BINDING OF CHOLINERGIC SUBSTANCES BY PROTEOLIPIDS FRACTIONATED AT −60° C FROM QUENCHED RAT CEREBRAL CORTEX

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1. Introduction

The stimulating papers of E. De Robertis and his associates [1] proposing proteolipids as possible 'specific cholinergic receptors' make it timely to reconsider the properties of proteolipids, by definition being proteins extractable by a mixture of chloroform–methanol (2:1 v/v). When solutions of proteolipids are dialyzed against more of this solvent, they lose appreciable quantities of their lipid components [2]. The question naturally arises as to what extent such dissociation occurs during extraction and treatments by the solvent.

This solvent for lipids is well known for its modification of protein structure, not infrequently to the extent of irreversible denaturation. Even milder reversible changes induced by the solvent may lead to properties of the proteolipids of uncertain relevance for physiological correlations.

On the other hand, though membrane enzymes such as Na^+, K^+-ATPase and acetylcholinesterase were completely denatured in a similar organic solvent at the usual temperatures, they retained their enzymatic activities at subzero temperatures [3]. To obtain preparations of proteolipids in better approximation to in vivo structures, extraction of the brain tissue, previously quenched at −196°C was carried out with the same solvent when the temperature of the tissue was raised to −60°C. At lower temperature, dissociation of lipid components would be less probable in both energy- and rate-determined processes.

We describe preparation at −60°C or proteolipid fractions that bind acetylcholine and cholinergic blocking agents. Since the proteolipids extracted at low temperature are presumably also extracted at higher temperature along with additional proteolipids, they constitute a fraction that on the average is less tightly bound to the membranes of origin than those previously studied. Fractions of successively higher binding energy can, of course, be extracted by stepwise increase in temperature. We overlook in this approximation the dissociation of lipid components, particularly with increase of temperature.

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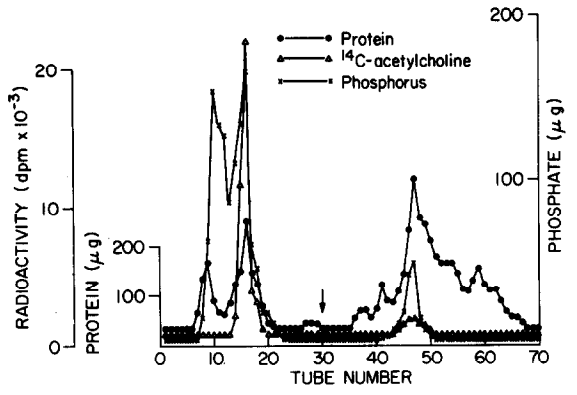

Fig. 1. Elution of total lipid extract incubated with [^{14}C]-acetylcholine (5 X 10^{-6} M) at −60°C. Further description under Materials and methods.

2. Materials and methods

For obtaining data of fig. 1, the cerebral cortex of Sprague-Dawley rats was quenched at −196°C in liquid nitrogen and pulverized at this temperature. The brain powder was warmed up only to −60°C. The following procedures were all carried out at or below −60°C except the column chromatography, at −60°C. Extraction of tissue to give the total lipid extract (TLE) was done with chloroform–methanol (2:1 v/v, 100 ml for 100 mg of brain powder). The extract was freed from residue by filtration. The organic solvent was completely removed under high vacuum by molecular distillation [4]. The dried material was dissolved with chloroform–methanol (2:1 v/v) to become the TLE solution for chromatography, after dilution with the same solvent to give 5 mg protein per 5 ml and was submitted for incubation with 2.5 X 10^{-8} moles [^{14}C] acetylcholine and then applied to a Sephadex LH-20 column (1.8 cm diam., 40 cm high). This was packed in chloroform and washed with 120 ml of the same solvent. Preparation and operation of the columns was done in a refrigerating cabinet (Model ULT657, Revco, Inc., West Columbia, South Carolina). Attached to the columns, also in the cabinet, was a sample collector (Model 272, Instrumentation Specialties Co., Lincoln, Nebraska), after removal of grease and application of slight heat at the bearing, thermally insulated from surroundings. Elution was carried out in pure chloroform followed by chloroform–methanol (4:1 v/v) as indicated by the arrow. Fractions were 3.5 ml per tube. Protein content in each tube was measured at 280 mμ with a Cary 14 spectrophotometer (Varian Associates, Palo Alto, California). Radioactive counting was done by a standard scintillation method. Total lipid phosphorus was determined by the method of Martin and Doty [5] after digestion of dried material with perchloric acid. All operations for data of fig. 2 were carried out at −60°C. Proteolipids were precipitated from the TLE solution by adding twice its volume of diethyl ether.

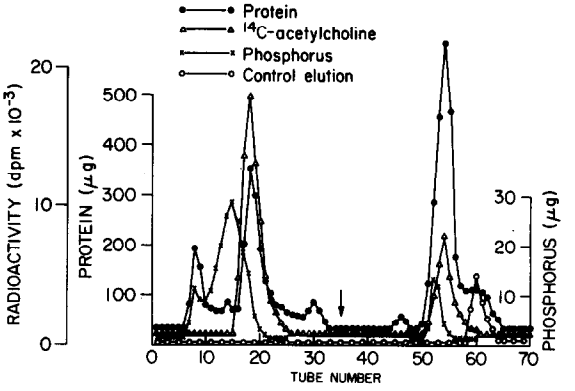

Fig. 2. Delayed application of proteolipids. Elution from LH-20 Sephadex column pre-loaded with [^{14}C] acetylcholine (5 X 10^{-6} M) at −60°C. Further description under Materials and methods.

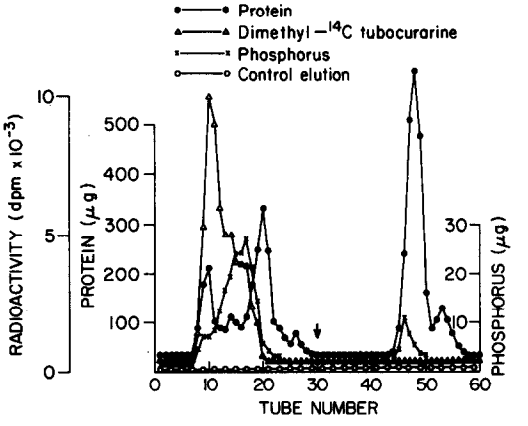

Fig. 3. Elution of proteolipids incubated with dimethyl-tubocurarine (2 X 10^{-6} M) at −60°C. Further descriptions under Materials and methods.

The precipitate was gathered by centrifugation and the organic solvent was removed under high vacuum. The proteolipids were dissolved with chloroform–methanol (2:1 v/v) and applied to the column which had been preloaded with 2.5×10^{-8} moles of [^{14}C] acetylcholine. Subsequent procedures were the same as for fig. 1. Proteolipids for data of fig. 3 were dissolved in chloroform–methanol (2:1 v/v) and incubated with 10^{-8} moles dimethyl-^{14}C tubocuranine and applied to Sephadex LH-20 columns at –60°C. Subsequent procedures were the same as for fig. 1.

3. Results and discussion

E. De Robertis with his associates found that after incubation with the proteolipids from cerebral cortex, cholinergic blocking agents were eluted from Sephadex LH-20 column in coincident peaks with some of the proteolipids. Similar coincidences occurred with acetylcholine and some of the proteolipids derived from the electric organ of electric fishes.

Confirmation that the coincidences arose from binding had emerged clearly from the work of Weber et al. [6] with aqueous and organic biphasic equilibria where proteolipids in the organic phase induced marked shift of a cholinergic drug to this phase. The drug was soluble only in the aqueous phase in the controls.

Fig. 1 represents the elution profile of total lipid extract (TLE) incubated with [^{14}C] acetylcholine at –60°C applied to the Sephadex LH-20 column. Clearly coincidence occurs in the peak of acetylcholine with the second peak of proteolipids eluted with chloroform and also with the main protein peak eluted with chloroform–methanol (4:1 v/v), the more polar solvent. The proteolipids from the tissue have been subjected to practically minimal treatment with solvent. In the application of the extract to the column, they are still in the presence of various components of the membrane with which the proteolipids may be in equilibrium. However, the extract had required moderate dilution to render good resolution in the profile.

Radioactive acetylcholine (2.5×10^{-8} moles in 0.5 ml of methanol) applied at –60°C with 5 ml of chloroform to a Sephadex LH-20 column were totally bound to Sephadex and remained uneluted by chloroform. If to such a bound column, after being washed with chloroform, a bolus of proteolipid dissolved in 5 ml of chloroform–methanol (2:1 v/v) was similarly applied, subsequent passage of chloroform gave a profile showing coincidence of [^{14}C] acetylcholine with the second peak of proteolipid (fig. 2). When 5 ml of chloroform–methanol (2:1 v/v) was substituted for proteolipid solution, the [^{14}C] acetylcholine still remained tightly bound to the column in the course of elution by chloroform. Virtually the same elution pattern of column chromatography was produced by a solution of proteolipids incubated with [^{14}C] acetylcholine for 1 hr at –60°C.

The binding of [^{14}C] acetylcholine to Sephadex LH-20 column was dependent on the polarity of the eluting solvent. Even in the absence of added proteolipids, small amounts of [^{14}C] acetylcholine were loosened from the column by the more polar solvent, chloroform–methanol (4:1 v/v). But in the presence of proteolipids, the elution peak of [^{14}C] acetylcholine was shifted forward showing higher activity and good coincidence with the main proteolipid peak eluted by this solvent mixture. Considerable variability in the shape and sometimes even in the number of peaks was found in the elution profile of proteolipids. Good reproducibility could be obtained when two Sephadex LH-20 columns were run side by side using the same LH-20 and proteolipid preparations. Despite variabilities, the specific coincidence of the peaks of radioactive acetylcholine and those of proteolipids showed excellent reproducibility.

The question arises whether cholinergic blockers are also bound to the proteolipids binding acetylcholine or are they bound to different proteolipid fractions. Dimethyl-^{14}C tubocurarine (10^{-8}–2×10^{-8} moles in 0.5 ml methanol) was incubated with a solution of proteolipids in chloroform–methanol (2:1 v/v, 5 ml) and applied to the column as in the case of acetylcholine. Dimethyl-^{14}C tubocurarine, in this amount, had been completely bound to the column in the absence of proteolipid and no radioactivity was detected in the eluate with chloroform followed by chloroform–methanol (4:1 v/v). But in the presence of proteolipids almost all the radioactive dimethyltubocurarine was recovered in the eluate with chloroform, showing excellent coincidence with the first peak of proteolipid (fig. 3). It is evident that the specificities in binding of [^{14}C] acetylcholine and of