PURIFICATION OF MEMBRANE FRAGMENTS DERIVED FROM THE NON EXCITABLE SURFACE OF THE EEL ELECTROPLAX

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Received 17 March 1970

1. Introduction

The monocellular electroplax, the elementary unit of the electric organ of various fishes, presents, in the Gymnotid: *Electrophorus electricus*, two different types of plasma membranes [1]. One, the innervated membrane, receives nerve terminals and responds to both chemical and electrical stimuli. The other, the non-innervated membrane, is not excitable but specialized in active transport. A purification procedure of membrane fragments derived from the innervated surface and based upon their particularly high content in acetyl-cholinesterase (AcChE) has been previously reported [2]. In this letter, we describe the preparation of another type of membrane fragments, which do not contain appreciable amounts of AcChE, but are extremely rich in another enzyme: the ouabain sensitive, Na^+ K^+ activated, adenosine triphosphatase (ATPase). Evidence is given that these fragments are derived from the non-innervated membrane of the electroplax.

2. Material and methods

Enzyme assays: The ouabain sensitive ATPase was assayed following exactly the procedure of Bonting, Simon and Hawkins [3]. The incubations were carried at 37° in samples of a total volume of 1 ml and the three reagent blanks and the two sets of inorganic phosphate standards suggested by Bonting et al. were included in each experiment.

AcChE was measured by the method of Ellman [4] in a medium containing: 5×10^{-4} M acetylthiocholine, 5×10^{-4} M sodium -5,5′-dithiobis-2-nitrobenzoate and 5×10^{-2} M sodium phosphate pH 7.0.

Chemicals: Acetylthiocholine chloride, sodium -5,5′-dithiobis-2-nitrobenzoate, were from Sigma Chemicals Co.; Ouabain was from Serlabo.

3. Results

Membrane fragments rich in ATPase were routinely purified by the following method:

10 g of fresh electric tissue, cut with scissors into fragments of about 1 cm, are suspended in 50 ml of 0.2 M sucrose in distilled water and homogenized in a Virtiss apparatus for 1 min 30 sec at 75% of its maximal speed. The homogenate (H) is first centrifuged at 5000 *g* (20 min in a refrigerated Servall centrifuge rotor SS34 at 6,500 rpm). This slow centrifugation eliminates unbroken cell fragments, nuclei and most of the connective tissue. The supernatant S is then centrifuged at high speed (105,000 *g*) in a SW 25 rotor of a Beckman preparative centrifuge. The best separations were obtained with discontinuous gradients established immediately before centrifugation in the following manner. From the bottom to the top are carefully layered, in a 25 ml lusteroid tube: 8 ml of 1.4 M sucrose, 12 ml of 1.0 M sucrose and 5 ml of S. The gradients are centrifuged at 4°

* We thank Dr. L. Benedetti for the electron micrograph published in this letter. Research was aided by grants from the "Collège de France", the "Centre National de la Recherche Scientifique", the "Délégation Générale à la Recherche Scientifique et Technique", the "National Institutes of Health" and the "Commissariat à l'Energie Atomique".

Table 1
Separation of membrane-bound ATPase.

	Total ATPase activity (μmoles/hr at 37°)	% recovery	Specific ATPase activity (μmoles/hr/mg protein)	Purification from S
H	2380		30	-
S	900	100	24	-
Heavy Peak	412	45.7	360	X 15.2
Light Peak	258	28.7	51.5	X 2.1
H + L	670	74.5		

for 3 hr at 25,000 rpm and the fractions collected immediately afterwards by perforation of the bottom of the tube with a 1 mm thick injection needle. Fig. 1 shows the distribution of ATPase, AcChE and proteins in the tube after centrifugation.

It is clear that membrane bound ATPase separates readily from both membrane-bound AcChE and soluble proteins. The high efficiency of separation comes from the remarkable difference of density of both classes of membrane fragments: using continuous sucrose density gradients we found that the *apparent* equilibration occurs, in these conditions of centrifugation, around 1.15 ± 0.10 sucrose (d = 1.142) for the ATPase rich fragments and around only 0.65 ± 0.10 M sucrose (d = 1.085) for the AcChE rich ones. The recovery in the experiment of fig. 1 was 77.7% and 72.5% of the total activity recovered was in the high density peak. The specific activity of ATPase in the peak was 500 μmoles of ATP hydrolysed per hour and per mg of protein at 37°, which corresponds to a 20.8

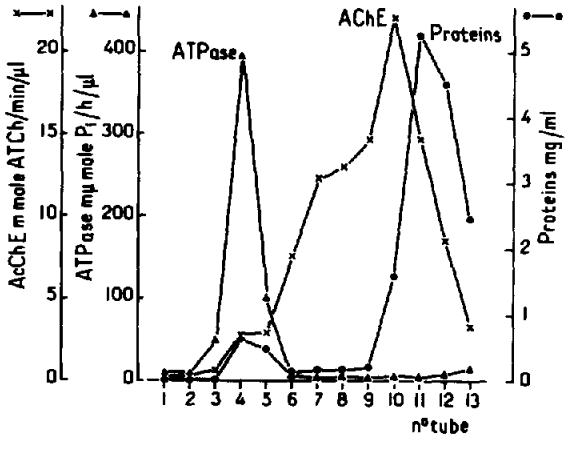

Fig. 1. Distribution of ATPase, AcChE and proteins after centrifugation.

fold purification, in one step, from S. The preparation then obtained was about 3 times purer than that of Post and Sen (1967). The ATPase activity in the peak was almost completely (98%) inhibited by 10^{-4} M ouabain.

If homogenization is done at higher speeds (85% of the maximal speed on the dial of the Virtiss apparatus) but for the same length of time, a second peak of ATPase appears at the interface between 1.0 and 0.2 M sucrose. 10^{-4} M Ouabain inhibits ATPase from both peaks (100% inhibition in the heavy peak, 90% in the light one) (table 1). The most plausible interpretation for the appearance of this second peak is that the conditions of vigorous homogenization lead either to a partial solubilization of the enzyme or to a fragmentation of the membrane into smaller pieces which show an *apparent* density, lighter than that of the larger fragments.

As indicated earlier, ATPase activity in the membrane fractions is sensitive to ouabain and activated by Na^+ and K^+ (table 2); it thus corresponds to the enzyme which has been shown to be directly involved in the active transport of Na^+ and K^+ ions [5]. In the top fraction of the soluble proteins a different ATPase which is not activated by Na^+ and K^+ is present but in a much smaller quantity (7.5% of the total activity).

Fig. 2 shows an electron micrograph taken by Dr. L. Benedetti of the membrane fragments rich in ATPase in the high density peak. These fragments make closed vesicles of a much larger size (0.1 - 1 μm) than the AcChE rich fragments (in the present conditions of homogenization). In addition their ultrastructure strikingly differs from that of the AcChE rich ones: in particular they do not show a structure with "repeating subunits" like the innervated membrane but, instead, a typical "unit" membrane (L. Benedetti and J.P. Changeux, unpublished results).

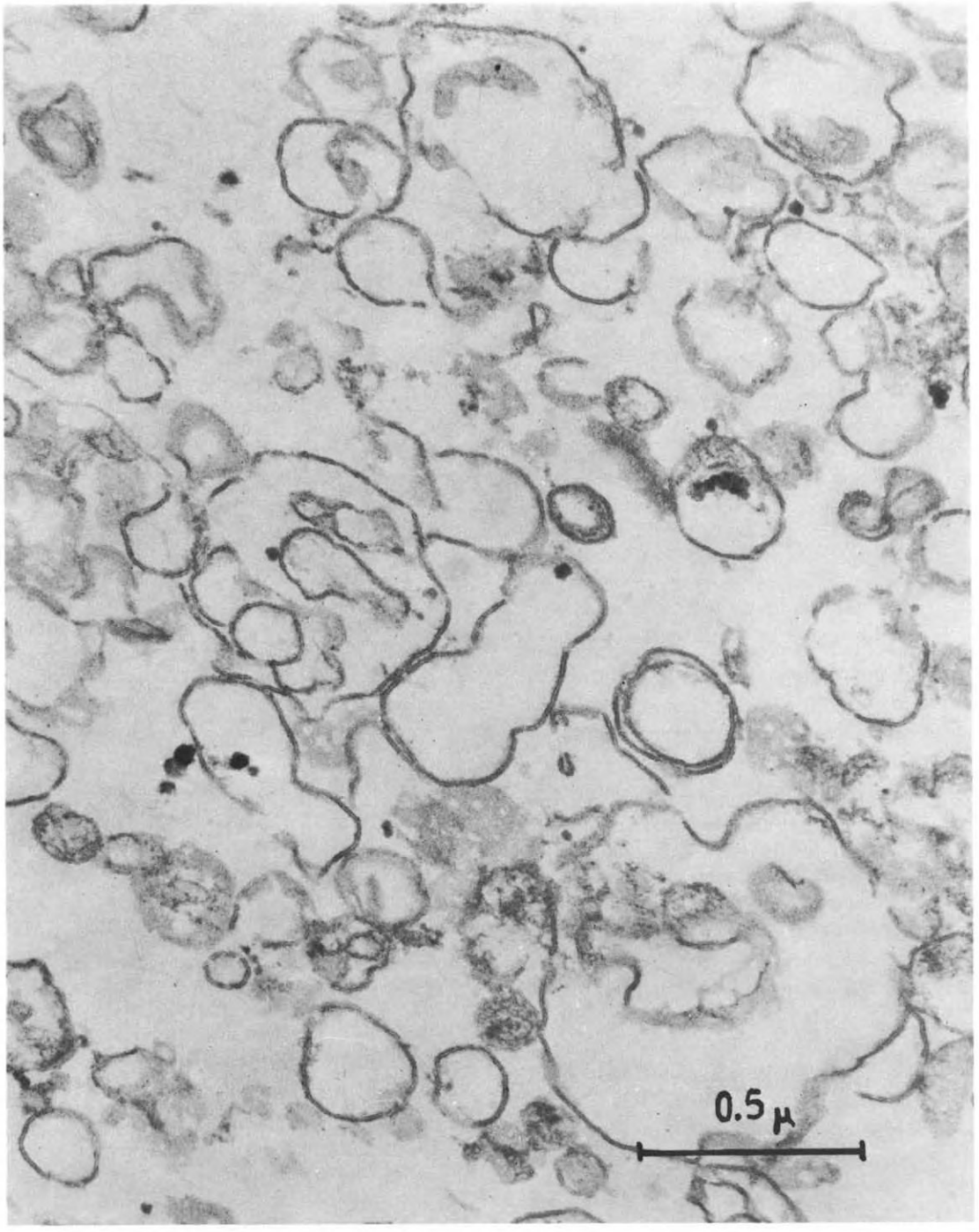

Fig. 2. Electron micrograph of membrane fragments rich in ATPase. Taken by L.Benedetti.

Table 2
Properties of membrane-bound ATPase.

Medium:	ATP	2	2	2	2	2	2
	Mg^{2+}	1	1	1	1	1	1
	K^+	5	5	-	-	5	5
	Na^+	58	58	14	14	58	58
	CN^-	10	10	10	10	10	10
	EDTA	0.1	0.1	0.1	0.1	0.1	0.1
	Tris	92	91	146	144	91	90
	Ouabain	-	0.1	-	0.1	-	0.1
	Ca^{2+}	-	-	-	-	1	1
	pH	7.5	7.5	7.5	7.5	7.5	7.5
Inhibition (%)		0	98	97	99	76.6	98

Assay media are the same as in Bonting et al. [3]. Concentrations are mM.

4. Discussion

From homogenates of electric tissue, membrane fragments rich in ATPase are easily separated from fragments rich in AcChE. This particularly favorable situation, which has not up to now been found with other tissues, brain in particular [6], is due to the unusual differentiation of the cytoplasmic membrane of the electroplax into two different surfaces. As previously discussed [2,7] the AcChE rich fragments originate from the innervated, excitable, surface of the cell. Various physiological observations, in particular those of Schoffeniels [8] and the more recent ones, although less convincing, of Karlin [9], suggest that the non-innervated membrane is specialized in active transport Na^+K^+ ATPase. Since most of the ouabain sensitive of Na^+ and K^+ and thus is rich in ouabain sensitive ATPase from a crude homogenate of electric tissue is recovered in the high density fragments, it is reasonable to say that these fragments originate from the non-innervated membrane of the electroplax. The present method of separation might be particularly valuable as a first step for a purification of Na^+K^+ ATPase. In addition, the purified vesicles constitute a material well designed for the study of active transport in an acellular system. Finally, these non-excitable fragments offer an excellent control, they constitute some kind of "mutant" membrane, for the specification of the physicochemical properties characteristic of membrane excitation [10].

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