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Identification of a Local Anesthetic Binding Site in Nicotinic Post-Synaptic Membranes Isolated from *Torpedo marmorata* Electric Tissue

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SUMMARY

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Studies are presented of the interaction of aromatic amine noncompetitive antagonists with the receptor-rich (post-synaptic) membranes isolated from *Torpedo marmorata* electric organ. When present at micromolar concentrations, methyl iodide quaternary derivatives of the potent non-competitive antagonists proadifen and dimethisoquin increase the affinity of the membrane-bound nicotinic receptor for [^{3}H]acetylcholine and [^{14}C]dimethyltubocurarine. The equilibrium binding of [^{14}C]meproadifen (2-(diethylmethylamino)ethyl-2,2-diphenyl valerate) to the receptor-rich membranes is characterized by an ultracentrifugation assay. When all acetylcholine binding sites are occupied by the agonist carbamylcholine or the antagonist tubocurarine, [^{14}C]meproadifen is bound with a dissociation constant $K_D = 0.5$ μM to a number of sites equal to one-quarter the number of α-neurotoxin binding sites. That high affinity binding site is found in the receptor-rich membranes and not in other membrane fractions isolated from *Torpedo* electric organ. Other non-competitive aromatic amino antagonists including dimethisoquin, proadifen, and prilocaine displace [^{14}C]meproadifen, as does perhydrohistrionicotoxin. It is concluded that there is a specific site of binding for the aromatic amine non-competitive antagonists in the isolated nicotinic post-synaptic membrane that is distinct from the site of binding of acetylcholine. Analysis of the interaction of [^{14}C]meproadifen with the receptor-rich membranes in the absence of cholinergic ligands indicates that under these circumstances the ligand binds weakly to both the anesthetic binding site and the acetylcholine binding site ($K_D = 5$ μM). The functional significance of the specific binding site for the aromatic amine non-competitive antagonists is discussed in terms of its possible relation to the site of ion translocation and to the mechanism of receptor desensitization.

INTRODUCTION

Permeability control by nicotinic cholinergic receptors involves a minimum of two distinct cation recognition sites: the site of binding of AcCh[2] and the site of ion translocation. While numerous quaternary am-

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[2] Abbreviations used are: AcCh, acetylcholine; carb, carbamylcholine; proadifen, 2-(diethylamino)ethyl-2,2-diphenylvalerate (also known as SKF-525a); meproadifen, 2-(diethylmethylamino)ethyl-2,2-diphenylvalerate; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; DFP, diisopropylfluorophosphate; C_{50}, concentration of competitor required to reduce specific binding by 50%; P, membrane buffer partition coefficient expressed in units nmoles ligand per gram of membrane protein/nmole ligand per ml of buffer.

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monium compounds are known that interact with the AcCh binding site either as agonists or antagonists, it has proven difficult to identify ligands interacting with high specificity and affinity with the site of ion translocation. If such compounds existed, they would be classified pharmacologically as non-competitive antagonists; that is, they would alter the steady state agonist dose-response relation by decreasing the maximum response without altering significantly the apparent agonist dissociation constant.

Several classes of compounds including detergents (1, 2), fatty acids (2), and aromatic amines (3–6) have been shown to act as potent non-competitive antagonists of the action of AcCh on the isolated *Electrophorus* electroplax. Because all these compounds are amphiphilic, and they all are known to partition rather indiscriminately into biological and model membranes, it is possible that they act at the nicotinic post-synaptic membrane, as elsewhere, as membrane stabilizers (7, 8). In this case, they would alter the coupling between the binding of acetylcholine and the permeability response in the absence of any specific binding site in the post-synaptic membrane. Of the non-competitive antagonists, however, the aromatic amines are of particular interest because they might function by binding to a specific cation recognition site in the post-synaptic membrane. The identification of such a specific binding site provides no direct information about its functional significance, but for a cationic non-competitive antagonist that site could be a part of the site of ion translocation. Alternatively, it could be a distinct regulatory site (5, 9, 10).

Historically, many of the aromatic amines were developed and first characterized as local anesthetics, compounds that block reversibly the action potential of nerve and muscle (11). Although there is no direct correlation between the potency of the aromatic amines as local anesthetics and nicotinic antagonists (3, 12), we (6) have referred to the aromatic amine nicotinic non-competitive antagonists as local anesthetics and we will continue to do so in this paper. We wished to study directly the interaction of radioactively-labeled local anesthetics with the acetylcholine receptor-enriched membranes isolated from *Torpedo marmorata* electric tissue (13). The cholinergic receptor constitutes about 20–40% of the protein in these membranes, and the nicotinic response remains functional both in terms of ligand binding and in terms of the agonist associated increase of membrane permeability (for recent reviews, see 14, 15). It is reasonable to assume that the concentration of a specific binding site for a non-competitive antagonist in those membranes would be similar to the concentration of AcCh binding sites (1–3 μmoles AcCh bound per gram of protein). Under these circumstances it is possible to measure directly the equilibrium binding of a local anesthetic binding with a dissociation constant (K_D) smaller than 5–10 μM.

The most potent nicotinic non-competitive antagonists that have been identified are aromatic amines such as dimethisoquin and proadifen (2-(diethylamino)ethyl-2,2-diphenylvalerate), and histrionicotoxin, a spiropiperidine alkaloid isolated from the skin of *Dendrobates histrionicus* (16). These compounds are active at micromolar concentrations as noncompetitive antagonists of the action of carbamylcholine on the isolated *Electrophorus* electroplax (6, 12). Trimethisoquin, the methyl iodide quaternary derivative of dimethisoquin, is actually an order of magnitude more potent that its tertiary precursor (10). The action of these ligands has also been characterized *in vitro* in terms of their effect on the binding properties of the receptor-rich *Torpedo* membranes. At concentrations that block the electroplax permeability response, these ligands increase by a factor of two or three the affinity of the receptor for [^{3}H]AcCh (6, 17). At ten to one hundred-fold greater concentrations, these ligands decrease the amount of [^{3}H]AcCh bound at equilibrium (5, 6). The fact that low concentrations of these non-competitive antagonists increase the affinity of the cholinergic receptor suggested that they bound to a site distinct from, but coupled to, the acetylcholine binding site. Since higher concentrations of the ligands were associated with reduced binding of cholinergic

ligands, they might bind weakly to the AcCh binding site itself. Although the mechanism by which the local anesthetics increase the receptor affinity is unknown, the effect provides a useful assay to aid in the identification of ligands best suited for direct binding studies.

Radioactive derivatives have been prepared of several of these noncompetitive antagonists and utilized for equilibrium binding studies. [^{3}H]Perhydrohistrionicotoxin prepared by catalytic tritiation of histrionicotoxin was shown to bind to a site in receptor-rich membranes of *T. californica* (18) and *T. ocellata* (19) that is distinct from the acetylcholine binding site. [^{14}C]-Trimethisoquin was shown to interact with a distinct binding site in the receptor-rich membranes of *T. marmorata* (10). We report here a more detailed characterization of the local anesthetic binding site in the *T. marmorata* receptor-rich membranes in terms of the equilibrium binding properties of the quaternary amine non-competitive antagonist, [^{14}C]meproadifen (2-(diethylmethylamino)ethyl-2,2-diphenylvalerate). A preliminary report of this work has been presented (20).

MATERIALS AND METHODS

Preparation of receptor-rich membrane fragments. The preparation of membrane fragments rich in cholinergic receptor from fresh *T. marmorata* electric tissue has been described (13), and the preparation was followed with several modifications: The tissue was homogenized in 100 g portions in two volumes of water at 4° for 2 min in a Virtis apparatus at 95% maximal speed, allowed to rest one minute, and then homogenized for 1 min longer. The homogenate was centrifuged at 5000 × *g* for 10 min. The pellet was homogenized again according to the same procedure. The supernatants from these centrifugations were combined and centrifuged at 10,000 × *g* for 90 min. The supernatant was discarded, the pellet was resuspended, homogenized in 10% sucrose in a hand-held ground glass homogenizer, and 23 ml aliquots applied to sucrose density gradients (14 ml) formed by two cycles of freezing and thawing of a 1.2 M sucrose solution. The gradients were centrifuged for 4 hours at 80,000 × *g* in the SW 27 rotor of a Beckman L5-50 ultracentrifuge.

Equilibrium binding of radioactive ligands. Binding of [^{14}C]meproadifen at 4° was measured by centrifugation. The membrane suspension was diluted in *Torpedo* physiological saline solution (250 mM NaCl; 5 mM KCl; 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Na phosphate, pH 7.0) and homogenized with a Potter teflon-glass homogenizer before the addition of [^{14}C]meproadifen. The membrane suspension was equilibrated with the ligand(s) for 10–30 min at 4°, and separation of bound from free ligand was then accomplished by centrifuging 100 μl samples in polyethylene tubes for 15 min in a Beckman Airfuge at 130,000 × *g*. Radioactivities of the media before and after centrifugation were determined by counting aliquots in 10 ml of scintillation cocktail (2 parts toluene, 1 part Triton X-100, 0.4% 2-(4′-t-butylphenyl)-5(4″-biphenylyl)-1,3,4-oxadiazole). The final sample volume to be counted was always adjusted to 1 ml by the addition of *Torpedo* physiological saline. Radioactivity in the pellet was determined by the following procedure. After centrifugation, the supernatant was removed from the centrifuge tube, which was then wiped with a cotton swab, and left inverted on the swab for 20 min. One hundred microliters of 10% (w/v) sodium dodecylsulfate (Sigma) was then added to the tubes, which were capped and allowed to stand overnight. The next day the solution was transferred to a counting vial and counted as described above.

The binding of the [^{3}H]AcCh and tubocurarine [^{14}C]dimethyliodide to the *Torpedo* membrane fragments in *Torpedo* physiological saline solution was measured by an ultracentrifugation assay (21). For studies with [^{3}H]AcCh, a concentrated membrane suspension was pretreated for 30 min with the potent acetylcholinesterase inhibitor diisopropylfluorophosphate (DFP, 3.3 mM), and binding studies were then performed in the presence of 0.1 mM DFP. Bound [^{3}H]AcCh or tubocurarine [^{14}C]dimethyliodide was determined from the difference between the radioactivities of the media before and after centrifugation at 100,000 × *g* for 90 min in a Beckman

Rotor 50. All studies were performed at 4°.

Assays. Proteins were estimated by the method of Lowry *et al.* (22) using bovine serum albumin as the standard. Acetylcholinesterase activity was assayed with acetylthiocholine as substrate by the method of Ellman *et al.* (23). In order to convert from the observed change in optical density per time per volume of membrane suspension to concentration units, it was assumed that acetylcholinesterase had a molecular weight of 260,000 and that the acetylthiocholine turnover number for the pure enzyme was 750 moles per hour per gram protein (24).

Na^+-K^+-dependent ATPase was assayed using p-nitrophenyl phosphate (di-tris salt) as the substrate (25). The rate of appearance of p-nitrophenol was determined in a reaction mixture containing 4 mM $MgCl_2$-50 mM Tris, pH 7.8. The increased phosphatase activity associated with the presence of 10 mM KCl was taken as a measure of Na^+-K^+ ATPase activity, since control experiments established that greater than 90% of that activity was inhibited by ouabain.

The binding of [^{3}H]α-toxin from *N. nigricollis* was used to estimate the concentration of cholinergic receptor sites. The assay was performed as described (21), except that the Millipore HA filters used to retain the membrane-[^{3}H]α-toxin complex were first soaked in *Torpedo* physiological saline solution supplemented with 0.1% bovine serum albumin. This procedure decreased nonspecific retention of radioactivity on the filters.

Initial rate of [^{3}H]α-toxin binding to membrane fragments. This rate was measured at 23° by filtration on Millipore filters as described by Weber and Changeux (21). The concentration of [^{3}H]α-toxin was 0.8 nM and that of [^{3}H]α-toxin binding sites was 4 nM. Under these conditions the amount of toxin bound was linear with time for at least 5 min, and the initial rate of binding was determined by filtering aliquots at 45 sec intervals after mixing.

Preparation of [^{14}C]meproadifen iodide. [^{14}C]Meproadifen was prepared by reaction of 2-(diethylaminoethyl)-2,2-diphenyl valerate with [^{14}C]methyliodide (56 mCi/mmole, New England Nuclear Batch #08-093-7). The product was purified by preparative thin layer chromatography on silica gel with 1:9 ethanol:CH_3NO_2. The R_F of the product was 0.55, and the purified material cochromatographed with unlabeled meproadifen, while the R_F for proadifen was 0.05.

Chemical products. Cholinergic ligands and local anesthetics obtained commercially were used without further purification. The hydrochloride salts of dimethisoquin and proadifen were gifts from Smith, Kline, and French (Philadelphia, Pa.). Trimethisoquin and meproadifen were prepared from the free base tertiary amine precursor as described for [^{14}C]meproadifen. Lidocaine, prilocaine, and the ethyl bromide derivative of lidocaine were a gift from Astra Pharmaceuticals (Worcester, Mass.). Perhydrohistrionicotoxin (H_{12}-HTX) was a gift of Dr. Y. Kishi (Cambridge, Mass.) and his colleagues who had synthesized it (26). Structures of these noncompetitive antagonists are presented in Fig. 1. The [^{3}H]α-neurotoxin of *Naja nigricollis* was a gift of Drs. A. Menez, J. L. Morgat, P. Fromageot, and P. Boquet. [^{3}H]Acetylcholine chloride (TRA. 277, Batch 11) was purchased from Amersham (Arlington Heights, Ill.). On the basis of an isotope dilution assay using the membrane bound cholinergic receptor, it was concluded that the reported specific activity (147 mCi/mmole) was underestimated by a factor of 3.4. Tubocurarine di[^{14}C]methyl iodide (Batch 7, 89 mCi/mmole) was purchased from Amersham. Although the material was described as tubocurarine di[^{14}C]methylether iodide, in view of the recent appreciation of the correct structure of tubocurarine chloride (27), the structure of this methyl iodide adduct may be incorrect. It is probably O,O,N-trimethyltubocurarine iodide.

RESULTS

Choice of non-competitive antagonists for direct binding studies. Dimethisoquin and proadifen were known to be active at micromolar concentrations both *in vivo* on the isolated *Electrophorus* electroplax and *in vitro* as modifiers of the equilibrium

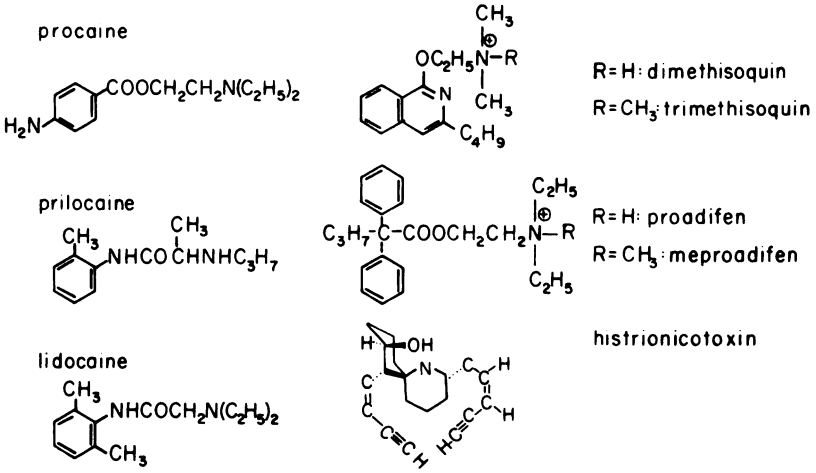

FIG. 1. *Structures of nicotinic non-competitive antagonists.*

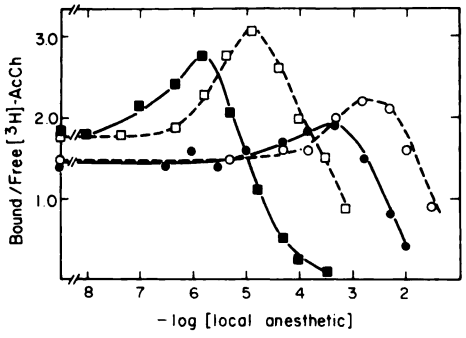

FIG. 2. *Effects of tertiary amine local anesthetics and their quaternized derivatives on the binding of [3H]acetylcholine to Torpedo receptor-rich membranes*

Binding of [3H]AcCh (total concentration, 7 nM) to a membrane suspension (30 nM Naja α-toxin sites, .03 g of protein per liter) was measured by ultracentrifugation after preincubation with 0.1 mM DFP. □—□, dimethisoquin; ■—■, trimethisoquin; ○—○, lidocaine; ●—●, lidocaine ethyl bromide.

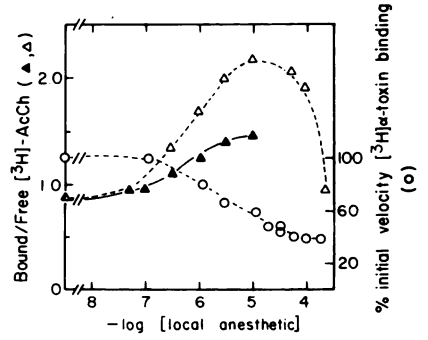

FIG. 3. *Effects of proadifen and meproadifen on the equilibrium binding of [3H]AcCh and the kinetics of binding of [3H]α-toxin to Torpedo receptor-rich membranes*

Binding of [3H]AcCh (total concentration 17 nM) to a membrane suspension (30 nM Naja α-toxin binding sites) was measured by ultracentrifugation in the presence of 0.1 mM DFP. In the absence of local anesthetic, the concentration of bound ligand was 8 nM, and greater than 90% of that binding was displaced by α-neurotoxin. △—△, proadifen; ▲—▲, meproadifen. Initial rate of binding of [3H]α-toxin to a membrane suspension (4 nM Naja α-toxin sites) in physiological saline incubated for 20 min in the presence of proadifen (○—○) prior to the addition of [3H]α-toxin of *Naja nigricollis* (0.8 nM). Association kinetics were determined by a Millipore filtration assay (21).

binding of [3H]AcCh to *Torpedo* post-synaptic membranes (6). Several methods were possible for the preparation of radioactive derivatives of these compounds. A simple direct approach involves the quaternization of suitable parent compounds with radioactive methyl or ethyl iodide. This reaction would result in products of high specific activity with little danger of multiple reaction products. However, one has to be aware that a quaternary ammonium analogue of any particular tertiary amine non-competitive antagonist could act itself as a competitive rather than non-competitive blocking agent. In order to determine whether quaternized derivatives of local anesthetics still acted as non-competitive antagonists, we examined the effect of several such derivatives on the equilibrium binding of [3H]AcCh by the *Torpedo* post-synaptic membranes.

In Figs. 2 and 3 it is shown that the

methyliodide derivatives of dimethisoquin (trimethisoquin) and proadifen (meproadifen) and the ethyl bromide derivative of lidocaine behave like other non-competitive antagonists in that they regulate the affinity of the membrane-bound *Torpedo* receptor for AcCh. In their presence there is an increase in the amount of [^{3}H]AcCh bound at equilibrium that is due to an increased affinity of the receptor for AcCh and not due to any change in the number of sites. It is striking that the quaternary derivatives of dimethisoquin and lidocaine caused an increased binding of AcCh when present at slightly lower concentrations than the tertiary amine precursors. At higher concentrations both the tertiary and quaternary amines decrease the binding of AcCh, possibly due to a direct interaction with the AcCh binding site. Since meproadifen and proadifen were associated with increased binding of AcCh over wider concentration ranges than dimethisoquin or trimethisoquin, [^{14}C]meproadifen was prepared for use in direct binding studies.

Interaction of [^{14}C]meproadifen with surfaces and Torpedo membranes. Aromatic amines are well known for the diversity of their effects as general surface active compounds (8). Before attempting to study the interaction of meproadifen with isolated *Torpedo* membranes, we first examined its interaction with the materials necessary for the binding assay. Meproadifen interacts strongly with cellulose nitrate centrifuge tubes, and that interaction is superficially analogous to a specific binding process in that high concentrations of non-radioactive ligand reduce retention on the tube walls. Even at the high ionic strength characteristic of *Torpedo* physiological saline, at 2 μM [^{14}C]meproadifen 20% of the ligand was adsorbed on the sides of the tube, and that adsorption was reduced by 80% in the presence of 100 μM non-radioactive meproadifen. To make matters worse, amines such as proadifen and carbamylcholine, as well as α-neurotoxin, also reduced the amount of adsorbed radioactivity. For polyethylene tubes, however, the problem was less serious: the adsorbed meproadifen was still displaced by high concentrations of non-radioactive meproadifen, but less than 3% of the radioactivity was adsorbed.

The interaction of [^{14}C]meproadifen with *Torpedo* membrane fractions was measured in polyethylene centrifuge tubes by the Airfuge ultracentrifugation assay (see MATERIALS AND METHODS). The ligand interaction with two different membrane fractions was measured: a receptor-rich fraction (1.3 μmoles α-toxin sites per gram of protein) and a fraction containing smaller amounts of receptor (0.1 μmoles α-toxin sites per gram of protein) but rich in acetylcholinesterase. Total binding of [^{14}C]meproadifen (2 μM) was determined as well as the nonspecific interaction of the ligand with the membranes in the presence of 100 μM meproadifen (Fig. 4). The specifically bound [^{14}C]meproadifen was determined from the difference between the total binding and the binding in the presence of 100 μM meproadifen. For the receptor-rich membrane fraction in the presence of 2 μM [^{14}C]meproadifen, the specifically-bound li-

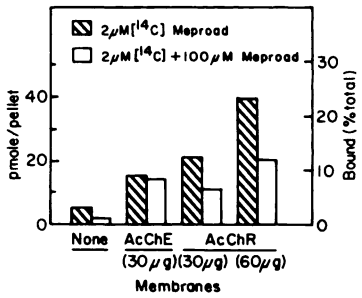

FIG. 4. *Interaction of [^{14}C]meproadifen with Torpedo membrane fragments and polyethylene tubes*

2 μM [^{14}C]meproadifen (5900 cpm/25 μl) in *Torpedo* physiological saline solution was equilibrated for 20 min in polyethylene centrifuge tubes: in the absence of any added membranes ("None"); in the presence of a membrane fraction containing few nicotinic receptor sites but enriched in acetylcholinesterase ("AChE," 0.3 g of protein per liter containing 0.1 μmoles Naja α-toxin sites/g protein), and a fraction enriched in nicotinic receptors ("AcChR," 0.3 and 0.6 g of protein per liter containing 1.3 μmoles α-toxin sites/g protein). Solutions were equilibrated with 2 μM [^{14}C]meproadifen (hatched bars) or 2 μM [^{14}C]meproadifen supplemented with 100 μM non-radioactive meproadifen (open bars). The radioactivity in the pellets obtained from 0.1 ml aliquots after centrifugation at 130,000 × *g* for 15 min was recovered as described in MATERIALS AND METHODS.

gand amounted to 0.3 μmoles/g protein, while the ligand bound specifically to the esterase rich fraction was less than 0.04 μmoles/g protein. Hence a specific binding component of [^{14}C]meproadifen was identified that was characteristic of the receptor-rich but not the acetylcholinesterase-rich membrane fractions of *Torpedo marmorata* electric tissue. It was also established that both the specific and non-specific interaction of [^{14}C]meproadifen with the *Torpedo* membranes was proportional to the mass of protein over a range from 0.1–0.7 g protein/l.

While no significant specific binding of [^{14}C]meproadifen to the esterase-rich membranes was detected, the non-specific interaction with those membranes was essentially the same as with the receptor-rich membranes. At 100 μM meproadifen, that non-specific interaction was characterized by a partition coefficient, P = 220 nmoles per g protein/nmole per ml for the receptor-rich membranes, and for the esterase-rich membranes, P = 270. For comparison purposes we also determined the partition coefficients characterizing the non-specific interaction with the *Torpedo* receptor-rich membranes in physiological saline of other non-competitive and competitive nicotinic antagonists. [^{14}C]Trimethisoquin was characterized by a similar partition coefficient (at 100 μM, P = 300), and a similar value for [^{3}H] H_{12}-histrionicotoxin (at 40 μM, P = 200–400) can be estimated from published data (18) by assuming that the receptor-rich membranes used in that study contained 1–1.5 μmoles α-toxin sites/g of protein). These values are an order of magnitude higher than the partition coefficients for the tertiary amine local anesthetic [^{14}C]lidocaine (at 1 mM, P = 7) or the competitive antagonist [^{3}H]tubocurarine (at 100 μM, P = 30).

To further characterize the specific binding of [^{14}C]meproadifen to the *Torpedo* receptor-rich membranes, the binding was determined in the presence of radioactive anesthetic concentrations varying from 0.1 to 3 μM (Fig. 5). The binding was determined in the presence of concentrations of cholinergic ligands sufficient to occupy all receptor sites. Whether the acetylcholine binding site was occupied by the agonist carbamylcholine (30 μM) or the antagonist tubocurarine (10 μM), the membranes bound the same total amount of [^{14}C]meproadifen and the same nonspecific partitioning was observed in the presence of 100 μM non-radioactive meproadifen. At each concentration of free [^{14}C]meproadifen, specifically bound [^{14}C]meproadifen was determined by the difference between total binding and the non-specific partitioning. Double reciprocal analysis of the specific binding component established that the concentration of [^{14}C]-meproadifen binding sites was 0.5 μmoles/g protein, and the dissociation constant, K_d = 0.4 μM. The site concentration was equivalent to (0.25) per α-toxin site. Although the same high affinity binding component was detected whether the acetylcholine binding sites were occupied by a reversible agonist or antagonist, when the nicotinic receptor was occupied by α-Bungarotoxin, very little, if any, specific binding of [^{14}C]-meproadifen was detected (Fig. 5). More detailed analyses of the meproadifen binding function will be presented later (Fig. 8 and 9) when a comparison is made of the ligand binding in the presence and absence of cholinergic ligands.

Distribution of [^{14}C]meproadifen binding sites in Torpedo membranes fractionated on a sucrose density gradient. In order to determine whether the high affinity meproadifen binding site identified in the isolated post-synaptic membranes was characteristic of those membranes in particular, the distribution of high affinity meproadifen binding sites was determined in *Torpedo* membranes fractionated on a sucrose density gradient. For each fraction, specific binding was determined from the difference between total and non-specific binding of [^{14}C]meproadifen (0.5 μM) to a membrane suspension (0.3 g protein/1). All binding assays were performed in the presence of 30 μM carbamylcholine, and the concentration of [^{14}C]meproadifen was sufficient to occupy about half the anesthetic sites present in the receptor-rich membranes. The distribution of [^{14}C]meproadifen binding sites paralleled closely the distribution of [^{3}H]α-neurotoxin binding sites with the highest concentration of both sites

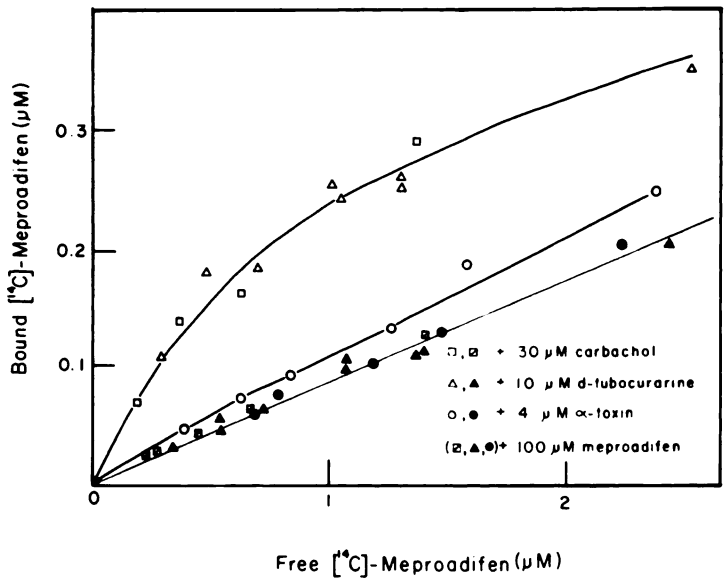

FIG. 5. *Equilibrium binding of [^{14}C]meproadifen to Torpedo receptor-rich membranes in the presence of cholinergic ligands.*

A membrane suspension (0.8 μM Naja α-toxin sites, 0.4 g of protein per liter) in *Torpedo* physiological saline was divided into three portions. An ultracentrifugation assay was used to measure the binding of [^{14}C]-meproadifen to suspensions containing 30 μM carbamylcholine (□, ▨), 10 μM tubocurarine (△, ▲), or 4 μM α-Bungarotoxin (○, ●). Non-specific interaction of [^{14}C]meproadifen was determined by the inclusion of 100 μM non-radioactive meproadifen (▨, ▲, ●). Each data point is the mean of duplicate samples.

recovered at 38% (w/w) sucrose (Fig. 6A). Such a distribution of anesthetic binding sites is distinct from the distribution of membrane protein (peak distribution at 33% (w/w) sucrose) and from the distribution of (Na^+-K^+)-ATPase activity (33% (w/w) sucrose, peak activity) or acetylcholinesterase (24% (w/w) sucrose, peak activity) (Fig. 6B). In all membrane fractions, the non-specific interaction of [^{14}C]meproadifen with the membranes remained roughly constant and was characterized by a partition coefficient P = 200 ± 30. These results established that the specific [^{14}C]meproadifen binding site is characteristic of the isolated post-synaptic membranes themselves. Of course, the assay procedure used does not exclude the possibility that specific binding sites occur in other membrane components that are present either at a much lower concentration per gram of protein or are characterized by a weaker binding constant.

Effect of local anesthetics and histrionicotoxin on the binding of [^{14}C]meproadifen. The specific binding of [^{14}C]meproadifen (0.3 μM) was determined in the presence of various local anesthetics in order to characterize the pharmacological specificity of the meproadifen site. Once again, experiments were first carried out in the presence of 30 μM carbamylcholine, to prevent any possible binding of [^{14}C]meproadifen to the acetylcholine binding site. All five local anesthetics studied (Fig. 7) as well as perhydrohistrionicotoxin (Fig. 8) displace meproadifen in a dose-dependent manner. Perhydrohistrionicotoxin and proadifen were the most potent ligands tested. The concentration of each which displaced 50% of the specifically bound meproadifen (C_{50}) was approximately micromolar. Trimethisoquin was slightly more potent than dimethisoquin, both being characterized by C_{50} about 10 μM. Prilocaine and procaine displaced meproadifen only when present at concentrations in excess of millimolar. The C_{50}'s for the various anesthetics are summarized in Table 1, along with values for similar displacement experiments using

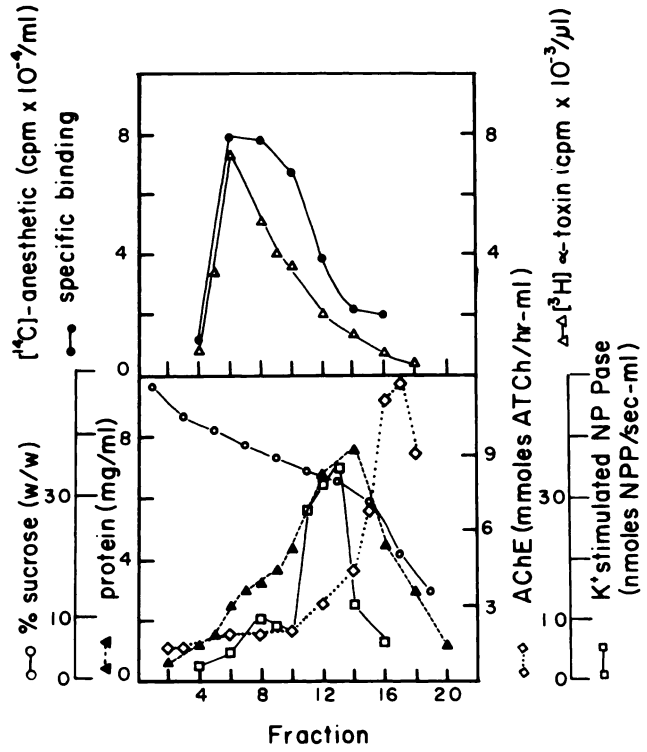

FIG. 6. *Distribution of [^{14}C]meproadifen (local anesthetic) binding sites, [^{3}H]α-neurotoxin binding sites and plasma membrane enzymes in membrane fragments isolated from an homogenate of Torpedo marmorata electric tissue by ultracentrifugation on a sucrose density gradient*

Membranes were prepared as described in METHODS. A. Distribution of [^{3}H]α-toxin of *N. nigricollis* (Δ) and specifically bound [^{14}C]meproadifen (●). For each gradient fraction, the binding of [^{14}C]-meproadifen (0.5 μM total concentration) to a membrane suspension containing 0.3 g of protein per liter was determined in the presence of 30 μM carbamylcholine as described in METHODS. B. Distribution of proteins (▲), acetylcholinesterase (◇), (Na^+-K^+)-ATPase (□) and sucrose (○) are indicated.

receptor-rich membranes isolated from *Torpedo californica* electric tissue.

Binding of [^{14}C]meproadifen to Torpedo receptor-rich membranes in the absence of cholinergic ligands. Since it was shown previously (6) that in the presence of local anesthetics, [^{3}H]AcCh is bound at equilibrium with an affinity two or three times higher than in their absence, it was of interest to determine the specific binding of [^{14}C]meproadifen in the absence of cholinergic ligands as well as in their presence. The binding of [^{14}C]meproadifen to *Torpedo* receptor-rich membranes was thus determined over concentrations ranging from 0.1 μM to 8 μM (Fig. 9). The total binding in the absence of carbamylcholine differed strikingly from that observed in its presence. For concentrations less than micromolar meproadifen, enhanced binding was observed in the presence of carbamylcholine, but at higher meproadifen concentrations, the opposite was true. The nonspecific partitioning of [^{14}C]meproadifen determined in the presence of 100 μM non-radioactive meproadifen was the same in the presence or absence of 30 μM carbamylcholine. Inspection of the data shown in Fig. 9 reveals that meproadifen is apparently bound with higher affinity to a smaller number of sites in the presence than in the absence of carbamylcholine.

The concentration dependence of the specific component of the meproadifen binding function is shown in Fig. 10. To a first approximation, both in the absence

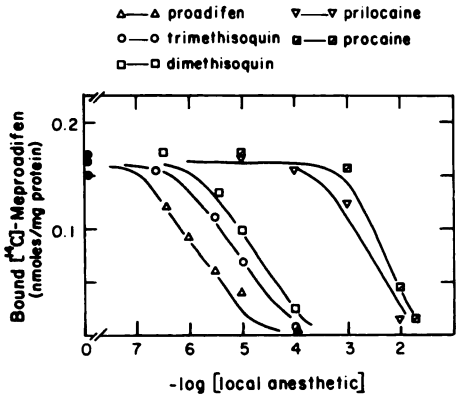

FIG. 7. *Effect of local anesthetics on the binding of [^{14}C]meproadifen to Torpedo receptor-rich membranes*

A membrane suspension (0.4 μM Naja α-toxin sites, 0.2 g of protein per liter) in *Torpedo* physiological saline was equilibrated with [^{14}C]meproadifen (0.27 μM), 30 μM carbamylcholine and the indicated concentration of local anesthetics. Specifically bound [^{14}C]-meproadifen was determined by subtracting the binding in the presence of 100 μM meproadifen from the total binding. In the absence of other local anesthetics, the total amount of [^{14}C]meproadifen bound was 54 nM (270 nmoles/g protein) of which 34 nM (170 nmoles/g protein) was displaced by 100 μM non-radioactive meproadifen (●). Each data point is the mean of duplicate samples. Specific binding in the presence of proadifen (△); trimethisoquin (○); dimethisoquin (□); procaine (▨); prilocaine (▽).

and in the presence of carbamylcholine, the meproadifen binding was consistent with a single class of sites. In the absence of carbamylcholine, meproadifen was bound to a number of sites equal to 0.9 ± 0.2 times the number of α-toxin sites with a dissociation constant, K_d = (4.4 ± 0.9) μM. In the presence of carbamylcholine, meproadifen was bound an order of magnitude more tightly, K_d = (0.5 ± 0.2) μM to a smaller number of sites, n = 0.26 ± .05 the number of α-toxin sites. In direct binding studies with [^{3}H]acetylcholine, we have determined that the number of acetylcholine sites in these preparations is the same as the number of sites for the [^{3}H]α-toxin of *Naja nigricollis*.[3]

It is striking that in the absence of carbamylcholine there was no component of the meproadifen binding characterized by the high affinity observed in the presence of carbamylcholine. Increased precision of the binding data is necessary to determine whether the meproadifen binding function in the absence of carbamylcholine may be better characterized by two sites possessing slightly different dissociation constants. The binding constants estimated for this preparation (A) and a second preparation (B) of *Torpedo marmorata* receptor-rich membranes are summarized in Table 2.

Several experiments were performed to provide further characterization of the nature of the anesthetic binding function in the absence of carbamylcholine. First, the effect of carbamylcholine on the anesthetic binding function is a result of the binding of carbamylcholine to the acetylcholine binding site. In Fig. 11 is shown the binding of [^{14}C]meproadifen (0.2 μM) to the receptor-rich *Torpedo* membranes in the presence of concentrations of carbamylcholine

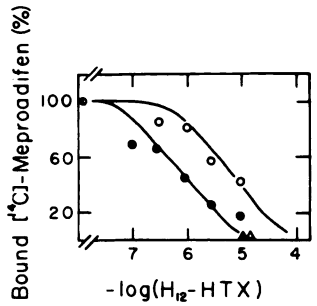

FIG. 8. *Specific binding of [^{14}C]meproadifen to Torpedo receptor-rich membranes: effect of H_{12}-histrionicotoxin in the presence and absence of carbamylcholine*

Torpedo marmorata membranes (2.8 μM Naja α-toxin sites, 1.3 g of protein per liter) were diluted 4.2-fold in *Torpedo* physiological saline and divided into two portions. A: [^{14}C]Meproadifen was added to 0.38 μM and carbamylcholine was added to 33 μM. B: [^{14}C]Meproadifen was added to 3.7 μM. Specific binding was determined by subtracting the amount bound in the presence of 100 μM meproadifen from the total binding. Specific binding in the absence of H_{12}-HTX was taken to be 100% for each sample (◑). Binding in the presence of H_{12}-HTX: sample A (●); sample B (○). Binding was also determined in the presence of 10 μM H_{12}-HTX plus 100 μM meproadifen: sample A (▲); sample B (△). Each data point is the mean of duplicate samples. In the absence of carbamylcholine, specifically bound [^{14}C]meproadifen was 0.78 μmoles/g protein; in the presence of carbamylcholine, 0.28 μmoles/g protein.

[3] Neubig, R. and J. B. Cohen, unpublished observations; see also (21).

TABLE 1

Displacement of [^{14}C]meproadifen from Torpedo marmorata receptor-rich membranes by local anesthetics and perhydrohistrionicotoxin

C_{50} is the concentration of anesthetic required to reduce specific binding by 50%. C_{50} for ligands in the presence of carbamylcholine was determined from the data in Figs. 7 & 8. The displacement of [^{14}C]meproadifen by ligands in the absence of carbamylcholine was determined for a membrane suspension (0.4 μM α-toxin sites) equilibrated with 2 μM [^{14}C]meproadifen. K_P is the protection constant characterizing the anesthetic concentration required to reduce the initial rate of [^{3}H]α-toxin binding by 50% (Figs. 3 and 13).

Anesthetic	C_{50} + carbamylcholine	C_{50} − carbamylcholine	K_P against [^{3}H]α-toxin binding
	μM	μM	μM
Meproadifen	0.5[a]	5[a]	
Proadifen	1, 0.3[b]	20	—[c]
Dimethisoquin	10, 6[b]	50	25 (18 ± 4)[d]
Trimethisoquin	8, 6[b]	—	12
H_{12}-HTX	0.7	6	—
Prilocaine	2000	—	>5000[d]
Procaine	5000	—	1000[d]

[a] Direct binding [^{14}C]meproadifen.

[b] Displacement from *T. californica* receptor-rich membrane.

[c] Ligand effect inconsistent with hyperbolic protection function (see Fig. 3).

[d] From Weber and Changeux (5).

ranging from 0 to 30 μM. In the presence of carbamylcholine the specific binding of [^{14}C]meproadifen was increased from 40 nmoles/g protein to 140 nmoles/g protein, and 0.3 μM carbamylcholine was associated with a half maximal effect, a concentration similar to the dissociation constant characteristic of the binding of carbamylcholine to the membrane-bound nicotinic receptor (21). Furthermore, when the receptor-rich membranes were pretreated with α-Bungarotoxin, carbamylcholine no longer altered the [^{14}C]meproadifen binding function. Second, experiments established that local anesthetics and perhydrohistrionicotoxin displaced meproadifen bound in the absence of carbamylcholine (Table 1 and Fig. 8). Because meproadifen was bound with a lower affinity in the absence of carbamylcholine, higher concentrations of meproadifen were used. However, the concentrations used were always less than those necessary to occupy 50% of the ligand binding sites. In the absence of carbamylcholine, proadifen and dimethisoquin both displaced specifically-bound [^{14}C]meproadifen, although the concentrations necessary to displace half the specifically bound ligand were an order of magnitude greater than those active in the presence of carbamylcholine. For dimethisoquin the C_{50} (50 μM) is the same as the dissociation constant of the cholinergic receptor for dimethisoquin calculated from its competitive inhibition of the binding of [^{3}H]AcCh (5). Similarly, in the absence of carbamylcholine 6 μM perhydrohistrionicotoxin displaced 50% of the meproadifen bound specifically, while 0.7 μM perhydrohistrionicotoxin displaced that fraction of the specifically bound meproadifen in the presence of carbamylcholine (Fig. 8).

Effects of local anesthetics on the binding of [^{14}C]tubocurarine and of [^{3}H]α-neurotoxin. [^{14}C]Meproadifen was bound with high affinity whether the acetylcholine binding site was occupied by carbamylcholine or tubocurarine. However, in the presence of α-neurotoxin, no equivalent high affinity binding was observed. The similarity of the meproadifen binding function in the presence of reversible cholinergic agonists and antagonists is not surprising in view of the fact that local anesthetics regulate the equilibrium binding of tubocurarine to an extent similar to their regulation of the [^{3}H]acetylcholine binding function (10). [^{14}C]Dimethyltubocurarine is bound at equilibrium with a K_d = 1 μM to a number of sites equal to the number of acetylcholine binding sites (10). Furthermore, the local anesthetic prilocaine (3 mM) increases by a factor of three the affinity of the membrane bound *Torpedo* receptor for [^{14}C]dimethyltubocurarine (10). We extended these studies to the ligands emphasized in this work, proadifen and dimethiosoquin. In Fig. 12 it is shown that both these ligands increase the affinity of the receptor for dimethyltubocurarine when they are present at concentrations between 1 and 10 μM. It is also shown that procaine, perhaps the prototypic local anesthetic nicotinic non-compet-

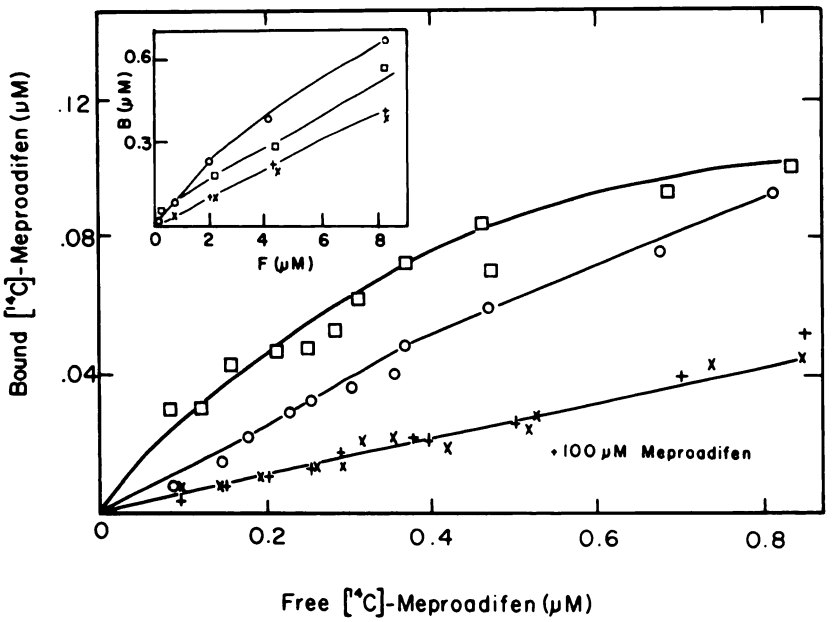

FIG. 9. *Equilibrium binding of [¹⁴C]meproadifen to Torpedo marmorata receptor-rich membranes in the presence and absence of carbamylcholine*

Torpedo membrane fragments (3.8 μM Naja α-toxin sites, 1.9 g of protein per liter) were diluted 9.4-fold in physiological saline (○——○); dilution into the same medium containing 30 μM carbamylcholine (□——□); containing 100 μM meproadifen (+——+); containing 30 μM carbamylcholine and 100 μM meproadifen (×——×). Each data point is the mean of duplicate samples.

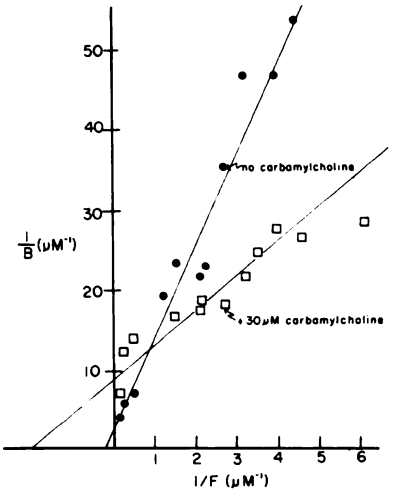

FIG. 10. *Analysis of the specific binding of [¹⁴C]-meproadifen to Torpedo membrane fragments in the presence* (□——□) *and absence* (●——●) *of carbamylcholine (30 μM)*

The data were taken from Fig. 9. The specific binding of [¹⁴C]meproadifen was determined from the difference between the total binding and that observed in the presence of 100 μM meproadifen. The lines were drawn according to the results of a weighted linear regression analysis where the weighting factors were determined by the variances of the amount bound expressed as the percent of the bound ligand. In the absence of carbamylcholine, K_D = 4.4 ± 0.9 μM, n = 0.9 ± .2 anesthetic sites/α-toxin sites; in the presence of carbamylcholine, K_D = 0.5 ± 0.2 μM; n = 0.26 ± .05 anesthetic/α-toxin site.

itive antagonist, does not increase the affinity of the nicotinic receptor for tubocurarine. Furthermore, procaine did not increase the affinity with which [³H]AcCh was bound at equilibrium (data not shown). These results indicate that while not all non-competitive antagonists regulate the affinity of the membrane-bound cholinergic receptor, those that do actually control the affinity for both agonists and antagonists.

The effects of the non-competitive antagonists proadifen and dimethisoquin on the kinetics of binding of [³H]α-neurotoxin were studied to provide further information about the relationship between the binding of non-competitive antagonists and cholin-

ergic ligands. Weber and Changeux (5, 21) studied the α-toxin association kinetics in the presence of a variety of ligands. For nicotinic agonists and competitive antagonists, they found that the capacity of a ligand to decrease the initial rate of binding of α-toxin was characterized by a hyperbolic protection function, and the protection constant (K_P) equivalent to the ligand concentration reducing the rate of binding by 50% was the same as the dissociation constant of the ligand for the acetylcholine binding site determined by direct binding studies. They also reported that local anesthetic non-competitive antagonists reduced the rate of binding of α-toxin, but only at concentrations one to two orders of magnitude higher than those necessary to block the nicotinic response of the isolated *Electrophorus* electroplax. The concentration dependence of the effect of certain of the local anesthetics (dibucaine) was characterized by a hyperbolic protection function, while for others such as tetracaine this was not so. We used the Millipore filtration assay of Weber and Changeux (5) to determine the initial rate of binding of the [^{3}H]α-neurotoxin of *N. nigricollis* to *Torpedo* receptor-rich membranes that had been pre-equilibrated with the non-competitive antagonists. The effects of trimethisoquin and dimethisoquin on the rate of binding of α-toxin were characterized by hyperbolic protection functions characterized by protection constants, K_P = 13 μM and 25 μM, respectively (Fig. 13). The protection constants are summarized in Table 1, where they may be compared with the anesthetic concentrations necessary to displace [^{14}C]-meproadifen. The concentration dependence of the effect of proadifen on the [^{3}H]-α-toxin association kinetics was more complicated than for the other ligands (Fig. 3). In the presence of 10 μM proadifen, the rate of [^{3}H]α-toxin binding is decreased by 50% but that same ligand concentration is as-

TABLE 2

Equilibrium binding of [^{14}C]meproadifen to Torpedo marmorata receptor-rich membranes

Parameters were determined by a weighted linear regression of the double reciprocal plot of the specific component of the anesthetic binding function.

Preparation	K_D		Anesthetic site / α-toxin site	
	+carb	−carb	+carb	−carb
	μM	μM		
A	0.5 ± 0.2	4.4 ± 0.9	0.26 ± .05	0.9 ± 0.2
B	0.3 ± 0.2	5 ± 2	0.35 ± .05	1 ± 0.4

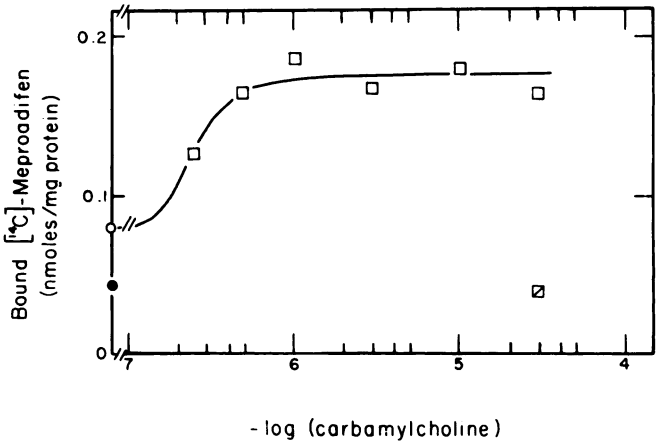

FIG. 11. *Concentration dependence of the effect of carbamylcholine on the binding of [^{14}C]-meproadifen to T. marmorata receptor-rich membranes*

Torpedo membranes (3.8 μM Naja α-toxin sites, 1.8 g of protein per liter) were diluted 9.4-fold in *Torpedo* physiological saline solution, and [^{14}C]meproadifen was added to 0.24 μM. Equilibrium binding was determined in the absence of other ligands (○); in the presence of 100 μM meproadifen (●); in the presence of the indicated concentrations of carbamylcholine (□); and in the presence of 30 μM carbamylcholine and 100 μM meproadifen (▨). Data are means of duplicate samples.

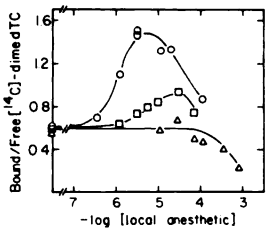

FIG. 12. *Effect of local anesthetics on the equilibrium binding of [^{14}C]dimethyltubocurarine to receptor-rich membranes of Torpedo marmorata*

Membrane fragments (0.6 μM Naja α-toxin sites, 1.1 g of protein per liter) in physiological saline were incubated with [^{14}C]dimethyltubocurarine (0.4 μM) and the indicated concentrations of proadifen (○); dimethisoquin (□), or procaine (△). The free ligand in equilibrium with the membrane fragments was determined from the supernatant radioactivity after centrifugation at 82,000 × g for 90 min at 15°. Bound dimethyltubocurarine was determined from the difference between total and free ligand. In the absence of added local anesthetics, bound ligand = 0.15 μM, free ligand = 0.25 μM, and greater than 95% of the bound ligand was displaced by 5 μM α-Bungarotoxin.

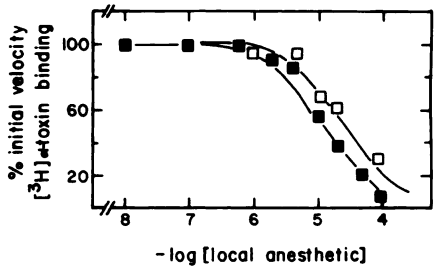

FIG. 13. *Effect of dimethisoquin and trimethisoquin on the initial rate of binding of [^{3}H]α-neurotoxin of Naja nigricollis to Torpedo membrane fragments*

Membrane fragments (4 nM α-toxin sites) in *Torpedo* physiological saline were incubated 20 min in the presence of the indicated concentrations of dimethisoquin (□) or trimethisoquin (■) prior to the addition of [^{3}H]α-toxin (0.8 nM). Initial rate of binding of [^{3}H]α-toxin was determined by a Millipore filtration assay (21). The solid line through the data for trimethisoquin was calculated for K_P = 12 μM, that through the dimethisoquin data was calculated according to K_P = 25 μM.

sociated with a maximal increase of the affinity of the nicotinic receptor for [^{3}H]-AcCh (Fig. 3) or [^{14}C]dimethyltubocurarine (Fig. 12). In the presence of 1 μM proadifen, the rate was decreased by 20%, but in the presence of 200 μM proadifen it was decreased only by 55%. Such a concentration dependence is not consistent with a hyperbolic protection function. This result is not in disagreement with earlier studies (5, 28) where it was reported that 1 mM proadifen reduced the rate of binding of α-toxin by 40–50%. The effect of lower concentrations of proadifen was not examined. Hence, on the basis of the effect of proadifen on the rate of binding of [^{3}H]α-toxin, it is necessary to conclude that some small reversible ligands decrease the rate of binding of α-toxin by binding to a site distinct from the acetylcholine binding site.

DISCUSSION

Meproadifen was initially identified as a potent nicotinic non-competitive antagonist on the basis of an *in vitro* assay: the capacity of a ligand to increase the affinity with which cholinergic ligands are bound at equilibrium to isolated *Torpedo* post-synaptic membrane. It had been shown previously (6) that the concentrations at which tertiary aromatic amines such as proadifen and dimethisoquin regulated receptor affinity *in vitro* were the same as those at which they acted as non-competitive antagonists at the isolated *Electrophorus* electroplax. We have now extended these studies to show that not only meproadifen but also quaternary derivatives of the aromatic non-competitive antagonists dimethisoquin and lidocaine also act *in vitro* as non-competitive antagonists.

The fact that the quaternary amines were active at lower concentrations than their tertiary amine precursors suggested to us that they might be interacting with a specific site in the isolated post-synaptic membrane, since quaternary aromatic amines are characterized by smaller partition coefficients than their tertiary precursors. For example, chlorpromazine methiodide was 75-fold less potent than chlorpromazine as a non-specific membrane stabilizer as judged by the ligand concentrations necessary to protect erythrocytes from osmotically-induced hemolysis (29). Furthermore, electrophysiological studies of the action of quaternized local anesthetics at the neuromuscular junction provided evidence that these ligands may block by binding to the

site of ion translocation itself (12, 39).

[^{14}C]Meproadifen was synthesized in order to study by direct means the interaction of aromatic amine non-competitive antagonists with the nicotinic receptor-rich membranes isolated from *Torpedo* electric tissue. Analysis of the interaction of [^{14}C]-meproadifen with biological membranes and various plastic surfaces established that, as expected, meproadifen is a highly surface active compound. Considerable care is necessary in the analysis of these interactions. For example, cellulose nitrate centrifuge tubes cannot be used because of the very strong interaction of [^{14}C]meproadifen with that surface. However, we have defined conditions under which it is possible to study with reasonable precision the equilibrium binding of [^{14}C]meproadifen to biological membranes.

The interaction of [^{14}C]meproadifen with *Torpedo* membranes consists of two components, a specific binding component and a non-specific partitioning into the membrane phase. The non-specific interaction of meproadifen with *Torpedo* membrane fragments in physiological salt solution is characterized by a partition coefficient (P = 200 nmoles per g protein/nmole per ml) that is an order of magnitude greater than that of a ligand such as tubocurarine, but similar to that for the potent non-competitive antagonists trimethisoquin (10) and perhydrohistrionicotoxin (17-19). While the non-specific interaction of meproadifen was relatively constant for different membrane fractions isolated from *Torpedo* electric tissue, a specific high affinity binding site was identified in the receptor-rich membranes that was not found in other membrane fractions.

The binding of [^{14}C]meproadifen to the isolated post-synaptic membranes in *Torpedo* physiological salt solution was first studied in the presence of high concentrations of cholinergic ligands, concentrations sufficient to assure occupation of all acetylcholine binding sites. In this manner any meproadifen bound specifically must be bound to a site other than the acetylcholine binding site. The total binding of meproadifen consists of a saturable component and a linear partitioning of the ligand into the membrane (Figs. 3 and 9). The similarity of the linear component of the total binding determined at relatively low concentrations of meproadifen (1–2 μM) with the ligand partitioning determined in the presence of 100 μM meproadifen indicates that the membrane-buffer partition coefficient of meproadifen does not change significantly over that range of concentrations. This result is surprising if one considers the number of molecules of anesthetic that partition into the membrane per phospholipid at the higher concentrations. That value can be estimated from the anesthetic partition coefficient and the 2:1 protein to lipid ratio of the *Torpedo* receptor-rich membranes (30, 31). With an average phospholipid molecular weight of 800, at 1 μM meproadifen there is about 1 anesthetic per 1000 lipid molecules but at 100 μM meproadifen about 1 anesthetic per 10 phospholipids. At these higher concentrations, it is quite possible that the anesthetic can cause nonspecific alterations of membrane structure.

The saturable component of the total binding function establishes that in the presence of cholinergic ligands, [^{14}C]meproadifen is bound with a dissociation constant, K_d = 0.5 μM to a number of sites equal to about 0.25 times the number of α-toxin or acetylcholine binding sites. Furthermore, [^{14}C]meproadifen is displaced from that binding site by local anesthetic noncompetitive antagonists such as dimethisoquin and prilocaine, and also by perhydrohistrionicotoxin. The concentration of H_{12}-HTX necessary to displace half the meproadifen (C_{50} = 0.7 μM) is similar to the dissociation constant, K_d = 0.3 μM, reported for the interaction of [^{3}H]H_{12}-HTX with receptor-rich membranes isolated from *Torpedo californica* (18) and *Torpedo ocellata* (19). These results establish that meproadifen as well as aromatic amine noncompetitive antagonists and histrionicotoxin bind to a common site on the isolated *Torpedo* post-synaptic membranes that is distinct from the acetylcholine binding site.[4]

[4] We have recently characterized the binding of [^{14}C]meproadifen to *Torpedo* post-synaptic membranes from which non-receptor peptides have been extracted (43). Since the ligand binding properties are

The results reported here indicate that the regulation of receptor affinity by the non-competitive antagonists is in fact due to the specific binding of the non-competitive antagonists and not to their non-specific partitioning into the receptor-rich *Torpedo* membranes. If the effect on the acetylcholine or tubocurarine binding function is due to occupation of the local anesthetic binding site identified in this report, then the principles of thermodynamics necessitate that nicotinic cholinergic ligands must regulate the binding of the local anesthetics. Direct analysis of the binding of [^{14}C]meproadifen established (Fig. 10) that meproadifen is bound more weakly but to a larger number of sites in the isolated *Torpedo* post-synaptic membranes in the absence of cholinergic ligands than in their presence. It is not simply a matter that meproadifen binds with high affinity to the site identified in the presence of cholinergic ligands and also to a second site. All the meproadifen bound in the absence of carbamylcholine is bound more weakly than in its presence. As the data in Table 2 show, in the absence of carbamylcholine the number of meproadifen binding sites is roughly equal to or slightly greater than the number of α-toxin sites (acetylcholine sites). The simplest interpretation of these data is that in the absence of cholinergic ligands meproadifen is bound with low affinity both to a local anesthetic binding site and to the acetylcholine binding site. Although the meproadifen binding function in the absence of carbamylcholine was analyzed in terms of a single class of binding sites (Table 2), further refinement of that binding function may be possible with binding data of greater precision. It is clear, however, that occupation of the acetylcholine binding site by cholinergic agonists (carbamylcholine) or antagonists (tubocurarine) does regulate the affinity of the anesthetic site. The specifically bound meproadifen regulates the equilibrium binding of cholinergic ligands and *vice versa*.

Meproadifen bound specifically in the absence of cholinergic ligands is displaced by other non-competitive antagonists including histrionicotoxin (Table 1 and Fig. 8). On the basis of the competition between dimethisoquin and [^{3}H]AcCh, a dissociation constant, $K_d = 60\ \mu M$, was calculated (5) for the binding of dimethisoquin to the cholinergic receptor site. That is the concentration necessary to displace half the [^{14}C]meproadifen bound in the absence of cholinergic ligands and is a factor of two greater than the dimethisoquin protection constant determined from the association kinetics of [^{3}H]α-toxin (Fig. 13). These results support the interpretation that local anesthetics do bind weakly to the AcCh binding site. The results reported here concerning the effects of H_{12}-HTX on the binding of meproadifen in the absence of cholinergic ligands suggest that H_{12}-HTX may also interact weakly with the AcCh binding site. However, the number of sites of [^{3}H]H_{12}-HTX in *Torpedo californica* receptor-rich membranes was reported to be 0.25 times the number of α-toxin sites not only in the presence of carbamylcholine but also in its absence (18). For receptor-rich membranes of *T. ocellata*, on the other hand, the number of [^{3}H]H_{12}-HTX sites was 1.9 times the number of [^{3}H]acetylcholine sites (19). Further work will be necessary to resolve these discrepancies.

The site of binding of the local anesthetics may function in one of two ways: as part of the ion channel itself or as a distinct regulatory site controlling the linkage between agonist binding and channel opening. In discussing the possible function of the local anesthetic binding site, two points about the functional state of the membranes in these studies must be kept in mind. First, the high affinity binding site for meproadifen in the presence of carbamylcholine must be a structural feature of the desensitized nicotinic post-synaptic membrane. This is necessary because it is known that *in vitro* in the isolated *Torpedo* membranes (33, 34), as *in vivo* at the vertebrate neuromuscular junction (35, 36), nicotinic receptors equilibrated with cholinergic agonists are desensitized. That is, they are no longer associated with a permeability response, even though they bind agonists.

unaltered, we conclude that the anesthetic binding site resides within the peptides of the cholinergic receptor protein itself.

Second, the fact that meproadifen is bound with the same high affinity in the presence of a reversible antagonist (tubocurarine) as in the presence of an agonist indicates that: 1) the high affinity binding of meproadifen is independent of channel activation and 2) the equilibrium structure of the isolated receptor-rich membranes is similar whether the receptor sites are occupied with agonist or antagonist. This latter conclusion may appear surprising since it is normally recognized that an antagonist such as tubocurarine does not cause receptor desensitization (34, 36). However, spectroscopic studies of the interaction of the non-competitive antagonist quinacrine with the *Torpedo* receptor-rich membranes provide indirect evidence that at equilibrium both agonists and antagonists do result in a similar change of membrane structure (32). Furthermore, analysis of the kinetics of binding of [^{3}H]AcCh to the receptor-rich membranes shows directly that tubocurarine as well as cholinergic agonists stabilize a receptor conformation binding [^{3}H]AcCh with high affinity, i.e., a desensitized receptor conformation (10).

While [^{14}C]meproadifen is bound with high affinity in the presence of reversible antagonists, no comparable binding was detected in the presence of α-Bungarotoxin. This fact could indicate either that the α-toxin stabilizes a distinct receptor conformation or that because of its large size, α-toxin occludes the sites of binding of both cholinergic ligands and local anesthetics. The latter interpretation is consistent with the observation that concentrations of proadifen that decrease the rate of binding of [^{3}H]α-toxin actually increase the receptor affinity for [^{3}H]AcCh and [^{14}C]d-tubocurarine (Figs. 3 (Figs. 3 and 12).

Studies of the kinetics of binding of [^{14}C]meproadifen to the *Torpedo* receptor-rich membranes will provide information to complement spectroscopic studies of the action of fluorescent noncompetitive antagonists at these membranes (37, 38), and it is these kinetic studies that will be necessary to clarify the relationship between the occupancy of the local anesthetic binding site and the distinct processes of channel blockade and receptor desensitization. Without such studies it is not possible to relate in a direct manner the biochemical studies reported here to the phenomena of channel blockade (39, 40) and receptor desensitization (41, 42) characteristic of the action of local anesthetics at the vertebrate neuromuscular junction.

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REFERENCES

1. Bartels, E., & Rosenberg, P. (1972) Correlation between electrical activity and splitting of phospholipids by snake venom in the single electroplax. *J. Neurochem.* **19**, 1251–1265.
2. Brisson, A., Devaux, P. & Changeux, J. P. (1975) Effet anesthésique local de plusieurs composés liposolubles sur la réponse de l'électroplaque de Gymnote à la carbamylcholine et sur la liaison de l'acétylcholine au récepteur cholinergique de Torpille. *C.R. Acad. Sci.* **280**, 2153–2156.
3. Podleski, T. R., & Bartels, E. (1963) Difference between tetracaine and d-tubocurarine in the competition with carbamylcholine. *Biochem. Biophys. Acta* **75**, 387–396.
4. Bartels, E., & Nachmanson, D. (1965) Molecular structure determining the action of local anesthetics on the acetylcholine receptor. *Biochem. Z.* **342**, 359–374.
5. Weber, M., & Changeux, J. P. (1974) Binding of *Naja nigricollis* [^{3}H]α-toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs. 3. Effect of local anesthetics on the binding of the tritiated α-neurotoxin. *Mol. Pharmacol.* **10**, 35–40.
6. Cohen, J. B., Weber, M. & Changeux, J. P. (1974) Effects of local anesthetics and calcium on the interaction of cholinergic ligands with the nicotinic receptor protein from *Torpedo marmorata*. *Mol. Pharmacol.* **10**, 904–932.
7. Shanes, A. M. (1958) Electrochemical aspects of physiological and pharmacological action in excitable cells. I. The resting cell and its alteration

by extrinsic factors. II. The action potential and excitation. *Pharmacol. Rev.* **10**, 59–164, 165–275.

8. Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**, 583–655.
9. Albuquerque, E. X., Barnard, E. A., Chiu, T. H., Lapa, A. J., Dolly, J. O., Jansson, S. E., Daly, J. & Witkop, B. (1973) Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction: Evidence from specific toxin reactions. *Proc. Nat. Acad. Sci. USA* **70**, 949–953.
10. Cohen, J. B. (1978) Ligand binding properties of membrane bound cholinergic receptors of *Torpedo marmorata*, in *Molecular Specialization and Symmetry in Membrane Function* (A. K. Solomon and M. Karnovsky, Eds.). Harvard University Press, Cambridge, Mass., 99–128.
11. Ritchie, J. M. & Cohen, P. J. (1975) Local anesthetics. Cocaine; procaine and other synthetic local anesthetics, in *Pharmacological Basis of Therapeutics* (L. G. Goodman and A. Gilman, Eds.). Macmillan, New York, 379–403.
12. Steinbach, A. B. (1968) Alteration by xylocaine (lidocaine) and its derivatives of the time course of the end plate potential. *J. Gen. Physiol.* **52**, 144–161.
13. Cohen, J. B., Weber M., Huchet, M. & Changeux, J. P. (1972) Purification from *Torpedo marmorata* electric tissue of membrane fragments particularly rich in cholinergic receptor. *FEBS Letters* **26**, 43–47.
14. Cohen, J. B. & Changeux, J. P. (1975) The cholinergic receptor protein in its membrane environment. *Ann. Rev. Pharmacol.* **15**, 83–103.
15. Heidmann, T. & Changeux, J. P. (1978) Structural and functional properties of the acetylcholine receptor protein in its purified and membrane bound states. *Ann. Rev. Biochem.* **47**, 371–411.
16. Daly, J. W., Karle, I., Myers, C. W., Tokuyama, T., Waters, J. A. & Witkop, B. (1971) Histrionicotoxins: Roentgen-ray analysis of the novel allenic and acetylenic spiro alkaloids isolated from a Colombian frog, *Dendrobates histrionicus*. *Proc. Natl. Acad. Sci. USA* **68**, 1870–1875.
17. Kato, G. & Changeux, J. P. (1976) Studies on the effect of histrionicotoxin on the monocellular electroplax from *Electrophorus electricus* and on the binding of [^{3}H]acetylcholine to membrane fragments from *Torpedo marmorata*. *Mol. Pharmacol.* **12**, 92–100.
18. Elliott, J. & Raftery, M. A. (1977) Interactions of perhydrohistrionicotoxin with post-synaptic membranes. *Biochem. Biophys. Res. Commun.* **77**, 1347–1353.
19. Eldefrawi, A. T., Eldefrawi, M. E., Albuquerque, E. X., Oliveira, A. C., Mansour, N., Adler, M., Daly, J. W., Brown, G. B., Burgermeister, W. & Witkop, B. (1977) Perhydrohistrionicotoxin: A potential ligand for the ion conductance modulator of the acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **74**, 2172–2176.
20. Krodel, E. K. & Cohen, J. B. (1978) Identification of a local anesthetic binding site in cholinergic post-synaptic membrane. *Fed. Proc.* **37**, 578a.
21. Weber, M., & Changeux, J. P. (1974) Binding of *Naja nigricollis* [^{3}H]α-toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs. I. Binding of the tritiated α-neurotoxin in the absence of effector. II. Effect of cholinergic agonists and antagonists on the binding of the tritiated α-neurotoxin. *Mol. Pharmacol.* **10**, 1–14, 15–34.
22. Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
23. Ellman, G. L., Courtney, K. D, Andres, V. & Featherstone, R. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.
24. Leuzinger, W., Goldberg, M. & Cauvin, E. Molecular Properties of acetylcholinesterase. *J. Mol. Biol.* **40**, 217–225, 1969.
25. Robinson, J. (1969) Kinetic studies on a brain microsomal adenosine triphosphatase. II. Potassium-dependent phosphatase activity. *Biochemistry* **8**, 3348–3354.
26. Fukuyama, T., Dunkerton, L. V., Aratani, M. & Kishi, Y. (1975) Synthetic studies on histrionicotoxins. II. A practical synthetic route to (±)-perhydro and (±)-octahydrohistrionicotoxin. *J. Org. Chem.* **40**, 2011–2012.
27. Everett, A. J., Lowe, L. A. & Wilkinson, S. (1970) Revision of the structures of (+)-tubocurarine chloride and (+)-chondrocurine. *Chem. Commun.* 1020–1021.
28. Franklin, G. I. & Potter, L. T. (1972) Studies of the binding of α-Bungarotoxin to membrane bound and detergent-dispensed acetylcholine receptors from *Torpedo* electric tissue. *FEBS Letters* **28**, 101–106.
29. Seeman, P. W. & Kwant, K. O. (1969) Membrane expansion of the erythrocyte by both the neutral and ionized forms of chlorpromazine. *Biochim. Biophys. Acta* **183**, 512–519.
30. Popot, J. L., Demel, R. A., Sobel, A., Van Deenen, L. L. M. & Changeux, J. P. (1978) Interaction of the acetylcholine (nicotinic) receptor protein from *Torpedo marmorata* electric organ with monolayers of pure lipids. *Eur. J. Biochem.* **85**, 27–42.
31. Schiebler, W. & Hucho, F. (1978) Membranes rich in acetylcholine receptor: Characterization and reconstitution to excitable membranes from exogeneous lipids. *Eur. J. Biochem.* **85**, 55–63.

32. Grünhagen, H. H. & Changeux, J. P. (1976) Studies on the electrogenic action of acetylcholine. IV. Quinacrine: a fluorescent probe for the conformational transitions of the cholinergic receptor protein in its membrane bound state. V. Qualitative correlation between pharmacological effects and equilibration processes of the cholinergic receptor protein as revealed by the structural probe quinacrine. *J. Mol. Biol.* **106,** 497–516, 517–535.
33. Popot, J. L., Sugiyama, H. & Changeux, J. P. (1976) Studies on the electrogenic action of acetylcholine with *Torpedo marmorata* electric organ. II. The permeability response of the receptor-rich membrane fragments to cholinergic agonists *in vitro*. *J. Mol. Biol.* **106,** 469–483.
34. Sugiyama, H., Popot, J. L. & Changeux, J. P. (1976) Studies on the electrogenic action of acetylcholine with *Torpedo marmorata* electric organ. III. Pharmacological desensitization *in vitro* of receptor-rich membrane fragments by cholinergic agonists. *J. Mol. Biol.* **106,** 485–496.
35. Katz, B. & Thesleff, S. (1957) A study of the desensitization produced by acetylcholine at the motor end-plate. *J. Physiol.* **138,** 63–80.
36. Rang, H. P. & Ritter, J. M. (1970) On the mechanism of desensitization at cholinergic receptors. *Mol. Pharmacol.* **6,** 357–382.
37. Grünhagen, H., Iwatsubo, M. & Changeux, J. P. (1977) Fast kinetic studies on the interaction of cholinergic agonists with the membrane-bound acetylcholine receptor from *Torpedo marmorata* as revealed by quinacrine fluorescence. *Eur. J. Biochem.* **80,** 225–242.
38. Quast, V., Schimerlik, M. & Raftery, M. A. (1978) Stopped flow kinetics of carbamylcholine binding to membrane-bound acetylcholine receptor. *Biochem. Biophys. Res. Commun.* **81,** 955–964.
39. Neher, E. & Steinbach, J. H. (1978) Local anesthetics transiently block currents through single acetylcholine channels. *J. Physiol.* **277,** 153–176.
40. Adams, P. R. (1977) Voltage jump analysis of procaine action at frog end-plate. *J. Physiol.* **268,** 291–318.
41. Vyskočil, F. & Magazanik L. G. (1972) The desensitization of post-junctional membrane after intracellular application of membrane stabilizers and snake venom polypeptides. *Brain Res.* **48,** 417–419.
42. Magazanik, L. G. & Vyskočil, F. (1973) Desensitization at the motor endplate, in *Drug Receptors* (H. P. Rang, Ed.), MacMillan, London, 105–120.
43. Neubig, R. R., Krodel, E. K., Boyd, N. D. & Cohen, J. B. (1979) Acetylcholine and local anesthetic binding to *Torpedo* nicotinic post-synaptic membranes after removal of nonreceptor peptides. *Proc. Natl. Acad. Sci. USA* **76,** in press.