Isolation of a Cholinergic Proteolipid Receptor from Electric Tissue

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Abstract. A proteolipid protein having a high affinity for methyl 14C-hexamethonium, 3H-p-(trimethylammonium)-benzene diazonium fluoroborate, and (acetyl-1-14C)choline chloride was isolated and purified from the electric tissue of Torpedo marmorata and Electrophorus electricus. At variance with the "receptor proteolipid" from the brain the one from electric tissue apparently does not bind atropine sulfate.

A proteolipid protein having high affinity binding for dimethyl 14C-d-tubocurarine has been separated from the isolated nerve ending membranes of the cerebral cortex. A similar "receptor proteolipid" extracted from basal ganglia was found to bind adrenergic blocking agents and serotonin. Furthermore, the binding of atropine sulfate to the proteolipid has been studied with equilibrium dialysis, light scattering, and polarization of fluorescence. All these studies lead us to postulate that in the subsynaptic membrane of central synapses, there are receptor proteolipids for the various transmitters and that, once separated from the membrane, such proteins are able to undergo conformational changes with the binding of drugs.

The isolation of receptors from the central nervous system had the disadvantage of dealing with a very complex population of nerve endings involving different transmitters. This led us to try to isolate a proteolipid from a peripheral and purely cholinergic tissue, such as the electric organ of Torpedo marmorata and Electrophorus electricus. This tissue has an extremely high density of synapses present in the electroplaque; however, even in such favorable conditions, the amount of receptor material they contain is extremely low. Based on a figure of 107 receptors per end plate it was estimated that the electric tissue should contain 10–20 mg of receptor per kilogram of fresh tissue. Here we will describe the results obtained with a proteolipid separated from lyophilized electroplaxes of Torpedo and Electrophorus which shows high binding affinity for acetylcholine (ACh), methylhexamethonium (MHM), and p-(trimethylammonium)-benzene diazonium fluoroborate (TDF). This last compound irreversibly blocks the reaction of the electroplaque toward ACh and other receptor activators and probably forms covalent bonds with side chains of proteins.

Materials and Methods. Electric tissue from Torpedo marmorata and of Electrophorus electricus was dissected at the Marine Biological Laboratory of Woods Hole,
Mass, and obtained frozen or lyophilized. For each experiment with Torpedo, 0.16 gm of dry tissue (equivalent to 2 gm of fresh tissue) was extracted with 15 ml of chloroform-methanol (2:1, v/v). The amount of protein obtained was 2.5 mg. To obtain the same amount of proteolipid protein from Electrophorus it was necessary to use 1 gm of lyophilized tissue (equivalent to 12.5 gm fresh tissue). The extract was filtered and to maintain the original proportion of the solvents after evaporation, 7.5 ml of chloroform were added and the total lipid extract was concentrated under a stream of nitrogen and at room temperature to a final volume of 5 ml. The lipid extract was submitted to binding by addition of the radioactive drug and left standing at room temperature for 20–30 min and then put on a Sephadex LH20 column (2.1 × 18 cm) previously equilibrated with chloroform (for details on this technique see ref. 9). The elution was carried out with 80 ml of chloroform, followed by chloroform-methanol 15:1 (20 ml), 10:1 (20 ml), 6:1 (20 ml), and 4:1 (40–80 ml). The eluate, monitored with an LKB ultraviolet absorption meter at 280 mμ, was collected into 2–5 ml fractions at a flow rate of 0.5 ml/min.

In each tube lipid phosphorus (P) and protein content were determined and the radioactivity measured in a Nuclear-Chicago liquid scintillation counter as previously described. Control experiments were performed with the free radioactive drug passed through the Sephadex LH20 column. The radioactive drugs used were: 14C-MHM, specific activity 1.52 mCi/mM (New England Co.); 4H-TDF, specific activity 255 mCi/mM (kindly provided by Dr. Changeux); acetylcholine (acetyl-1-14C)choline chloride specific activity 9.2 mCi/mM (Radiochemical Center, Amersham).

Results. Binding of 14C-MHM: This labeled drug was added to the lipid extract of Torpedo in concentrations of 1.4 and 5 × 10⁻⁶ M corresponding, respectively, to 6,600 and 24,000 dpm/ml. The blank experiment for the highest concentration of 14C-MHM gave a retention of 92 per cent of the free drug in the Sephadex LH20 column. Figure 1 shows the pattern of protein, lipid P, and radioactivity obtained in the experiment with 5 × 10⁻⁶ M 14C-MHM; essentially similar results were obtained with the lower concentration of 14C-MHM. The proteolipid protein is eluted in a rather sharp peak in the chloroform and two or three peaks at the end of the column with the chloroform-methanol (4:1 v/v). The total recovery of Lowry positive material varied between 30 and 40 per cent.

![Fig. 1](image-url)
The peak in the 4:1 chloroform-methanol gave no ultraviolet absorption at 280 nm and, upon evaporation under air, produced whitish needle-shaped crystals. Control experiments performed with an extract without binding gave essentially a similar pattern of elution of the protein. About 90 per cent of the lipid phosphorus was recovered and appeared in a sharp peak at the beginning of the elution, starting after the exclusion volume of chloroform (i.e., 20 ml). It is interesting that the maximum of lipid P appeared slightly earlier (i.e., at 40 ml) than that of protein (i.e., at 45 ml). A very flat peak of lipid P was also eluted in the 4:1 chloroform-methanol. The 14C-MHM appeared in a sharp peak, between 40 and 50 ml of chloroform, which contained most of the radioactivity (i.e., 18,000 dpm/ml). At this point the amount 14C-MHM bound to the proteolipid was $1.3 \times 10^{-10}$ moles per microgram protein. Assuming a molecular weight of 20,000 for the proteolipid, about 3 molecules of 14C-MHM would be bound per molecule of protein. Similar results were obtained with the binding of 14C-MHM to the proteolipid extracted from Electrophorus.

![Graph](image)

**Fig. 2.**—Similar description as in Fig. 1 but from an extract submitted to binding with $3 \times 10^{-6} M$ 3H-TDF. Observe that also in this case the peak of radioactivity coincides with the first peak of proteolipid protein.

Binding of 3H-TDF was also performed in Torpedo and as indicated above. The concentrations used were $8 \times 10^{-7} M$, corresponding to 150,000 dpm/ml and $3 \times 10^{-6} M$ having 500,000 dpm/ml. In both cases an essentially similar chromatographic pattern was obtained. The recovery for the first concentration was 91 per cent in the binding experiments, and 80 per cent of the free drug in the blank experiments was retained. The recovery for the second concentration was 50 per cent, and 75 per cent of the free drug was retained. As shown in Figure 2, corresponding to the $3.0 \times 10^{-6} M$ concentration, the pattern of elution of the proteolipid protein and lipid phosphorus is essentially similar to that described for Figure 1. The peak of radioactivity is also coincident with the first protein peak eluted in the chloroform and which also binds the 14C-MHM. Some free 3H-TDF was also eluted with the chloroform but the peak was very low. For example, with the proteolipid peak there are 230,000 dpm/ml of 3H-
TDF (Fig. 2), while with the free drug only about 60,000 dpm/ml appear in the same region.

The binding of 14C-ACh was done with the extract of Electrophorus and as indicated above. The concentration used was 5 × 10⁻⁷ M, corresponding to 15,000 dpm/ml. The chromatographic pattern obtained with Sephadex LH20 was similar to the one obtained with Torpedo using 14C-MHM or 3H-TDF. The recovery of the bound counts was 80 per cent and all appeared in a sharp peak between 35 and 40 ml of chloroform in coincidence with a peak of protein. With free 14C-ACh only 6 per cent of the counts appeared in the eluate and these were in the chloroform-methanol (4:1), i. e., at a volume of 150 ml.

Discussion. In the past, several attempts have been made to isolate the cholinergic receptor from the electric organ and to learn about its organization and molecular nature (see ref. 11). They involved the extraction of soluble proteins and the use of high concentrations of d-tubocurarine, at a range in which the unspecific binding masks the specific high affinity one. In our work on nerve ending membranes1: 2 we have shown that: (a) the receptor proteolipid is intimately bound to the lipoprotein structure of the membrane and is mainly localized in the subsynaptic membrane12; (b) it shows a high affinity binding for endogenous transmitters and other drugs active in synaptic transmission; (c) upon interaction with the drug, it may undergo some kind of conformational change which is revealed by light scattering, polarization of fluorescence,3 and electronmicroscopy.13 The proteolipid extracted from the electric tissue shows a high affinity binding for 14C-MHM and 3H-TDF and 14C-ACh, as demonstrated by column chromatography.

This confirms the previous observations made with brain proteolipids, in which the proteolipid extracted from nerve-ending membranes showed a much higher affinity binding than that from myelin.10 The proteolipid from the electroplaque has not yet been purified as much as that from brain10 but already shows some striking differences. In fact it is eluted from the Sephadex LH20 column with chloroform, while the brain proteolipid is eluted with chloroform-methanol (4:1).

Of more pharmacological interest is the fact that the proteolipid from electric tissue does not give the light scattering reaction with atropine sulfate, which is so characteristic of brain proteolipid and results from the cooperative aggregation of the macromolecules.3, 13 The lack of this reaction indicates that this proteolipid probably does not bind the atropine sulfate. This should be correlated with the purely nicotinic properties of this cholinergic receptor. Observations with electron microscopy indicate that both types of receptor proteolipids are elongated macromolecules.13

As was mentioned in the introduction, the amount of receptor protein must be extremely small. Based on the fact that from about 2 gm of fresh tissue of Torpedo we could separate some 130 µg of proteolipid, having high affinity for 14C-MHM, it is possible to calculate that this tissue has about 65 mg of receptor proteolipid per kilogram of fresh tissue. The amount of proteolipid extracted from the electroplaques of Electrophorus is lower that that from Torpedo (i.e., about 33 mg per kilogram fresh tissue). The higher content in Torpedo may be related
to the richer innervation\textsuperscript{14} and to the fact that in this case the electroplaques are electrically inexcitable. In fact in \textit{Torpedo} bioelectrogenesis only results from from postsynaptic potentials.\textsuperscript{15}

In this work the "receptor" proteolipid was isolated from the total electric tissue. It remains to be demonstrated whether it is present in the membranes of the electroplaques. At present, work is being pursued in our laboratory to isolate purified membrane fractions, to study their binding properties, and to extract from them the receptor proteolipid.

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\textsuperscript{7} Changeux, J.-P., T. Podleski, and L. Wofsy, these \textit{PROCEEDINGS}, \textbf{58}, 2063 (1967).


\textsuperscript{9} We are grateful to Dr. Francisco Ruiz from Columbia University for kindly providing the frozen electric tissue of \textit{Electrophorus electricus}.


\textsuperscript{12} For literature on the general problem of the isolation of the cholinergic receptor, see Hasson Voloch, A., \textit{Nature}, \textbf{218}, 330 (1968).


\textsuperscript{14} Unpublished work from our laboratory (Vásquez, Barrantes, La Torre, and De Robertis) shows that the receptor proteolipid from brain is an elongated macromolecule about 15 Å in width which aggregates under the action of atropine sulfate.

\textsuperscript{15} Although we have not found quantitative references about the innervation of electroplaques in both species, by observing the published electronmicrographs it is evident that there is a more dense packing of nerve endings in the electroplaque of \textit{Torpedo}. See Sheridan, M. N., \textit{J. Cell Biol.}, \textbf{24}, 129 (1965); Bloom, F. E., and R. J. Barnnett, \textit{J. Cell Biol.}, \textbf{29}, 475 (1966).