Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 216-222

ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANES FROM RABBIT SKELETAL MUSCLE

E. ROCK [a], L. LEFAUCHEUR [b] and J. CHEVALLIER [a]

[a] Institut de Biochimie Cellulaire et Neurochimie, 1 rue Camille Saint-Saens, 33077 Bordeaux cedex FRANCE

[b] ENSA-INRA, 9 place Pierre Viala, 34060 Montpellier cedex FRANCE

Received July 17, 1984

An improved procedure was developed for the isolation of skeletal muscle plasma membranes. This method includes a DNAse treatment of the homogenate prior to the isolation of membranes by differential and sucrose gradient centrifugation techniques. We obtained two light fractions which were highly enriched in many biochemical and chemical plasma membrane markers. These fractions were shown to be mostly inside-out vesicles containing a Ca^{2+}-ATPase activity. These results suggested that this enzyme could participate in the extrusion of calcium ions from the muscle cells.

Introduction. The understanding of the role played by the skeletal muscle surface membranes (i.e. T-tubule and sarcolemma) has been restricted by the difficulty of preparing pure fractions of these membranes.

Several purification schemes for sarcolemma and/or t-tubule membranes have been published (1-5). These two membranes have been distinguished on the basis of different amounts of specific markers (for a review, see in 2 and 6).

In this communication, we present a technique for the isolation of skeletal muscle surface membrane fractions by a sucrose gradient centrifugation technique. Two light fractions were shown to originate from the surface membranes by using several biochemical and chemical markers. Measurements of specific Mg^{2+}-ATPase activities showed that these membranes were implicated in the control of Na^{+} and K^{+} ions and also of Ca^{++} ions movements.

This last result combined with the marker study will be discussed with respect to the actual source of these membrane fractions.

0006-291X/84 $1.50

Materials and Methods

Determination of enzymatic activities. The 5' nucleotidase activity was measured as in (7); Ca^{2+}-ATPase was determined in 10 mM Hepes-Tris, pH 7.3, 100 mM KCl, 2 mM $MgCl_2$, 2 mM ATP 10 µg/ml of protein in the presence of either 5.10^{-6} M $CaCl_2$ or 1 mM EGTA at 37°C; ($Na^+ + K^+$)-ATPase was carried out in 10 mM Hepes-Tris pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 4 mM ATP) in the presence of either 120 mM NaCl plus 40 mM KCl or 1 mM ouabain. For these activities the released inorganic phosphate was measured as in (8).The acetylcholinesterase activity was determined according to Ellman (9).

Binding assays. Insulin receptors were evaluated using 350 µg of membrane proteins and 2.23 10^{-11} M [^{125}I] insulin prepared as in (10) (specific radioactivity 900 Ci/mmol). Non-specific binding was measured in the presence of 5.5 10^{-7} M of unlabelled insulin (Novo). Ouabain binding was performed after 15 min incubation at 37°C with 60 µg of protein in 1 ml of 10 mM Hepes-Tris buffer pH 7.4, 100 mM NaCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 µM [^{3}H]ouabain (Amersham : specific radioactivity 37 Ci/mmol) with or without 4 mM ATP.

Chemical analysis. Lipid phosphorus was determined according to Napias (11). Cholesterol was extracted in $CHCl_3$/EtOH 2:1 and measured by the cholesterol oxidase-catalase procedure (Boehringer n° 139050). Sialic acid contents was measured according to Warren (12). Proteins were determined by the Lowry method (13) or by the fluorescamine reaction (14) using bovine serum albumin as the standard. SDS polyacrylamide gel electrophoresis was performed using the method of Laemmli (15).

Vesicle sidedness. Acetylcholinesterase activity and ouabain binding assays for the determination of the vesicle sidedness were performed in the presence of increasing amounts of deoxycholate as already described (2).

Purification of surface membranes. The entire procedure was done at 4°C. Rabbit white muscles were ground in a meat grinder and mixed with 10 volumes of buffer I (20 mM Tris-HCl, pH 7.8, 2 mM EGTA, 300 mM sucrose, 1 mM DTT and 300 mM NaCl). 30 g of minced meat were homogenized in a Waring blendor twice for 15 sec at half maximal speed. The crude homogenate (H) was centrifuged for 50 min at 39,000g. The pellet was washed with 5 volumes of buffer I and centrifuged 50 min at 39,000g. The pellet was then resuspended in 3 volumes of buffer II (20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 300 mM sucrose, 1 mM DTT) and treated by 5 µg ml^{-1} of DNAse I (Sigma) for 30 min at 20°C. The reaction was stopped by addition of 2 mM EDTA. This solution was homogenized twice for 10 sec in a Polytron homogenizer PT 20S at 21, 000 rpm and centrifuged for 20 min at 13,000g. The resultant pellet was re-homogenized under the same conditions. The pooled supernatants were collected and centrifuged for 90 min at 105,000g. The pellet was resuspended in 36 ml of 10 mM Hepes pH 7.4, 300 mM sucrose, 600 mM KCl and maintained on ice for 1 hour. The microsomal fraction (M) was obtained after a 90 min centrifugation at 105,000g. After resuspension (16-11 mg protein ml^{-1}) in 11 % (w/v) sucrose, 3.2 ml of this fraction were layered onto a sucrose density gradient consisting of 20 % (w/v) sucrose (3 ml), 30 % sucrose (4,5 ml), 35 % sucrose (1,5 ml) and 45 % sucrose (3 ml) buffered with 10 mM Hepes pH 7.4. The gradients were centrifuged in a MSE SW 6x16.5 rotor at 105 000g for 12-14 hours. Four particle fractions banding at the 11:26 (F1), 26/30 (F2), 30/35 (F3), 35-35 (F4) interfaces were collected, then diluted in 10 mM Hepes buffer pH 7.4 to bring them at 300 mM sucrose, centrifuged at 105,000g for 60 minutes and finally resuspended in 0.5 to 0.9 ml of 10 mM Hepes pH 7.4, 300 mM sucrose for further analysis.

Results. Microsomal fractions (M) obtained from muscle homogenate (H) by means of the procedure described above allowed us to isolate four membrane fractions. Their characteristics are presented in Table 1.

Two light fractions F1 and F2 show considerable enrichment in plasma membrane enzymatic and binding activities as compared to the homogenate. In addition, ACHE and ouabain binding specific activities can be enhanced by addition of detergent (Deoxycholate) as shown in Table 2.

Their high cholesterol to phospholipid ratio and their sialic acid content (Table 1) confirmed their surface origin. They are very different from the fractions F3 and F4 which contain more than 94 % of the total microsomal proteins loaded onto the gradient.

The presence of the mitochondrial membranes in the light fractions is tested by measuring the oligomycin and the aurovertin sensitivity of Mg^{2+}-ATPase. Such measurements indicate a tiny contamination (less than 8 %; not shown).

We have determined the sidedness of these membranes by a slight modification of the method similar to that described in (2). Such measurements (Table 2), similar for F1 and F2, indicate that about 75 % of the membranes are sealed. This sealed material is actually composed of about 55 % and 20 % of inside-out and right side-out

TABLE 1 CHARACTERISTICS OF THE FRACTIONS OBTAINED AS DESCRIBED IN MATERIALS AND METHODS

Fractions	Yield mg/g	ACHE nmol/min/mg	5'-nucleotidase nmol Pi/min/mg	Ouabain binding pmol/mg	Insulin binding fmol/mg	Cholesterol/PL molar ratio	Sialic acid µg/mg
H	155	14	2.9	0.07	n.d.	-	-
M	3.1	57	14	0.39	0.29	0.06	23
F1	0.04	136	186	4.84	2.42	0.30	101
F2	0.06	143	92	4.52	3.71 (a)	0.20	58
F3	1.2	76	15	0.57	0.89 (a)	0.04	14
F4	0.6	52	8	0.55	0.9	0.04	22

n.d. = not detectable. Average of 3 determinations of 2 to 6 different preparations.
(a) Bmax is 62.2 and 16.7 fmol/mg for F2 and F3 respectively.

TABLE 2 SIDEDNESS OF PURIFIED PLASMA MEMBRANE VESICLES

		F1	F2
Acetylcholinesterase*	- DOC$^{\circ}$	135.7 (5)	142.6 (6)
	+ DOC$^{\circ}$	310.5 (3)	315.1 (5)
	% (RO + L)	43.7	45.2
	% (IO) $\neq$	56.3	54.7
Ouabain binding■	- DOC	4.84 (7)	4.52 (6)
	+ DOC	20.4 (2)	16.1 (5)
	% (L)	23.7	28.1
	% (RO + IO)x	76.3	71.9

(*) nmol of acetylthiocholine hydrolyzed min^{-1} mg $protein^{-1}$; (■) pmol (H^3) ouabain bound mg $protein^{-1}$; ($\neq$) the increased acetylcholinesterase activity deducted from 100 % gives the percentage of (IO) vesicles; (x) the increased ouabain binding deducted from 100 % gives the percentage of (RO + IO) vesicles; (o) 0.45 mg ml^{-1}.
RO : right-side out vesicles; IO : inside-out vesicles; L : leaky material; DOC : sodium deoxycholate.

vesicles respectively. The leaky material is estimated to be about 25 %.

In order to assess the functional state of the fractions, we measured the $(Na^+ + K^+)$-dependent Mg^{2+}-ATPase which is known to be associated with the surface membranes and the Ca^{2+}-dependent Mg^{2+}-ATPase (Table 3).

The $(Na^+ + K^+)$ATPase activity is found in both light fractions, even though F2 is less sensitive to cations, the specific

TABLE 3 ATPase ACTIVITY IN SUCROSE GRADIENT FRACTIONS.

Fractions	$(Na^+ + K^+)$ ATPase		(Ca^{2+})-ATPase	
	µmol Pi/mg/h		µmol Pi/mg/h	
	Basal ATPase	$(Na^+ + K^+)$activity	Basal ATPase	(Ca^{2+})-activity
F1	45.6	52.2	0.91	1.02
F2	50.4	40.2	0.71	1.79
F3 + 4	-	-	0.26	3.95

Basal ATPase represents the activity measured in the presence of 1 mM ouabain or 1 mM EGTA for $(Na^+ + K^+)$ ATPase and Ca^{2+}-ATPase respectively. The ion dependent activity represents the activity measured in the presence of specific ions minus basal ATPase. The concentrations of the cations are 120 mM KCl, 40 mM NACl and 5.10^{-6} M $CaCl_2$.

activities are higher than those already published (1, 3, 16, 17). F1,F2 and the combined fractions F3+F4 show a high affinity Ca^{2+}-dependent ATPase activity. The activity of F3+F4 is clearly distinguishable from that of the fractions F1 and F2 in that the former is much more activated by calcium ions than the latters. This enzymic activity, the cholesterol to phospholipid ratio (Table 1) and the pattern in SDS electrophoresis (not shown) of F3+F4 are identical to that described for SR membrane fractions (18).

Discussion

Our method of purification is based on extraction of microsomes from homogenate which was treated with 0.15 M NaCl to solubilize the actomyosin complex, 1 mM DTT to protect the -SH groups of the proteins and 5 µg/ml of DNAse to decrease the medium viscosity. After one hour incubation with 0.6 M KCl to solubilize the remaining contractile proteins, the microsomes were fractionated through a discontinuous sucrose gradient.

Using this procedure, we demonstrated clearly that two light fractions F1 and F2 were composed of surface membranes while SR membrane fractions, F3 and F4, banded in the dense regions of the gradient (See Tables 1 and 3).

The prominent result of this study is the presence of an ATPase activity having high affinity for calcium ions in F1 and F2 which showed at the same time high (NA+K)ATPase activity (Table 3). Ca^{2+}-ATPase activity has been found in T-tubule (1, 19) and this activity was supposed to be absent in the sarcolemma (2) or present with a small specific activity (17, 20).

In this study, it is difficult to assert that F1 and/or F2 were composed of T-tubule or SL membranes: (i) because of the high (Na-K)ATPase, 5'-nucleotidase activities and sialic acid content of F1 and insulin binding, ACHE activity of F2, since all these markers

have been considered as characteristics of sarcolemmal membrane (2, 5, 7, 16); (ii) we found a Na-F dependent adenylate cyclase activity of 1.46 and 0.95 nmol/min/mg for F2 and F1 respectively, and no basal ATPase activity (Table 3), both activities which have been considered to be T-tubule markers (Table 5 in ref. 6).

In conclusion, the highest specific activities for all positive surface membrane markers in fractions F1 and F2 strongly suggested that these membrane markers were originating from the cell surface. Although some markers might indicate that F1 is mostly composed of sarcolemma, one cannot exclude the presence of T-tubule in this fraction. The same reasonning is valuable for F2. Anyway, the high affinity Ca^{2+}-ATPase activity observed in F1 and F2 cannot be attributed only to SR contaminating membranes. Thus, this activity is intrinsically associated with muscle cell surface membrane. Its presence in our fractions which were shown to be mostly oriented inside-out (Table 2) suggest that this enzyme could participate in the extrusion of calcium ions out of the cell as it was demonstrated in erythrocyte (21), cardiac muscle (22) or liver cell (23) plasma membranes.

Acknowledgements We wish to thank Dr. P. Vigneron for helpful discussions, Dr. C. Napias for his critical reading of the manuscript and T. Volker for carrying out the adenylate cyclase assay.

References

1. Rosemblatt, M., Hidalgo, C., Vergara, C. and Ikemoto, N. (1981) J. Biol. Chem. 256, 8140-8148.
2. Seiler, S. and Fleischer, S. (1982) J. Biol. Chem. 257, 13862-13871.
3. Kidwai, A.M., Radcliffe, A., Lee, Y.E. and Daniel, E.E. (1973) Biochim. Biophys. Acta 298, 593-607.
4. Schapira, G., Dubocz, J., Piau, J.P. and Delain, E. (1974) Biochim. Biophys. Acta 245, 348-358.
5. Desnuelle, C., Lombet, A., Liot, D., Maroux, S. and Serratrice, G. (1983) Biochem. Biophys. Res. Commun. 112, 521-527.
6. Mitchell, R.D., Volpe, P., Palade, P. and Fleischer, S. (1983) J. Biol. Chem. 258, 9867-9877.
7. Wallis, I., Koenig, E. and Rose, S. (1980) Biochim. Biophys. Acta 559, 505-517.

8. Ottolenghi, P. (1975) Biochem. J. 151, 61-66.
9. Ellman, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88-95.
10. Posner, B.I. (1974) Diabets 23, 209-217.
11. Napias, C. (1975) Thèse de Doctorat d'Etat, Bordeaux.
12. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975.
13. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.S. (1951) J. Biol. Chem. 193, 265-275
14. Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220.
15. Laemmli, U.K. (1970) Nature 227, 680-685;
16. Smith, P.B. and Appel, S.H. (1977) Biochim. Biophys. Acta 466, 109-122.
17. Barchi, R.L., Bonilla, E. and Wong, M. (1977) Proc. Natl. Acad. Sci. USA 74, 34-38.
18. Meissner, G., Conner, G.E. and Fleischer, S. (1973) Biochim. Biophys. Acta 298, 246-254.
19. Lau, V.H., Caswell, A.H. and Brunschwig, J.P. (1977) J. Biol. Chem. 252, 5565-5574.
20. De Boland, R.R., Gallego, S. and Boland, R. (1983) Biochim. Biophys. Acta 733, 264-273.
21. Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955-9958.
22. Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263-3270.
23. Lotersztajn, S., Hanoune, J. and Pecker, F. (1981) J. Biol. Chem. 256, 11209-11215.