Allosteric Interactions between the Membrane-Bound Acetylcholine Receptor and Chemical Mediators: Equilibrium Measurements†

Juian-juian L. Fu,‡ David B. Donner,§ Douglas E. Moore,¶ and George P. Hess*

ABSTRACT: An approach to equilibrium dialysis measurements has been developed which enables one to study the interaction of chemical mediators with the membrane-bound acetylcholine receptor and to gain information of a type previously obtainable only with soluble proteins. Equilibrium dialysis experiments conducted at pH 7.0, 4 °C, and $\mu = 0.18$ M, with electroplax membrane preparations from *Electrophorus electricus* revealed apparently homogeneous binding isotherms for decamethonium with dissociation constants in the range of 0.2–0.4 μM. The following new information has been obtained. (1) The activators of neural transmission, decamethonium and carbamylcholine, occupy overlapping binding sites. (2) These activators and the inhibitors, α-bungarotoxin and *d*-tubocurarine, compete for only one-half of the sites available to them even though the stoichiometry of these sites is 1:1 as measured with decamethonium (a reversibly binding activator) and α-bungarotoxin (an irreversible specific inhibitor). Different receptor molecules, preexisting nonequivalent binding sites, or an allosteric mechanism involving ligand-induced conformational changes are often considered to account for such observations.

The acetylcholine receptor protein, found in synaptic membranes of many nerve fibers, at the neuromuscular junction, and in the electric organ of a number of fish, plays a key rôle in the transmission of nerve impulses (Nachmansohn, 1973, 1975). The properties of the acetylcholine receptor, a protein of 90 000 molecular weight per binding site (Klett et al., 1973) and an estimated total molecular weight ranging from 240 000 (Biesecker, 1973) to 330 000 (Edelstein et al., 1975), have been investigated with intact cells (Schoffeniels, 1957, Schoffeniels and Nachmansohn, 1957; Higman et al., 1963; Karlin, 1967), membrane preparations (Kasai and Changeux, 1971a,b; Miledi et al., 1971; Eldefrawi and Eldefrawi, 1973) and the isolated receptor (Karlsson et al., 1972; Schmidt and Raftery, 1972; Biesecker, 1973; Eldefrawi and Eldefrawi, 1973; Klett et al., 1973; Chang, 1974; Meunier et al., 1974). An important primary event in regulation of ion flux across excitable membranes is believed to be the interaction between the membrane-bound receptor and chemical mediators. The mechanism of this interaction is the subject of our present studies.

Membrane-bound rather than purified receptor was chosen for these studies because we are mainly interested in the molecular processes involved in receptor-mediated changes in permeability of nerve membranes to inorganic ions (Hess et al., 1975a,b, 1976; Hess and Andrews, 1977). The isolated acetylcholine receptor does not have a measurable function in solution and changes in ligand-binding properties are known to accompany the isolation process (Eldefrawi and Eldefrawi, 1973; Meunier et al., 1974; Raftery et al., 1975). Membrane preparations from the electric organ (electroplax) of *Electrophorus electricus* were chosen for the studies because they appear uniquely suitable for finding a correlation between the interactions of chemical mediators with the receptor and changes of the membrane permeability to inorganic ions. The chemical properties of the membrane (Nachmansohn, 1973, 1975) and the electrophysiological properties of single electroplax cells (Schoffeniels and Nachmansohn, 1957; Higman et al., 1963; Karlin, 1967) have been investigated extensively. Recently, Kasai and Changeux (1971a,b) reported the preparation of electroplax membrane vesicles which exhibited receptor-mediated ion flux. A kinetic analysis of the ion flux observed with these membrane vesicle preparations indicated that on average only 15% of the vesicles are responsible for this receptor-mediated flux (Hess et al., 1975a, 1976) and the successful isolation of the functional vesicles has recently been achieved (Hess and Andrews, 1977).

In this paper, we report on the binding properties of two compounds (decamethonium and carbamylcholine) which are known to initiate changes in the permeability of neural membranes to inorganic ions, in the presence and absence of inhibitors of this process, *d*-tubocurarine and the snake neurotoxin α-bungarotoxin (Chang and Lee, 1963; Lee et al., 1967; Lee, 1972).

These equilibrium measurements of the binding of effectors and inhibitors to the membrane-bound receptor differ from all previous investigations in that a number of problems which are often encountered in equilibrium dialysis studies are carefully considered and investigated. Two of the problems are the exclusion of ligand from the volume occupied by the membrane preparation, and unspecific binding of the ligand (Donner et al., 1976). Important and interesting information (which was obscured in previous studies) is obtained as a result of these investigations of the ligand-binding reaction. The data indicate that the activators and inhibitors occupy different binding sites on the membrane-bound receptor and that these binding sites interact only partially. These properties of the receptor-chemical mediator interaction could be due to distinct and separate binding sites for effectors and inhibitors, or to properties of the receptor which are very similar to those encountered in regulatory enzymes which exhibit an allosteric

† From the Section of Biochemistry, Cornell University, Ithaca, New York 14853. *Received October 17, 1975.* This work was supported by National Science Foundation Grant BMS 72-01908, and National Institutes of Health Grants NS 08527 and GM 04842.

‡ Recipient of National Institutes of Health Postdoctoral Fellowship GM53-265. Present address: Research and Development Department, Miami Valley Laboratory, Procter and Gamble Co., Cincinnati, Ohio 45239.

§ Present address: Sloan-Kettering Institute for Cancer Research, New York, N.Y.10021.

¶ Present address: Department of Pharmacy, University of Sydney, Australia.

mechanism involving ligand-induced conformational changes (Koshland et al., 1966; Koshland, 1970; Hammes and Wu, 1974). A preliminary account of some of these data has appeared (Fu et al., 1974).

Materials and Methods

Electrophorus electricus were purchased from Paramount Aquarium, Ardsley, N.Y. "Excitable" membrane fragments were prepared using a modified version of the sucrose-density gradient method of Kasai and Changeux (1971a). The gradient consisted of 5 ml of 1.5 M sucrose layered on the bottom of a centrifuge tube, followed by 5 ml of 0.4 M sucrose and then 28 ml of the suspension of membrane fragments in 0.2 M sucrose. The acetylcholinesterase-rich fragments, which concentrated at the interface between the 1.5 and 0.4 M sucrose layers, were collected and diluted with 180 ml of physiological eel Ringers' solution (Keynes and Martins-Ferreira, 1953). The suspension was then centrifuged for 1 h at 100 000*g* and 4 °C. The sediment was resuspended in cold eel Ringers' solution with a Dounce no. 23 hand homogenizer. The preparation was further dialyzed against eel Ringer's solution overnight at 4 °C and then sonicated (Heat Systems-Ultrasonics, Plainview, N.Y.) for 10 s at a 4–5 A setting. Usually, each preparation (starting with 80 g of fresh tissue) contained approximately 150 mg of proteins, determined by the method of Lowry et al. (1951). The specific activity of the membrane-bound acetylcholinesterase, determined by the method of Ellman et al. (1961), was in the range 1–4 mmol mg of protein^{-1} h^{-1}. The (Na^+, K^+)ATPase was assayed following the procedure of Bonting et al. (1961). On the average, a value of 13 μmol of ATP hydrolyzed h^{-1} mg of membrane protein^{-1} was obtained. The comparable value obtained by Kasai and Changeux (1971a) with "nonexcitable" electroplax membranes was 500 μmol h^{-1} mg^{-1}. The activity of acetylcholinesterase and the cholinergic ligand-binding properties of the "excitable" membrane preparations were checked during storage at 4 °C and found to be constant for 1 week.

Salt-treated membrane fragment preparations were obtained by treating the native membrane preparation, as described earlier, with 1 M NaCl and 0.01 M Na_2HPO_4 at pH 7.0, 4 °C (Silman and Karlin, 1967) for 0.5–3 h, depending on the extent of extraction of acetylcholinesterase desired. The suspension was then centrifuged for 1 h at 100 000*g*. The pellet was resuspended in the desired volume of Ringers' solution and sonicated for 3 s at a 5-A setting.

3-Hydroxylphenyltrimethylammonium iodide (3-HOPTA[1]) was prepared by methylation of *N,N*-dimethyl-3-hydroxyaniline (Eastman Organic Chemicals, Rochester, N.Y.) with methyl iodide in acetone. After recrystallization from a methanol–ether mixture, the compound had a mp of 187 °C (lit. mp 182 °C) (Hantsch and Davison, 1896). Anal. calculated: C, 38.8; H, 5.0; N, 5.3. Found: C, 39.1; H, 5.0; N, 5.1 (Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.). The compound was catalytically tritiated by the New England Nuclear Co. (Boston, Mass.) as described by Taylor and Singer (1967). After three recrystallizations from methanol–ether, the mp of the final compound was 185–186 °C. The compound was 98% pure as determined by paper chromatography in two solvent systems (Bray, 1950; Augustinsson, 1953). The specific radioactivity of the compound was 228 mCi/mmol.

[*methyl*-^{3}H]Decamethonium chloride (Code TRA 254, specific radioactivity either 500 or 418 mCi/mmol, radiochemical purity 99%) was purchased from Amersham Searle Corp. (Arlington Heights, Ill.). The purity of the commercially obtained compounds was further tested by paper chromatography, as described by Amersham Searle, and found to be 99% pure.

Decamethonium bromide, lot 1010-A, carbamylcholine chloride, lot 78854, and *d*-tubocurarine chloride, lot 4223-A, were obtained from K & K Laboratories (Plainview, N.Y.) and were used directly.

Tetram (*O,O*-diethyl *S*-(β-diethylaminoethyl)phosphorothiolate) was prepared as described previously (Calderbank and Ghosh, 1955; Tammelin, 1957).

α-Bungarotoxin from *Bungarus multicinctus* venom was prepared and tested as described in the following paper of this issue (Bulger et al., 1977).

Acetylthiocholine bromide, 99% purity, obtained from the Sigma Chemical Co. (St. Louis, Mo.) was used directly. 5,5′-Dithiobis(2-nitrobenzoic acid), A grade, lot 901 588, acetylcholine bromide, and crystalline bovine serum albumin were obtained from Calbiochem (La Jolla, Calif.), the Fisher Scientific Co. (Rochester, N.Y.), and the Sigma Chemical Co. (St. Louis, Mo.), respectively.

1,4-Bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Me_2-POPOP) and Biosolv solubilizer Formula BBS-3 (both scintillation grade) were supplied by the Packard Instrument Co. Inc. (Downers Grove, Ill.) and the Beckman Instrument Co. (Palo Alto, Calif.), respectively. All other chemicals were reagent grade and were used without further purification.

Cellulose dialysis tubing (3787-D40, 1¾ in. flat width for the microcells, and 3787-D10 ⅜ in. flat width when used to form a tube) was obtained from A. H. Thomas Co. (Philadelphia, Pa.). The dialysis tubing was first treated twice with 50% ethanol for 1 h at boiling temperature, twice by stirring in 10 mM sodium bicarbonate solution for 1 h, and then transferred to 1 mM EDTA solution and stirred for another 1 h. After rinsing and soaking with double-distilled water several times, it was stored in a closed jar at 4 °C in double-distilled water. A few drops of toluene were added to prevent the growth of bacteria.

Inhibition of Catalytic Activity of Membrane-Bound Acetylcholinesterase. Ten microliters of 0.01 M Tetram in eel Ringers' solution was incubated with 1 ml of the membrane fragments for 30 min at 4 °C. This gave an optimum concentration of Tetram for enzyme inhibition (without interfering with the binding of cholinergic ligands to the receptor (Eldefrawi et al., 1971b)). The acetylcholinesterase activity in the membrane preparations was determined at pH 7.0, 25 °C, by pH-stat titration (Radiometer) with 0.01 M NaOH as titrant and 2 mM acetylcholine as substrate. The Tetram-treated membrane fragments contained 0.015% of the original acetylcholinesterase catalytic activity. When 3-HOPTA was used as an enzyme inhibitor, appropriate amounts of 6×10^{-4} M 3-HOPTA stock solution were introduced into the membrane preparations to give a final concentration of 6 μM.

Equilibrium Dialysis. Specially constructed Lucite microcells (Furlong et al., 1972) consisting of two 100-μl compartments separated by a single thickness of the pretreated dialysis tubing were used. Eighty microliters of the membrane preparation (protein content 10–14 mg/ml) and 80 μl of radioactively labeled cholinergic ligand solution were allowed to equilibrate for 16 h at 4 °C with slow rotation (2 rpm). The half-life of equilibration for each ligand was measured to be less than 2 h; thus, complete equilibration was assured. More than 98% of the radioactivity was recovered. After equilibration, duplicate 20 μl aliquots from each chamber of the dialysis

[1] Abbreviations used are: 3-HOPTA, 3-hydroxylphenyltrimethylammonium iodide; EDTA, (ethylenedinitrilo)tetraacetic acid.

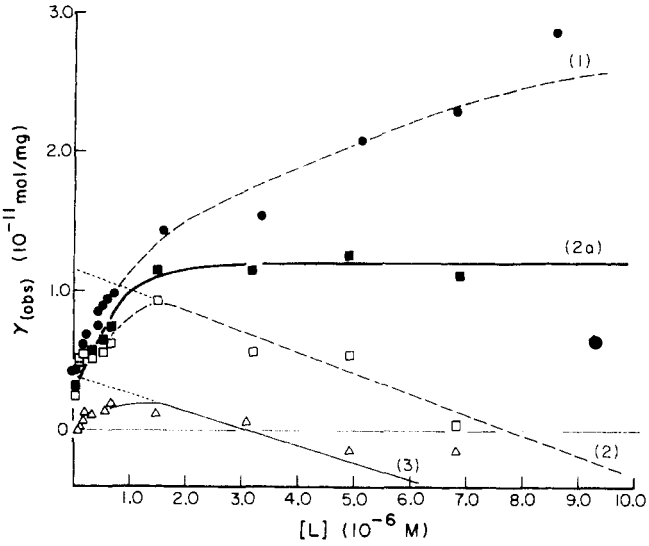


FIGURE 1: Binding of [^{3}H]decamethonium to electroplax membrane preparations; pH 7.0, 4 °C, μ = 0.18 M, eel Ringers' solution. (●) Decamethonium binding (curve 1). (□) Decamethonium binding in the presence of 6 μM 3-HOPTA (curve 2); r_0^s = 1.2 × 10^{-11} mol/mg; Φ = 1.5 μl/mg. (■) Data of curve 2 after corrections for unspecific binding and volume exclusion (curve 2a). (△) Decamethonium binding in the presence of 6 μM 3-HOPTA to membrane preparations pretreated with 2 μM α-bungarotoxin at 4 °C for 1 h; r_0^s = 0.4 × 10^{-11} mol/mg; Φ = 1.3 μl/mg.

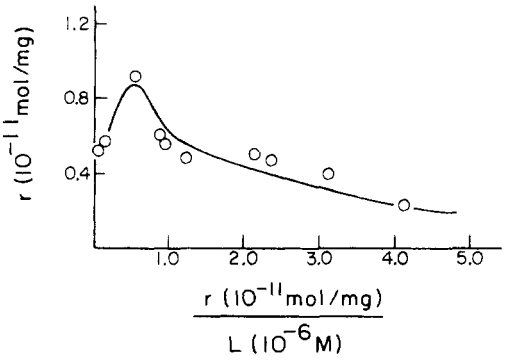


FIGURE 2: The data shown in curve 2 of Figure 1 are replotted, according to Scatchard (1949), r vs. r/L. Protein content was 14 mg of membrane protein per ml. The specific activity of the membrane-bound acetylcholinesterase was 2 mmol mg^{-1} h^{-1}, where mg represents mg of membrane protein. Equilibrium dialysis was used.

cell were transferred to vials containing 10 ml of scintillation fluid, and were counted in a liquid scintillation counter. Aliquots were also removed from each compartment for determination of protein concentrations by the method of Lowry et al. (1951). The number of moles of ligand bound per mg of membrane protein was calculated from the difference in concentration of radioactive ligand in the two chambers.

Counting of Radioactivity. Scintillation counting was used to determine concentrations of isotopically labeled compounds. Standard tritiated water was used to establish the efficiency of counting. The efficiency and quantity of known samples were determined under the same conditions as the experimental samples. The efficiency for tritium was found to be 35% using a Packard Tricarb scintillation counter, Model 3375, and 50% for a Beckman Liquid Scintillation counter, LS-230. The specific radioactivity of the commercially obtained compounds was used to convert the counts to concentrations.

Results

The complexity of the data obtained in equilibrium dialysis experiments with [^{3}H]decamethonium and membrane preparations is illustrated in Figure 1. The data are presented in the form of r(obs) vs. L plots, where r(obs) is the number of moles of ligand bound per mg of membrane protein and L is the concentration of free ligand. Curve 1 was obtained in experiments in which the binding sites of the membrane-bound acetylcholinesterase were not blocked. When the enzyme sites were blocked by a specific inhibitor of acetylcholinesterase, 3-HOPTA (Wilson and Quan, 1958), curve 2 was obtained. Evidence is presented later to show that binding of decamethonium to the enzyme under these conditions is prevented. When the enzyme sites were blocked by 3-HOPTA and the receptor had been specifically and irreversibly reacted with α-bungarotoxin (Chang and Lee, 1963; Lee et al., 1967; Lee, 1972), curve 3 was obtained. The conditions of this experiment were such that more than 90% of the toxin sites were occupied (Bulger and Hess, 1973) at the beginning of the experiment, which lasted 16 h. Curve 2, therefore, indicates that decamethonium binds to sites on the membrane other than on the enzyme, and curve 3 indicates that decamethonium binds to membrane sites other than the enzyme or α-bungarotoxin binding sites. Two important problems are raised by curves 2 and 3. (1) Curve 2 indicates that at high decamethonium concentrations less ligand is bound than at low decamethonium concentrations. (2) Curve 3 shows that decamethonium does bind to the membrane preparations in presence of an enzyme inhibitor and stoichiometric amounts of α-bungarotoxin, but the value of r(obs) decreases.

We have shown (Donner et al., 1976) that three factors contribute to the r(obs) values in Figure 1. Specific binding and unspecific binding contribute positively to r(obs); the solution volume occupied by the membrane from which the ligand is excluded contributes negatively. It has been shown (Donner et al., 1976) by independent measurements that the appropriate correction terms can be obtained from the straight-line portions of the graphs (Figure 1, curves 2 and 3). The ordinate intercept of these lines gives the concentration of specific binding sites; r_0 = 1.2 × 10^{-11} mol of decamethonium binding sites/mg of membrane protein in the case of curve 2, and 0.4 × 10^{-11} mol of decamethonium binding sites/mg of membrane protein in the case of curve 3.

The slope of each line gives the value of Φ, which contains correction terms for unspecific binding, and for volume occupied by membranes from which the ligand has been excluded. The average value of Φ determined for membrane preparations from six eels is 1.5 μl/mg of membrane protein. When the decamethonium binding isotherm (curve 2) was corrected appropriately, curve 2a was obtained. When the data (curve 2) were not corrected, and when presented in the form of an r vs. r/L plot (Scatchard, 1949) (Figure 2), the existence of more than one binding site or of cooperative effects was indicated.

The corrected data obtained with membrane preparations from two different eels are presented as Scatchard (1949) plots in Figure 3. The ordinate intercepts give r_0^s, and the slopes of the lines provide the value of the dissociation constant for the binding site–ligand complex. Either 0.1 mM Tetram (Figure 3) or 6 μM 3-HOPTA (Fu et al., 1974) was used to block the binding of decamethonium to membrane-bound acetylcholinesterase. The data presented in Figure 3 are consistent with one homogenous binding site. In the experiments performed in the presence of 0.1 mM Tetram (Figure 3), a K_D value of 0.4 μM and an r_0^s value of 1.4 (± 0.1) × 10^{-11} mol/mg of

TABLE I: Specific Binding of Decamethonium to Membrane Fragments, Eel Ringers' Solution, pH 7.0, 4 °C, μ = 0.18 M

Membrane Prep.	Treatment	r_0^s (10^{-11} mol/mg)	α-Bungarotoxin Sites (10^{-11} mol/mg)	K_D^s (10^{-6} M)	No. of Membrane Prep. Used[a]
Native	3-HOPTA (6 μM)	0.9 ± 0.03	0.9 ± 0.2[c]	0.3[b]	4
Native	3-HOPTA (6 μM), α-bungarotoxin (2 μM, 1 h)	0.4 ± 0.02		0.5[b]	4
Native	Tetram (0.1 mM, 0.5 h)	1.4 ± 0.1		0.4	2
Native	Tetram (0.1 mM, 0.5 h), α-bungarotoxin (2 μM, 1 h)	0.8 ± 0.8		0.7	2
Salt extracted	3-HOPTA (6 μM)	1.0 ± 0.1[b]		0.2[b]	2
Salt extracted	3-HOPTA (6 μM), α-bungarotoxin (2 μM, 1 h)	0.7[b]		0.2[b]	2
Native		5.9[d]	10.8[d] 1-2[e]		

[a] Each from a different eel. [b] Fu et al., 1974. [c] Bulger and Hess, 1973. [d] Determined from the amount of bound [^{14}C]decamethonium displaced by α-bungarotoxin. The acetylcholinesterase was not inhibited. An ultracentrifugal method was used (Kasai and Changeux, 1971c). [e] The number of α-bungarotoxin binding sites was estimated from the titration curve of [^{14}C]decamethonium binding sites with α-bungarotoxin (Kasai and Changeux, 1971c). [f] ^{3}H-labeled *Naja* toxin from *Naja nigricollis* and a centrifugal method were used (Meunier et al., 1972).

membrane protein were obtained. Similar results were obtained when 6 μM 3-HOPTA was used as enzyme inhibitor. The experimental results obtained with membrane preparations from four different eels, and with membrane preparations from which almost 90% of the acetylcholinesterase had been removed by salt extraction are summarized in Table I. In the absence of α-bungarotoxin, the dissociation constant of the decamethonium–enzyme site complex (K_D) is in the range of 0.2–0.4 μM. The average amount of decamethonium binding sites (1 × 10^{-11} mol/mg of membrane protein), determined using membrane preparations from eight different eels, corresponds, within experimental error, to the number of moles of α-bungarotoxin binding sites (0.9 (± 0.2) × 10^{-11} mol/mg of membrane protein) determined in studies with membrane preparations from 13 eels (Bulger and Hess, 1973).

It can also be seen from Figure 3 and Table I that decamethonium still binds to membrane fragments which have been allowed to react stoichiometrically with α-bungarotoxin. In the presence of both 3-HOPTA and Tetram, α-bungarotoxin reduces the number of decamethonium-binding sites by only about 50%, and changes the dissociation constants of the remaining sites by a factor less than 2 (Table I). One explanation of these results is that the enzyme inhibitors used did not prevent decamethonium binding to the enzyme present in the preparations. The following results are pertinent to this problem.

In our experiments, addition of 0.1 mM Tetram (Eldefrawi et al., 1971a,b) to the membrane preparations at 4 °C abolished more than 99% of the catalytic activity of the membrane-bound enzyme within 30 min, when tested with acetylthiocholine as substrate. The amount of decamethonium bound to the membrane preparation in the presence of 0.1 mM Tetram was found to be similar to that bound in the presence of 6 μM 3-HOPTA (Table I). Steady-state kinetic measurements indicate that 3-HOPTA competes with acetylthiocholine for the same binding sites in both free and membrane-bound acetylcholinesterase and that the competitive inhibition constants are the same within experimental error (0.15–0.17 (± 0.01) μM) (Moore et al., 1977).

The results of equilibrium dialysis experiments with 3-HOPTA and the membrane preparation are presented as plots of r vs. r/L in Figure 4. In these experiments, r represents the

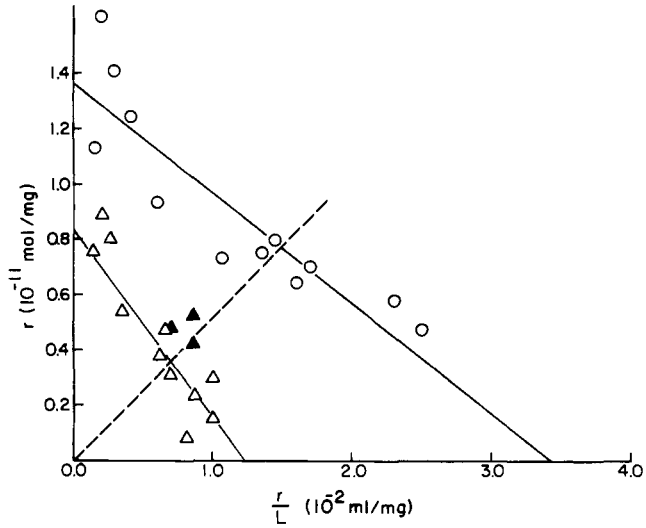


FIGURE 3: Binding data of [^{3}H]decamethonium to electroplax membrane preparations, pH 7.0, 4 °C, μ = 0.18 M, eel Ringers' solution, are presented in the form of Scatchard plots. (○) Decamethonium binding in presence of 0.1 mM Tetram; r_0^s = 1.4 ± 0.1 × 10^{-11} mol/mg; K_D = 0.4 ± 0.06 μM. (△) Decamethonium binding in the presence of 0.1 mM Tetram to membrane preparation pretreated with 2 μM α-bungarotoxin at 4 °C for 1 h; r_0^s = 0.8 ± 0.08 × 10^{-11} mol/mg; K_D = 0.7 ± 0.1 μM. (▲) Decamethonium binding in the presence of 6 μM 3-HOPTA to membrane preparations from which ~90% of the acetylcholinesterase originally present was removed by salt extraction (Silman and Karlin, 1967). The membrane preparations were then pretreated with 2 μM α-bungarotoxin at 4 °C for 1 h. The unbound decamethonium concentration was 0.5 μM. (- - -) Connects data points which were obtained at the same unbound decamethonium concentration of 0.5 μM. Duplicate determinations were made at each decamethonium concentration used. For each membrane preparation, Φ values were obtained as described previously (Donner et al., 1976) and the measurements were corrected for unspecific binding and volume exclusion. The data points shown represent the average value obtained from experiments with membrane preparations from two different eels. Protein content was 13–14 mg of membrane protein/ml in the various experiments. Equilibrium dialysis was used.

moles of ligand bound per milliliter of solution. These units were chosen so that we could present the results with the native membrane (upper line) and the acetylcholinesterase-depleted membrane (lowest line) on the same graph.

A single homogeneous binding isotherm is observed in the

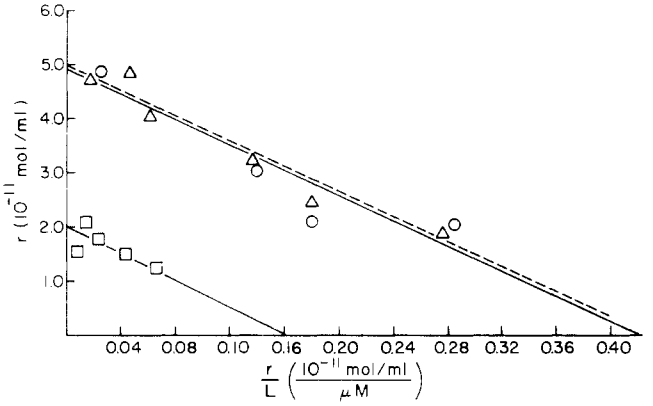


FIGURE 4: Binding data of [^{3}H]3-HOPTA to electroplax membrane preparations, pH 7.0, 4 °C, μ = 0.18 M, eel Ringers' solution, are presented in the form of Scatchard plots. (○) 3-HOPTA binding; $r = 5.0 \pm 0.5 \times 10^{-11}$ mol/ml (1.7 mg of membrane protein/ml); $K_H = 0.1 \pm 0.03$ μM. (△) 3-HOPTA binding to membrane preparations pretreated with 2 μM α-bungarotoxin at 4 °C for 1 h; $r = 5.0 \pm 0.2 \times 10^{-11}$ mol/ml (1.7 mg of membrane protein/ml); $K_H = 0.1 \pm 0.01$ μM. (□) 3-HOPTA binding to membrane preparation from which acetylcholinesterase has been removed by salt extraction (Silman and Karlin, 1967); $r = 2 \pm 0.2 \times 10^{-11}$ mol/ml (5.6 mg of membrane protein/ml); $K_H = 0.1 \pm 0.01$ μM. Equilibrium dialysis was used in the experiments.

binding of 3-HOPTA to the membrane-bound enzyme (Figure 4, upper line) and the dissociation constant obtained in these experiments (0.1 μM) is, within experimental error, the same as that determined from steady-state kinetic measurements. The experiments in Figure 4 also show that the concentration of α-bungarotoxin used in the experiments does not affect the equilibrium between 3-HOPTA and the membrane-bound enzyme. In other experiments, the membrane-bound acetylcholinesterase was removed from the membrane preparations by extraction with 1 M sodium chloride (Silman and Karlin, 1967). The concentration of the remaining membrane-bound acetylcholinesterase was determined by titration with 3-HOPTA (Figure 4). The data indicate that about 90% of the enzyme was removed from the membrane. These results are in agreement with measurements of the specific activity of membrane-bound acetylcholinesterase using the method of Ellman et al. (1961). However, the amount of decamethonium bound to the enzyme-depleted membrane preparation in the presence of 3-HOPTA is essentially the same as that bound to the native membrane (Table I). In other experiments (Moore et al., 1977), we established that decamethonium, in the concentrations used in our experiments, does not bind to purified acetylcholinesterase preparations in the presence of 6 μM 3-HOPTA.

Discussion

In investigation of the binding isotherms of membrane-bound receptors there are a number of problems which have not been considered sufficiently. (1) Measurements of binding isotherms using equilibrium dialysis and radioisotopes necessitate receptor concentrations which approach the value of the dissociation constant of the ligand under investigation. Membrane protein concentrations of 10 mg/ml or higher need to be used in experiments with electroplax membranes. Under these conditions, the volume occupied by the membrane from which the ligand is excluded must be considered in analyzing the data. In independent experiments using inulin, we found (Donner et al., 1976) that the exclusion volume, V_E, of the membrane preparation was 3 ± 0.3 μl/mg of membrane protein. The membrane preparation which we use forms vesicles which have an internal volume, determined with ^{22}Na (Kasai and Changeux, 1971), in the same range. This exclusion volume may be a major cause of error in evaluating equilibrium binding measurements with quaternary nitrogen compounds which are unable to penetrate the membrane vesicles (Nachmansohn, 1975). The observation (Figure 1, curves 2 and 3) that at high ligand concentration less ligand is bound than at low concentrations is accounted for by the exclusion volume (Donner et al., 1976). (2) The weak and possibly unspecific binding of chemical mediators to membrane components other than the receptor, which constitutes only a very small percentage of the membrane components, must also be considered.

An isotope dilution technique was used (Donner et al., 1976) to determine the unspecific binding of decamethonium to our membrane preparation. In the experiments shown in Figure 1, at a decamethonium concentration of 5 μM unspecific binding corresponds to about 40% of the specific binding.

In view of the corrections of the binding data which had to be made, the decamethonium binding isotherms do not rule out heterogeneous binding sites for this ligand. Nevertheless, when we average the moles of decamethonium binding sites per mg of membrane protein, determined using membrane preparations from eight different eels, we do obtain good agreement with the moles of α-bungarotoxin binding sites determined in independent experiments. The reason that ratios of effector to α-bungarotoxin sites other than 1 have been found (Kasai and Changeux 1971c) (Table I) is undoubtedly due, in part, to unspecific binding of either the effector or α-bungarotoxin (Bulger and Hess, 1973; Hess et al., 1975b; Bulger et al., 1977). (3) The binding of ligands to acetylcholinesterase must be considered. The observation that decamethonium binds to the membrane preparations in the presence of stoichiometric amounts of α-bungarotoxin (Figure 1, curve 3; Figure 3) has previously been made by Changeux et al. (Kasai and Changeux, 1971c; Weber and Changeux, 1974b). Their experiments were performed in the absence of enzyme inhibitors and the results were interpreted as being due to the binding of ligand to the membrane-bound acetylcholinesterase. We do not consider this as an explanation of our results.

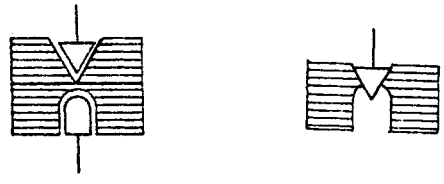
Our experiments show that we obtain the same decamethonium binding isotherms using two different enzyme inhibitors which block enzyme activity by more than 99% under the conditions used. This does not exclude the possibility that decamethonium binds to acetylcholinesterase at sites other than those blocked by these inhibitors. Therefore, we demonstrate that we obtain the same occupancy of decamethonium sites in the presence of enzyme inhibitors and stoichiometric amounts of α-bungarotoxin in native membrane preparations and in membrane preparations from which about 90% of the enzyme has been removed by salt extraction (Figure 3, Table I). With purified soluble acetylcholinesterase preparations, we showed (Moore et al., 1977) that 1 μM HOPTA prevents the binding of decamethonium to the enzyme at concentrations used in our experiments.

The data, therefore, show the occupancy by decamethonium of about half its sites in the presence of stoichiometric amounts of an irreversible acetylcholine receptor inhibitor, α-bungarotoxin. We have shown previously (Fu et al., 1974) that this interaction between binding sites is true not only for α-bungarotoxin and decamethonium, but also that *d*-tubocurarine, which competes with α-bungarotoxin for binding sites (Bulger and Hess, 1973), can displace only one-half of the bound decamethonium. Carbamylcholine, which is an activator, displaces bound decamethonium completely (Fu et al., 1974).

Further evidence of separate binding sites for *d*-tubocurarine and decamethonium was obtained from kinetic measurements of $^{22}Na^+$ efflux of excitable membrane vesicles (Hess et al., 1976). Using a fluorescent lanthanide, terbium, to probe the calcium- and activator-binding sites of the purified receptor prepared from *Torpedo ocellata*, we obtained additional evidence (Rübsamen et al., 1976a,b) for different binding sites of activators (acetylcholine, carbamylcholine, decamethonium) and inhibitors (α-bungarotoxin and *d*-tubocurarine).

In experiments with *Torpedo* spp. electroplax membranes, it was observed that in presence of d-tubocurarine only about 50% of the acetylcholine binding sites can be occupied without an apparent change in the dissociation constant of the acetylcholine–receptor site complex (Eldefrawi, 1974). Heterogeneous receptor sites have been suggested by O'Brien and Gibson (1974) and by Raftery et al. (1975). The experiments of Brockes and Hall (1975) indicate the existence of different receptor molecules in rat diaphragm muscles.

Kinetic experiments can distinguish between two interpretations of the data. Two forms of the receptor exist, A and B, as shown:



Form A has separate, noninteracting, binding sites for activators (▽) and inhibitors (♀). Form B has interacting sites for activators and inhibitors. According to one suggestion, forms A and B represent separate and distinct receptor molecules. An alternative possibility is that forms A and B represent two interconvertible conformations of the same molecule. The results of kinetic experiments are presented in the following paper of this issue (Bulger et al., 1977).

References

Augustinsson, K. B. (1953), *Acta Chem. Scand. 7,* 906.
Biesecker, G. (1973), *Biochemistry 12,* 4403.
Bonting, S. L., Simon, K. A., and Hawkins, N. M. (1961), *Arch. Biochem. Biophys. 95,* 416.
Bray, H. G., Thorpe, W. V., and White, K. (1950), *Biochemistry 46,* 271.
Brockes, J. P., and Hall, Z. W. (1975), *Biochemistry 14,* 2092, 2100.
Bulger, J. E., Fu, J.-j. L., Hindy, E. F., Silberstein, R. J., and Hess, G. P. (1977), *Biochemistry* (following paper in this issue).
Bulger, J. E., and Hess, G. P. (1973), *Biochem. Biophys. Res. Commun. 54,* 677.
Calderbank, A., and Ghosh, R. (1955), *Chem. Ind. (London) 118,* 637.
Chang, H. W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2113.
Chang, C. C., and Lee, C. Y. (1963), *Arch. Intern. Pharmacodyn. Ther. 144,* 241.
Donner, D., Fu, J.-j. L., and Hess, G. P. (1976), *Anal. Biochem. 75,* 454.
Edelstein, S. J., Beyer, W. B., Eldefrawi, A. T., and Eldefrawi, M. E. (1975), *J. Biol. Chem. 250,* 6101.
Eldefrawi, M. E. (1974), in The Peripheral Nervous System, John I. Hubbard, Ed., New York, N.Y., Plenum Press, p 181.
Eldefrawi, M. E., Britten, A. G., and Eldefrawi, A. T. (1971a), *Science 173,* 338.
Eldefrawi, M. E., Britten, A. G., and O'Brien, R. D. (1971b), *Pestic. Biochem. Physiol. 1,* 101.
Eldefrawi, M. E., and Eldefrawi, A. T. (1973), *Arch. Biochem. Biophys. 159,* 362.
Eldefrawi, M. E., Eldefrawi, A. T., Gilmore, L. P., and O'Brien, R. D. (1971c), *Mol. Pharmacol. 7,* 420.
Ellman, G. L., Courtney, K. D., Andrea, Jr., V., and Featherstone, R. M. (1961), *Biochem. Pharmacol. 1,* 88.
Fu, J.-j. L., Donner, D. B., and Hess, G. P. (1974), *Biochem. Biophys. Res. Commun. 60,* 1072.
Furlong, C. E., Morris, R. G., Kandrak, M., and Rosen, B. P. (1972), *Anal. Biochem. 47,* 514.
Hammes, G. G., and Wu, C.-W. (1974), *Annu. Rev. Biophys. Bioeng. 3,* 1.
Hantsch, A., and Davison, W. D. (1896), *Berichte 29,* 1533.
Hess, G. P., and Andrews, J. P. (1977), *Proc. Natl. Acad. Sci.* (in press).
Hess, G. P., Andrews, J. P., and Struve, G. E. (1976), *Biochem. Biophys. Res. Commun. 69,* 830–837.
Hess, G. P., Andrews, J. P., Struve, G. E., and Coombs, S. E. (1975a) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4371.
Hess, G. P., Bulger, J. E., Fu, J.-j. L., Hindy, E. F., and Silberstein, R. J. (1975b), *Biochem. Biophys. Res. Commun. 64,* 1018.
Higman, H. B., Podleski, T. R., and Bartels, E. (1963), *Biochim. Biophys. Acta 75,* 187.
Karlin, A. (1967), *J. Theor. Biol. 16,* 306.
Karlsson, E., Heilbron, E., and Widlund, L. (1972), *FEBS Lett. 28,* 107.
Kasai, M., and Changeux, J.-P. (1971a), *J. Membr. Biol. 6,* 1.
Kasai, M., and Changeux, J.-P. (1971b), *J. Membr. Biol. 6,* 24.
Kasai, M., and Changeux, J.-P. (1971c), *J. Membr. Biol. 6,* 58.
Keynes, R. D., and Martins-Ferreira, H. (1953), *J. Physiol. (London) 119,* 315.
Klett, R. P., Fulpius, B. W., Cooper, D., Smith, M., Reich, E., and Possani, L. D. (1973), *J. Biol. Chem. 248,* 6841.
Koshland, D. E. (1970), *Enzymes 1,* 347.
Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 365.
Lee, C. Y. (1972), *Annu. Rev. Pharmacol. 12,* 265.
Lee, C. Y., Tseng, L. F., and Chiu, T. H. (1967), *Nature (London) 215,* 1177.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Meunier, J.-C., Olsen, R. W., Menez, A., Fromageot, P., Boquet, P., and Changeux, J.-P. (1972), *Biochemistry 11,* 1200.
Meunier, J.-C., Sealock, R., Olsen, R., Changeux, J.-C. (1974), *Eur. J. Biochem. 45,* 371.
Miledi, R., Molinoff, P., and Potter, L. T. (1971), *Nature (London) 229,* 554.
Moore, E., Fu, J.-j. L., and Hess, G. P. (1977), *Biochemistry* (in press).
Nachmansohn, D. (1973), *Struct. Funct. Muscle 2nd Ed., 1972–1973 3,* 31–116.
Nachmansohn, D. (1975), Chemical and Molecular Basis of Nerve Activity, New York, N.Y., Academic Press.
O'Brien, R. D., and Gibson, R. E. (1974), *Arch. Biochem.*

Biophys. 165, 681.
Raftery, M. A., Bode, J., Vandlen, R., Chao, Y., Deutsch, J., Duguid, J. R., Reed, K., and Moody, T. (1975), Proceedings of the Mosbach Colloquium, L. Jaenicke, Ed., Springer-Verlag, Berlin, p 541.
Rübsamen, H., Hess, G. P., Eldefrawi, A. T., and Eldefrawi, M. E. (1976a) *Biochem. Biophys. Res. Commun. 68,* 56-63.
Rübsamen, H., Montgomery, M., Hess, G. P., Eldefrawi, A. T., and Eldefrawi, M. E. (1976b), *Biochem. Biophys. Res. Commun. 68,* 1020-1027.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Schmidt, J., and Raftery, M. A. (1972), *Biochem. Biophys. Res. Commun. 49,* 572.
Schoffeniels, E. (1957), *Biochim. Biophys. Acta 26,* 585.
Schoffeniels, E., and Nachmansohn, D. (1957), *Biochim. Biophys. Acta 26,* 1.
Silman, I., and Karlin, E. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1664.
Tammelin, L. E. (1957), *Acta Chem. Scand. 11,* 1340.
Taylor, P. T., and Singer, S. J. (1967), *Biochemistry 6,* 881.
Weber, M., and Changeux, J.-P. (1974a), *Mol. Pharmacol. 10,* 1.
Weber, M., and Changeux, J.-P. (1974b), *Mol. Pharmacol. 10,* 15.
Wilson, I. B., and Quan, C. (1958), *Arch. Biochem. Biophys. 77,* 286.

Allosteric Interactions between the Membrane-Bound Acetylcholine Receptor and Chemical Mediators. Kinetic Studies†

James E. Bulger,‡ Juian-juian L. Fu,§ Ellen F. Hindy, Richard L. Silberstein,¶ and George P. Hess*

ABSTRACT: The kinetics of the specific irreversible reaction of a snake neurotoxin, α-bungarotoxin, with the acetylcholine receptor of electroplax membrane preparations have been investigated. The effects of activators (decamethonium, carbamylcholine) and inhibitors (α-bungarotoxin, *d*-tubocurarine) of neural transmission on this reaction have been measured and the following new information obtained. (1) The irreversible reaction is preceded by the reversible formation of toxin-receptor complexes. (2) Two types of receptor binding site exist. *d*-Tubocurarine directly competes with the toxin for one type of binding site. Decamethonium and carbamylcholine are noncompetitive inhibitors of the toxin reaction. (3) The data are *inconsistent* with binding sites on separate and distinct molecules or with preexisting nonequivalent binding sites. A simple model is proposed to explain both the kinetic data and equilibrium measurements which indicated that activators and inhibitors of neural transmission compete for only one-half of the receptor sites available to them. The model proposes that for the compounds investigated the binding sites of activators do not overlap with those of inhibitors and that ligand-induced conformational changes of the receptor result in changes in the affinities of the binding sites. The model is simple and is based on mechanisms which have been found to be valid for many well-characterized regulatory enzymes.

After appropriate corrections of equilibrium measurements for unspecific binding of ligands to electroplax membrane preparations, and for the volume occupied by the membranes, we found that compounds (carbamylcholine, decamethonium) which initiate changes in the permeability of nerve and electroplax membranes to inorganic ions, and compounds which inhibit this permeability change (*d*-tubocurarine, α-bungarotoxin) occupy different binding sites on the membrane-bound receptor (Bulger and Hess, 1973). These binding sites interact only partially with each other (Fu et al., 1974, 1977). Different molecules, preexisting nonequivalent binding sites (MacQuarrie and Bernhard, 1971), or an allosteric mechanism which involves ligand-induced conformational changes (Koshland et al., 1966; Koshland, 1970; Conway and Koshland, 1968), are often invoked to account for such observations with well characterized enzymes. The kinetic investigations described in this paper were undertaken in the hope of discovering whether these mechanisms also apply to the more complex acetylcholine receptor-mediated processes.

In this paper, we describe our studies of the kinetics of the specific and irreversible reaction of [^{125}I]iodo-α-bungarotoxin (Chang and Lee, 1963; Lee and Chang, 1966; Lee et al., 1967; Lee, 1972) with the membrane-bound receptor of electroplax. Our investigations indicated the existence of two types of receptor-binding site, and provided information about the interconversion of the sites induced by α-bungarotoxin and about the competition of reversibly binding ligands with the toxin sites.

Several investigators have reported the reaction of α-bungarotoxin with the membrane-bound receptor (Barnard et al., 1971; Kasai and Changeux, 1971; Raftery et al., 1971; Bosmann, 1972; Franklin and Potter, 1972; Weber et al., 1972). Preliminary reports of some of our studies have appeared (Bulger and Hess, 1973; Hess et al., 1975).

† From the section of Biochemistry, Cornell University, Ithaca, New York 14853. *Received October 17, 1975.* This work was supported by Grants GM 704842 and NS08527 from the National Institutes of Health, and Grant BMS572-01908 from the National Science Foundation.

‡ National Institutes of Health Postdoctoral Fellowship NS008527. Present address: Department of Chemistry, Memphis State University, Memphis, Tenn. 38152.

§ National Institutes of Health Postdoctoral Fellowship GM53265. Present address: Miami Valley Laboratory, Research and Development, Procter & Gamble Co., Cincinnati, Ohio 45239.

¶ Some of this work formed an Honors Thesis for A.B. Cornell University, May 1974. Present address: Stanford University Medical School, Palo Alto, Calif. 94303.