HALF-OF-THE-SITES REACTIVITY OF THE MEMBRANE-BOUND ELECTROPHORUS ELECTRICUS ACETYLCHOLINE RECEPTOR

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Received August 22,1974

Summary: Equilibrium binding studies of the interaction of activators (decamethonium, carbamylcholine) and inhibitors (d-tubocurarine, α-bungarotoxin) of membrane electrical potential changes in electroplax membrane preparations from Electrophorus electricus have been carried out at 4°C, in eel Ringer solution, pH 7.0. The properties of the interaction of these chemical mediators with the membrane-bound receptor appear to be similar to those observed with regulatory enzymes which exhibit an allosteric mechanism involving ligand-induced conformational changes. The data presented here show that activators and inhibitors compete for only one-half the available membrane sites. The experiments also provide additional support for the interpretation of kinetic studies which indicated that electroplax membranes contain two different binding sites, one for activators and one for inhibitors of electrical membrane potential changes.

Interaction between chemical mediators and the membrane-bound acetylcholine receptor is believed to be the important primary event in regulation of nerve impulse transmission (1, 2). The mechanism by which such binding triggers electrical membrane potential changes is being investigated extensively in intact cells (3, 4), membrane preparations (5-11) and purified receptor (12-17). Measurements of electrical properties of membranes exhibit sigmoidal dose-response curves for acetylcholine, carbamylcholine, decamethonium, and other compounds (4, 18), and have been interpreted in terms of a Monod-type allosteric mechanism (19). Published data concerning the binding of chemical mediators to membrane p[illegible]rations from Torpedo marmorata (7-9, 11), Electrophorus electricus (6, 11) and Torpedo californica

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(15), and to various purified receptor preparations, do not exhibit the marked positive cooperativity observed in electrophysiological measurements (4, 18, 19). Therefore, the relationship between the electrophysiological response and the binding of chemical mediators to the receptor is not known.

In this paper we report on the binding properties of decamethonium, alone and in the presence of carbamylcholine and d-tubocurarine to the membrane-bound acetylcholine receptor of Electrophorus electricus in the presence and absence of a snake toxin, α-bungarotoxin (20, 21), in eel Ringer solution, pH 7.0, at 4°C. These data, together with kinetic studies (22), indicate that the ligand-receptor interaction appears similar to those observed in regulatory enzymes which exhibit an allosteric mechanism involving ligand-induced conformational changes (23).

RESULTS Equilibrium binding experiments were performed with electroplax membrane preparations from Electrophorus electricus. The data obtained with decamethonium as a ligand are presented as Scatchard plots (24) in Figure 1. The number of moles of ligand bound per milligram of membrane protein (r) is plotted versus r/[free ligand]. The ordinate intercept gives n, the number of moles of binding sites per milligram of membrane protein. The slope of the lines provides the value of the dissociation constant for the binding site-ligand complex.

Either 6 μM 3-HOPTA (3-hydroxyphenyltrimethylammonium iodide) or 0.1 mM Tetram (0,0-diethyl S-(β-diethylamino)ethyl phosphorothiolate)was used in the experiments to block the binding of decamethonium to membrane-bound acetylcholinesterase (25). The data presented in Figure 1 show only one homogeneous binding site in either native membrane preparations or membranes from which 80% of the enzyme has been removed by salt extraction (26). The dissociation constant of the binding site-decamethonium complex is 0.3 ± 0.02 μM. The number of binding sites is $0.9 \pm 0.03 \times 10^{-11}$ moles/mg protein. This

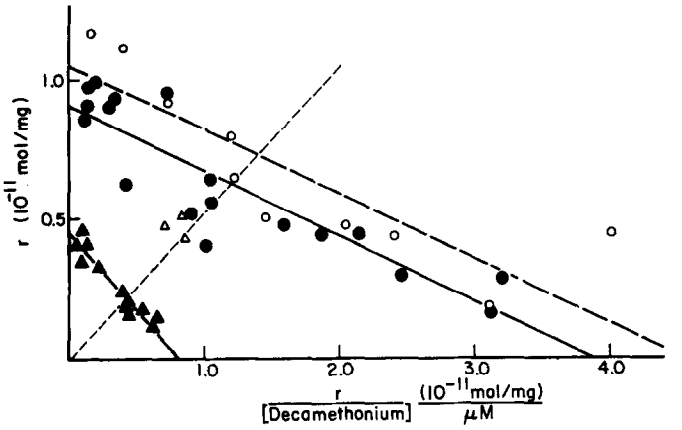

Figure 1: Binding of decamethonium (methyl-^{3}H) chloride to native (closed symbols) and salt-extracted membrane preparations (open symbols) from Electrophorus electricus electroplax at 4°C, pH 7.0, and ionic strength 0.189.

The data are plotted as r versus r/[decamethonium], where r is the number of moles of decamethonium bound per mg membrane protein.

●, ○, Binding of decamethonium (0.1 to 10 μM) to membrane preparation in the presence of 3-hydroxyphenyltrimethylammonium iodide (3-HOPTA) (6 μM).

▲, △, Binding of [^{3}H]-decamethonium (0.0 to 10 μM) to membrane preparations pretreated with 2 μM α-bungarotoxin for 1 hour at 4°C in the presence of 3-HOPTA (6 μM).

The salt extracted membrane preparations were obtained by treating the native membrane preparation with 1 M NaCl and 0.01 M Na_2HPO_4, pH 7.0, for 3 hours, at 4°C (26). The amount of remaining membrane-bound enzyme was determined by binding with [^{3}H]-3-HOPTA. Equilibrium binding of [^{3}H]-decamethonium (418 mCi/mmol. Amersham/Searle) to electroplax membrane preparations (6) was carried out in eel Ringer solution (0.169 M NaCl, 5.0 mM KCl, 3.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0, ionic strength 0.189) (30). Specially constructed Lucite microcells consisting of two 100-μl compartments separated by Visking dialysis tubing were used. 80 μl membrane preparation (protein content 10-14 mg/ml) and 80 μl of the desired decamethonium solution were allowed to equilibrate across the dialysis tubing for 16 hr at 4°C. For construction of binding curves, 20-μl samples from both compartments were withdrawn in duplicate and counted for radioactivity in 10 ml toluene-based scintillation counting fluid using a Beckman Liquid Scintillation Counter LS-230. Protein concentrations were determined at the same time by the method of Lowry (32). The thin diagonal line connects data points which were obtained at the same unbound decamethonium concentration (0.5 μM). A linear least square computer program was used to treat the data.

Native membrane preparations: The points shown are average values from 4 different electric eels.

●, α-Bungarotoxin absent. $r = (0.9 \pm 0.03) \times 10^{-11}$ mol/mg
$K_D = 0.3 \pm 0.02$ μM

▲, α-Bungarotoxin present $r = (0.44 \pm 0.02) \times 10^{-11}$ mol/mg
$K_D = 0.5 \pm 0.05$ μM

Salt-extracted membrane preparations: The points shown are average values from 2 eels.

○, α-Bungarotoxin absent $r = (1.0 \pm 0.1) \times 10^{-11}$ mol/mg
$K_D = 0.23 \pm 0.05$ μM

△, α-Bungarotoxin present An insufficient number of experiments was performed to determine r or the apparent dissociation constant.

corresponds, within experimental error, to the number of moles of α-bungarotoxin-binding sites ($0.9 \pm 0.2 \times 10^{-11}$ moles/mg protein) determined in studies with membrane preparations from thirteen eels (22).

The data also show that decamethonium binds to membrane fragments pretreated for one hour with 2 μM α-bungarotoxin. Under these reaction conditions, more than 90 per cent of the α-bungarotoxin sites are irreversibly blocked (22). The least square line defined by the decamethonium binding data gives an ordinate intercept which is 50 per cent of the value obtained in the experiment in which the membrane was not pretreated with α-bungarotoxin.

The experiments with the salt-extracted, enzyme-depleted membrane preparations indicate that these results are not due to decamethonium binding to acetylcholinesterase. In Figure 1, the ordinate intercepts and the slopes of the lines pertaining to native and salt-extracted membrane preparations are essentially the same, indicating that salt extraction removed the enzyme but did not measurably alter the binding properties of the receptor site. At equal initial concentrations of decamethonium, the ratio of decamethonium bound in the absence and presence of α-bungarotoxin is the same for both the native and the enzyme-depleted membrane preparations (Fig. 1).

Experiments were performed to determine whether the half-of-the-site occupancy by decamethonium, in the presence of α-bungarotoxin, is restricted to these two compounds. In the experiments shown in Figure 2, the concentration of unbound decamethonium (0.5 μM) was the same in all experiments. (The concentration of free decamethonium for those experimental points in Figure 1 which are connected by the dotted line is also 0.5 μM.) The amount bound in the presence or absence of carbamylcholine, d-tubocurarine, or α-bungarotoxin and d-tubocurarine, was determined. The amount of decamethonium bound in the absence of a particular compound was taken as 100 per cent.

Figure 2(a) shows that d-tubocurarine, at concentrations 100 times the value of its apparent dissociation constant, displaces only 50 per cent of

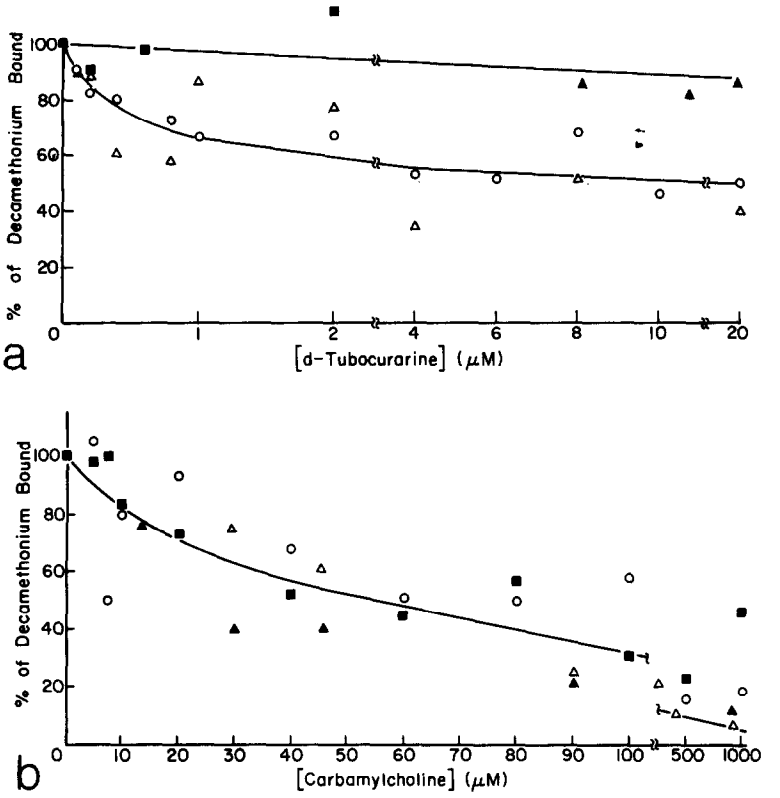

Figure 2

the bound decamethonium. In similar experiments in which the membrane preparations were pretreated for one hour with α-bungarotoxin (2 μM), d-tubocurarine did not displace decamethonium. This is to be expected if the two compounds, d-tubocurarine and α-bungarotoxin, occupy overlapping binding sites.

Figure 2(b) illustrates a similar experiment, except that carbamylcholine was used instead of d-tubocurarine. In these experiments, the same percentage of [^{3}H]-decamethonium was displaced whether or not the membrane preparations was pretreated with 2 μM α-bungarotoxin. At high concentrations of carbamylcholine, most of the decamethonium was displaced from the membrane sites. These results are to be expected if the two compounds, decamethonium and carbamylcholine, occupy overlapping binding sites. For a simple mechanism, the dissociation constant of carbamylcholine

can be estimated from the data shown by using the equation given in Figure 2. The value of 20 μM agrees with the dissociation constant determined in electrophysiological experiments using single electroplax (4).

DISCUSSION

The data in this paper are in good agreement with previous results. An apparently homogeneous decamethonium binding site, and adherence of the data

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Figure 2: Displacement of 0.5 μM decamethonium (methyl-^{3}H) chloride bound to membrane preparations by activators or inhibitors, at 4°C, pH 7.0 and ionic strength 0.189.
0.2 ml membrane preparation together with decamethonium and either d-tubocurarine or carbamylcholine were placed in a dialysis sac (1 cm wide, 15 cm long). The sac was dialyzed against a large excess (5 ml) of eel Ringer (3_0) solution containing the same concentration of carbamylcholine (0 to 1 mM) or d-tubocurarine (0 to 20 μM) as the solution inside the sac. The dialysis was carried out inside test tubes which were shaken for 16 hr at 4°C to ensure complete equilibration. Triplicate aliquots (20 λ) from both the sac and the test tubes were taken for radioactivity counting. Protein concentrations were determined by the method of Lowry (32). The amount of decamethonium bound in the absence of d-tubocurarine or carbamylcholine was set as 100%. The other data are reported as percentages of the control. For a simple mechanism involving competition between decamethonium and carbamylcholine for the same binding site, the amount of decamethonium, D, bound to the membrane in presence of various concentrations of carbamylcholine, C, is given by:

$$DM = \frac{M_0 D}{D + K_D \left(1 + \frac{C}{K_C}\right)}$$

DM represents the concentration of the decamethonium-membrane complex, M_0 the moles of binding site per mg membrane protein, and K_D and K_C the dissociation constants of the decamethonium and carbamylcholine complexes respectively.

(a) Displacement by d-tubocurarine
○, The membrane was pretreated with 0.1 mM Tetram at 4°C for 30 min.
△, 6 μM 3-HOPTA present.
■,▲,6 μM 3-HOPTA present. The membrane was pretreated at 4°C for 1 hr with 2.0 μM α-bungarotoxin. (The two symbols refer to membrane preparations obtained from different eels.)

(b) Displacement by carbamylcholine
The closed symbols refer to experiments in which the membrane was pretreated with 2 μM α-bungarotoxin at 4°C for 1 hr.
○, Membrane pretreated with 0.1 mM Tetram at 4°C for 30 min.
△, 6 μM 3-HOPTA present.
■,▲, 6 μM 3-HOPTA present. The membrane was pretreated at 4°C for 1 hr with 2.0 μM α-bungarotoxin. (The two symbols refer to membrane preparations obtained from different eels.) The solid line was computed on the basis of a dissociation constant for decamethonium of 0.3 μM and for carbamylcholine of 20 μM.

to a classic Langmuir isotherm, has been reported previously (11, 15). The stoichiometry of the decamethonium and α-bungarotoxin sites in electroplax is believed to be one to one. Deviations from this value are assumed to arise from binding of decamethonium to membrane-bound acetylcholinesterase (27). The value which we determined for the dissociation constant of decamethonium, 0.3 μM, is similar to the values obtained with the same membrane preparation by Changeux et al. (11, 27).

The new findings reported here are that 50 per cent of the membrane sites can be occupied by decamethonium in the presence of a large excess of d-tubocurarine, or when more than 90 per cent of the α-bungarotoxin sites have reacted irreversibly with the toxin. While this half-of-the-sites occupancy was shown directly only for decamethonium, the experiments suggest that the phenomenon is also true for carbamylcholine which competes for all the decamethonium sites.

In the presence of α-bungarotoxin or saturating amounts of d-tubocurarine, half-of-the-sites occupancy of decamethonium sites without major changes in the dissociation constant can occur only if the decamethonium sites on one hand, and the α-bungarotoxin and d-tubocurarine sites on the other, do not completely overlap.

These new observations and conclusions are in agreement with the previously published kinetic measurements of Bulger and Hess (22) which indicated the existence of two different binding sites, one for activators (decamethonium, carbamylcholine) and one for inhibitors (d-tubocurarine, α-bungarotoxin) of changes in membrane electrical potential.

In these experiments the specific α-bungarotoxin reaction was followed to within 90 per cent of completion without obtaining evidence for heterogeneous toxin sites. This result, together with the 1:1 stoichiometry of decamethonium and α-bungarotoxin sites, suggests that the half-of-the-sites occupancy by decamethonium in presence of α-bungarotoxin or of saturating amounts of d-tubocurarine involves one and the same membrane-bound receptor

molecule. Separate binding sites for activators and inhibitors, and half-of-the-sites reactivity, are being observed with an increasing number of regulatory enzymes, particularly those which exhibit sequential ligand induced conformational changes (28,29). To the best of our knowledge, this repor is the first documented case in which a membrane-bound binding protein is shown to have these properties.

Acknowledgements: We are grateful to Drs E. Bartels-Bernal and W. Niemi, Department of Neurophysiology, Columbia University, for performing the electrophysiology, and to the National Institutes of Health and the National Science Foundation for financial support. J.L.F. is grateful to the National Institutes of Health for the award of a Postdoctoral Fellowship and for support from an Institutional award.

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