ISOLATION OF PLASMA MEMBRANES FROM CHICK EMBRYO CHORIOALLANTOIC MEMBRANES AND IDENTIFICATION OF MAIN NUCLEOLYTIC ACTIVITIES

Svetlana PRISTAŠOVÁ

Institute of Virology,
Slovak Academy of Sciences, 809 39 Bratislava

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The method of isolating plasma membranes from chick embryo chorioallantoic membranes is described along with some morphological and chemical properties of these membrane preparations. Besides 5'-nucleotidase, alkaline phosphodiesterase, alkaline phosphatase, ribonuclease-endonuclease and 2',3'-cyclic nucleotide 3'-phosphohydrolase activities, the plasma membranes possesse a high activity of 2',3'-cyclic nucleotide 2'-phosphohydrolase. They exert negligible activities for glucose 6-phosphatase and succinate dehydrogenase.

Since the isolation of plasma membranes from the nuclear fraction of the liver by Neville[1] and from the microsomal fraction of Ehrlich ascites tumour cells[2], numerous studies on the characteristics of plasma membranes isolated from various tissues were published[3]. A simple method for the isolation of plasma membranes from chick embryo chorioallantoic membranes along with some properties of the enzymes associated with the plasma membrane preparations are described in the present paper.

EXPERIMENTAL

Isolation of Plasma Membranes

Solution for isolation of plasma membranes A) 1 mM-Tris-HCl buffer, pH 7·4, containing 130 mM-NaCl, 5 mM-KCl and 1 mM-$CaCl_2$, and B) 1 mM-Tris-HCl buffer, pH 8·5, containing 1 mM-$MgCl_2$ were used. Plasma membranes were isolated from 10—11 days old chick embryo chorioallantoic membranes (CAM) by a slight modification of the method of Križanová and Čiampor[4]. CAM were rinsed several times with cold 0·14M-NaCl, minced and allowed to settle in an ice bath for 60 min. They were then suspended in solution A (45—50 minced CAM in 300 ml), disintegrated in a glass homogenizer (80—100 strokes of the pestle) and centrifuged at 2500 to 3000 rpm for 30 min. The supernatant was centrifuged for 20 min at 50000*g*. The pellet was resuspended in solution A, layered on the top of a 35% (w/v) surcrose solution in solution B and sedimented for 80 min at 27000*g*. The interface was suspended in solution B and centrifuged for 20 min at 50000*g*. The pellet obtained was resuspended in a small volume of solution B and centrifuged twice on a discontinuous sucrose gradient for 90 min at 24000 rpm (Spinco L2 50B

centrifuge, SW 25·1 rotor). The gradient was prepared by overlaying 5, 20 and 10 ml of 45%, 35% and 25% sucrose (w/v) in solution B, respectively. The plasma membranes were collected from the surface of the 35% sucrose layer. The plasma membranes after the second centrifugation were resuspended in solution A and centrifuged for 20 min at 50000*g*. The pellet was suspended in solution A. The final membrane preparations, containing about 2 mg of protein per ml, were stored at —20°C. They were thawed only once.

Enzyme Assays

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase) was determined in a reaction mixture which contained 0·1 ml of adenosine 5′-monophosphate (1 mg/ml), 0·3 ml of $3 \cdot 3 \cdot 10^{-2}$M-Tris-HCl buffer, pH 7·5, 0·05 ml of 0·2M-$MgCl_2$, and 0·05 ml of membrane proteins (2 mg protein/ml). The mixture was incubated for 30 or 60 min at 37°C. Mg^{2+}-ATPase was determined according to Emmelot and Boss[5]. The reaction mixture contained 0·2 mg of membrane proteins.

Alkaline phosphatase (β-glycerophosphatase) was assayed by the procedure of Hinton and coworkers[6] with sodium β-glycerophosphate as the substrate. The reaction was carried out at pH 8·5 in the presence of 0·2 mg of membrane proteins. Glucose 6-phosphatase was assayed also according to Hinton and coworkers[6]. The activities of these four enzymes were expressed in terms of μmol . l^{-1} P_i released per 1 mg of protein during 60 min of incubation.

Succine dehydrogenase was assayed as described[6]. Ribonuclease activity was followed by the formation of acid-soluble products according to Rosenbergová and Pristašová[7]. 50—60 μg of Ehrlich ascites tumour cell RNA and 0·2 mg of membrane proteins were used in the reaction mixture.

Alkaline phosphodiesterase (phosphodiesterase I) was determined in a total volume of 1·1 ml containing 0·2 ml of membrane protein solution (2 mg/ml), 0·3 ml of 0·1M-Tris-HCl buffer, pH 8·5, 0·1 ml of 0·1M-$MgCl_2$, and 0·5 ml of 10^{-3}M bis-*p*-nitrophenylphosphate (sodium salt). After incubation for 60 min at 37°C the reaction was stopped by the addition of 1·1 ml of 0·25M-NaOH. Precipitated proteins were centrifuged and the optical density of the supernatant was read at 405 nm.

Decyclizing 2′,3′-phosphodiesterases were determined in a total volume of 0·2 ml containing 0·1 ml of guanosine 2′,3′-cyclic nucleoside monophosphate (1·5 mg/ml) and 0·1 ml of membrane protein solution (2 mg/ml). The reaction was carried out in the presence of $5 \cdot 10^{-2}$M-Tris-HCl buffer, pH 7·2. After incubation for 120 min at 30 or 60°C the reaction was stopped by heating in a boiling water bath for 5 min. Proteins were removed by extraction with 3 volumes of chloroform and centrifugation (4000 rpm 20 min). The reaction products were separated by descending chromatography in a solvent system 1-butanol–ethanol–water (312 : 198 : 90) on paper Whatman No 1. For the determination of nucleoside 2′- and 3′-monophosphates the corresponding spots were eluted with water and further separated chromatographically in a solvent system composed of ammonium sulphate saturated with water: 1M-ammonium acetate: isopropanol (79 : 19 : 2) on paper Whatman No 1. The spots of digestion products were cut out, eluted with 3 ml of 0·1M-HCl, and their absorbance was measured at 256 nm.

Chemical Assays

The released anorganic phosphorus (P_i) was determined by the Fiske–Subbarow method[8]. Proteins were assayed according to Lowry and coworkers[9] with bovine serum albumin as the standard. RNA was determined by the orcinol method[10]. Lipids and cholesterol were determined as described by Križanová and Čiampor[4].

RESULTS AND DISCUSSION

The plasma membranes from chick embryo chorioallantoic membranes sedimented in a discontinuous sucrose gradient (25%–45%) in two sharp zones. The light fraction of plasma membranes remained in the upper part of 25% sucrose. The heavy fraction was concentrated on the 35% sucrose layer. The ratio of light to heavy plasma membrane fractions was about 1 : 2 and was dependent on the age of chick embryos. The yield of the heavy fraction as measured by protein concentration decreased with increasing age of the embryos.

Electron microscope examination of the final plasma membranes demonstrated clear flat membranes, free of any particulate or granular material. The light fraction consisted of small membrane fragments; the heavy one was composed of large oval membrane profiles. The small plasma membrane fragments may very likely have originated from large membranes disrupted during the homogenization and purification procedures.

The yield of plasma membranes obtained by this procedure was around 25–30 mg of total proteins from 100 chick embryo chorioallantoic membranes routinely used. Of the total protein present in the cellular homogenate 2·1–2·6% were recovered in the final plasma membrane preparations. The plasma membranes contained RNA (27–30 μg/mg protein), the amount of cholesterol was about 240 μg/mg protein and that of phospholipids 810 μg/mg protein. The molar ratio of cholesterol to phospholipids in our membrane preparations was 0·6–0·63.

Although the enzyme activities are not always sufficient to resolve the question of purity of plasma membrane preparations, an increase in the specific activity of some enzymes over the cell homogenate is considered as a criterion of purity of plasma membranes. Some enzyme activities of CAM plasma membranes are compared with those of CAM cell homogenate in Table I.

It can be seen that a 4·0–4·5-fold increase in the specific activity of 5'-nucleotidase over the CAM cell homogenate was obtained. The enzyme was frequently used as a positive plasma membrane marker enzyme[11–14]. The specific activity of ribonuclease increased 1·8–2·5-fold over the CAM cell homogenate. Also the specific activity of alkaline phosphatase was increased about 17-fold over that of the cell homogenate. A somewhat higher increase (22-fold) was observed in plasma membranes from chick embryo cells[4]. It was suggested that a marked increase in alkaline phosphatase activity may be used as one of the criteria of plasma membrane purity. However, plasma membranes isolated from other animal cells show either a lower (2-fold in plasma membranes from rat heart[15]) or no increase of this enzyme (plasma membranes from guinea pig pancreas[16]).

Alkaline phosphodiesterase (phosphodiesterase I) was assayed with bis-*p*-nitrophenylphosphate as the substrate at pH 8·5. Its specific activity was about 6-fold over that of the CAM cell homogenate. The enzyme has been studied in most detail

on rat liver plasma membranes and the relative specific activity varied considerably[17,18]: Nevertheles, phosphodiesterase I, along with 5'-nucleotidase, is generally used as a marker enzyme for the rat liver plasma membranes[19].

In CAM plasma membranes preparations two decyclizing phosphodiesterase activities were determined. 2',3'-Cyclic nucleotide 3-phosphohydrolase (producing 2-nucleotides) and 2',3'-cyclic nucleotide 2'-phosphohydrolase (producing 3'-nucleotides). The relative specific activity of the 2',3'-cyclic nucleotide 3'-phosphohydrolase was 1·6–2·1. A similar phosphodiesterase was determined in plasma membranes prepared from several animal cells[20–22]. Decyclizing phosphodiesterase hydrolysing 2',3'-cyclic nucleotides to the corresponding 3'-nucleotides were found in some bacteria[23] and in higher plants[24,25]. However, among the animals, similar enzyme was detected only in the venom of *Crotalus adamanteus* and in the calf intestinal mucosa[24]. As concerns 2',3'-cyclic nucleotide 2'-phosphohydrolase we are not aware of any report on its presence in plasma membrane preparations of animal cells.

The CAM plasma membranes possess high activity of 2',3'-cyclic nucleotide 2'-phosphohydrolase. In the present experiments the specific activity of CAM

TABLE I

Activities of Some Enzymes Associated with Plasma Membranes from Chick Embryo Chorioallantoic Membranes

Enzyme mg protein/h	Specific activity		Relative specific activity
	plasma membranes	homogenate	
5'-Nucleotidase, μmol . l^{-1} P_i	1·50	0·35	4·2
Phosphodiesterase (bis-*p*-nitrophenylphosphatase), A_{405}	15·70	2·65	6·0
Alkaline phosphatase (β-glycerophosphatase), μmol . l^{-1} P_i	1·40	0·08	17·5
Ribonuclease, A_{260}	6·20	2·50	2·5
2',3'-Cyclic nucleotide 3'-phosphohydrolase, A_{256}	0·90	0·64	1·4
2',3'-Cyclic nucleotide 2'-phosphohydrolase, A_{256}	1·39	0·05	26·2
Mg^{2+}-ATPase, μmol . l^{-1} P_i	8·10	2·70	3·0
Glucose 6-phosphatase, μmol . l^{-1} P_i	0·015	0·17	0·05
Succinate dehydrogenase, μmol . l^{-1}/mg	0·02	0·31	0·05

plasma membrane was increased over the cell homogenate about 20-fold. The high relative specific activity suggests that the enzyme occures mainly in plasma membranes. The CAM plasma membrane preparations possessed negligible activities of Mg^{+2}-ATPase, glucose 6-phosphatase (an enzyme generally accepted as a microsomal marker) and succinate dehydrogenase (the marker enzyme for mitochondria).

The described method for the isolation of CAM plasma membranes is simple and gives reproducible results. CAM plasma membranes possess enzymatic activities found in plasma membranes of other animal cells. Moreover they showed a high 2′ 3′-cyclic nucleotide 2′-phosphohydrolase activity which has so far been reported mainly for plant and bacterial cells.

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REFERENCES

1. Neville D. M., jr: J. Biophys. Biochem. Cytol. *8*, 413 (1960).
2. Wallach D. F. H., Kamat V. B.: Methods Enzymol. *8*, 164 (1966).
3. De Pierre J. W., Karnovsky M. L.: J. Cell. Biol. *56*, 275 (1973).
4. Križanová O., Čiampor F.: Acta Virol. *15*, 19 (1971).
5. Emmelot P., Bos C. J.: Biochim. Biophys. Acta *120*, 369 (1966).
6. Hinton R. H., Dobrota M., Fitzsimons J. T. R., Reid E.: Eur. J. Biochem. *12*, 349 (1970).
7. Rosenbergová M., Pristašová S.: Acta Virol. *16*, 1 (1972).
8. Fiske C. H., Subbarow Y.: J. Biol. Chem. *66*, 375 (1925).
9. Lowry O. H., Rosenbrough M. J., Farr A. L., Randal R. J.: J. Biol. Chem. *193*, 265 (1951).
10. Schneider W. C.: Methods Enzymol. *3*, 680 (1957).
11. Brake E. T., Will P. C., Cook J. S.: Membrane Biochem. *2*, 17 (1977).
12. Solyom A., Trams E. G.: Enzymes *13*, 329 (1972).
13. Cotman C. W., Mathews D. A.: Biochim. Biophys. Acta *249*, 380 (1971).
14. Evans W. H.: Biochem. J. *116*, 833 (1970).
15. Kidwai A. M., Radcliffe M. A., Duchon G., Daniel E. E.: Biochem. Biophys. Res. Commun. *45*, 901 (1971).
16. Meldolesi J., Jamieson J. D., Palade G. E.: J. Cell. Biol. *49*, 150 (1971).
17. House P. D., Weidemann M. J.: Biochem. Biophys. Res. Commun. *41*, 541 (1970).
18. Brown A. E., Lok M. P., Elovson J.: Biochim. Biophys. Acta *426*, 418 (1976).
19. Yannarell A., Aronson N. N., jr: Biochim. Biophys. Acta *311*, 191 (1973).
20. Kurihara T., Tsukada Y.: J. Neurochem. *14*, 1167 (1967).
21. Volpe J. J., Fujimoto K., Marasa J. C., Agraval H. C.: Biochem. J. *152*, 701 (1975).
22. Morgan I. G., Wolfe L. S., Mandel P., Gombos G.: Biochim. Biophys. Acta *241*, 737 (1971).
23. Anraku Y.: J. Biol. Chem. *239*, 3412 (1964).
24. Whitfeld P. R., Heppel L. A., Markham R.: Biochem. J. *60*, 15 (1955).
25. Lin P. P. C., Varner J. E.: Biochim. Biophys. Acta *276*, 454 (1972).