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Transport of sodium into apical membrane vesicles prepared from fetal sheep alveolar type II cells *

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A method is described for isolating apical plasma membrane vesicles from fetal alveolar type II cells. The procedure yields purified apical membranes which are enriched 24-fold with the brush-border enzyme marker, alkaline phosphatase. Contamination of this fraction by basolateral membranes and organelles is minimal. Evidence for transport of Na^+ into an intravesicular space is demonstrated by: (1) time-dependent uptake of Na^+ with release of accumulated Na^+ by treatment with detergent; (2) a linear inverse correlation between Na^+ uptake and medium osmolarity. In addition, Na^+ uptake is shown to be anion dependent ($SCN^- > Cl^- >$ gluconate$^-$) and sensitive to amiloride inhibition at a concentration of 1 mM.

Introduction

Although the primary function of the lung is that of gas exchange, available evidence indicates that the pulmonary epithelium, in common with other epithelia, possesses ion transport systems for the regulation of cell homeostasis and the vectorial flow of solutes and water. These properties are of fundamental importance for the normal functioning of the lung both pre- and post-natally. Thus, an active secretory process generates the liquid template around which the developing air spaces grow [1,2] and in the air filled lung a specialised secretion forms the aqueous interior lining layer – the surfactant subphase at alveolar level and the sol-phase of the airway's mucocillary 'escalator' [3–5]. Active absorption brings about the removal of lung liquid at birth [6] and keeps the lung 'dry' thereafter. Additionally, it provides a potential safety mechanism for the removal of fluid in conditions of lung water overload [7].

In the progression from fetus to adult, the lung is first a predominantly secretory organ and then principally absorptive, the switch occurring at birth. Whatever the direction of net volume flow at any particular stage of development, the lung epithelium (or at least part of it) retains the facility for both absorption and secretion. It is this balance between secretory force (Cl^- driven) and absorptive force (Na^+ driven) which is of crucial importance to function.

Although study of the naturally liquid filled lung of the fetus [1,8] has provided extensive data on bulk fluid and electrolyte transport by the prenatal alveolar epithelium in vivo (much of it relevant to the function of the air filled lung) it remains true that the epithelium of the lung at alveolar level has been studied much less extensively than that of the airways. Strategies designed to circumvent the difficulties of inaccessibility and inconvenient gross morphology include fluid filling of the lobes in vivo, isolation of alveolar cells and cell culture. Each preparation has its own strengths and weaknesses but all suffer from the disadvantage that they are complex systems in which it is often difficult to identify and locate the functional properties of the individual membrane components, apical and basolateral, responsible for ion and water transport. Despite the difficulties of precise localisation of membrane transport properties, data from isolated cell preparations [9,10] indicate that the type II alveolar cell is a site of active sodium transport.

The study described here represents a novel application of established techniques for cell isolation and membrane vesicle preparation. We have obtained highly purified apical membrane vesicles from fetal alveolar type II cells which show anion dependent uptake of Na^+ into an intravesicular space. This preparation offers

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new opportunities for investigation of the molecular mechanisms of alveolar ion transport, its control and ontogeny.

Methods and Materials

Methods

Twin fetal lambs, the product of Grey Face Ewes/Suffolk Tups, were studied as gestational ages of between 136 and 142 days. Anaesthesia in the ewe was induced by intravenous injection of sodium thiopentone (70–90 ml of a 2.5% solution) and maintained with inhaled halothane, 1–2% in N_2O/O_2 using a rebreathing circuit. The fetuses were delivered through an oblique flank incision and heparinised by injection of 1000–2000 units of heparin sulphate into the umbilical vein. After an interval of 5 minutes, the fetuses were killed by intravenous injection of Euthital (2.5 ml of a 25% solution). The lungs were perfused in-situ via the pulmonary artery (following ligation of the Ductus Arteriosus) with 2.5 litres of a balanced salt solution (140 mM NaCl, 5 mM KCl, 2.5 mM sodium phosphate buffer, 10 mM Hepes, 2.0 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 6 mM glucose). The lungs were then excised to allow harvesting of type II cells and preparation of apical membrane vesicles according to an adaptation of methods previously published [10–14]. Following excision, the lungs were lavaged 6 times at total lung capacity with balanced salt solution (see above). After as much lavage solution as could be aspirated was removed, 50 ml of a protease solution containing 0.3 mg/ml elastase (48 units/mg protein) and 0.025 mg/ml trypsin (10000 BAEE units/mg protein) was added via the trachea and the lungs incubated at 37°C for 20 min (topped up with a further 25 ml of proteinase solution after 10 minutes incubation). Proteolytic digestion of the lung epithelium was terminated by addition of soybean trypsin inhibition (10000 units/mg protein) in 8 ml fetal bovine serum also containing 10 μg/ml DNAase (from bovine pancreas). The lung parenchyma was stripped off the large airways and chopped with scissors into approximately 1 cm cubes. Lung tissue used to provide material for subsequent cell count and morphology was further reduced in size to approx. 1 mm^3 by use of scissors and, although handled separately, was processed in a way identical to the remainder of the lung. For convenience and speed the bulk of the lung was reduced to fragments of approx. 1 mm^3 in size by means of a Mouli food mincer. Balanced salt solution was added to a final volume of 175 ml per lung and the mince divided between two siliconised 25 ml conical flasks and shaken in a reciprocating water bath at 130 cycles/min for 2 min at 37°C. The resulting slurry was filtered sequentially through nylon bridal net and 120 μm/80 μm/40 μm mesh nylon fabric. No pressure, positive or negative, was used during the filtering steps. Additional balanced salt solution was added to a final volume of 310 ml. Cells were centrifuged in plastic round-bottomed tubes at 200 × *g* for 8 min and the supernatant liquid, which contained debris, discarded. At this stage the pellet derived from the sections of lung prepared for cell count and morphology was resuspended in balanced salt solution. Total cell count was measured by means of a haemocytometer and a differential cell count performed on a separate aliquot after staining by a modification of the Papanicolou method [15]. The remainder of the cell pellet was resuspended in an ice-cold 50 mM mannitol, 5 mM Hepes-Tris solution at pH 7.4 containing 0.25 mM $MgCl_2$ and 1 mM dithiothreitol (homogenisation medium). All subsequent procedures were performed at 0–4°C.

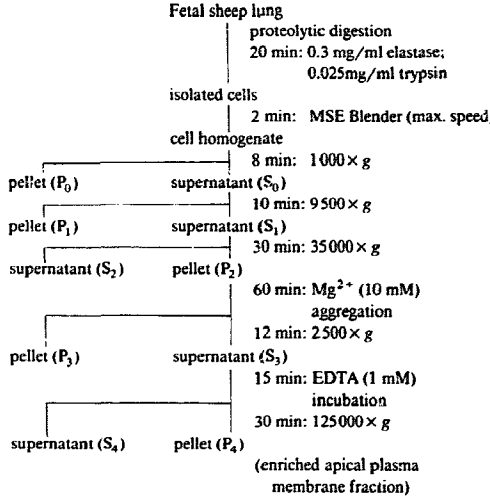

Fig. 1. Preparation of apical plasma membrane vesicles from fetal sheep alveolar type II cells.

Homogenisation and membrane isolation

The cell suspension was homogenised in an MSE Blender at maximum speed (14000 rpm) for 2 min. The homogenate was centrifuged at 1000 × *g* for 8 min and the supernatant (S_0) collected. The pellet (P_0) contained nuclei and large debris. S_0 was centrifuged at 9500 × *g* for 10 min to form a dense buff coloured pellet (P_1). Centrifugation of the supernatant (S_1) at 35000 × *g* for 30 min yielded a mixed plasma membrane fraction (P_2).

P_2 was further resolved to yield an apical membrane fraction by the following procedure (see Fig. 1): P_2 was resuspended in isolation medium (100 mM mannitol, 5 mM Hepes-Tris, pH 7.4) and $MgCl_2$ was added as a 1 M solution to a final concentration of 10 mM. The suspension was incubated on ice with occasional stirring for 1 hour, during which time an increase in turbidity was observed. Subsequent low speed centrifugation

(2500 × *g* for 12 min) sedimented a fraction (P_3) enriched in Na^+/K^+-ATPase activity, leaving a supernatant which contained apical membranes (S_3). The supernatant was diluted with isolation medium containing 1 mM EDTA to remove excess Mg^{2+} and centrifuged at 125000 × *g* for 30 min. The resulting pellet (P_4) containing the apical membranes was resuspended in appropriate buffer at a protein concentration of 0.2–0.4 mg/ml and was not purified further. Depending on the total vesicle yield (0.4–1.0 mg protein) and the complexity of the experimental design, material from a single lung was either used alone or pooled with that from the twin fetus to make a single preparation. In no case were the lungs from each twin used independently as separate preparations within a particular experiment (i.e., one pregnancy yielded one preparation containing vesicles from one or both twins).

Enzyme assay procedures

The membrane isolation procedure was followed analytically using standard markers for nuclei, mitochondria, endoplasmic reticulum and apical and basolateral membranes. Na^+/K^+-ATPase was used as a marker for basolateral membranes and measured as the fraction of Na^+/K^+-ATPase activity inhibited by 10^{-4} M ouabain [16]. Alkaline phosphatase activity (a brush-border marker) was measured at 37 °C using 2-amino, 2-methyl, 1-propanol buffer (pH 10.3) and 7 mM *p*-nitrophenyl phosphate as the substrate. The colour developed on addition of 10 ml of 50 mM NaOH was determined at 410 nm. DNA served as a marker for nuclei and was measured according to the method of Chambers and Rickwood [17]. Succinate dehydrogenase activity, a marker for mitochondria, was determined as described by Chambers and Rickwood, [17] using 2 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride as substrate with the addition of an ethyl acetate extraction step [18]. NADH dehydrogenase activity, an endoplasmic reticulum marker, was assayed as described by Hochstadt et al. [19].

Protein was estimated according to Lowry et al. [20] using bovine serum albumin as a standard.

Transport measurements

We used Dowex 50W-X8 (50–100 mesh, Tris form) columns to assay ^{22}Na influx as described by Gasko et al. [21]. 1.5 ml cation exchange resin was packed into 2 ml disposable plastic pasteur pipettes supported by a dacron wool plug. Columns were equilibrated with 100 mM mannitol, 2 mM Tris sulphate (pH 7.4). Apical membrane vesicles were resuspended in the same medium, using 20 strokes through a 26 G needle.

Uptake of ^{22}Na was measured following incubation of vesicles in medium comprising 2 mM Tris sulphate (pH 7.4), 50 mM KSCN and 1 mM $^{22}NaSCN$. In some experiments SCN^- was replaced by Cl^- or gluconate$^-$.

Uptake was initiated by addition of 30 μl vesicles to 90 μl incubation medium at 20 °C. Drugs were added to the medium prior to starting the experiment. At predetermined times 100 μl of the reaction mixture was placed on a Dowex column and immediately washed into the column with 0.25 ml of ice-cold 100 mM mannitol 2 mM Tris sulphate solution to stop the uptake of ^{22}Na, then eluted with 1.8 ml of the same solution. Transit time through the columns was less than 30 seconds. The eluent was counted for radioisotope content. The specific activity of ^{22}Na was determined from a standard sample of the incubation medium. Uptake was measured as μmol Na^+ transported per mg protein. Zero time points were obtained from matched incubation media at 0 °C. Typically, six data points could be obtained from vesicles prepared from a single fetus. Each uptake measurement was performed in triplicate.

Materials

Sources of supply were as follows – Sigma: elastase, trypsin inhibitor, DNAase, Dowex, amiloride, Hepes, Tris. Amersham: ^{22}Na. British Drug Houses: all standard laboratory chemicals (Analar grade).

Results

Isolation of apical membranes

Tracheal instillation of proteolytic enzymes released 10^6–10^8 granular pneumocytes per lung. We used the recovery of enzyme markers during the isolation procedure to assess the purity of the preparation at each stage (Table I). The final membrane fraction showed a 24-fold enrichment of alkaline phosphatase activity compared to the crude homogenate. It was essentially free of mitochondrial, nuclear and endoplasmic reticulum contamination. The specific activity of Na^+/K^+-ATPase was 3.6-times that of the homogenate, indicating the presence of a relatively small component of basolateral membrane material in the vesicle preparation.

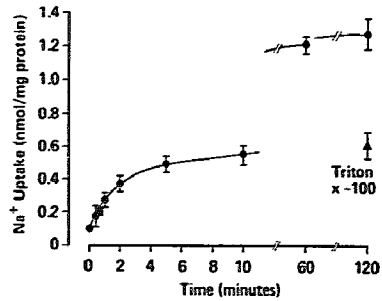

Fig. 2. Uptake of Na^+ by alveolar type II cell apical membrane vesicles. Vesicles were equilibrated with 2 mM Tris sulphate, 100 mM mannitol (pH 7.4). The incubation medium was 1 mM $^{22}NaSCN$, in 2 mM Tris sulphate, 50 mM KSCN (pH 7.4). Each point is mean ± S.E. of triplicate assays using vesicles from five preparations.

TABLE I

Recovery of enzyme markers during a representative membrane isolation procedure

The data relate to the preparation of vesicles from a single fetal sheep lung. The specific activity and percentage yield of protein and all subcellular markers are given for fractions recovered at each stage of the isolation procedure. Yields are presented as percentage of the respective homogenate (H) values. Alkaline phosphatase and Na^+/K^+-ATPase activities are expressed as μmol inorganic phosphate produced per hour per mg protein; succinate dehydrogenase and NADH dehydrogenase activities are expressed as nmol/mg protein per hour.

Fraction	Protein		DNA		Alkaline phosphatase		Na^+/K^+-ATPase		Succinate dehydrogenase		NADH dehydrogenase	
	mg	yield	μg/mg protein	yield	activity	yield	activity	yield	activity	yield	activity	yield
H	121	100	78	100	1.0	100	0.17	100	2.1	100	7.5	100
S_0	100	83	68	72	1.0	83	0.14	68	1.6	62	6.8	75
P_0	24	20	178	45	0.9	18	0.23	27	3.8	36	7.5	20
S_1	81	67	–	–	0.8	54	0.06	24	0.3	9	5.4	48
P_1	27	22	252	72	1.2	27	0.39	51	5.1	54	9.0	27
S_2	60	50	–	–	0.3	14	0.00	0	0.2	5	4.6	30
P_2	8	7	–	–	6.1	40	0.64	25	0.6	2	20.0	18
S_3	4	3	–	–	6.7	22	–	0	–	–	9.0	4
P_3	6	5	–	–	3.6	18	0.72	21	2.0	5	26.0	17
S_4	2.5	2	–	–	5.0	10	–	–	–	–	–	–
P_4	0.6	0.5	–	–	24.0	12	0.60	2	1.0	< 0.1	–	–

Na^+ transport studies

We found uptake of Na^+ by type II apical membrane vesicles to be time-dependent, reaching a value close to equilibrium at 60 min (Fig. 2).

In order to differentiate between the two components of Na^+ uptake, transport into the intravesicular space and binding to the vesicle membrane, we manipulated the transport component by varying intravesicular volume in response to osmotic gradients. Fig. 3 shows that the uptake of Na^+ at (or near) equilibrium is inversely related to increasing osmolarity of the incubation medium as the vesicles shrink. The portion of uptake that is independent of the intravesicular volume (i.e. binding) is given by extrapolation of the regression line to infinite osmolarity (the theoretical point of obliteration of the intravesicular spaces). This value represents 49% of the total Na^+ uptake (1.84 nmol/mg protein) measured in 100 mosM transport medium. A similar value for the proportion of Na^+ uptake non-specifically membrane bound was obtained following disruption of vesicles by means of detergent, Triton X-100 (Figs. 2 and 3).

Na^+ uptake was inhibited by amiloride only at millimolar concentration. The reduction in uptake at 1 min represents 24% of total uptake (Fig. 4) and is due

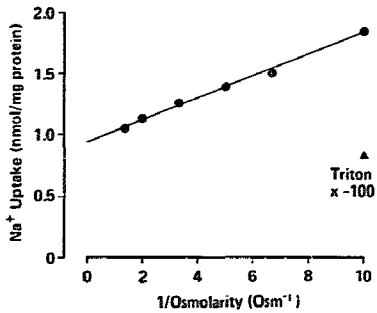

Fig. 3. Effect of medium osmolarity on Na^+ uptake. Vesicles were equilibrated with 2 mM Tris sulphate, 100 mM mannitol (pH 7.4). Incubation medium was 1 mM ^{22}NaSCN in 2 mM Tris sulphate, 50 mM KSCN (pH 7.4) with mannitol added to increase the osmolarity. The columns were pre-incubated with solutions of appropriate osmolarity. Incubation period was 6 minutes. The line, drawn by linear regression, has a correlation coefficient of 0.998. Also shown is the effect of addition of Triton X-100 (0.1%). Each point is mean of triplicate assays using vesicles from four preparations.

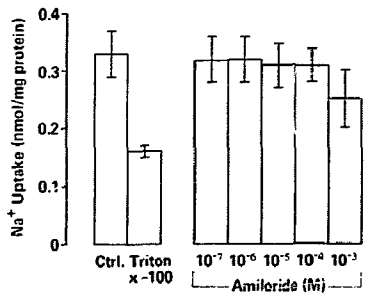

Fig. 4. Effect of increasing concentrations of amiloride on Na^+ uptake at 1.0 minute. Vesicles were equilibrated with 2 mM Tris sulphate, 100 mM mannitol (pH 7.4). The incubation medium was ^{22}NaSCN in 2 mM Tris sulphate, 50 mM KSCN (pH 7.4). Amiloride was added to the incubation medium at preselected doses at the start of the experiment. Each point is mean ± S.E. of triplicate assays using vesicles from four preparations. Total uptake in the presence of 10^{-3} M amiloride is significantly different to control ($P < 0.01$, paired t-test). Residual uptake after Triton X-100 is a measure of membrane bound ^{22}Na.

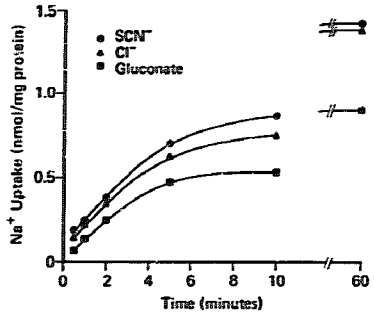

Fig. 5. Anion-dependence of Na^+ uptake by type II cell apical membrane vesicles. Vesicles were equilibrated with 2 mM Tris sulphate, 100 mM mannitol (pH 7.4). Incubation medium contained 1 mM ^{22}NaX in 2 mM Tris sulphate, 50 mM KX (pH 7.4), where X = SCN^-, Cl^- or gluconate$^-$. Each point is mean of triplicate assays using vesicles from two preparations.

entirely to blocking of specific uptake since the membrane bound fraction (after Triton X-100) was found to be insensitive to amiloride. Fig. 5 shows the effect of anion-substitution on the time-course of Na^+ uptake into apical membrane vesicles. The curves for SCN^- and Cl^- are similar, suggesting that Na^+ transport may be supported by either anion. Gluconate$^-$, however, significantly retards Na^+ uptake.

Discussion

Apical membrane vesicle isolation and preparation

There exists a considerable body of literature describing the use of proteases to disaggregate epithelial cells in the lung. Most proteolytic enzymes preferentially release type II cells, albeit with varying degrees of efficiency. Concern about possible damage to membrane constituents has led to a move towards the use of pure enzymes in low concentration with elastase as the predominant (or only) constituent. Mason et al. [22] showed that elastase digestion of the lung released epithelial cells with intact membrane receptors. Subsequently Finklestein and Shapiro [11], working with concentrations of elastase (0.3 mg · ml^{-1}) and trypsin (0.025 mg · ml^{-1}), much lower than previously employed, showed that it was possible to obtain a substantial yield of type II pneumocytes which were viable and intact by criteria of vital dye exclusion and cellular enzymatic integrity. This technique, which was subsequently modified for use on fetal lung by Bland and Boyd [10], has been used in the experiments reported here.

The purity of the apical membrane fraction and the efficiency of the separation procedure may be assessed by comparison with established procedures for isolating brush-border membranes from other epithelia. Apical plasma membranes, including those of granular pneumocytes [23–25], are rich in alkaline phosphatase which can thus serve as a discriminatory marker. Our apical membrane fraction is markedly enriched (24-fold) with alkaline phosphatase, an enrichment which exceeds that measured in renal and intestinal tissue (10–15-fold) [26–28] but is not dissimilar to the enhanced activity (19-fold) reported by Langridge-Smith, Field and Dubinsky in tracheal apical plasma membranes [14]. Furthermore, the specific activity of the enzyme in our preparation lies within the range of those reported for intestinal, renal and tracheal brush borders (8.3–165.0 μmol per mg protein) for a variety of species [26,29–31].

The apical membranes showed little enrichment (3.6-fold) of Na^+/K^+-ATPase. Our observation that Na^+ uptake by vesicles is not inhibited by ouabain (10^{-4} M) is consistent with our view that the Na^+/K^+-ATPase activity in the apical membrane fraction is functionally insignificant. A 2–5-fold enrichment of Na^+/K^+-ATPase activity has been considered acceptable in renal epithelia in which the enrichment of alkaline phosphatase (10–15-fold) was lower than in the present study [29,31–33]. The lack of appreciable contamination of the final fraction by intracellular organnelles is borne out by the absence or low activity of succinate dehydrogenase (a mitochondrial marker) and NADH dehydrogenase (an endoplasmic reticulum marker) and by the low DNA content.

Therefore, with respect to several criteria, the isolation procedure employed here yields purified apical membrane vesicles which compare favourably with luminal preparations from a variety of other epithelia.

Na^+ transport studies

This study has established the existence of Na^+ uptake across the apical membranes of fetal sheep type II alveolar cells. That our Na^+ uptake measurements represent transport into a membrane limited space is shown by the linear inverse relationship between medium osmolarity and Na^+ uptake. Further evidence in support of Na^+ entry into vesicles was provided by experiments in which accumulated Na^+ was shown to be released following disruption of the vesicular structure with the detergent Triton X-100 (0.1%).

Extrapolation of uptake to infinite osmolarity, or vesicle disruption with detergent indicates that, at equilibrium, a significant portion (48–49%) of uptake represents binding. Although high, this proportion of membrane binding at equilibrium is not unique to our preparation. In rat colon Na^+ binding to the membrane is 50–60% of total uptake at equilibrium [34].

Na^+ uptake was relatively insensitive to the diuretic amiloride as is the case in the resting fetal sheep lung in vivo [8].

The rate of Na^+ uptake varied with the anion in solution with a rank order of $SCN^- > Cl^- >$ gluconate$^-$ during the initial period of observation. While Na^+ uptake at 60 minutes was similar for SCN^- and Cl^-,

that for gluconate$^-$ was considerably lower. These findings are broadly in agreement with the observations of Langridge-Smith et al. in tracheal epithelium [14]. Explanations for the relatively low Na^+ uptake at 60 min in the presence of gluconate$^-$ include the possibilities that (1) Na^+ uptake is not at equilibrium at 60 minutes; (2) there is an altered Donnan equilibrium effect. A further possibility, that vesicle volume is diminished due to the different ionic composition of the incubation medium, has not been observed in vesicles derived from tracheal epithelium [14].

Taken together, the results indicate that Na^+ transport across the walls of apical membrane vesicles prepared from resting (unstimulated) mature fetal lungs occurs via pathways of molecular dimensions. The results show no evidence for any substantial Na^+ transport via specific conductive channels since amiloride, in micromolar concentrations, was without effect. The finding of amiloride inhibition only at high (10^{-3} M) concentration could be due to a non-specific effect but preliminary data [35] indicate blocking of a Na^+/H^+ antiport. This phenomenon will be the subject of further experiments.

In conclusion, the membrane isolation procedure described in this paper yields highly purified, efficiently transporting apical membrane vesicles from alveolar type II cells. These purified membrane vesicles provide a new and simplified approach with which to characterise the mechanism and intracellular regulation of alveolar epithelial transport.

Acknowledgements

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