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Determination of pathways for sodium movement across corneal endothelial cell derived plasma membrane vesicles

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Abstract

A bovine corneal endothelial cell plasma membrane vesicle preparation was used to investigate passive Na^+ transport across the plasma membrane of these cells. Sodium accumulation rate into the vesicle was not dependent on the presence of HCO_3^- or a HCO_3^- gradient, but was stimulated by a trans-vesicle pH gradient. Amiloride, furosemide and DIDS all reduced the rate of Na^+ accumulation. The data indicate the presence of at least two independent pathways for passive sodium movement across the vesicle: the first probably via a Na^+/H^+ exchanger and the second a furosemide inhibitable Na^+ entry mechanism. No evidence was found for direct Na^+-HCO_3^- coupled transport.

Keywords: Corneal endothelium; Bicarbonate transport; Sodium transport; Membrane vesicle; Drug effect

1. Introduction

Corneal hydration and transparency are regulated by a metabolically active transport process located in the endothelial cell monolayer that covers the posterior surface of the cornea [1–3].

Inhibition of endothelial Na^+/K^+-ATPase by ouabain causes complete inhibition of the endothelial transport process [4,5]. This taken together with the baso-lateral location of the Na^+/K^+-ATPase [6] and the absence of a net trans-endothelial Na^+ flux when the endothelium is short-circuited [7], has led to the suggestion that in some way Na^+ gradients across the basolateral membrane are used to drive the physiologically important net transendothelial HCO_3^- flux [8].

A number of models have been proposed to explain the Na^+/HCO_3^- coupling process but none is yet agreed. It seems likely that part of the difficulty in reaching an unambiguous interpretation of ion coupling mechanisms across endothelial cell membranes is associated with working on whole cells, where the complexities of multiple pathways have been suggested [9–11].

To resolve further the transport pathways present in the plasma membranes of these cells and to gain more insight into coupling mechanisms between the ions, we made membrane vesicles from enriched plasma membrane fractions taken from bovine corneal endothelial cells. By controlling the internal and external environments of the vesicles we were able to monitor the factors that regulate rates of ion entry, in particular Na^+, across the membranes. Although no previous study of this type has been reported to date on corneal endothelium, similar studies on renal and hepatic tissues have proved useful in the elucidation of ion coupling mechanisms [12–15].

2. Materials and methods

2.1. Reagents and materials

Percoll was obtained from Pharmacia LKB, Uppsala, Sweden; membrane filters from Millipore UK; radioisotopes from Amersham, UK; Ecoscint from National Diagnostics, USA. Amiloride, furosemide, DIDS, sorbitol, and all other reagents except where noted in the text were obtained from Sigma, Poole, UK.

2.2. Membrane vesicle preparation

Fresh bovine eyes were obtained from a local abattoir 2–4 h post-mortem. The eyes were transported corneal

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side up in an ice-packed cooler box, then refrigerated at 4°C for use within 24 h.

Corneal endothelial cell plasma membranes were isolated using self-forming Percoll density gradients under isopycnic centrifugation [16]. When viewed in transverse light against a black background, three distinct light scattering bands were visible in the Percoll gradient, previously identified as; band 1, the nuclear enriched fraction; band 2, the plasma membrane enriched fraction and band 3, the mitochondrial enriched fraction [16]. Purity of the plasma membrane fraction used in these experiments was determined by assay of Na^+/K^+-ATPase activity in the various fractions. Protein in the fractions was measured by the method of Bradford [17], using bovine serum albumin as a standard. Table 1 shows the considerable enrichment of Na^+/K^+-ATPase activity in the plasma membrane fraction (band 2) used in these experiments.

The plasma membrane fraction was then centrifuged at 35 000 rpm ($90\,000 \times g_{avg}$; $118\,000 \times g_{max}$) for 1 h at 4°C in a fixed angle rotor to separate out the Percoll and eliminate problems of precipitation between Percoll and Coomassie blue in the protein assay (a problem identified subsequent to the work described in [16]).

The plasma membrane fraction (which sedimented as a soft layer above the Percoll pellet), plus a minimal amount of supernatant was diluted with a buffered sorbitol solution to give a final composition of (mM); 250 sorbitol, 0.1 $CaCl_2$, 10 $MgCl_2$, 10 Hepes, pH set at 7.5 with KOH. Plasma membrane vesicles were formed by passing the membrane suspension four times through a 19G hypodermic needle. The vesicle suspension was stored on ice with aliquots taken as required and pre-incubated at 25°C.

The homogeneity of the membrane vesicles was assessed by scanning electron microscopy. Vesicles captured on nitrocellulose membrane filters (0.45 μm pore size) were fixed in 2.5% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in ethanol and critical point dried before viewing. Mean vesicle diameter was 0.68 ± 0.02 μm, $n = 15$.

Vesicle integrity was checked by measurement of radiolabelled L-alanine uptake into vesicles prepared from bovine corneal endothelial and hepatic cell plasma membranes, in the presence of Na^+ or K^+ containing solutions [18].

2.3. Na^+ transport in the presence of an inward HCO_3^- gradient

The technique of rapid microfiltration through a nitrocellulose membrane filter was used to measure solute trapped in the vesicles [12–14]. To eliminate problems with maintaining pH in these HCO_3^- containing solutions (the tendency to become progressively alkaline when exposed to air due to loss of CO_2), high buffer concentrations were used. This strategy was effective in the short time scale used here. The extravesicular medium contained 50 mM Tris and Mops and solutions were used within 30 min of preparation.

To initiate the transport reaction, 10 μl of vesicle suspension and 10 μl of radio-labelled substrate were mixed in a water bath at 25°C to give a final composition of (mM); 250 sorbitol, 10 $MgCl_2$, 0.1 $CaCl_2$, 25 $KHCO_3$, 0.2 $^{22}NaCl$ (activity = 300 cpm/pmol), 10 Hepes, 50 Trizma base, 50 Mops, at pH 7.5. The reaction was terminated by adding 1 ml of ice-cold buffered sorbitol (stop) solution containing (mM); 250 sorbitol, 120 mM sodium gluconate, 10 $MgCl_2$, 0.1 $CaCl_2$, 10 Hepes, 10 Trizma base at pH 7.5. The suspension was immediately filtered through a pre-wetted membrane filter (HAWP 02500, 0.45 μm pore size) which was washed three times with 1 ml of stop solution, removed and placed in 10 ml of scintillation fluid (Ecoscint). After approx. 30 min (to allow the filter to dissolve) the activity of the solution was measured in a liquid scintillation counter. The activities were used to calculate uptake per mg protein. All Na^+ uptake experiments were performed in triplicate at 25°C.

The $KHCO_3$ in the external solution produced the inwardly directed HCO_3^- gradient to enable the detection of any HCO_3^- dependent $^{22}Na^+$ uptake. Bicarbonate independent $^{22}Na^+$ uptake was determined by substituting $KHCO_3$ with potassium cyclamate (cyclamate) or potassium thiocyanate (thiocyanate). Non-specific binding of $^{22}Na^+$ to the membranes and filters was estimated by exposing the vesicles to ice-cold hypotonic medium at time zero. Buffered sorbitol and $^{22}NaCl$ solutions were mixed and diluted with 1 ml of ice-cold de-ionised water. The vesicle suspension (10 μl) was then added and the mixture filtered and washed with the stopping solution as described earlier.

Table 1
Total protein and Na^+/K^+-ATPase activity in bovine corneal endothelial cell homogenate and sub-cellular fractions isolated on a Percoll self forming density gradient

Particulate fraction [16]	Total protein (mg)	Na^+/K^+-ATPase activity (μmol P_i per mg protein per h)
Homogenate	0.732 ± 0.012	8.16 ± 1.3
Supernatant	0.505 ± 0.016	1.02 ± 0.28
Nuclear rich	0.023 ± 0.002	10.91 ± 1.73
Plasma membrane rich	0.074 ± 0.008	31.17 ± 3.44
Mitochondrial rich	0.024 ± 0.002	4.02 ± 1.24

Values are the mean ± S.E., $n = 6$.

2.4. Sodium transport in the presence of an inside-acid gradient

The effect of an inside-acid gradient on ^{22}Na uptake was determined in experiments where vesicles were formed in solutions at pH 6.0. Extravesicular pH was 7.5 as before. Intravesicular buffering was increased in these experiments by the addition of 50 mM Mops and Tris to the membrane solution. The purpose of these studies was to (a) maintain trans-membrane HCO_3^- gradients in the presence of the ubiquitous trans-membrane CO_2 equilibration and (b) to look for pH dependent Na^+ fluxes.

2.5. Inhibition of traffic proteins

The rate of accumulation of Na^+ inside the vesicles was determined in the presence of the traffic protein inhibitors; amiloride (with and without a pH gradient across the vesicle), furosemide and DIDS, all at 1 mM in both intra and extra-vesicular solutions.

3. Results

3.1. Viability

The ionic dependence of L-alanine accumulation was used to check the vibility of the vesicles. The ratio of uptake in Na^+ solution compared to K^+ after 30 s incubation was 2.6 for the hepatic vesicles and 1.8 for the endothelial vesicles.

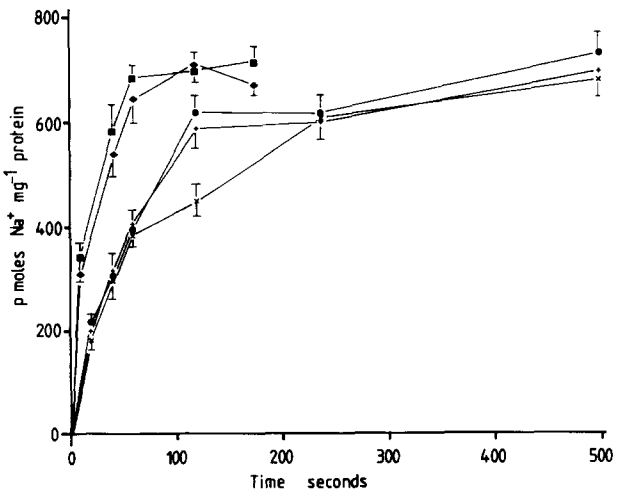

Fig. 1. Accumulation rate of $^{22}Na^+$ into corneal endothelial cell plasma membrane derived vesicles. The lower three curves demonstrate uptake where anion gradients across the vesicle were provided by cyclamate (●–●), bicarbonate (+–+) and thiocyanate (×–×). The upper two curves demonstrate Na^+ accumulation with a pH gradient across the vesicle ($pH_o = 7.5$, $pH_i = 6.0$) and with bicarbonate (◆–◆) and thiocyanate (■–■) providing the anion gradient.

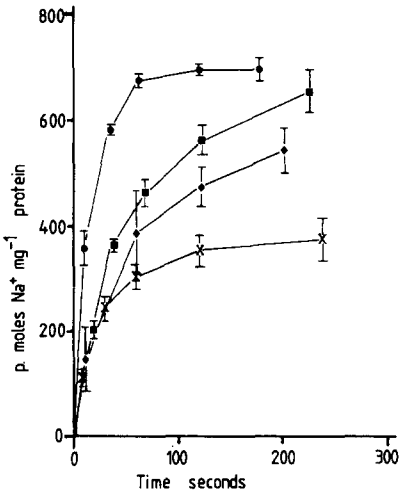

Fig. 2. Accumulation rate of $^{22}Na^+$ into corneal endothelial cell plasma membrane derived vesicles, with HCO_3^- providing the anion gradient and in the presence of 1 mM amiloride. Control uptake with no pH gradient (■–■), with a pH gradient ($pH_o = 7.5$, $pH_i = 6.0$; ●–●). Uptake in the presence of 1 mM amiloride with no pH gradient (×–×), and with a pH gradient (◆–◆).

3.2. Mean free equilibrium concentrations

The equilibrium concentration inside our vesicle calculated from final $^{22}Na^+$ uptake was in the range 500–700 pmol Na^+ per mg protein. The variation in this figure between batches was, we believe, due to differences in mean vesicle size (Lane, Hodson and Wigham, unpublished data). Typical protein concentration in the vesicle samples was 0.2 mg ml^{-1}.

3.3. Anion dependence

The rate of accumulation of Na^+ into the vesicles was not dependent upon the nature of the accompanying anion.

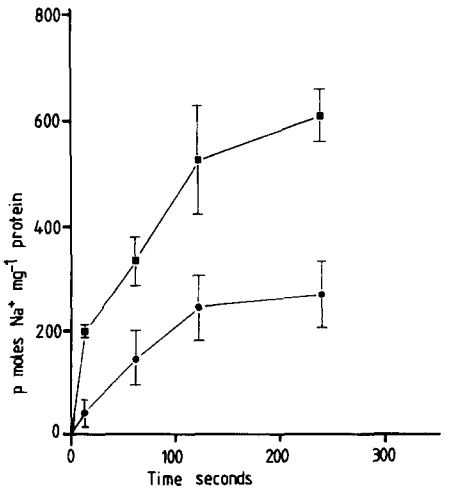

Fig. 3. Accumulation of $^{22}Na^+$ into corneal endothelial cell plasma membrane derived vesicles, with HCO_3^- providing the anion gradient and in the presence of 1 mM furosemide. Control uptake (■–■), uptake in the presence of 1 mM furosemide (●–●).

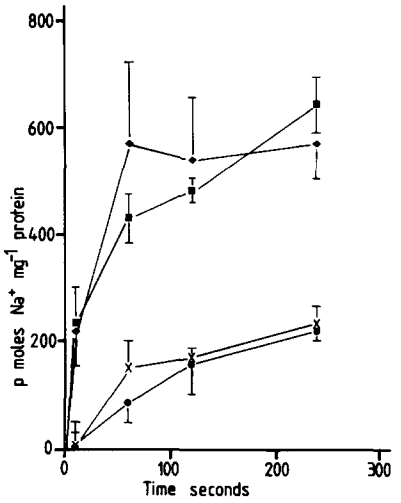

Fig. 4. Accumulation of $^{22}Na^+$ into corneal endothelial cell plasma membrane derived vesicles with HCO_3^- providing the anion gradient in the presence of DIDS and DIDS + amiloride + furosemide. The curves show the effect of 1 mM DIDS (×–×) and its control (■–■) and DIDS + amiloride + furosamide (1 mM of each; ●–●) and its control (◆–◆).

Accumulation rates were not significantly different with bicarbonate or cyclamate or thiocyanate. The presence of a pH gradient across the vesicle did not affect this relationship, (Fig. 1). A pH gradient ($pH_o = 7.5$, $pH_i = 6.0$) did however increase the rate of Na^+ entry into the vesicles; from Fig. 1, the half-time of entry ($t_{1/2}$) without a pH gradient is 47.5 ± 3 s (mean ± S.E., $n = 4$, separate vesicle preparations) with a pH gradient of this magnitude $t_{1/2} = 10 \pm 2$ s (mean ± S.E., $n = 4$).

3.4. Inhibitor effects

In the presence of 1 mM amiloride Na^+ accumulation into the vesicles was slowed. This effect occurred with and without a pH gradient across the vesicle (Fig. 2). The reduction in accumulation rate was significant (at 10 s, $P < 0.01$, Student's t-test).

The addition of 1 mM furosemide also reduced the rate of Na^+ accumulation (Fig. 3).

The addition of 1 mM DIDS caused an apparently greater reduction in Na^+ entry rate than either furosemide or amiloride alone. All three inhibitors together caused no further reduction in the accumulation rate than with DIDS alone (Fig. 4). It was interesting to note that DIDS or a combination of inhibitors including DIDS, reduced Na^+ entry in the first 10 s to a level not significantly different from zero.

4. Discussion

It has not yet been possible to isolate apical and basolateral membranes from corneal endothelial cells, however stereological analysis shows that approx. 2/3 of the plasma membranes are basolateral [16]. Probably the major proportion of the plasma membrane vesicles used in this study would be of basolateral origin, but there would also be apical membrane present. The Na^+ dependent alanine uptake shows that a proportion of the vesicles possess their normal orientation and the procedures we adopted did not cause excessive osmotic disruption of the vesicles. The technique is useful for identifying qualitatively, pathways for Na^+ entry, but confidence on quantitative assessment could only be placed upon relative values and not upon absolute values.

4.1. Anion specificity

The rate of Na^+ accumulation into these vesicles was found to be independent of the nature of the anion.

This result was surprising, firstly because a Na^+/HCO_3^- coupling mechanism has been proposed in these cells [9,19] and secondly because thiocyanate and cyclamate are unable to substitute for HCO_3^- in maintaining corneal transparency. An ultra-rapid passage of CO_2 molecules into the vesicle followed by conversion to HCO_3^- could have negated the HCO_3^- gradient, however this possibility was eliminated by using a pH gradient to buffer intravesicular [HCO_3^-]. Even under these conditions Na^+ accumulation rate was independent of the nature of the anion. These findings were contrary to our expectations, but reproducible. Rate of accumulation of Na^+ into corneal endothelial cell membrane vesicles was independent of the presence of HCO_3^- or a HCO_3^- gradient. Whilst the pH gradient ($pH_o > pH_i$) promoted the passage of Na^+ into the vesicles, the effect was again independent of the nature of the anion.

The above data seems inconsistent with the concept of a direct Na^+-HCO_3^- coupling traffic protein acting in corneal endothelial cells. Similar experiments conducted in this laboratory with hepatic and renal cell plasma membrane vesicles produced a pattern of Na^+ accumulation (down a HCO_3^- gradient) where the concentration inside the vesicle overshot the eventual equilibrium value. This phenomena has been interpreted as a demonstration of Na^+-HCO_3^- co-transporter activity [20]. The lack of overshoot with Na^+ accumulation into corneal endothelial derived vesicles seems, we believe, to be further evidence against Na^+-HCO_3^- coupling.

4.2. Amiloride effect

The addition of amiloride reduced the rate of accumulation of Na^+. We conclude from this that a component of Na^+ flux into the vesicles is via a Na^+/H^+ antiport. Stimulation of accumulation rate by a Na^+ or H^+ gradient (in the appropriate orientation) we believe to be due to increased Na^+/H^+ antiport activity. The H^+ dependence and absence of effect on uptake rate with μM concentra-

tions of amiloride (even after prolonged pre-incubation), suggests that amiloride is acting on the antiport rather than a Na^+ channel.

4.3. Effect of furosemide

When we added furosemide to the preparation we noted a significant reduction in Na^+ accumulation rate. The data suggests a second (independent) route for passive Na^+ entry into the plasma membrane vesicle.

4.4. DIDS effect

As a consequence of the amiloride and furosemide inhibitions we were interested to see if we could impose a condition whereby Na^+ entry into the vesicles was completely inhibited. Exposure to DIDS prevented Na^+ entry for the first 10 s, followed by a slow accumulation ($t_{1/2}$ approx. 600 s, whereas in control experiments $t_{1/2}$ was 50 s). The accumulation rate in the presence of DIDS was slower than in the presence of amiloride or furosemide.

A combination of amiloride, furosemide and DIDS provided a pattern of effect similar to DIDS alone. Our inability to completely inhibit Na^+ entry into the vesicles over a prolonged period suggests that the vesicles may possess leak pathways, or that low permeability pathways (for Na^+) not affected by this selection of inhibitors are present.

If DIDS inhibits most, possibly all, of the passive (non-leak) Na^+ entry into the vesicles (the combination of transport blockers produced no more inhibition than DIDS alone), it is unlikely that DIDS is acting specifically on the putative Na^+-HCO_3^- co-transporter as has been claimed in the literature [10,21] because the results presented here show DIDS inhibition of Na^+ into the vesicles but no evidence of direct Na^+/HCO_3^- transport.

Our evidence indicates that endothelial plasma membranes (basolateral) possess at least two routes for passive Na^+ entry. The first an amiloride inhibitable Na^+-H^+ exchanger, also suggested by other authors using different techniques [11,21]. The second involves a Na^+ entry mechanism which is inhibited by furosemide.

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