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Multiple Binding Sites for Acetylcholine in a Proteolipid from Electric Tissue

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SUMMARY

A special proteolipid protein fraction having a high affinity for binding to acetylcholine and other cholinergic drugs was isolated from electroplax of *Electrophorus electricus*. After extraction in chloroform–methanol (2:1 by volume) and column chromatography on Sephadex LH-20, the proteolipid appeared in a special peak together with the radioactive acetylcholine and increased in proportion with the amount of protein applied to the column. A saturation curve of this peak with respect to concentrations of ^{14}C-acetylcholine between 7×10^{-7} and 5×10^{-5} M was constructed. The shape of this curve and the double-reciprocal plot of the data suggested the presence of more than one type of binding site. Assuming a homogeneous population of molecules having a molecular weight of 40,000, the use of the Scatchard equation suggested that there is a single high-affinity binding site with an apparent dissociation constant of 1×10^{-7} M and a group of low-affinity sites with a dissociation constant of 1×10^{-5} M. Since this proteolipid is present only in the electroplax membranes, these results are discussed in relation to the possible quantity of this material per electroplax or synaptic junction and its possible physiological significance as a receptor in cholinergic synapses.

INTRODUCTION

A proteolipid soluble in organic solvents and having a high affinity for binding to ^{14}C-dimethyl-*d*-tubocurarine (1) and atropine sulfate has been separated from nerve ending membranes of the cerebral cortex and named "receptor" proteolipid (2, 3). Another proteolipid extracted from basal ganglia of brain showed a similar type of binding for serotonin (4) and for adrenergic blocking agents (5, 6). This study was extended to the electric organs of *Torpedo* and *Electrophorus*, to gain the advantage of having a purely cholinergic innervation and nicotinic type of receptor. Using column chromatography on Sephadex LH-20, a proteolipid peak was separated from the electroplax (7) which showed a high affinity for binding of methyl-^{14}C-hexamethonium and for the irreversible inhibitor of the electroplax ^{3}H-[trimethylphenylammonium]diazonium fluoroborate (8) at a drug concentration range from 1×10^{-7} to 5×10^{-6} M. In this same work some preliminary experiments involving the use of [^{14}C-methyl]-acetylcholine chloride were also

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described (7). These investigations were extended to isolated electroplax membranes, either intact or experimentally depleted of acetylcholinesterase, from both of which the same proteolipid, having a high affinity for binding to labeled acetylcholine, was separated (9).

In the present report the binding of [^{14}C-methyl]-acetylcholine by proteolipids extracted from the electroplax will be considered in greater detail, and quantitative information on the characteristics of the drug-proteolipid interaction will be presented.

METHODS

Specimens of *Electrophorus electricus* were kept frozen at −30°. The electroplaques were dissected from the frozen fish and homogenized in distilled water (10%, w/v) in a Waring Blendor for three 1-min periods at the highest speed. The homogenate was frozen as a thin film in glass vessels and immediately lyophilized for at least 24 hr. The dry material was stored under vacuum over a desiccant. Lyophilized electroplaques (1 g was equivalent to 12.5 g of fresh tissue) were homogenized in 15 ml of chloroform–methanol (2:1 by volume) for 1 min in an Ultra-Turrax (Karl Kolb, Frankfurt). After standing at room temperature for 5 min, the extract was filtered through Whatman No. 2 filter paper. The residue was washed with chloroform–methanol (5 ml; 2:1 by volume), the volume of the extract was noted, and a volume of chloroform equal to half that of the extract was added. This was then evaporated under vacuum at room temperature to a final volume of 5 ml. Binding was carried out by the addition of [^{14}C-methyl]-acetylcholine chloride (9.2 mCi/mmole, Radiochemical Centre, Amersham, England). This was dissolved in 0.01 N methanolic HCl at a concentration of 5×10^{-4} M. Volumes of 1–500 µl were added to the extract to give the desired final concentration.

After standing at room temperature for 20 min, the extract was loaded onto a Sephadex LH-20 column (2.1 × 18 cm) that had been equilibrated overnight in chloroform. The standard elution procedure previously reported (10) was slightly modified as follows: chloroform, 80 ml; chloroform–methanol (15:1 by volume), 30 ml; and chloroform–methanol (4:1 by volume), 70 ml. The eluate was monitored at 278 nm with an LKB Uvicord ultraviolet absorption meter at a flow rate of 0.5 ml/min. The first 60 ml eluted were collected in fractions of approximately 2 ml, and subsequently fractions of 5 ml were collected.

In each tube the lipid phosphorus (11) and protein content (12) were determined, and the radioactivity was measured in a Nuclear-Chicago liquid scintillation counter as described previously (13). Control experiments were performed by passing through the column concentrations of ^{14}C-acetylcholine equivalent to those used in the binding experiments, but without protein.

RESULTS

Figure 1 shows the typical elution pattern obtained from the total lipid extract of *Electrophorus*. In this particular experiment an initial concentration of 5×10^{-7} M ^{14}C-acetylcholine was used. The proteolipid protein appeared in three peaks, which were eluted with the chloroform, and two peaks, eluted with chloroform–methanol (4:1), at the end of the chromatogram. The total recovery of protein was 70–80%. Control experiments performed with the lipid extract without ^{14}C-acetylcholine gave the same pattern of elution of the proteolipid. About 90% of the lipid phosphorus was recovered, appearing in two peaks in the chloroform extract and in another at the end of the chromatogram. The maximum amount of lipid phosphorus appeared 7 ml earlier than the elution volume containing maximal radioactive protein. The recovery of radioactivity at this concentration was 80% and appeared only in a sharp peak (peak 3) between 35 and 40 ml of chloroform, coinciding with the maximum recovery of proteolipid protein. In this peak the lipid phosphorus to protein ratio was 0.2. In control experiments with free acetylcholine (5×10^{-7} M) no radioactivity was eluted with the chloroform, and only

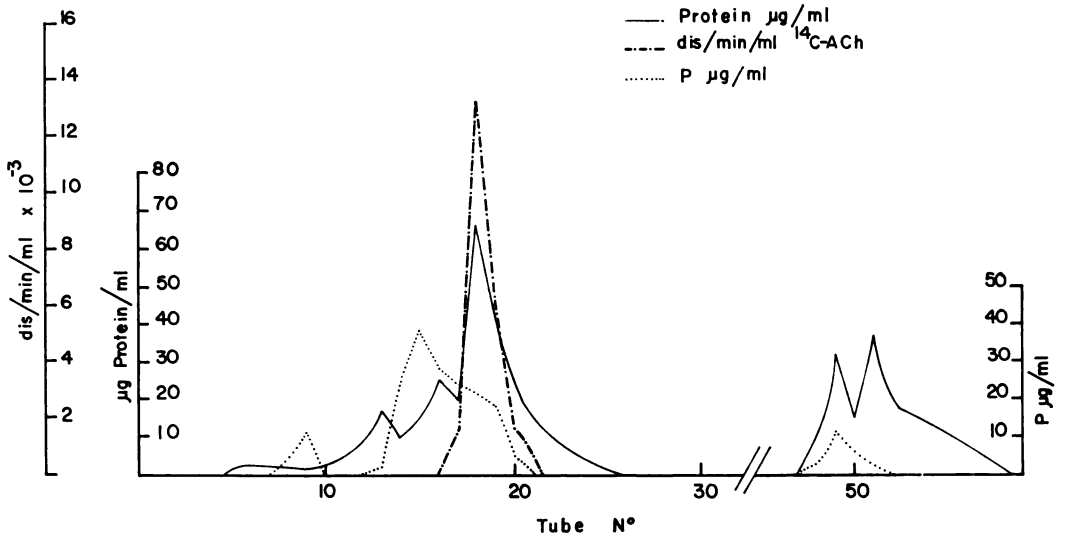

FIG. 1. *Chromatographic pattern obtained from lyophilized electric tissue of Electrophorus*

The chloroform–methanol (2:1 by volume) extract was subjected to binding with 5×10^{-7} M [^{14}C-methyl]-acetylcholine (^{14}C-ACh) and passed through a column of Sephadex LH-20 (2.1 × 18 cm). The bound radioactivity was eluted together with peak 3 of proteolipid protein (see description in the text).

6% of the radioactivity was eluted with chloroform–methanol (4:1).

Saturation of binding sites of proteolipid. Binding experiments were carried out using 6×10^{-7} M ^{14}C-acetylcholine and varied amounts of protein. Table 1 shows that the protein recovered in peak 3, which carried the label, increased in parallel with the amount of protein applied to the column. The ^{14}C-acetylcholine bound to peak 3 was quantitatively the same at all concentrations of protein and represented about 60% of the radioactivity of the original extract. This indicates that the available acetylcholine was bound to the 220 µg of proteolipid eluted as peak 3 and that more acetylcholine would be necessary to achieve saturation with this amount of proteolipid. Correspondingly, the specific radioactivity in peak 3 decreased in parallel with the increased amount of protein (Table 1).

In other experiments the protein concentration was kept constant and the concentration of ^{14}C-acetylcholine was varied between 7×10^{-7} and 5×10^{-5} M. In each case controls were performed with the same concentration of ^{14}C-acetylcholine without proteolipid. In all the experiments the radioactivity appeared in peak 3 together with the protein. Table 2 shows the various concentrations of ^{14}C-acetylcholine used, the results obtained for binding, and also the data used to construct the curves of Figs. 2 and 3.

TABLE 1

Binding of [^{14}C-methyl]-acetylcholine in receptor proteolipid as a function of total protein applied to Sephadex LH-20 column

Peak 3 corresponds to the one carrying the peak of radioactivity (see Fig. 1). The ^{14}C-acetylcholine added to the column in these experiments was at a final concentration of 6×10^{-7} M, equivalent to 61,000 dpm.

Protein on column	Protein in peak 3	Radioactivity in peak 3
µg	*µg*	*dpm/µg protein*
1100	220	180
1950	384	100
3300	480	83
6200	1396	33

With increasing concentrations of acetylcholine an increasing number of counts appeared in the chloroform–methanol (4:1) extract. Since no difference was found between test and control experiments, such counts were interpreted as being due to

TABLE 2

Binding at various concentrations of [^{14}C-methyl]-acetylcholine

[ACh$_i$], initial concentration of acetylcholine; [ACh$_f$], concentration of free acetylcholine; $\bar{\nu}$ and $\bar{\nu}$/[ACh$_f$], the terms used in the Scatchard plot (Fig. 3), are defined in the text. At saturation $\bar{\nu}$ = 10, assuming a molecular weight for the proteolipid of 40,000.

[ACh$_i$]	Acetylcholine bound to peak 3	[ACh$_f$]	Radioactivity in peak 3	$\bar{\nu}$	$\bar{\nu}$/[ACh$_f$]
M	%	*M*	*dpm/μg protein*		
7.0×10^{-7}	80	5.6×10^{-8}	176	0.36	6.32×10^{6}
1.1×10^{-6}	80	2.2×10^{-7}	334	0.68	3.06×10^{6}
2.3×10^{-6}	50	1.1×10^{-6}	570	1.15	1.04×10^{6}
4.5×10^{-6}	47	2.4×10^{-6}	880	1.78	7.40×10^{5}
7.3×10^{-6}	48	3.8×10^{-6}	1465	2.96	7.70×10^{5}
1.2×10^{-5}	39	7.3×10^{-6}	1750	3.55	4.85×10^{5}
2.6×10^{-5}	25	1.9×10^{-5}	3000	6.05	3.18×10^{5}
5.0×10^{-5}	15	4.2×10^{-5}	3890	7.84	1.87×10^{5}
∞			4895	10.00	

free acetylcholine. At high concentrations free acetylcholine also appeared in the chloroform eluent. Table 2 indicates the percentage of bound radioactivity in peak 3 at each concentration of acetylcholine.

The saturation curve obtained by plotting radioactivity against the concentration of free acetylcholine did not show the characteristic rectangular hyperbola expected for the saturation of a single set of binding sites. When a double-reciprocal plot was constructed, the multiple nature of the binding became more evident. As shown in Fig. 2, two sets of binding sites having different dissociation constants, i.e., $K_1 = 1 \times 10^{-7}$ and $K_2 = 1 \times 10^{-5}$, may be calculated by extrapolation of the curve.

A plot of acetylcholine bound against the reciprocal of the initial acetylcholine concentration indicated the degree of saturation of the proteolipid. By extrapolation it could be demonstrated that, at full saturation, about 4950 dpm/μg of protein may be bound (Table 2). This amount corresponds to 2.4×10^{-10} mole of acetylcholine per microgram of protein, or approximately 10 moles/mole of proteolipid, assuming that peak 3 consists of a homogeneous population of molecules having a molecular weight of 40,000 (see DISCUSSION).

To analyze these data further, the Scatchard equation (14) was introduced.

$$\frac{\bar{\nu}}{[\mathrm{ACh}_f]} = K(n - \bar{\nu})$$

Here $\bar{\nu}$ is the average number of moles of acetylcholine bound per mole of proteolipid, [ACh$_f$] is the concentration of free acetylcholine, K is the association constant, and n is the number of binding sites in the proteolipid molecule. The Scatchard plot of $\bar{\nu}$/[ACh$_f$] against $\bar{\nu}$ gives a straight line if only one type of binding site is present. Because of the shape of the curve it is evident that there is more than one type of site present in the molecule (14), thus confirming the analysis of the saturation curve. By extrapolation of the curve to both abscissa and ordinate intercepts, it is possible to make an approximate evaluation of the number of binding sites and of the apparent dissociation constant for each species of site (Fig. 3). Taking into account the assumptions made above, we may confirm that there is a single high-affinity binding site per molecule of proteolipid, having an apparent dissociation constant of 1×10^{-7}, and another group of sites with much less affinity, having

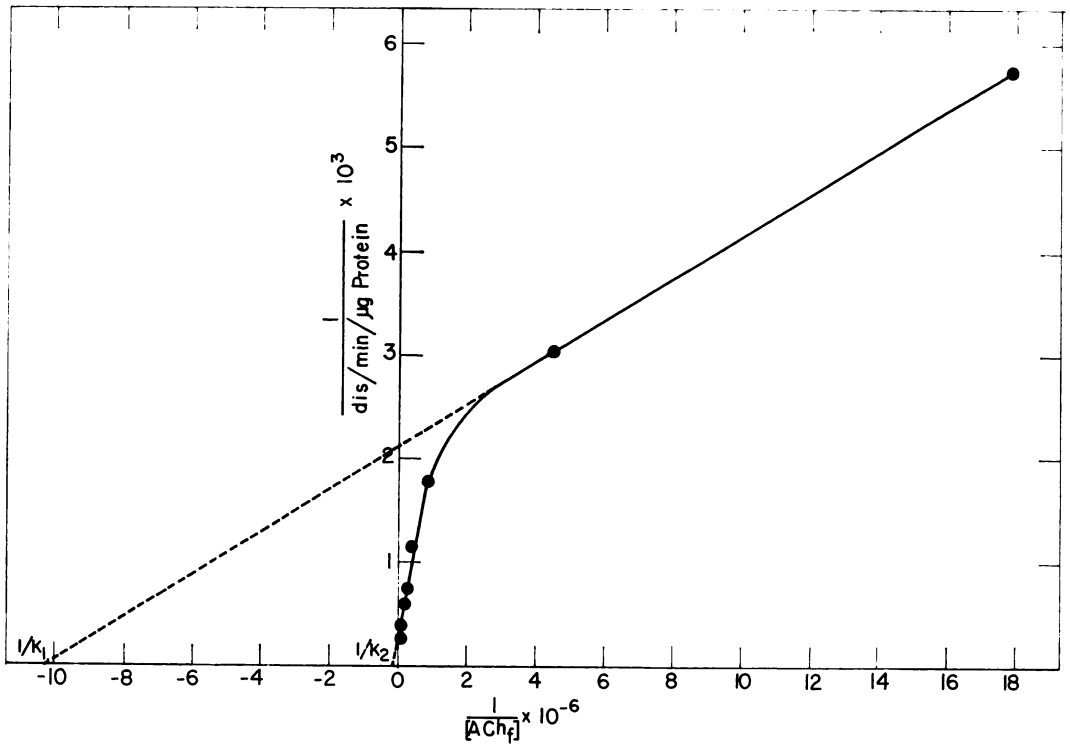

FIG. 2. *Double-reciprocal plot of data obtained from saturation curve*

In each experiment the same amount of proteolipid (i.e., 1600 μg) in 5 ml of chloroform–methanol was loaded onto the column. [ACh_f], concentration of free acetylcholine. K_1 and K_2 are association constants resulting from extrapolation of the curve to the left on the abscissa.

an apparent dissociation constant of 1×10^{-5}.

DISCUSSION

The results presented here indicate that, as previously demonstrated for methyl-^{14}C-hexamethonium and ^{3}H-[trimethylphenylammonium]diazonium fluoroborate (7), ^{14}C-acetylcholine binds to a special proteolipid protein fraction extractable with chloroform–methanol (2:1) and purifiable by chromatography through a Sephadex LH-20 column. With all these cholinergic drugs the bound radioactivity appeared with the same proteolipid peak (peak 3). This peak contains mainly phosphatidylcholine, phosphatidylethanolamine, and a small amount of phosphatidylserine and phosphatidylinositol; however, these phospholipids are also common to protein peak 2, which shows no binding. These findings suggest that it is the protein of peak 3 which is responsible for the binding, although the participation of phospholipids cannot be excluded at present. From the amount of protein present in peak 3 it is possible to calculate that, at the most, 26 mg of this type of proteolipid having cholinergic properties are present in 1 kg of electric tissue of *Electrophorus*. We have previously reported that in *Torpedo* there may be more than twice that amount of proteolipid with a similarly high binding affinity (7).

Assuming a molecular weight for the proteolipid of 40,000 (see below), it is possible to estimate that a single electroplax of 35 mg, wet weight, having some 35,000 synapses, should contain a maximum of 1.2×10^{13} of these proteolipid molecules; this corresponds to some 3.4×10^{8} molecules/end plate. This value is not far from the number of acetylcholine molecules

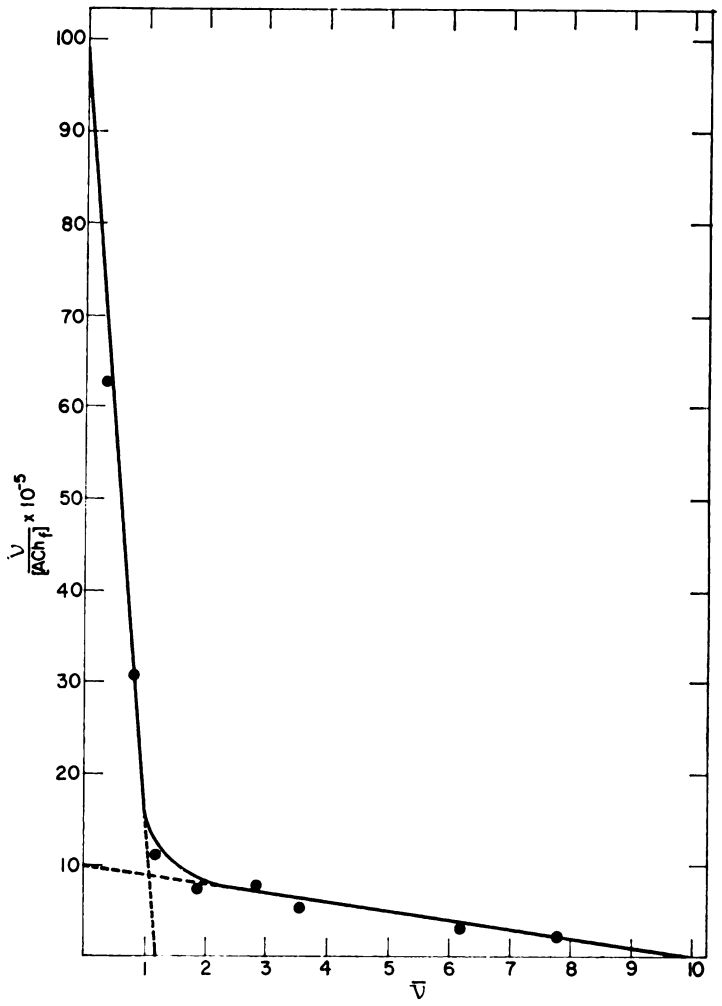

FIG. 3. *Scatchard plot of data shown in Table 2 (see description in the text)*
Intercepts on the abscissa indicate the number of binding sites. Intercepts on the ordinate correspond, according to Scatchard, to n/K_{ass}.

(i.e., 6×10^7 - 6×10^8) needed to cause depolarization of the myoneural junction (15).

For these calculations an exact knowledge of the molecular weight of the proteolipid of peak 3 would be indispensable. Such information is unfortunately not available at the present time. Previous studies were done on the proteolipid isolated from nerve ending membranes of the cerebral cortex. This was eluted in chloroform–methanol (4:1) with the Sephadex LH-20 column and bound ^{14}C-dimethyl-*d*-tubocurarine (1) and atropine sulfate (2, 3). Electron microscopy demonstrated that this macromolecule is rod-shaped with a minimum size of 15 × 150 A (16), from which a molecular weight of about 20,000 may be calculated. Similar morphological studies being conducted on the peak 3 proteolipid of *Electrophorus* bound to cholinergic drugs suggest that the size of this molecule may be larger. Another crucial problem in these binding studies is the degree of homogeneity of the proteolipid in peak 3. However, in view of its special, constant chromatographic properties and the smoothness of the saturation curve, we are inclined to believe that the

peak 3 proteolipid fraction is a fairly pure molecular species. This conclusion is supported by electron microscopic observations of these macromolecules, which show a rather homogeneous appearance.

The Scatchard equation requires a minimum molecular weight of 40,000 to give a 1:1 molecular ratio between the proteolipid molecule and the high-affinity binding site for acetylcholine. The conclusion drawn from the double-reciprocal plot (Fig. 2) suggesting that the binding is not simple but multiple, involving several sites of binding with more than one apparent dissociation constant, is confirmed by the Scatchard plot (Fig. 3). If we assume a homogeneous population of proteolipid molecules for peak 3, there would be only one site with high-affinity binding, the remaining species of low affinity, being less specific anionic sites on the protein molecule. Since the affinity of the first site is about 100 times greater than that of the nonspecific sites, we may postulate that within the physiological range of acetylcholine (15) only the high-affinity binding site would be effective in synaptic transmission.

The special binding properties of this molecule and the fact that it is tightly bound to the electroplax membrane (9) suggest that it may be the cholinergic receptor molecule. Experiments to prove this by other techniques are now under way.

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