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Sodium-proton exchange across the apical membrane of the alveolar type II cell of the fetal sheep

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In order to detect and characterise Na^+-H^+ countertransport in the fetal lung epithelium we have studied under a variety of conditions the effect of an outward facing H^+ gradient on Na^+ uptake into purified apical membrane vesicles prepared from alveolar type II cells. Kinetic analysis of the data reveals both a diffusional and a saturable component of total Na^+ uptake. Evidence for the presence of a Na^+-H^+ exchanger is demonstrated by (1) stimulation of Na^+ uptake by proton loading of vesicles both in the presence and absence of chemical voltage clamping; (2) saturation kinetics with respect to external Na^+ with a K_m of 16 mM and a V_{max} of 2.1 nmol / mg protein per min; (3) amiloride inhibition of Na^+ uptake driven by pH gradient. We conclude that although diffusion may be the major component of total Na^+ uptake at physiological external Na^+ concentration, Na^+-H^+ countertransport provides a possible mechanism for the acidification of fetal lung liquid in-vivo in addition to its established role in intracellular pH and volume regulation.

Introduction

The lung of the mammalian fetus secretes a specialised liquid which acts as a template around which developing airspaces grow [1,2]. By virtue of its low bicarbonate content [3], lung liquid pH is low relative to plasma (6.3 vs. 7.4). The concentrations of HCO_3^- across the fetal lung epithelium are far removed from equilibrium and after experimental elevation of lung liquid bicarbonate its concentration in the alveolar space is driven down to baseline levels (2–3 mM) in the absence of appropriate transepithelial passive forces [1]. These findings are consistent with active bicarbonate extrusion from the lung lumen or proton secretion into lung liquid involving ion transporters in the apical and basolateral membranes of alveolar epithelial cells.

The recent development in this laboratory of a preparation of apical plasma membrane vesicles derived from fetal alveolar type II cells [4] has made it feasible to address the problem of alveolar pH regulation directly. Using this approach we describe here a study which demonstrates, for the first time, the presence of a Na^+-H^+ exchanger in the alveolar apical membrane. We speculate that in addition to the well established cell homeostatic functions of such a transporter, its particular importance in the developing lung may be its permissive role in cellular proliferation.

A preliminary account of this work was given at the meeting of the Physiological Society in Aberdeen, U.K., September 1988.

Methods and Materials

Surgical procedures

Twin fetal lambs, the product of Grey Face Ewes/Suffolk Tups, were studied at 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 2–3 min, 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 2–2.5 litres of warm balanced salt solution (BSS) with the following composition: 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 at pH 7.4.

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Cell isolation and purification of apical membranes

The lungs were excised to allow harvesting of type II cells and purification of apical membrane vesicles according to published methods [5–9] adapted in this laboratory [4]. In the experiments reported here the proteolytic solution instilled into the lung consisted of 500 ml BSS containing 0.09 mg/ml elastase (12 orcein units/ml) and 0.03 mg/ml trypsin (37 BAEE units/ml) at 37°C. After 20 min incubation, proteolytic digestion was terminated by addition of soybean trypsin inhibitor (30 000 BAEE units) in 15 ml fetal calf serum containing DNAase (72 biuret units per ml). Isolation of type II cells from lung mince and apical membrane purification by differential centrifugation/$MgCl_2$ precipitation were carried out as previously described [4]. This technique yields a final membrane fraction which is essentially free of nuclear, mitochondrial and endoplasmic reticulum contamination as judged by DNA, succinate dehydrogenase and NADH dehydrogenase activity. The activity of Na^+/K^+-ATPase is 3–4-times that of the crude homogenate indicating the presence of a relatively small component of basolateral membrane material in the vesicle preparation. Total apical membrane yield averaged 2 mg protein per lung and alkaline phosphatase, the brush border marker, was enriched by 18–24-fold. Vesicles were formed by resuspending the membranes in the desired intravesicular medium by repeated passage through a 26 gauge needle (for pH 7.5: 86 mM mannitol, 6 mM Mes, 10 mM Hepes, 13 mM Tris; for pH 5.5: 73 mM mannitol, 30 mM Mes, 14 mM Hepes and 18 mM Tris).

Transport measurements

As before [4] we used Dowex 50W-X8 (50–100 mesh, Tris form) columns to assay ^{22}Na influx according to the method of Gasko et al. [10]. With the exception of chemical voltage clamping and Na^+ saturation experiments (see below) ^{22}Na uptake was measured after a timed period of incubation in a medium identical to that described above for an intravesicular pH of 7.5 with the addition of 1 mM $^{22}NaSCN$. 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 s. Chemical voltage clamping of vesicles was achieved by adding potassium gluconate (10 mM) and valinomycin (10 μM) to the intravesicular (pH 5.5) and incubation (pH 7.5) media. In those experiments designed to examine the kinetics of the exchanger with respect to external Na^+, the incubation medium was modified by addition of NaCl with intra- and extravesicular osmolarity maintained at 350 mosmol/l by manipulation of D-mannitol concentration. Non-specific binding of ^{22}Na was determined by measuring the radioactivity associated with vesicles disrupted with 0.1% Triton X-100. Specific uptake (total uptake less bound ^{22}Na) was measured as pmol or nmol Na^+ transported per mg protein. Zero time points were obtained from matched incubation media at 0°C, all other uptake measurements were performed at room temperature. Typically 24 data points could be obtained from vesicles prepared from a single lung. Each uptake measurement was performed in triplicate.

Curve fitting

By means of a computer programme (Multifit [11]) using an iterative non-linear regression technique, the diffusional and saturable components for Na^+ transport were determined by calculation of the best fit of the data to the kinetic equation:

$$y = \frac{(K_1 \cdot x) + (K_3 \cdot x)}{(K_2 + x)}$$

where y = rate of specific Na^+ uptake, x = external Na^+ concentration, K_1 = maximum rate of the saturable component of Na^+ uptake, K_2 = Michaelis-Menten constant and K_3 = apparent permeability coefficient. Reworking the data using estimated Na^+ activity [12] rather than concentration does not significantly alter the calculated parameters of the saturable component of transport but increases the apparent permeability coefficient by a third. For convenience, and because of the difficulty of ascribing an accurate activity coefficient to Na^+ in a complex buffer solution, we have used concentration throughout.

Materials

Sources of supply were as follows-Sigma: amiloride, valinomycin, trypsin, trypsin inhibiter, DNAase, Hepes, Mes, Trizma Base, Dowex. Gibco: fetal calf serum. Elastin Products Company Inc., Missouri: elastase. Amersham: ^{22}Na. British Drug Houses: all standard laboratory chemicals (analar grade).

Results

Effect of outward facing proton gradient on Na^+ uptake

In order to detect Na^+-H^+ exchange activity we provided a thermodynamic driving force for Na^+ uptake in the form of an outward facing proton gradient. Fig. 1 shows that the initial uptake of ^{22}Na from the incubation medium (pH 7.5) into vesicles pre-loaded with protons (pH 5.5) was markedly enhanced when compared to controls (no gradient, internal and external pH 7.5).

In no single experiment was an overshoot of Na^+ uptake above equilibrium seen at any time point up to 60 min. Chemical voltage clamping of proton loaded

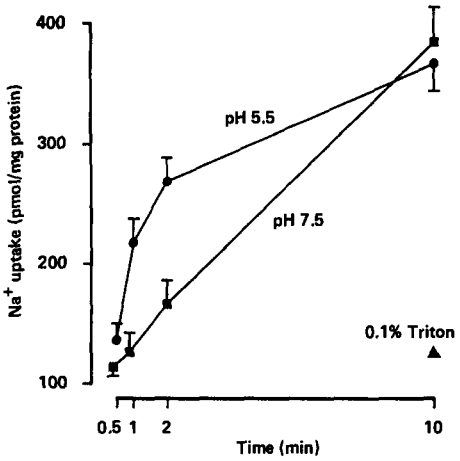

Fig. 1. Time dependent uptake of $^{22}Na^+$ by vesicles with and without an outward facing proton gradient. Vesicles were equilibrated with the appropriate medium (see Methods) to give an intravesicular pH of 7.5 or 5.5. Na^+ uptake was initiated by the addition of vesicles to incubation medium (pH 7.5) containing $^{22}NaSCN$ (1 mM). The uptake was terminated at the times shown. Each time point represents the means ± S.E. from 6–10 experiments conducted in triplicate. Total uptake in the presence of a proton gradient is significantly greater than control at 1 and 2 min ($P < 0.01$, paired t-test). Uptake at 60 min averaged (± S.E.) 810 ± 180 pmol/mg protein per min (with pH gradient) and 790 ± 194 pmol/mg protein per min (without pH gradient). Residual uptake after Triton X-100 represents membrane bound $^{22}Na^+$.

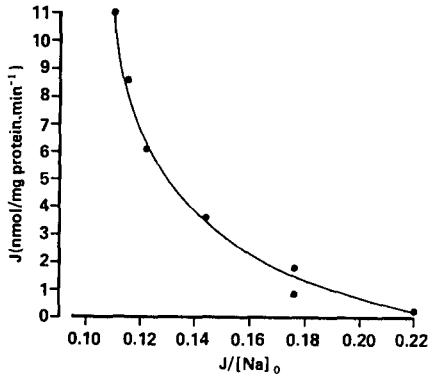

Fig. 2. Eadie-Hofstee plot of sodium kinetics in the presence of a 2 unit outward facing proton gradient. One min specific Na^+ uptake (J) was measured as a function of external Na^+ concentration ($[Na]_o$) between 1 mM and 100 mM at a constant osmolarity of 350 mosmol/l (specific uptake defined as total uptake of $^{22}Na^+$ minus membrane-bound $^{22}Na^+$). In this plot the diffusional flux is a vertical line with an intercept on the abcissa giving the apparent permeability coefficient. The curvilinear format indicates more than one transport process. Curve fitting according to a two-component model as described in Methods and Materials gives a saturable component with a K_m of 16 mM and a maximum rate of saturable flux, J_{max} (V_{max}), of 2.1 nmol/mg protein per min plus a diffusional flux with an apparent permeability coefficient of 0.09 nl/mg protein per min. Each point represents the mean of four experiments conducted in triplicate.

vesicles at zero PD failed to show any evidence that Na^+ uptake was driven by vesicle membrane potential. In three experiments the uptake of Na^+ in the presence of valinomycin and potassium at 1 and 2 min averaged 139 and 173 pmol/mg protein, respectively. Control values (pH gradient but no ionophore or K^+) averaged 125 and 142 pmol/mg protein, respectively.

Kinetics of Na^+ uptake

Increasing the Na^+ concentration of the incubation medium resulted in an increase in Na^+ uptake into proton loaded vesicles. Mean values ± S.E. of specific Na^+ uptake (nmol/mg protein per min) in four experiments, with Na^+ concentration in parentheses, were as follows: 0.22 ± 0.02 (1 mM), 0.88 ± 0.15 (5 mM), 1.76 ± 0.14 (10 mM), 3.6 ± 0.28 (25 mM), 6.10 ± 0.31 (50 mM), 8.60 ± 0.64 (75 mM), 10.96 ± 0.98 (100 mM). The pattern of uptake suggested the operation of more than one transport process (Fig. 2). Kinetic analysis of the data according to a model comprising a single exchanger plus a diffusional component (see Methods and Materials) yielded the following kinetic parameters (± S.E.): K_m 16 ± 4.6 mM, V_{max} 2.1 ± 0.37 nmol/mg protein per min and an apparent diffusion coefficient of 0.09 ± 0.0029 nl/mg protein per min. Extrapolation of these data to the external Na^+ concentration prevailing in the fetal lung in vivo (150 mM) suggests that exchanger mediated Na^+ flux is likely to be small in relation to the diffusional component (2.1 vs. 13.5 nmol/mg protein per min).

Effect of amiloride on Na^+-H^+ exchange

The pyrazine diuretic amiloride is a known competitive inhibitor of Na^+-H^+ exchange acting at or near the external Na^+ binding site. In the presence of an external sodium concentration of 1 mM and a 2 unit pH gradient (conditions designed to optimise exchanger

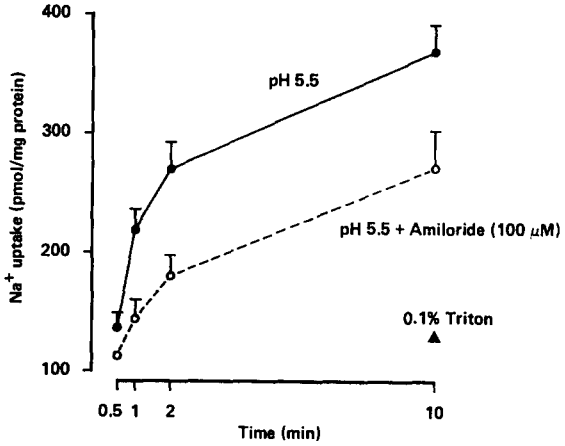

Fig. 3. Time dependent total uptake of $^{22}Na^+$ by vesicles in the presence of an outward facing proton gradient with and without amiloride. Vesicles were added to incubation medium (pH 7.5) containing $^{22}NaSCN$ (1 mM) ± amiloride (100 μM). Uptake was terminated at the times shown. Each time point represents the mean values for total uptake of $^{22}Na^+$ ± S.E. from 3–6 experiments conducted in triplicate.

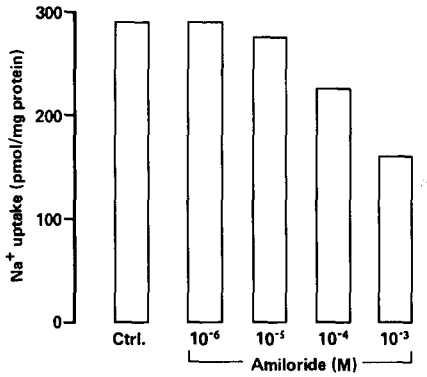

Fig. 4. Effect of increasing concentrations of amiloride on specific uptake of $^{22}Na^+$ by vesicles in the presence of an outward facing proton gradient (specific uptake defined as total uptake of $^{22}Na^+$ minus membrane bound $^{22}Na^+$). Vesicles were added to incubation medium (pH 7.5) containing $^{22}NaSCN$ (1 mM) and amiloride at the concentrations shown. Uptake was terminated after 1 min. The results are the means from triplicate values and were similar to results obtained in other sets of experiments. A Hill plot of the data yields a value for K_i of 100 μM.

mediated transport (60%) in relation to diffusional flux (40%)), 10^{-4} M amiloride reduced total Na^+ uptake by 32% at 1 min and 30% at 2 min (Fig. 3). Inhibition was dose-dependent and kinetic analysis by means of a Hill plot of the data in Fig. 4 yields a value for K_i of $1.0 \cdot 10^{-4}$.

Discussion

The Na^+-H^+ exchanger is an ubiquitous transporter found in the plasma membrane of a variety of cells including the isolated alveolar type II cell of the adult rat [13]. Since the exchanger has been described in basolateral as well as apical cell membranes, data on Na^+-H^+ exchange from isolated cells with asymmetrical plasma membrane properties in vivo can pose problems of interpretation.

The evidence for a Na^+-H^+ exchanger located in the apical membrane of the fetal alveolar type II cell can be summarised as follows:

(1) An outward facing proton gradient stimulates Na^+ uptake both in the presence and absence of chemical voltage clamping.

(2) Na^+ uptake has a saturable component showing kinetics similar to other epithelial Na^+-H^+ exchangers.

(3) Na^+ uptake driven by pH gradient is inhibited by amiloride, a known competitive inhibitor of Na^+-H^+ exchangers.

Effect of proton gradient on Na^+ uptake

Our data clearly demonstrate that proton loading of vesicles leads to an increase in the rate of Na^+ uptake at the early time points without an overshoot above equilibrium (this is also true of vesicles prepared from adult sheep lung, unpublished observation). Although overshoot is frequently taken as a hallmark of Na^+-H^+ countertransport it is by no means universal [14,15]. Possible explanations for its absence include the existence of leak pathways for Na^+ and H^+ which would tend to speed up the dissipation of the H^+ gradient and mask proton driven Na^+ uptake by the transporter. Analysis of the Na^+ uptake kinetics indicates that there exists a substantial diffusive pathway for Na^+. If there is significant diffusional efflux of H^+, there is no evidence that the PD thereby created promotes Na^+ uptake since voltage clamping at zero PD with valinomycin and potassium does not reduce Na^+ uptake (if anything there is a tendency for it to rise).

Kinetics of Na^+ saturation and amiloride blocking

The data are consistent with a model comprising a single exchanger plus a diffusional flux. The calculated K_m for external Na^+ of 16 mM is within the range reported for a variety of epithelial cells including gall bladder, intestine and kidney [16–19] but lower than that found in adult rat type II cells (62 mM) which is more akin to values described for non-epithelial cells [20]. With a K_m of this magnitude the exchanger in the adult rat is unlikely to be saturated with respect to external Na^+ under physiological conditions (see below). This decreased affinity for Na^+ at the external binding site of the adult type II cell may be a normal maturational change but equally it may represent an artefact due to the presence of other Na^+ transport pathways in the intact type II cell which are either absent or inactive in the apical membrane vesicle preparation.

Proton-driven Na^+ uptake is substantially inhibited by 10^{-4} M amiloride, a concentration previously shown by us to be without inhibitory effect in the absence of a pH gradient [4]. 10^{-4} M amiloride would appear to approximate to the K_i for the exchanger (Fig. 4) and is close to values reported for brush border vesicles prepared from upper small intestine [17], the organ from which the lung is embryologically derived.

Functional role of the Na^+-H^+ exchanger

The thermodynamic driving force for H^+ exchange is the energy of the inwardly directed chemical Na^+ gradient which results from the activity of Na^+-K^+ ATPase at the basolateral membrane. The exchanger has three major interrelated functions which vary in importance depending on cell type (see Refs. 21 and 22 for reviews). First, it regulates intracellular pH and thereby regulates those cellular metabolic processes which are dependent on intracellular pH. Second, Na^+-H^+ exchange may bring about net transepithelial transport of ions. Third, it acts as a cell entry mechanism for Na^+ and thereby has important consequences for cell volume regulation. Of these, the first two may be of special importance in the fetal lung.

The apical (luminal) membrane of the fetal alveolar type II cell in vivo is bathed by lung liquid containing Na^+ at a concentration of 150 mM [3]. At this level the Na^+-H^+ exchanger can be expected to be saturated with respect to Na^+ and it is likely that the activity of the exchanger is regulated principally by intracellular pH. In a variety of cells it has been demonstrated that the activity of the exchanger exhibits greater than first order dependence on internal H^+ concentration and that there is a set point below which the exchanger becomes quiescent. In the adult rat type II cell [13] the set point appears to be around physiological intracellular pH (7.07) which implies that the exchanger is able to dissipate an acid load but is not otherwise active. However, in the majority of epithelia studied, Na^+-H^+ countertransport occurs at physiological pH (i.e. the threshold is set lower). The set point is not invariant and can be shifted upwards or downwards by a variety of hormones, growth factors and intracellular messengers. Of these, epidermal growth factor (EGF), thyroid and steroid hormones have particular relevance to the developing lung, being powerful stimuli for differentiation and maturation – both morphological and functional [23–27]. These factors are mitogenic in a variety of cell types and have been shown to act, at least in part, by elevating intracellular pH to a level permissive for DNA synthesis and cell growth [28]. Whether this is true of the developing fetal lung remains to be determined.

We have demonstrated that the apical membrane of the fetal alveolar type II cell possesses a mechanism capable of acidifying lung liquid. If, in a manner analagous to that described for the proximal renal tubule [29], protons generated from a CO_2/HCO_3^- buffer system within the cell are extruded by the Na^+- H^+ exchanger they will react with HCO_3^- to yield CO_2 and H_2O. The overall effect will be net absorption of $NaHCO_3$. Although intracellular pH appears to be the principal regulator of Na^+-H^+ exchanger activity, lung liquid [H^+] may be expected to exert an inhibiting effect on the exchanger – competing with Na^+at the external binding site, reducing Na^+-H^+ exchange in the forward mode and so preventing an overshoot in lung liquid acidification.

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