-HTX ($K_d = 3 \times 10^{-7}$ M) (Figure 6) and drug specificity (Table II). Nonspecific binding of [^{3}H]H_{12}-HTX to detergent is absent when 0.5% cholate is used for solubilization, thus avoiding the complication of nonspecific binding to detergent micelles, which pertained with Triton.

The data suggest that the ACh receptor and its ionic channel are two separate molecules. Thus, antibodies against the ACh receptor precipitate all the [^{3}H]ACh-binding molecules from the cholate extract while precipitating only 64% of the [^{3}H]-H_{12}-HTX-binding molecules (Table IV). Although the receptor protein purified from Triton X-100 extracts and used as an immunogen does not bind H_{12}-HTX up to 1 μM (Table VI), we do not exclude the possibility that it may contain some ionic channel proteins which no longer bind the toxin. Thus, it is possible that antireceptor immune sera may contain antibodies against the ionic channel protein. The titer of these antibodies will depend on the concentration and immunogenicity of the ionic channel protein. Therefore it is possible that the 64% fraction of H_{12}-HTX binding precipitated by the antiserum may be in part due to direct interaction between antibodies and ionic channel protein. The other possibility is coprecipitation of the ionic channel protein with the receptor–antibody complex because of the coupling between the two. The *Naja* toxin affinity gel, which adsorbs all of the [^{3}H]-ACh-binding molecules from the cholate extract, adsorbs only 54% of the [^{3}H]H_{12}-HTX-binding molecules, 25% of which is lightly adsorbed and removed by washing (Table V). The cholate-solubilized HTX-binding fraction, which is not adsorbed on the affinity gel and not precipitated by the antireceptor antibodies, has a similar affinity for [^{3}H]H_{12}-HTX and slightly lower drug sensitivity than the original cholate extracts. Furthermore, the ACh-receptor molecule purified by affinity adsorption from the cholate extract binds only 0.4 nmol of [^{3}H]H_{12}-HTX per mg of protein (Figure 6). This binding would probably have been higher, possibly 2 nmol/mg of protein, if the receptor–channel proteins had not been incubated with 1 M carbamoylcholine for 4 h at 21 °C or the affinity gel–protein complex had not been washed with 1 M NaCl prior to desorption with carbamoylcholine (Table V). Both of these treatments reduce [^{3}H]H_{12}-HTX binding. The average ratio of [^{3}H]ACh binding sites to [^{3}H]H_{12}-HTX binding sites is 1.4 in the electric organ membrane (Table I) and becomes 25 in the proteins purified by affinity adsorption from the cholate extract (Figure 6). Thus, the majority of [^{3}H]H_{12}-HTX binding sites can be separated from the [^{3}H]ACh binding ones in the cholate extract by affinity adsorption of the receptor and are accounted for during the various steps of affinity chromatography including losses due to denaturation by NaCl and carbamoylcholine (Table V). The higher specific binding of [^{3}H]ACh (i.e., per mg of protein) obtained for the ACh receptor purified from the Triton extract, as compared with the cholate extract, is possibly due to the presence of some ionic channel protein in the latter.

The molecules in the intact electric organ membrane, or in the cholate extract, that bind [^{3}H]H_{12}-HTX do so with the same affinity constant of $1\text{–}4 \times 10^{-7}$ M (Figures 3 & 6). The [^{3}H]H_{12}-HTX binding sites associated with the ACh receptor purified from the cholate extract have a similar affinity. Binding of [^{3}H]H_{12}-HTX to these various extracts also has the same drug sensitivity. The data suggest that all the [^{3}H]-H_{12}-HTX binding sites (K_d of $1\text{–}4 \times 10^{-7}$ M) in the electric organ membrane and its cholate extracts are on one kind of entity tentatively called the ionic channel. It is possible that at any time some ionic channel molecules are tightly associated with the ACh receptor, while others are less tightly associated or even dissociated.

If the receptor and its ionic channel are separate entities they must be coupled in the membrane to account for the time

course of end-plate currents. Accordingly, one would expect that ligands which bind to one might allosterically affect the binding of ligands to the other. The best example of this is tetracaine, which was found to enhance binding of ACh to the *Torpedo* receptor only in the intact membrane, but had no such effects on Triton X-100 extracts of electric organs (Cohen et al., 1974). It was suggested that tetracaine was binding to an allosteric site on or near the ACh receptor. Our data on tetracaine lead to a similar suggestion. Tetracaine inhibits [^{3}H]ACh binding to its membrane-bound ACh receptor (Figure 7) but does not inhibit its binding to the ACh receptor purified from the Triton extract (Figure 8). This receptor preparation does not bind [^{3}H]H_{12}-HTX.

In conclusion, the present study suggests that [^{3}H]H_{12}-HTX (at concentrations of 0.01–1 μM) binds to the ionic channel of the ACh receptor as judged by the correspondence of effects of drugs in inhibiting this binding with their effects in modulating the ionic channel in situ. This putative ionic channel has the properties of a protein and, when solubilized with cholate, retains its affinities for toxins and drugs. By the use of different solubilization and purification procedures, it may be possible to obtain a homogeneous ACh receptor free of ionic channel, a purified receptor–channel complex and a homogeneous ionic channel protein. Such progress would set the stage for the reconstruction of a reconstituted, lipid bilayer-supported receptor–channel complex from solubilized components that may produce a meaningful selective cation flux on stimulation with cholinergic agonists.

After submission of this manuscript for publication in *Biochemistry*, a paper by Sobel et al. ((1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 510–514) was published, in which it was reported that a protein was separated from the *Torpedo* ACh-receptor protein, which bound both HTX and quinacrine and consisted of a 43 000 subunit. However, in another recent report (Neubig et al. (1978) *Soc. Neurosc. Abst. 4,* 517) a 43 000 subunit was quantitatively extracted from *Torpedo* membranes while leaving behind in the membranes 80–90% of the [^{3}H]ACh binding sites and unchanged binding affinity of the local anesthetic [^{14}C]trimethisoquin and its inhibition by μM HTX, leading to the conclusion that the 43 000 protein was unrelated to the site of binding of local anesthetics or HTX. Further investigations are needed to resolve this issue.

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Resonance Raman Spectra of Whole Mitochondria†

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ABSTRACT: The resonance Raman spectra of reduced cytochromes *b* and *c* and cytochrome oxidase in whole mitochondria have been recorded without any instrument modifications. The contributions of the individual cytochromes have been identified by comparison with the characteristic features observed in partially purified preparations including: (i) the strong dependence of the intensity patterns on excitation wavelength relative to the peak positions of the α, β, and γ absorption bands of the cytochromes; and (ii) the presence of marker bands for heme type. Since the Raman spectra can be used as an intrinsic indicator of interaction between hemes, the ability to record spectra in intact mitochondria opens the possibility to study heme-heme interactions in the functioning membrane in situ.

Reports of the resonance Raman (RR) spectra of hemeproteins have proliferated since the initial investigation of Strekas and Spiro on cytochrome *c* (Strekas & Spiro, 1972a) and hemoglobin (Strekas & Spiro, 1972b) and their elegant analysis of the vibronic origin of the scattering mechanism (Spiro & Strekas, 1972) which is enhanced by resonance between the laser frequency and the porphyrin $\pi \rightarrow \pi^*$ transitions. More detailed descriptions of the scattering phenomenon in both cytochrome *c* (Friedman & Hochstrasser 1973, 1976; Collins et al., 1973; Nafie et al., 1973) and other metalloporphyrins (Verma et al., 1974; Mendelsohn et al., 1975; Woodruff et al., 1975; Asher & Sauer, 1976; Shelnutt et al., 1976, 1977; Kitawagawa et al., 1976; Mayer et al., 1973) have since appeared. Correlations have been made between the RR band frequencies of various heme proteins and (i) their oxidation and spin states (Loehr & Loehr, 1973; Yamamoto et al., 1973; Spiro & Strekas, 1974), (ii) the porphyrins' geometry (Spaulding et al., 1975), and (iii) axial ligands (Kitagawa et al., 1975, 1976). RR spectra of cytochrome oxidase have also been reported (Salmeen et al., 1973; Kitagawa et al., 1977; Salmeen et al., 1978). The observation that the overall RR intensities of heme proteins are inversely correlated with the line widths of the absorption bands was explained by recognizing the role of the iron electronic state in providing paths for radiationless decay out of the porphyrin $\pi \rightarrow \pi^*$ excited state which has the effect of decreasing the probability for the resonance Raman process to occur (Adar et al., 1976). Helium temperature excitation profiles of the intensities of the Raman bands of ferrous cytochromes *c* and b_5 as a function of excitation wavelength demonstrated the usefulness of the technique in making inferences about optically inactive d-d transitions of the iron atom which have direct bearing on the redox properties of the hemes (Friedman et al., 1977). Moreover, in a study of the RR line widths of paramagnetic hemes it has been shown that there is nonradiative coupling between the porphyrin vibrational levels and low lying electronic levels of the iron (Adar, 1978). This latter suggestion may prove important in the study of heme proteins where subtle changes in bonding of axial ligands is thought to have an important role in biological function.

We have proposed that the relatively high resolution capabilities of the Raman technique could be exploited in studying interactions between hemes in biological membranes. A model study of the μ-oxo dimer indicated that this proposal was feasible (Adar & Srivastava, 1975). A spectroscopic analysis of RR data in a series of samples of the purified cytochrome b-c_1 complex from pigeon breast mitochondria, which had been trapped in well-defined redox states, indicated that the effects of membrane organization on some of the Raman bands can be substantial (Adar & Erecińska, 1977). We have since been able to excite RR spectra in whole mitochondria and identify them with the various cytochrome components by exploiting the known wavelength dependence of the intensities and by following marker bands for heme type. These data are reported here.

Materials and Methods

Pigeon breast mitochondria were prepared as described previously (Erecińska et al., 1973) and kept frozen at −30 °C. The frozen mitochondria were rapidly thawed at 30 °C and washed once in 10 mM phosphate buffer, pH 7.4, and twice in 100 mM phosphate buffer, pH 7.4, and finally suspended in the same medium. Final concentration as measured by cytochrome *a* content [$\Delta\epsilon_{mM}$ at 605-630 nm (red-oxid) = 26.4 cm^{-1}] was of the order of 20-30 μM.

Samples reduced with dithionite were contained in 1-mm melting point glass capillaries. At these high protein concentrations, oxygen cannot diffuse into samples contained in the sealed capillaries. To prevent denaturation by the focused laser beam, the samples were cooled with a flow of cool nitrogen gas. (Previous experience with particulate preparations has indicated that samples will not denature if adequately cooled.) It was assumed that RR spectra can be attributed to the reduced

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