MOLECULAR PHARMACOLOGY, 7, 420–428

Multiple Affinities for Binding of Cholinergic Ligands to a Particulate Fraction of *Torpedo* Electroplax

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(Received February 8, 1971)

SUMMARY

The binding of four radioactive cholinergic ligands to a particulate fraction of *Torpedo* electroplax was measured by equilibrium dialysis at ligand concentrations ranging from 10^{-9} to 10^{-4} M. Multiple affinities for the four ligands were found, two each for muscarone, nicotine, and dimethyl-*d*-tubocurarine, and three for decamethonium. Binding was reversible in every case.

The two agonists, muscarone and nicotine, each showed two kinds of binding, a low-affinity type of approximately 0.5 nmole/g, and a high-affinity type of approximately 0.1 nmole/g. Binding occurred to phospholipoproteins or to a phospholipid-protein complex, as judged from sensitivity to hydrolases. The antagonist dimethyl-*d*-tubocurarine also showed two kinds of binding. The low-affinity type was insensitive to hydrolases, however, and therefore probably was to a different macromolecule; it was almost 10 times more extensive: 3.6 nmoles/g of electroplax. Decamethonium showed three kinds of binding, all sensitive to the hydrolases, amounting to 0.6, 1.4, and 3.2 nmoles/g.

It is suggested that muscarone and nicotine bind to two different sites, which exhibit binding properties similar to the acetylcholine receptor, and that decamethonium and dimethyl-*d*-tubocurarine bind to other distinct sites and perhaps also to the sites binding muscarone and nicotine.

INTRODUCTION

Before attempting to isolate and characterize the acetylcholine receptor, we first embarked on studies to identify the binding characteristics *in vitro* of crude preparations of these macromolecules in a particulate fraction of *Torpedo* electroplax (1–4) and a supernatant fraction (100,000 × *g*, 1 hr) of housefly brain (5, 6), guided in these efforts by the physiological behavior of the acetylcholine receptor. The high affinity and amount of muscarone binding, as well as its reversibility and its competitive blockade by appropriate drugs, strongly suggested that the binding macromolecules were acetylcholine receptors.

When the study of the *Torpedo* electroplax was extended to encompass the binding of other cholinergic ligands (at 0.1–1 μM), a similar number of sites (1–2.1 nmoles/g of tissue) was found to bind muscarone, nicotine, decamethonium, and dimethyl-*d*-tubocurarine (dimethylcurare) (3). This confirmed earlier hypotheses (7–9) proposing that cholinergic agonists and antagonists bind to a common receptor site; these hypotheses had been used to calculate dissociation constants for inhibitors by their competition with activators in electrophysiological experiments (10, 11). On the other hand, we

This work was supported by United States Public Health Service Grants NS 09144 and GM 07804 and Training Grant ES 98.

have also reported discrepancies in the binding data that might suggest multiple binding sites in *Torpedo* electroplax. Examples are the fact that trypsin and chymotrypsin inactivated the materials that bind muscarone, nicotine, and decamethonium but had little effect on the binding of dimethylcurare, and several instances in which the competitive ability of a drug (with respect to another) did not correspond to its binding affinity (3).

Interaction between two ligands may also occur even when they bind at two different sites that regulate each other (12, 13); one would act as an allosteric (functional) antagonist of the other. In fact, several reports suggest the presence of more than one site for action in the acetylcholine receptor. Experiments on the monocellular preparation of the eel electroplax showed that decamethonium and carbamylcholine provoked different maximal responses of the innervated membrane, and their depolarizing action was affected in different ways by reducing, oxidizing, and alkylating agents (14–16). This suggested that these binding sites, which also affected each other cooperatively, were topographically distinct. Again, evidence from recovery of the response of rat intestine to various cholinergic agonists and partial agonists, as well as from the efflux of the latter, suggested the presence of two binding sites (17). In another study, on the uptake of labeled atropine by guinea pig intestine and its blocking effect on acetylcholine stimulation, two sites with different affinities were revealed (18).

It was therefore necessary to expand the range of ligand concentration at which binding by the particulate fraction of *Torpedo* electroplax is studied, in order to search for other binding sites that might be present but had previously been undetected. The present study was initiated to investigate such a possibility and, if more than one were discovered, to try to determine whether one or all are on the acetylcholine receptor macromolecule. Knowledge of the characteristics of binding and identification of the binding sites with the acetylcholine receptor would reduce the chances in subsequent preparative work of isolating nonspecific macromolecules. After obtaining this receptor in pure form, its binding could be studied more accurately and in more detail. A summary of these findings has formed part of a report previously presented (19).

METHODS

The lyophilized precipitate from *Torpedo* electroplax, the methods of equilibrium dialysis at 4° and counting of radioactivity, the replicates used, the tests for significance (3),

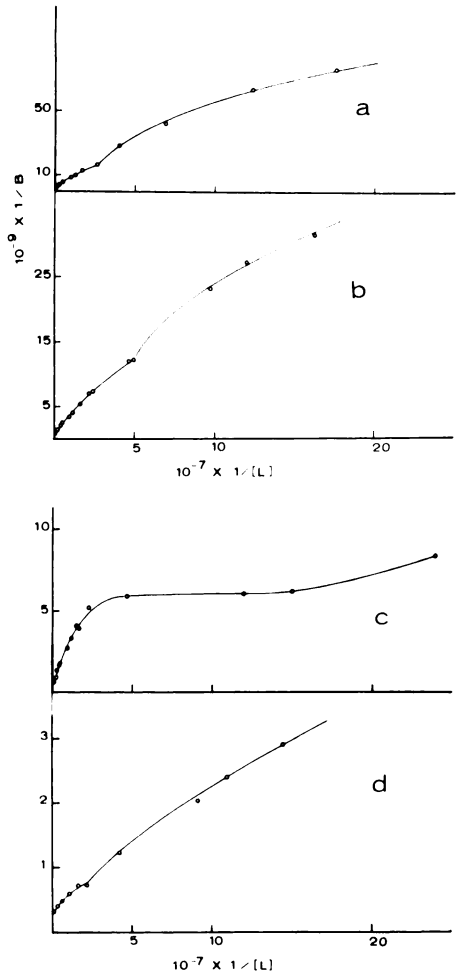
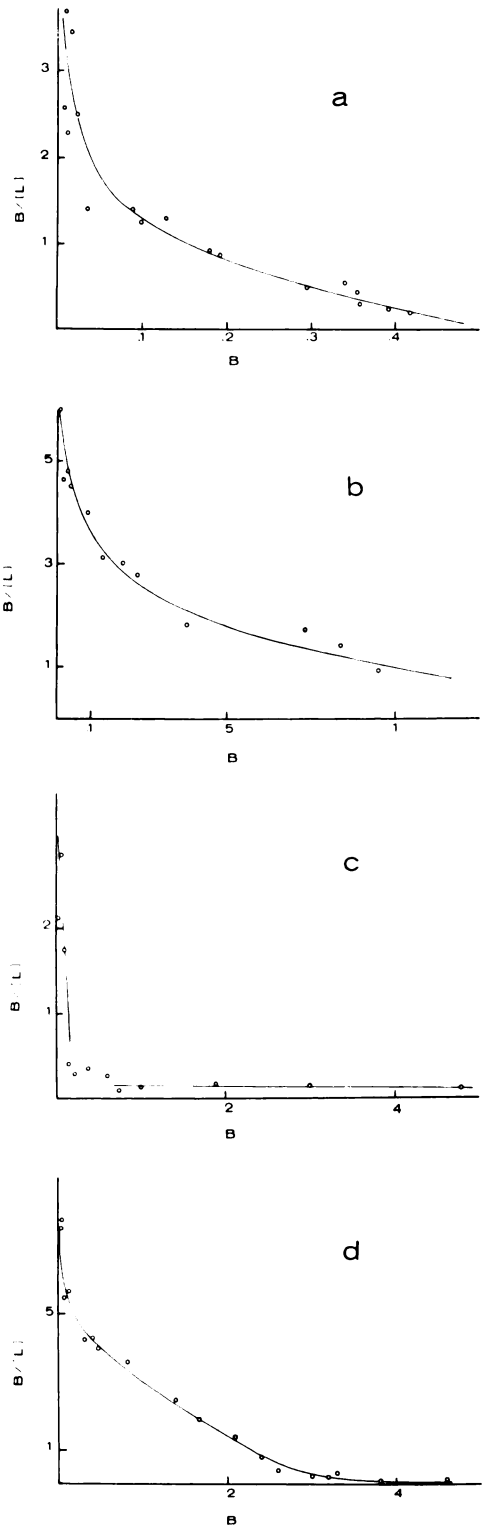


FIG. 1. *Lineweaver-Burk plots of binding (B) of cholinergic ligands to a particulate preparation of Torpedo electroplax as a function of concentration of ligands (L)*

a. Muscarone. b. Nicotine. c. Dimethylcurare. d. Decamethonium. Each point represents an average of two experiments, three samples each. *B* values are expressed in moles per gram of fresh tissue; *L* values, as molar concentration.



the testing of reversibility of binding, and the source of and treatment with hydrolases (2) have all been described previously. Also mentioned were the sources and specific activities of ^{3}H-muscarone (1), ^{3}H-nicotine (6), ^{3}H-decamethonium, and ^{14}C-dimethylcurare (3). In the present studies, however, except in the case of muscarone, when the desired ligand concentration was above 0.1 μM, its nonradioactive counterpart was used to raise the concentration.

The parameters of Table 1 were measured by an iterative procedure; approximate values of the binding constants (K) and the concentration of binding sites (B) were measured graphically and then systematically varied, and the values giving the least root-mean-square error, as measured by a computerized nonlinear regression program, were determined. Therefore, contributions of one site to K and B values of the other were eliminated.

RESULTS

When we previously studied the binding of cholinergic ligands at a range of concentration from 0.1 to 1 μM, straight Lineweaver-Burk plots were obtained (1, 3), but when the range of ligand concentration was extended 100,000-fold there were deviations from hyperbolae in the saturation curves, and from linearity in the Lineweaver-Burk plots (Fig. 1).

When binding was plotted on Scatchard plots (Fig. 2), each ligand displayed more than one straight line, indicating the presence of more than one kind of binding by the particulate fraction of *Torpedo* electroplax. Computer analysis of the data by an iterative procedure based on the Scatchard equation (20) gave two binding affinities each for muscarone, nicotine, and dimethylcurare, but three for decamethonium (Table 1). The

FIG. 2. *Scatchard plots of binding of ligands to a particulate preparation of Torpedo electroplax*

a. Muscarone. b. Nicotine. c. Dimethylcurare. d. Decamethonium. Each point represents an average of two experiments, three samples each. B values are expressed in nanomoles per gram of fresh tissue; L values, as molar concentration; B/L values multiplied by 10^{8}.

TABLE 1

Binding constants (K) and concentrations of binding sites (B) for different cholinergic ligands
Data were derived from iterative treatment of Scatchard plots.

Ligand	K	B	Error[a]
	μM	*nmoles/g electroplax*	%
Muscarone	$K_1 = 0.065$	$B_1 = 0.08$	
	$K_2 = 0.55$	$B_2 = 0.38$	21
Nicotine	$K_1 = 0.2$	$B_1 = 0.06$	
	$K_2 = 2.5$	$B_2 = 0.75$	9
Dimethylcurare	$K_1 = 0.037$	$B_1 = 0.18$	
	$K_2 = 1.0$	$B_2 = 3.6$	17
Decamethonium	$K_1 = 0.13$	$B_1 = 0.6$	
	$K_2 = 0.59$	$B_2 = 1.4$	
	$K_3 = 8.0$	$B_3 = 3.2$	14

[a] This is the root-mean-square percentage error (see METHODS).

concentrations of binding sites (B) were roughly similar for the two agonists, muscarone and nicotine, and much smaller than for the antagonist dimethylcurare and the long-acting depolarizer decamethonium. Muscarone showed 3–5-fold higher affinities than nicotine. It is interesting that although there are two affinities for dimethylcurare and three for decamethonium, the value of B_2 for the former is similar to B_3 for the latter.

A characteristic of the physiological acetylcholine receptor is the reversibility of its binding of agonists and antagonists. When the reversibility of binding at 0.1 μM was tested by dialysis in 100 volumes of ligand-free Ringer's solution, the amount that remained bound at equilibrium was reduced to almost the same level of binding at 0.001 μM (Table 2). This suggested the reversibility of binding of the ligands tested at 0.1 μM, as was previously found at 1 μM (3).

If the multiple binding sites presently revealed for each ligand are present on separate macromolecules, one means of distinguishing among them is to study possible differential sensitivities to enzymatic treatments. When the particulate fraction of *Torpedo* electroplax was treated with these hydrolases and its binding of cholinergic ligands was tested at low ligand concentrations (0.01 and 0.1 μM), the binding of all ligands except dimethylcurare was reduced to an extent similar to that observed at higher ligand concentration (1 μM) (Table 3). Only the higher-affinity binding of dimethylcurare was sensitive to the three enzymes, suggesting that this site also is on a phospholipoprotein; by contrast, the lower-affinity site was unaffected and thus is possibly on a different macromolecule.

TABLE 2

Reversibility of binding of cholinergic ligands (at 0.1 μM)

Ligand	Concentration bound at 0.1 μM	Reversed	
		Observed[a]	Calculated[b]
	nmoles/g electroplax	%	%
Muscarone	0.118	91	95
Nicotine	0.055	94	98
Decamethonium	0.55	85	95
Dimethylcurare	0.21	91	95

[a] Observed percentage reversed =

$$100 - \frac{\text{nmoles bound after redialysis in ligand-free Ringer's solution}}{\text{nmoles bound at 0.1 } \mu\text{M}} \times 100.$$

[b] Calculated percentage reversed =

$$100 - \frac{\text{nmoles bound at 0.001 } \mu\text{M as calculated from Lineweaver-Burk plots}}{\text{nmoles bound at 0.1 } \mu\text{M}} \times 100.$$

TABLE 3

Effect of enzyme treatment on binding of cholinergic ligands

Ligand	Concentration	Reduction of binding[a]		
		Trypsin	Chymotrypsin	Phospholipase C
	μM	%	%	%
Muscarone	0.01	64	72	64
	0.1	78	65	42
	1.0	67[b]	80[b]	48[b]
Nicotine	0.01	74	71	41
	0.1	87	78	54
	1.0	49[c]	58[c]	16[c]
Dimethylcurare	0.01	50	47	22
	0.1	44	47	21
	1.0	(7)[c, d]	12[c]	(5)[c, d]
Decamethonium	0.001	71	69	27
	0.01	76	71	21
	0.1	81	75	24
	1.0	80[c]	80[c]	25[c]

[a] Controls showed significant binding of decamethonium to the three enzymes, and of dimethylcurare to trypsin and phospholipase C. Values in the table were corrected accordingly.

[b] From O'Brien *et al.* (2).

[c] From Eldefrawi *et al.* (3).

[d] Values in parentheses are not significantly different from controls as judged by the Student's *t*-test.

To establish whether the four ligands tested compete for the same high-affinity site, the binding of ^{3}H-muscarone and ^{14}C-dimethylcurare was studied alone and in the presence of constant concentrations of the other ligands. To minimize contributions to binding by the low-affinity sites, a narrow range covering a low concentration of ligand (from 0.001 to 0.01 μM) was selected. The shifts to the right in the log concentration–binding curves of muscarone (Fig. 3a) and dimethylcurare (Fig. 3b) demonstrate a reduction in binding and consequently suggest that the ligands compete for the binding sites.

DISCUSSION

The low-affinity binding (K_2) reported in Table 1, based on Scatchard analysis of binding over a 100,000-fold concentration range, is in quite good agreement with our earlier data showing a single kind of binding (K), derived from Lineweaver-Burk analysis of data in the 10^{-6} M range. For instance, for muscarone K_2 was 0.55 and K was 0.72 μM, and for nicotine K_2 was 2.5 and K was 2.5 μM (3).

It is clear that macromolecules in the subcellular electroplax preparation of *Torpedo* bind each of the four cholinergic ligands studied with more than one type of affinity (Figs. 1 and 2 and Table 1). There are interesting similarities in the binding of muscarone and nicotine with regard to the number and concentration of their binding sites (Table 1) and the reversibility of their binding at 0.1 and 1 μM (Table 2). Both their lower- and higher-affinity binding sites are on phospholipoproteins or phospholipid-protein complexes, as judged from their sensitivity to proteinases and phospholipase C (Table 3).

The long-acting depolarizer decamethonium and the antagonist dimethylcurare present a different picture. Their binding does not saturate up to 100 μM, concentra-

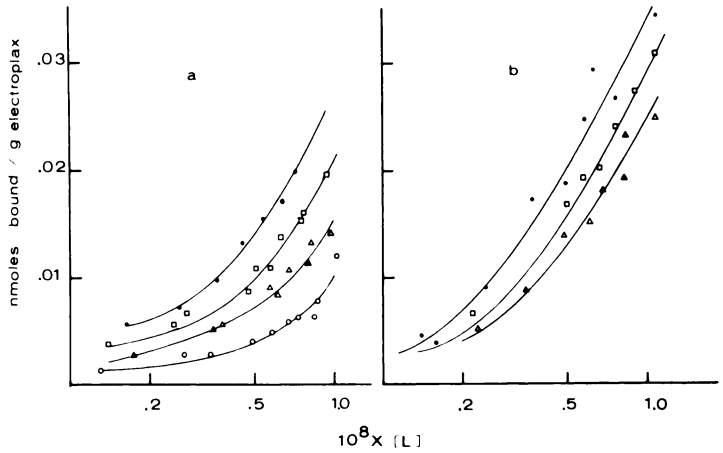


FIG. 3. *Antagonism among cholinergic ligands for binding macromolecules in a particulate preparation of Torpedo electroplax*

a. Binding of muscarone alone (●) and in the presence of 0.1 μM nicotine (□) 0.05 μM decamethonium (△), or 0.03 μM *d*-tubocurarine (○). b. Binding of dimethylcurare alone (●) and in the presence of 0.1 μM nicotine (□) or 0.05 μM decamethonium (△). *L*, molar concentration of ligand.

tion-binding curves display plateau regions, and they bind reversibly to 10 times the number of sites (Table 1). The sensitivity of their binding to the effect of enzymes (Table 3) indicates that the first two binding sites (K_1 and K_2) of decamethonium and the higher-affinity site (K_1) of dimethylcurare (Table 1) are on a phospholipoprotein (or a phospholipid-protein complex). On the other hand, the lower-affinity site of dimethylcurare (K_2) appears to be on a different molecule. The sum of the binding sites found presently for either ligand is higher than previously reported (3), when only a limited range of concentration was studied; possibly because these two ligands bind to other sites at concentrations above 1 μM.

The acetylcholine receptor molecule should satisfy several criteria *in vitro* before it is identified as the receptor. These criteria are based on its physiological behavior. It should bind cholinergic ligands reversibly and with high affinities, comparable to those determined physiologically. This binding should also be affected by the presence of other cholinergic ligands. Both sites binding muscarone or nicotine, the two higher-affinity sites binding decamethonium, and the higher-affinity site binding dimethylcurare all satisfy the above criteria equally well.

Another requirement is that the concentration of binding sites *in vitro* should be comparable to that *in vivo*; however, physiological values are hard to estimate. Recently a value of about 25 nmoles of receptor binding sites per gram of protein was reported for an extract of eel electroplax by Changeux *et al.* (21). It was measured by the interference of the snake poison α-bungarotoxin with binding of cholinergic ligands *in vivo* and *in vitro*. Since the *Torpedo* electroplax extract presently used contains 9.3 mg of protein per gram of tissue (2), the concentrations of the binding sites for muscarone (Table 1) become 8.5 and 41 nmoles/g of protein, and for nicotine, 6.5 and 80 nmoles/g of protein. These are in the same order of magnitude as the above data (21). A much higher value of 160 nmoles of hexamethonium bound per gram of electroplax was obtained for proteolipid extracted from *Torpedo* electroplax (22).

Very recently De Robertis *et al.* (23) have also reported multiple binding sites, in this case the existence of two binding affinities for acetylcholine in a proteolipid fraction from *Electrophorus* electroplax. However, these bindings occurred in an organic phase (methanol–chloroform), and it was not possible to examine reversibility or drug com-

petition. Consequently, comparisons with our data are not possible.[1]

A comparison of binding constants determined *in vitro* with those calculated from experiments *in vivo* should be made with due caution. *In vivo*, measured responses reflect the combined effect of affinity for the ligand as well as its "intrinsic activity" or ability to depolarize. It is generally assumed that agonists and antagonists bind at equal ratios to the same site, or, if to different sites, any cooperative effects are neglected in the calculations. On the other hand, direct measurement of dissociation constants can be made in binding studies. Therefore, affinities of ligands for the acetylcholine receptor *in vivo* may differ appreciably from those *in vitro*, in a manner analogous to that displayed by acetylcholinesterase of the eel electroplax (24).

The multiple affinity causing nonlinearity in the double-reciprocal (Fig. 1) and Scatchard plots (Fig. 2) of the four ligands may have one or more of the following explanations. The first and simplest possibility is that there exists in this particulate fraction of electroplax more than one molecule that binds cholinergic ligands. The binding of dimethylcurare may fit this category in binding to at least two different molecules, which differ in their sensitivity to hydrolases. Dimethylcurare and *d*-tubocurarine are known to bind to several macromolecules from the eel electroplax as well as other tissues, with affinities ranging from 0.01 μM to more than 100 μM (25, 26). It is also possible that there are two macromolecules, both of which bind nicotine and muscarone and show acetylcholine receptor properties as indicated by high-affinity binding and blockade by other cholinergic ligands. The two could bear a relationship to each other like that of isozymes.

A second possibility is that binding may occur at two sites on the same molecule, one present at a higher concentration than the other, and with negatively cooperative interactions (27). A third alternative is that the two kinds of binding (high- and low-affinity) represent nonidentical binding sites without cooperativity (28). This is supported by a model for the acetylcholine receptor proposed by Nastuk and Gissen (29), suggesting that it has a polyvalent anionic receptor site capable of binding several ligand molecules.[2]

Several models have been proposed for the acetylcholine receptor suggesting that it exists in two conformations. From studies on receptor desensitization at the motor end plate, Katz and Thesleff (30) proposed a cyclic model for the acetylcholine receptor, which was postulated to exist in two forms, effective and refractory. The ligand was suggested to combine rapidly and reversibly with both forms, but with different affinities. This view was also supported by Rang and Ritter (31). To explain the response of the monocellular electroplax to specific ligands, Changeux and Podleski (14, 15) also suggested that the acetylcholine receptor is an allosteric protein that exists in polarizing and depolarizing conformations. When Karlin (32) applied the allosteric model of Monod *et al.* (12) to the acetylcholine receptor of the monocellular electroplax, he found that treating the receptor mechanism according to the allosteric model had advantages over the classical treatment of dose-response relationship according to a simple saturation model. Because most proposals of protein conformational changes assume that the conformations are in rapid equilibrium, only an average of the affinities of the two sites would be observed under the experimental conditions we used above. Therefore, the possibility is remote that the two binding

[1] Since this manuscript was submitted, it has been reported [R. Miledi, P. Molinoff and L. T. Potter, *Nature* **229**, 554 (1971)] that a soluble material binding α-bungarotoxin was obtained from *Torpedo* electroplax. The concentration of binding sites which these authors found (1.1 nmoles/g of electroplax) is extremely close to that reported here and in previous work (2, 3).

[2] *Note added in proof*: According to a newly proposed model [N. Laiken and G. Nemethy, *Biochemistry 10*, 2101 (1971)], "flexible ligands cannot be expected to bind in accordance with the single-class model of the theory of multiple equilibria". The new model proposes that findings such as those described herein may reflect the binding of different ligand configurations to identical sites. As these sites are successively filled, subsequent ligand molecules are restricted in the number of multifunctional attachments which they can form, and so bind with lesser affinity.

affinities presently detected for both muscarone and nicotine are exhibited by the same site in two conformations of the macromolecule.

The large B_1 values for the low-affinity binding of decamethonium and dimethylcurare suggest that they bind totally or in part to sites other than those binding nicotine and muscarone. This agrees with evidence from physiological studies suggesting that decamethonium or suxamethonium binds to sites topographically distinct from those binding the agonist carbamylcholine in monocellular electroplax (14) and chick (31) and leach (33) muscles. The ligands still compete physiologically (14) and *in vitro* (Fig. 3), either by an allosteric interaction, if all binding sites are different, or perhaps because one of the sites is common to both.

As stressed in the introduction, we have not attempted purification in the present study. We have instead studied the binding characteristics in this crude preparation so that in subsequent isolation procedures (now under way) we can utilize the correct ligand and the concentration best suited to identify receptor activity.

In conclusion, the data indicate that there are phospholipoproteins or phospholipid-protein complexes in the particulate fraction of the *Torpedo* electroplax, which bind the four cholinergic ligands reversibly and with multiple affinities. The similarities between the binding characteristics of these macromolecules and the physiological response of the acetylcholine receptor strongly suggest that they are the same molecules. Only after isolation of these molecules in pure form can their identity with the acetylcholine receptor be confirmed.

ACKNOWLEDGMENT

The authors are grateful to Professor H. Howland for devising the program for computer analysis, according to the Scatchard equation.

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