BBA 75761

# A RAPID, SIMPLE METHOD FOR ISOLATING PINOCYTOTIC VESICLES AND PLASMA MEMBRANE OF LUNG

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(Received June 14th, 1971)

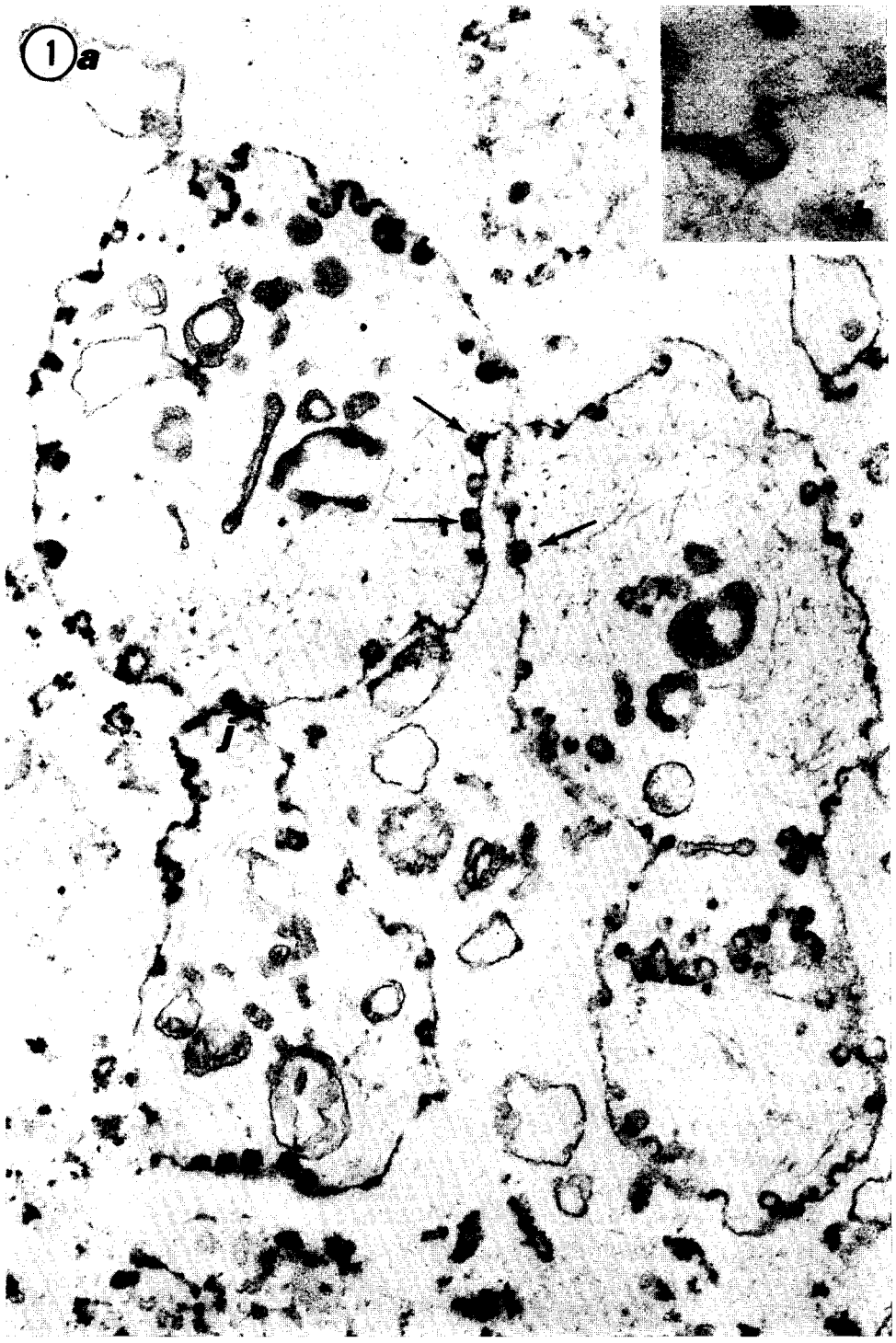
SUMMARY

We have found means of isolating pinocytotic vesicles and attached plasma membrane from the low speed (200 × *g*) supernatant of homogenates of lung. In lung, 5′-nucleotidase is restricted to pinocytotic vesicles and areas of incipient vesicle formation along the plasma membrane. In our method, $P_i$ released from AMP is precipitated as lead phosphate at the subcellular site of 5′-nucleotidase. The resulting increase in density allows collection of pinocytotic vesicles and attached plasma membrane as a pellet after centrifugation through sucrose ($d = 1.18$) at 250 × *g*. The final pellet contains long strands of plasma membrane, and the vesicles retain their characteristic morphology including the delicate diaphragm covering their stomata. The entire procedure can be performed in less than 90 min.

The growing interest in the properties of plasma membranes and pinocytotic vesicles has led to the development of several methods for membrane isolation (see refs. 1, 2 and 3 for example). However, none of the published methods appears to be useful for more than one or two solid tissues, and the reproducibility of some have been brought into question (see ref. 4 for discussion). All of the published techniques are tedious and time consuming and require ultracentrifugation equipment that is not available in a large number of laboratories. Plasma membrane fractions obtained by existing techniques vary considerably in terms of contamination with microsomes, and most methods do not take into account the fact that the bulk of the plasma membrane is lost in the nuclear fraction.

Here we report a rapid, simple method of harvesting plasma membrane and pinocytotic vesicles of lung. In lung, 5′-nucleotidase is largely restricted to pinocytotic vesicles and those areas of incipient vesicle formation along the plasma membrane[5-7]. Our method exploits the point that $P_i$ released from AMP can be precipitated as lead phosphate at or near the subcellular site of 5′-nucleotidase. The lead phosphate deposits vastly increase the density of pinocytotic vesicles and attached plasma membrane, allowing collection of these structures as a pellet after a low-speed centrifugation (250 × *g*).

Sprague–Dawley rats, weighing 0.2–0.3 kg, were anesthetized with an intraperitoneal injection of chloral hydrate, 300 mg/kg of body weight. Their lungs were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ventilated mechanically and were perfused with aerated Locke's solution at 38° until the venous effluent was free of blood. The perfusion circuit has been described[8]. Peripheral areas of lung were selected for further processing, thus avoiding contamination with tissues of large blood vessels and bronchi.

Lung tissue was cut into blocks of about 5 $mm^3$, which were homogenized (10 ml/g of tissue) in 50 mM Tris–maleate buffer, pH 7.4, containing 100 mM KCl, 5 mM $MgCl_2$ and 0.3 M sucrose at 0–4°. Homogenization was performed with a Teflon-glass Potter–Elvehjem homogenizer, having an average clearance of 0.13 mm, using 12 complete strokes. The homogenate was centrifuged at 200 × *g* for 10 min at 4° to remove nuclei and cell debris. The supernatant was decanted to a second tube and centrifuged at 250 × *g* for 10 min to assure complete removal of cell debris.

Ten ml of the supernatant of the second centrifugation was incubated at room temperature with AMP (disodium salt), 2 mg, and $Pb(NO_3)_2$, 6 mg, for 10 min. One- to three-ml aliquots of the reaction mixture were layered on 10 ml of cold sucrose, $d = 1.18$, in cellulose nitrate tubes (14.3 mm × 95.3 mm). Purer pellets were obtained using 1-ml aliquots. A thin, sheet-like, white pellet was collected after centrifugation at 250 × *g* for 30 min. After removing the supernatant by aspiration, the pellet was washed 3 times in the Tris–maleate buffer described above, the pellet being collected after each wash by centrifugation at 200 × *g* for 5 min. Sucrose was omitted from the wash solution when the pellet was to be used in analyses requiring chromatographic or electrophoretic separations.

Protein, cholesterol, phospholipid and 5'-nucleotidase were measured as described previously (see ref. 2). Pellets were prepared for electron microscopy by fixation in 0.05 M cacodylate buffer, pH 7.6, containing 0.25 M glutaraldehyde and 0.17 M sucrose. The fixative was layered over the pellets in the centrifuge tubes. Pellets were washed in several changes of cacodylate buffer containing 0.34 M sucrose. After washing, the pellets were removed from the tubes and cut into small pieces (approx. 1 mm in diameter), treated with 1 % $OsO_4$ in 0.14 M veronal acetate buffer, pH 7.4, dehydrated in an ethanol series and embedded in Araldite. Sections were cut on an LKB Ultrotome III, doubly stained in lead citrate and saturated uranyl acetate in 50% ethanol and examined in a Philips EM 100, 200 or 300.

The plasmalemmal pellet is shown in Fig. 1. The pellet contains long strands of plasma membrane with attached pinocytotic vesicles, many of which still retain the diaphragm covering the vesicle stoma. At high magnification it is evident that virtually all of the vesicles contain discrete lead phosphate deposits. By electron microscopy, the pellets are relatively pure, containing fewer than 7 mitochondria per 10 grid squares. Lysosomes and rough endoplasmic reticulum are not evident. Some amorphous material heavily coated with lead is encountered. This material may be over-reacted plasma membrane, but it may possibly be nucleic acid-containing material, as nucleic acids may bind lead.

By biochemical analysis, the pellet has properties like those described for cell membrane of other tissues. The cholesterol/phospholipid molar ratio is 0.93 and the

Fig. 1. Electron micrograph of the pellet showing long strands of plasma membrane and pinocytotic vesicles. The lead phosphate deposits are localized in the vesicles (arrows). Intercellular junctions (j) can be clearly distinguished. The characteristic morphology of the pinocytotic vesicles is preserved including the diaphragm covering the mouth of the vesicle (insert). (a) × 50000. (b) × 140000.

cholesterol/protein ratio is 0.24. The specific activity of 5'-nucleotidase is 9.6 μmoles/mg of protein per h and compares favorably with that of plasma membrane isolated from lymphocytes[9], suggesting that lead has little adverse effect on the enzymic capabilities of plasma membrane. In addition, the plasmalemmal fraction converts angiotensin I to angiotensin II (0.1 nmole/mg of protein per min) and hydrolyzes bradykinin to Pro–Pro and Phe–Arg, activities previously postulated for plasma membrane but not previously demonstrated[5–8]. Succinate dehydrogenase was not measurable.

Our results show that plasma membrane and pinocytotic vesicles of lung may be harvested in a highly purified state by a series of low-speed centrifugations, using equipment and materials available in virtually any laboratory. The technique as described is efficient in collecting plasma membrane except for that trapped in the nuclear and cell debris pellet. The plasma membrane protein represents 1.8% of the homogenate protein (see ref. 3 for comparison with liver). By including a second homogenization of the material sedimented in the first centrifugation, it is possible to use our technique to obtain a far greater yield. The technique is rapid, allowing collection of plasma membrane in less than 90 min. The principle of the technique is such that it can be used with all tissues in which 5'-nucleotidase is restricted to plasma membrane or pinocytotic vesicles. Furthermore, the ease of the preparation suggests that it could be scaled-up for use with kilogram quantities of lung.

The technique has the disadvantage of preventing characterization of plasma membrane in terms of true density. However, this is a point of little consequence to studies of the qualitative properties and enzyme capabilities of plasma membrane, and the simplicity of our technique is such that it should attract new workers to the field and increase the numbers of useful experiments that can be done.

ACKNOWLEDGEMENTS

This study was supported in part by grants from the National Institutes of Health (HE-12843 and HE-14360), the Florida Heart Association and the Council for Tobacco Research, U.S.A.

REFERENCES

1 D. M. NEVILLE, JR., *J. Biophys. Biochem. Cytol.*, 8 (1960) 413.
2 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND PH. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.
3 R. COLEMAN, R. H. MICHELL, J. B. FINEAN AND J. N. HAWTHORNE, *Biochim. Biophys. Acta*, 135 (1967) 573.
4 O. TOUSTER, N. N. ARONSON, JR., J. T. DULANEY AND H. HENDRICKSON, *J. Cell Biol.*, 47 (1970) 604.
5 U. SMITH AND J. W. RYAN, *J. Cell Biol.*, 47 (1970) 196.
6 U. SMITH AND J. W. RYAN, *Chest*, 59 (1971) 12S.
7 J. W. RYAN AND U. SMITH, *Trans. Assoc. Am. Phys.*, in the press.
8 J. W. RYAN, J. ROBLERO AND J. M. STEWART, *Biochem. J.*, 110 (1968) 795.
9 D. ALLAN AND M. J. CRUMPTON, *Biochem. J.*, 120 (1970) 133.