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Demonstration of a Na^+/H^+ exchanger NHE1 in fresh bovine corneal endothelial cell basolateral plasma membrane

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Abstract

Apical and basolateral plasma membranes of fresh bovine corneal endothelial cells were isolated using positively charged polyacrylamide beads. Marker enzyme assays demonstrated that the isolated apical and basolateral plasma membrane domains could be isolated and separated with relative purity. Western blotting with a polyclonal anti-NHE1 antibody detected a protein of 70 kDa in the basolateral plasma membrane isolate. NHE1 immunoreactivity was not detected in the apical membrane sample. This suggests that the Na^+/H^+ exchanger, NHE1, is strictly localised to the basolateral membrane of fresh bovine corneal endothelial cells.

Keywords: Corneal endothelium; Na^+/H^+ exchanger; NHE1; Basolateral plasma membrane isolation; Bicarbonate transport

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 [1,2]. A HCO_3^- flux has been identified as the predominant component of this active transport system [3]. There is a consensus that the metabolic energy required to actively move HCO_3^- across the endothelium is provided by plasma membrane-bound Na^+/K^+/ATPase which is coupled to the HCO_3^- movement. Inhibition of the basolateral Na^+/K^+/ATPase by ouabain causes complete inhibition of the endothelial transport process [4,5]. As HCO_3^- is a net transported ion, then either its movement from corneal stroma across the basolateral membrane or its exit from the cytoplasm into the aqueous humour across the apical membrane must be driven uphill. Localisation of the Na^+/K^+/ATPase to the basolateral membrane [6] taken together with the absence of a net transendothelial Na^+ flux, when the endothelium is short-circuited, suggests that the driven step (coupled to the transmembrane Na^+ gradient) takes place at the basolateral membrane [7,8]. At steady state, it has been proposed that Na^+ recycles across the basolateral membrane with the exit flux via Na^+/K^+/ATPase activity equal to its entry flux via the Na^+/H^+ exchanger and, it has been suggested by a Na^+/HCO_3^- cotransporter [9,10]. Although there is no dispute about the presence of Na^+/H^+ exchange activity in corneal endothelial cells, it has been suggested that the Na^+/H^+ exchanger is localised to the apical membrane.

To resolve whether a Na^+/H^+ exchanger is present in the basolateral or apical plasma membranes of corneal endothelial cells, we isolated both the apical

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and basolateral plasma membrane domains, adapting the published method of Soltau and coworkers in cultured cells for use in fresh cells [11]. For studies such as these, it is important to use fresh cells because it is established that cultured cells show different transport characteristics to fresh cells [12]. Corneal endothelial cells have a net negative surface charge [13,14] which allowed Soltau and coworkers to use positively charged polyacrylamide beads to bind covalently to the apical surface and effectively separate apical and basolateral membranes. This method was originally devised for membrane separation by Jacobson and coworkers [15–17].

2. Materials and methods

2.1. Reagents and materials

In this study, all reagents were obtained from Sigma (UK) unless otherwise stated. Acrylamide-Bis Acrylamide was used as a 30% stock solution obtained from Severn Biotech. Nitrocellulose membrane (0.45 μm pore size) was obtained from Bio-Rad, UK.

2.2. Plasma membrane isolations

Polyacrylamide beads (40–80 μm, Bio-Rad) were coated with polylysine according to the method of Cohen et al. [15]. Before use, the beads were removed from the fridge and washed three times in 0.2 M sodium chloride. The beads were then resuspended in sucrose acetate buffer (seven parts 310 mM sucrose to three parts 310 mM sodium acetate) to make a 50% bead suspension.

2.3. Isolation of apical plasma membrane

Fresh bovine eyes were obtained from a local abattoir within 1 h post-mortem. The eyes were transported in an ice-packed cooler box, then refrigerated at 4°C for use within 24 h. Fifteen corneas were removed from bovine eyes, leaving at least a 1 cm scleral rim. The corneas, endothelium uppermost, were covered with 1 ml bead solution. The beads were left to bind to the apical membranes for 10 min at room temperature. Then, the areas of the beads not covered by membranes were blocked by adding approximately 500 μl of 40 units ml^{-1} of heparin (sodium) in sucrose acetate buffer for 5 min. Following this, each cornea was held vertically over a collecting beaker and the bead solution (with attached apical membrane) was allowed to drain into the beaker. Each cornea was rinsed with 1 ml sucrose acetate buffer (over the beaker) to obtain maximum bead (and therefore membrane) retrieval. The beads were allowed to sediment at the bottom of the beaker for 10 min and the supernatant was removed. The beads were washed twice in 10 mM Tris-HCl (pH 7.4) at 4°C. The supernatant was pipetted off, leaving the beads (and attached membrane) in a small amount of Tris-HCl. Triton X-100 (0.1%) was added to the plasma membrane isolates prior to enzyme assay to separate the membrane proteins from the beads.

2.4. Isolation of basolateral plasma membrane

Ten bovine corneas were removed from fresh bovine eyes, leaving at least a 1 cm scleral rim. Each cornea was dipped twice in distilled water to disrupt the endothelial cells. The cornea was then laid flat, endothelium uppermost, and the cornea was covered with distilled water to ensure that all cells were disrupted. Once the cells were disrupted, the cell contents and most of the apical membrane were rinsed away by dipping the cornea in 10 mM Tris-HCl (pH 7.4) at 4°C. The remaining basolateral membranes were scraped off each cornea into 1.5 ml of 10 mM Tris-HCl using a plastic spatula. The membrane suspension was sonicated at 50 W for 15 s.

2.5. Isolation of total plasma membrane

The corneas from 15 bovine eyes were removed and each cornea was dipped three times in 37°C Hank's balanced salt solution (HBSS). The corneas were laid flat in a tray, endothelium uppermost, and 1 ml of 30 μM EDTA in HBSS (37°C) was pipetted onto each cornea and left for 10 min. After this time, the fluid was drained away and each cornea was dipped into ice-cold HBSS. The endothelial cells were scraped carefully from each cornea and were shaken into 1.5 ml of 250 mM sorbitol. The cell suspension was carefully transferred to a 100 ml ca-

pacity beaker. The bead suspension was added to the cell suspension to make a 50% cell/bead suspension. The suspension was gently agitated on a rotary shaker for 10 min to allow the beads to bind to the cells. Twenty vol of heparin in sucrose acetate buffer was added to the bead-cell suspension to inhibit non-specific binding. The beads (with attached cells) were allowed to settle to the bottom of the beaker and were then washed twice in sucrose acetate buffer. The cells (attached to the beads) were disrupted by sonication at the lowest setting for 10 s. This allowed the total cell membrane to remain attached to the beads and the cell contents to be released into the solution. The beaker was vortexed vigorously to ensure all cells were disrupted. The beads (with attached cell membrane) were allowed to settle to the bottom of the beaker and were washed twice with 10 mM Tris-HCl to remove the cell contents. The sample was treated with 0.1% Triton X-100 to solubilise the membrane protein and separate the membrane protein from the beads.

2.6. Isolation of whole cell homogenate

The corneas from five bovine eyes were removed and each rinsed in 250 mM sorbitol. The endothelial cells were scraped away using a plastic spatula which was shaken vigorously into a beaker containing 1.5 ml of 10 mM Tris-HCl, pH 7.4 to leave the cells in suspension. The cell suspension was transferred into a 1 ml Potter-Elvehjem homogeniser and homogenised with four strokes of the plunger.

2.7. Enzyme assays

Triton X-100 (0.1%) was added to the plasma membrane isolates prior to enzyme assay to separate the membrane proteins from the beads. A series of enzyme assays was performed with the isolated membrane protein samples, to determine relative purity of the membrane domains. Ouabain-sensitive Na^+/K^+ATPase activity [18] was measured as a marker for basolateral plasma membranes; alkaline phosphatase enzyme activity (ρ-nitrophenyl as substrate) as a marker for apical plasma membranes [19]; total protein by the assay method of Bradford [20]. The activity of alkaline phosphatase is determined as the rate of change of optical density at 410 nm (25°C) once the enzyme substrate has been added. The incubation mixture included a final concentration of 5 mM magnesium chloride, 0.25 mM calcium chloride, 0.2 mM zinc chloride, dissolved in 50 mM Tris-maleate buffer (pH 9.0).

2.8. Western blotting with anti-NHE1 antibody

SDS-gel electrophoresis was carried out according to the procedure of Laemmli [21] using 10% polyacrylamide gels. The samples were incubated in 2% SDS, 1% dithiothreitol, 15% glycerol, 0.1% bromophenol blue in 500 mM Tris-HCl, pH 6.8 for 5 min at 95°C. Approximately 10 μg of protein was applied to each lane on the SDS-polyacrylamide gel and the electrophoresis was performed in a Mini-Protean II electrophoresis cell (Bio-Rad) using a voltage of 150 V for 1 h. Each plasma membrane sample was electroblotted from the polyacrylamide gel onto nitrocellulose membrane filters. Each blotted sample was probed with anti-NHE1 antibody. This affinity-purified polyclonal antibody (designated #200) was generously donated by Professor Larry Fliegel, Department of Pediatrics and Biochemistry, University of Alberta, Edmonton, Alberta, Canada. This anti-NHE1 antibody has been extensively characterised by Professor Fliegel [22]. The antibody was made against the carboxyl terminal region (242 amino acids) of the Na^+/H^+ exchanger in human placental brush border membranes which had been fused to GST (glutathione S transferase). Polyclonal antibodies against the Na^+/H^+ exchanger, isoform 1 (NHE1) were raised in rabbits, affinity-purified and were used at a dilution of 1:200. The secondary antibody (goat anti-rabbit IgG with HRP conjugate) was used at a dilution of 1:500. Control blots for each sample were immunostained, omitting the primary antibody step. A control sample of human placental brush border membrane was also subjected to Western blotting.

3. Results

3.1. Enzyme assays

Table 1 show the results of experiments averaged from three different membrane isolations, with assays made in triplicate. The apical plasma membrane en-

Table 1
Presented are average ± S.E.M. of three determinations

Fraction	Na^+/K^+/ATPase (μmol/mg protein/30 min)	Alkaline phosphatase (μmol/mg protein/30 min)	Protein concentration (μg protein/ml)
Homogenate	3.23 ± 0.04	2.55 ± 0.08	903 ± 14
Total plasma membrane enriched fraction	4.27 ± 0.41	1.54 ± 0.17	776 ± 40
Apical membrane enriched fraction	0.19 ± 0.04	19.16 ± 1.61	40.6 ± 2.33
Basolateral membrane enriched fraction	5.06 ± 0.24	1.24 ± 0.10	805 ± 119

Enzyme activities expressed in terms of protein concentration for each of the plasma membrane domains, apical, basolateral, whole membrane and whole homogenate.

riched fraction showed a significantly higher degree of specific alkaline phosphatase enzyme activity over the basolateral membrane enriched fraction ($P < 0.001$), which was approximately 15 times higher in the apical membrane compared to the basolateral. The basolateral plasma membrane showed a significantly higher degree of specific activity of the Na^+/K^+/ATPase enzyme over the apical membrane ($P < 0.001$), and this activity was 25 times higher in the basolateral plasma membrane sample compared to the apical membrane sample.

3.2. Western blotting

The SDS-PAGE results in Fig. 1 show some distinct differences in protein constituents between the apical and basolateral plasma membranes. The total number of readily visualised protein bands using the current detection system in the apical membrane totalled approximately 30. For a weight matched sample, the total number in the basolateral sample was approximately 40. The molecular weights of the major protein bands visualised for each plasma membrane isolate are listed in Table 2.

3.3. Western blot analysis of basolateral and apical membrane domains

Western blotting was carried out on bovine corneal endothelial cell apical membrane and basolateral plasma membrane that had been subjected to SDS-PAGE as described earlier using polyclonal anti-NHE1 antibody. Fig. 2 shows the Western blot analyses for the apical and basolateral plasma membrane isolates with control NHE1 sample.

The anti-NHE1 antibody did not detect any pro-

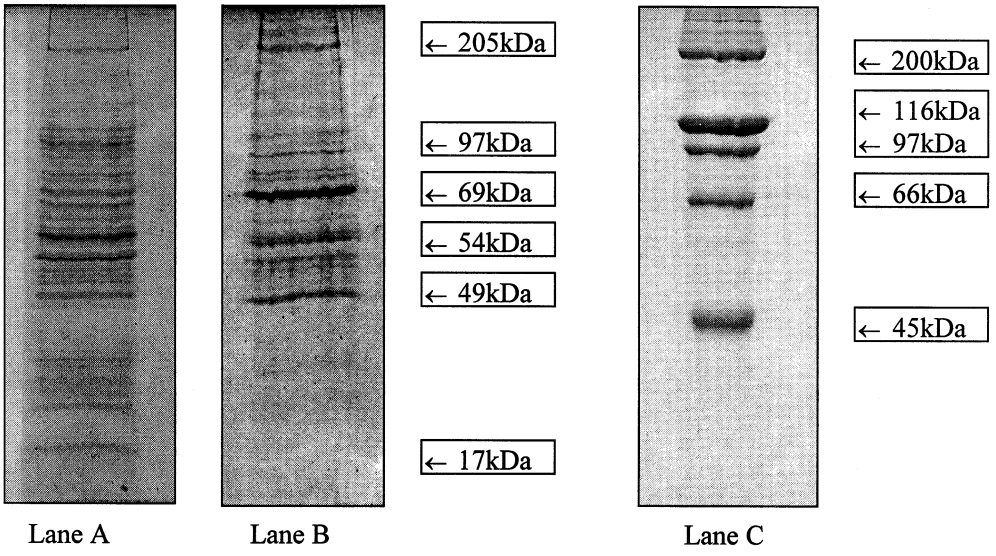

Fig. 1. Protein bands of both basolateral membrane (lane A), apical membrane (lane B) and molecular weight markers, Bio-Rad (lane C), resulting from SDS-gel electrophoresis (10% acrylamide). The protein bands have been stained with Coomassie blue dye.

Table 2
Molecular size of the prominent protein bands detected by gel electrophoresis of the apical and basolateral plasma membrane domains

Basolateral (kDa)	Apical (kDa)
97	205
70	69
58	54
49	49
17	40

tein bands by Western blotting in the apical plasma membrane samples, which suggests the absence of NHE1 activity in the apical membrane sample. We can say this with some certainty, since the apical sample was contaminated with less than 5% by basolateral membrane as shown from the enzyme assay results. Control blots were immunostained, omitting the primary antibody incubation step. No protein bands were detected in the control blot. The study demonstrates NHE1 activity in the basolateral plasma membrane sample. There was some contamination of the basolateral plasma membrane sample with apical membrane as shown from the enzyme assays earlier. However, the fact that the apical membrane did not show NHE1 activity, demonstrates that NHE1 activity is probably entirely restricted to the basolateral plasma membrane of fresh bovine corneal endothelial cells.

4. Discussion

In most polarised cells, the basolateral plasma membrane domain is characterised by a higher content of Na^+/K^+/ATPase and the apical plasma membrane domain is characterised by a higher ratio of alkaline phosphatase to Na^+/K^+/ATPase [19,23,24]. The data is in agreement with those studies and suggests that the basolateral and apical plasma membrane isolates from bovine corneal endothelium are similar in this respect to other polarised cell types and can be separated with relative purity. The only previous study of this kind on this cell type was on cultured bovine corneal endothelial cells which showed similar levels of purity for apical and basolateral plasma membrane domains [11]. The high level of Na^+/K^+/ATPase activity in the basolateral plasma membrane isolate agrees with numerous studies that have localised this enzyme to the basolateral membranes of bovine corneal endothelial cells [25–29]. From the Western blot analysis of apical and basolateral membrane isolates, it was demonstrated that effectively only the basolateral isolate exhibited Na^+/H^+ exchanger, NHE1, immunoreactivity. This is the first study to demonstrate the presence of NHE1 expression on the basolateral membrane of fresh bovine corneal endothelial cells, although its presence has been strongly implicated by evidence using pH electrodes, intracellular microelectrodes

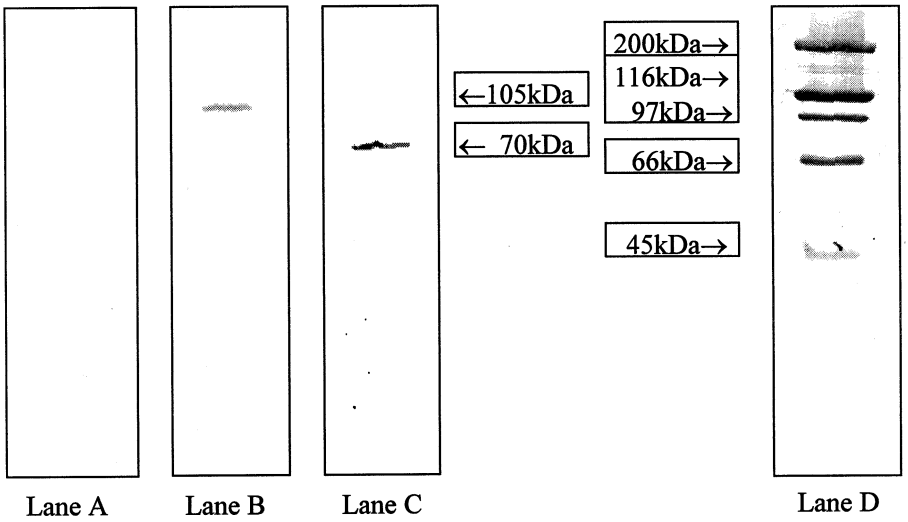

Fig. 2. Western blot analysis of NHE1 on bovine corneal endothelial cell plasma membrane isolates. Apical plasma membrane (lane A), control human placental brush border membrane (lane B), basolateral membrane (lane C). Molecular weight markers (Bio-Rad) are shown in lane D and were stained with Amido Black.

and transport protein drug inhibitors, such as amiloride. Such studies indicated the existence of a Na^+/H^+ exchanger and have proposed it to be localised to the basolateral membrane. The estimated molecular mass of NHE1 in the basolateral plasma membrane isolate in bovine corneal endothelium is shown to be $\sim$70 kDa.

These results add weight to the model proposed by our laboratory by which the Na^+/H^+ exchanger uses the energy stored in the Na^+ gradient across the plasma membrane (generated by the Na^+/K^+/ ATPase) to extrude protons from the cell. The fact that NHE1 is located on the basolateral membrane confirms the hypothesis that Na^+ entry and exit can occur along the basolateral surface of the corneal endothelium. This hypothesis came from findings whereby no net flux has been demonstrated across corneal endothelium [5] and the rate of Na^+ entry into both cultured [10] and fresh [30] rabbit corneal endothelial cells is reduced by half in the presence of amiloride. At steady state, Na^+ recycles across the basolateral membrane with exit flux via Na^+/K^+/ATPase equal to its entry flux via the Na^+/H^+ exchanger and, it has been suggested, by at least one other route [8,9]. The consequence is that passive Na^+ entry via the Na^+/H^+ exchanger is non-electrogenic: the Na^+ is simply recycling across the basolateral membrane. Therefore it is suggested that NHE1 probably has a regulatory role which could involve pH regulation or might provide a mechanism for Na^+ reentry into the cell that dissipates the Na^+ gradient across the basolateral membrane.

The results of this study also show, for the first time, that it is possible to separate and isolate apical, basolateral, and total plasma membrane domains from fresh bovine corneal endothelial cells, with relative purity using positively charged polyacrylamide beads.

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