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ISOLATION OF PLASMA MEMBRANES FROM RAT LUNGS

EFFECT OF AGE ON THE SUBCELLULAR DISTRIBUTION OF ADENYLATE CYCLASE ACTIVITY

M.S. NIJJAR and JAMES C.-H HO

Department of Pathology, Faculty of Medicine, University of Manitoba, Winnipeg, R3E OW3 (Canada)

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Summary

A simple and rapid method of isolating plasma membranes from rat lungs is described. The method involves homogenization of tissue in isotonic sucrose-buffered medium followed by differential and sucrose density gradient centrifugation. Plasma membranes obtained by this procedure were essentially free from other subcellular contamination. Plasma membranes isolated from 2-day-old rat lungs showed 6 to 7-fold purification of adenylate cyclase and 5′-nucleotidase activities compared to the original homogenate In contrast, plasma membranes from 35-day-old rat lungs showed no purification of adenylate cyclase activity although 5′-nucleotidase activity showed similar enrichment. These results suggest that adenylate cyclase activity is not a reliable marker for plasma membranes from adult rat lungs.

Adenylate cyclase is activated by many hormones, neurotransmitters and pharmaceutical agents [1] which are important both in health and disease. Most of adenylate cyclase activity is associated with the cell surface membranes of different tissues [2—4] although some of its activity has been reported in the intracellular membranes [4]. Due to the presence of adenylate cyclase mainly in plasma membranes, it is commonly used as a marker for plasma membranes during subcellular fractionation of different tissues [4]. In fact, Solyom and Trams [4] in their review on marker enzymes advocated the use of adenylate cyclase activity as the genuine marker for plasma membranes in most tissues.

In this paper, we describe a simple method for the isolation of plasma membranes from rat lungs and present evidence that adenylate cyclase is not a suitable marker for plasma membranes isolated from adult rat lungs.

All reagents were of analytical grade and purchased from either Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific locally. [8-^{14}C] ATP was purchased from New England Nuclear (Boston, MA).

Male Sprague-Dawley rats (Charles River Breeding Labs., Quebec) of 2- and 35-days of age were used in this study. The animals were killed by decapitation, and the lungs excised and freed from the tracheal and other extraneous tissue. All subsequent manipulations were carried out in a cold room at 2—4°C. The tissue was sliced and homogenized in 4 vols. of 0.27 M sucrose/10 mM Tris-HCl buffer, pH 7.4, by 15 hand-driven strokes in a loose-fitting Potter-Elvehjem homogenizer. The homogenate was filtered through two layers of cheesecloth and its final volume adjusted to 20% (w/v). The homogenate was centrifuged at 900 × *g* for 10 min in a refrigerated International centrifuge to sediment nuclei, unbroken cells and cell debris (P_1). The supernatant (S_1) was centrifuged at 6000 × *g* for 10 min. The pellet (P_2) contained mitochondria and lamellar bodies [5,6] and the supernatant (S_2) was centrifuged at 8000 × *g* for 10 min. The pellet (P_3) contained lysosomes [5,6] and the supernatant (S_3) was made 1 mM $MgCl_2$ to enhance binding of ribosomes to smooth endoplasmic reticulum, thereby allowing their separation from plasma membranes by sucrose density gradient centrifugation [7,8]. 2.3 ml of the supernatant (S_3) were layered on top of a discontinuous sucrose gradient made by layering from bottom to top with 2.7 ml 45%, 2.7 ml 35%, 2.3 ml 30% and 2.3 ml 25% sucrose solutions in a 12.5 ml centrifuge tube [9]. The gradient was centrifuged at 100 000 × *g* for 75 min in a swinging SB-283 rotor in an International centrifuge at 2°C. Starting from the top of gradient, fractions of 1.4 ml (F_1), 1.2 ml (F_2), 1.2 ml (F_3), 1.7 ml (F_4), 2.5 ml (F_5) and 4.0 ml (F_6) were carefully withdrawn, pooled from different tubes, diluted with ice-cold water to 10% sucrose and centrifuged at 140 000 × *g* for 60 min. The pellets were washed once with the homogenizing medium, centrifuged as before and suspended in a small volume of the homogenizing medium. These fractions were analysed for adenylate cyclase and glucose-6-phosphatase activities immediately whereas the other enzyme activities were estimated later in 1 week after storage at —75°C. Activities of 5′-nucleotidase [10] and adenylate cyclase [11] were used as markers for plasma membranes, glucose-6-phosphatase [12] for microsomes, acid phosphatase [13] for lysosomes, cytochrome *c* oxidase [14] for mitochondria and DNA [15] for nuclei.

Proteins were estimated by using the method of Lowry et al. [16] using bovine serum albumin as standard. Inorganic phosphate was analysed by using the method of Chen et al. [17].

About 60% of the 5′-nucleotidase and adenylate cyclase activities were present in the 8000 × *g* supernatant while the remaining activities were found in the low-speed pellets (P_1, P_2, P_3, Table I). These results indicate the presence of plasma membranes in the 8000 × *g* supernatant. About 37% of the glucose-6-phosphatase activity was also present in this fraction (Table II) suggesting the presence of microsomes as well. Activities of cytochrome *c*

TABLE I

Distribution of protein and plasma membrane marker enzymes, e.g., adenylate cyclase and 5′-nucleotidase in subcellular fractions prepared from rat lungs according to the procedure described in the text. The results are expressed as mean ± S.D. from the number (n) of observations. W.H., whole homegenate. F_{2P}, F_{3P}, F_{4P}, F_{5P} and F_{6P} refer to pellets from the sucrose gradient fractions F_2, F_3, F_4, F_5 and F_6, respectively.

Fraction	Protein (% of W.H., 35-day, n=5)	Adenylate cyclase (pmol cyclic AMP/mg protein per min)				5′-Nucleotidase (μmol P_i/mg protein per min)	
		2-day (n=3)		35-day (n=3)		35-day (n=5)	
		Activity	Recovery (%)	Activity	Recovery (%)	Activity	Recovery (%)
W.H.	100*	31.1 ± 2.5	100	220.8 ± 20.3	100	31.9 ± 1.6	100
P_1	23.7 ± 2.2	18.2 ± 1.6	13.9	15.3 ± 4.0	1.6	20.2 ± 1.4	15.0
P_2	7.5 ± 0.5	38.9 ± 4.1	9.4	41.3 ± 5.1	1.4	37.3 ± 3.0	8.8
P_3	3.9 ± 0.4	87.5 ± 5.6	11.0	81.3 ± 9.1	1.4	57.7 ± 2.3	7.1
S_3	52.4 ± 1.7	36.1 ± 2.7	60.8	235.0 ± 16.5	55.8	40.0 ± 1.6	65.7
F_{2P}	4.0 ± 0.4	196.3 ± 11.7	25.5	191.3 ± 8.8	3.5	108.1 ± 7.2	13.7
F_{3P}	2.9 ± 0.4	185.7 ± 13.9	17.1	225.1 ± 25.2	2.9	201.0 ± 6.4	18.1
F_{4P}	1.9 ± 0.2	216.3 ± 12.8	13.3	222.2 ± 20.3	1.9	222.7 ± 9.6	13.4
F_{5P}	1.7 ± 0.2	165.3 ± 10.0	9.0	165.2 ± 6.1	1.3	158.3 ± 8.7	8.4
F_{6P}	1.7 ± 0.2	135.7 ± 7.5	7.4	54.7 ± 16.5	0.4	73.9 ± 3.1	3.9
Total *recovery* (%)	87.3		106.6		14.0		88.4

*Whole homogenate contained 94.1 ± 8.9 mg protein/g lung weight.

TABLE II

Distribution of DNA and other enzyme activities in subcellular fractions prepared from 35-day-old rat lungs by the scheme described in the text. The results are expressed as mean ± S.D. from the number (n) of experiments shown in parenthesis. W.H., whole homogenate. $F_{2}P$, $F_{3}P$, $F_{4}P$, $F_{5}P$ and $F_{6}P$ refer to pellets from the sucrose gradient fractions F_{2}, F_{3}, F_{4}, F_{5} and F_{6} respectively.

Fraction	DNA (% of W.H., n=3)	Glucose-6-phosphatase		Acid phosphatase		Cytochrome c oxidase	
		μmol P_i/mg protein per min (n=3)	Recovery (%)	μmol P_i/mg protein per min (n=3)	Recovery (%)	nmol cyt. c oxidized/mg protein per min (n=3)	Recovery (%)
W.H.	100*	10.7 ± 0.6	100	28.7 ± 2.1	100	3.1 ± 0.2	100
P_1	74.1 ± 2.4	10.2 ± 1.1	22.6	27.1 ± 1.7	22.4	2.1 ± 0.2	16.0
P_2	6.2 ± 0.5	13.9 ± 1.4	9.8	191.0 ± 5.8	50.2	28.6 ± 1.1	69.6
P_3	3.6 ± 0.3	22.5 ± 2.0	8.3	164.2 ± 12.4	22.5	8.9 ± 0.8	11.2
S_3	7.2 ± 0.6	7.5 ± 0.5	36.7	negligible		negligible	
$F_{2}P$	0.3 ± 0.1	14.7 ± 1.4	5.6	none detected		0.8 ± 0.1	1.0
$F_{3}P$	0.2 ± 0.1	21.4 ± 1.7	5.7	none detected		0.8 ± 0.1	0.8
$F_{4}P$	0.4 ± 0.1	32.4 ± 2.5	5.8	29.7 ± 4.5	2.0	2.9 ± 0.2	1.8
$F_{5}P$	0.3 ± 0.1	41.1 ± 3.7	6.5	23.2 ± 2.6	1.4	2.5 ± 0.2	1.4
$F_{6}P$	0.5 ± 0.1	33.7 ± 2.9	5.4	none detected		6.8 ± 0.3	3.7
Total recovery (%)	85.6		69.7		98.5		105.5

*Whole homogenate contained 101 ± 3 mg DNA/mg protein.

oxidase, acid phosphatase and DNA were essentially absent from this supernatant (Table II) indicating the lack of mitochondria, lysosomes and nuclei. These data suggested the use of the 8000 × *g* supernatant (S_3) for the isolation of plasma membranes from rat lungs.

Mg^{2+} was added to the 8000 × *g* supernatant to effect the separation of plasma membranes from microsomes by sucrose density gradient centrifugation [7,8]. While the lighter fractions (F_1 and F_{2S}) contained most of the soluble proteins (about 40% of the total homogenate protein), F_{2P}, F_{3P}, F_{4P}, F_{5P} and F_{6P} contained predominantly plasma membranes and microsomes which together constituted about 15% of the total protein.

When lungs from 35-day-old rats were subjected to this scheme of subcellular fractionation, 5′-nucleotidase activity in F_{2P} and F_{3P} was enriched several-fold compared to the original homogenate activity and, together, F_{2P} and F_{3P} contained about 7% of the total protein and 32% of the 5′-nucleotidase activity (Table I). Some activity of 5′-nucleotidase was also present in the soluble fractions and in F_{4P}, F_{5P} and F_{6P} (Table I). In contrast, adenylate cyclase activity in F_{2P} and F_{3P} showed no purification compared to the whole homogenate activity and only 6% of the total enzyme activity was recovered in these fractions (Table I). F_{3P}, F_{4P}, F_{5P} and F_{6P} together contained about 3% of the total enzyme activity. Adenylate cyclase activity was absent from the soluble fractions and evidently, total recovery of the enzyme was very low. Glucose-6-phosphatase activity in F_{2P} and F_{3P} was comparable to that in the whole homogenate but was higher in F_{4P}, F_{5P} and F_{6P} (Table II). Activities of acid phosphatase and cytochrome *c* oxidase were absent from F_{2P} and F_{3P} whereas F_{4P}, F_{5P} and F_{6P} contained some activity of these enzymes (Table II). Similarly, DNA was not detected in F_{2P} and F_{3P} but was present in small amounts in F_{4P}, F_{5P} and F_{6P} (Table II). All these data indicate that F_{2P} and F_{3P} were rich in plasma membranes and relatively free from mitochondria, lysosomes and nuclei.

When lungs from 2-day-old rats were similar fractionated, about 40% of the adenylate cyclase activity was recovered in the plasma membrane fractions (F_{2P} and F_{3P}) and showed an enrichment of about 7-fold compared to the homogenate activity (Table I). The heavier fractions (F_{4P}, F_{5P} and F_{6P}) also contained some adenylate cyclase activity (Table I) but were contaminated with other subcellular organelles as indicated by the presence of glucose-6-phosphatase, acid phosphatase, cytochrome *c* oxidase and DNA. The subcellular distribution of these enzymes and 5′-nucleotidase was very similar to that found in 35-day-old rat lungs (data not shown).

The loss of adenylate cyclase activity in subcellular fractions prepared from the adult rat lungs was apparently due to the separation of membranes from the cytoplasm which contained some factor(s) activating the particulate adenylate cyclase (Fig. 1 and Ref. 18). These cytoplasmic factor(s) were absent from 2-day-old rat lungs (Fig. 1 and Ref. 18). The lack of adenylate cyclase purification in plasma membrane fractions from the adult rat lungs was due to the elevated adenylate cyclase activity in the homogenate of 35-day-old rat lungs as compared with the homogenate activity from 2-day-old rat lungs (Table I). These data clearly demonstrate that adenylate cyclase activity is not a suitable marker for plasma membranes from the adult rat

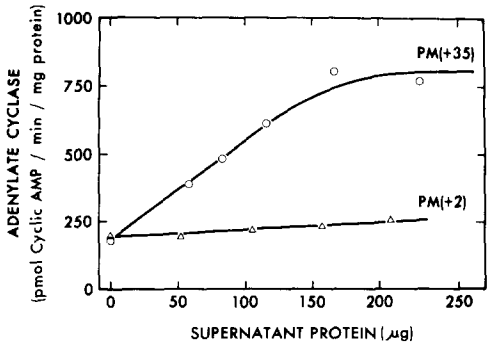

Fig. 1. Activation of adenylate cyclase in plasma membranes from 2- and 35-day-old rat lungs by their respective supernatant (F_1) fractions. Adenylate cyclase activity was estimated exactly as described in the text with about 60 μg of plasma membrane (F_2 and F_3) protein.

lungs. Addition of F_1 containing cytoplasmic proteins to the plasma membrane fraction ($F_{2P} + F_{3P}$) from the adult rat lungs enhanced adenylate cyclase activity markedly whereas F_1 from lungs of younger rats had little effect on adenylate cyclase activity in plasma membranes from the younger animals (Fig. 1). These data support our conclusion above and confirm our previous observations with the crude preparation of membranes from rat lungs [18].

Our method of isolating plasma membranes involves tissue homogenization in the isotonic buffered medium and allows the simultaneous preparation of plasma membranes and other subcellular fractions from the same homogenate. The method is simple and yields plasma membranes of reasonable purity within 5 h.

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References

1 Robinson, G.A., Butcher, R.W. and Sutherland, E.W. (1971) Cyclic AMP, Academic Press, New York
2 Davoren, P.R. and Sutherland, E.W. (1963) J. Biol. Chem. 238, 3016—3023
3 Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849—1856
4 Solyom, A. and Trams, E.G. (1972) Enzyme 13, 329—372
5 Sander, G.E, and Huggins, C.G. (1971) Nat. New. Biol. 230, 27—29
6 Hallman, M. and Gluck, L. (1975) Biochim. Biophys. Acta 409, 172—191
7 Hinton, R.H., Norris, K.A. and Reid, E. (1970) in Separation with Zonal Rotors (Reid, E., Ed.), Section S-2, pp. S5—S21 Wolfson Bioanalytical Centre, University of Surrey, Guildford
8 Nijjar, M.S. and Hawthorne, J.N. (1974) Biochim. Biophys. Acta 367, 190—201
9 Nijjar, M.S. and Pritchard, E.T. (1972) Arch. Oral Biol. 17, 1679—1690
10 Michell, R.H. and Hawthorne, J.N. (1965) Biochem. Biophys. Res. Commun. 21, 333—338
11 Drummond, G.I. and Duncan, L. (1970) J. Biol. Chem. 245, 976—983
12 DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appleman, F. (1955) Biochem. J. 60, 604—617
13 Hubscher, G. and West, G.R. (1965) Nature 205, 799—800
14 Wharton, D.C. and Txagoloff, A. (1967) Methods Enzymol. 10, 245—250
15 Ceriotti, G. (1952) J. Biol. Chem. 198, 297—303
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
18 Nijjar, M.S. (1979) Biochim. Biophys. Acta, 584, 43—50